Title: Comparative Genomic Epidemiology of Serotype 3 IPD and Carriage Isolates from Southampton, UK between 2005 and 2017

Authors: David W. Cleary1, 2, Stephanie W Lo3, Narender Kumar3, Stephen D. Bentley3, Saul N. Faust2, 4, 5 and Stuart C. Clarke2, 4, 6\*

1. Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom
2. Faculty of Medicine and Institute for Life Sciences, University of Southampton, Southampton, United Kingdom
3. Parasites and Microbes, Wellcome Sanger Institute, Hinxton, United Kingdom
4. NIHR Southampton Biomedical Research Centre, University Hospital Southampton Foundation NHS Trust, Southampton, United KingdomNIHR
5. Southampton Clinical Research Facility, University Hospital Southampton Foundation NHS Trust, Southampton, United Kingdom
6. Global Health Research Institute, University of Southampton, Southampton, United Kingdom

\*Corresponding: [S.C.Clarke@soton.ac.uk](mailto:S.C.Clarke@soton.ac.uk) Infectious Disease Epidemiology Group, University of Southampton, Mailpoint 814, Level C, Sir Henry Wellcome Laboratories, South Block, University Hospital Southampton Foundation NHS Trust, Southampton SO16 6YD, UK

Abstract

Background: Serotype 3 remains a significant cause of disease despite its inclusion in PCV13. Whilst clonal complex 180 (CC180) represents the major clone, recent studies have refined the population structure into three clades: Iα, Iβ and II, with the latter being a recent divergent and more antibiotic resistant. We present a genomic analysis of serotype 3 isolates from paediatric carriage and all-age invasive disease, collected between 2005 and 2017 in Southampton, UK.

Methods: Forty-one isolates were available for analysis. Eighteen were isolated during the annual cross-sectional surveillance of paediatric pneumococcal carriage. The remaining twenty-three were isolated from blood / CSF specimens at the University Hospital Southampton NHS Foundation Trust laboratory.

Results: All carriage isolates were CC180 GPSC12. Greater diversity was seen with IPD with three GPSC83 (ST1377: n=2, ST260: n=1) and one GPSC3 (ST1716). For both carriage and IPD Clade Iα was dominant (94.4 and 73.9% respectively). Two isolates were Clade II with one from carriage (a 34-month-old, October 2017), and an invasive isolate (49-year-old, August 2015). Four IPD isolates were outside the CC180 clade. All isolates were genotypically susceptible to penicillin, erythromycin, tetracycline, co-trimoxazole and chloramphenicol. Two isolates (one each from carriage and IPD; both CC180 GPSC12) were phenotypically resistant to erythromycin and tetracycline; the IPD isolate was also resistance to oxacillin.

Conclusion: In the Southampton area, carriage and invasive disease associated with serotype 3 is predominantly caused by Clade Iα CC180 GPSC12.

Keywords: serotype 3; IPD; Invasive Pneumococcal Disease; *Streptococcus pneumoniae*; carriage

Impact Statement

Pneumococcal conjugate vaccines (PCVs) have reduced the burden of invasive disease by targeting particular serotypes. Serotype 3 however, which is included in PCV13, continues to cause disease, and is still carried asymptomatically by some children. Using our long-running, cross-sectional paediatric carriage study we sought to answer key questions related this persistence. We combined carriage isolates with those from cases of invasive disease taken concurrently from hospital laboratories at the University Hospital Southampton NHS Foundation Trust. We highlight the on-going circulation of serotype 3 eight years after the introduction of PCV13 in both paediatric carriage and all age IPD, as well as an increase in the latter from 2012/13. We show this is not related to the expansion of a recently described clade (Clade II) of a particular clonal complex of serotype 3 strains (CC180) which was previously suggested as the driver for increases in associated IPD in the UK.

Introduction

The post-pneumococcal conjugate vaccine (PCV) era has been characterised by a remarkable global reduction in *Streptococcus pneumoniae* associated morbidity and mortality (1-5). There remains, however, a significant burden of both invasive (bacteraemia, meningitis, septicaemia) and non-invasive disease (pneumonia, otitis media). Although in part driven by serotype replacement (6-9) and the rise of non-vaccine serotype disease (10), a persistent challenge are those vaccine-type serotypes which have proven recalcitrant to immunisation programmes. Serotype 3 is one such example.

Despite its inclusion in the 13-valent PCV (PCV13), serotype 3 remains a particularly significant cause of disease globally (11). Following PCV13 introduction in the UK in 2010 serotype 3 has continued to circulate in paediatric carriage (7, 12). Vaccine effectiveness has been questionable given the fluctuating, albeit relatively low level, incidence of invasive pneumococcal disease (IPD) in children <5 years old (10). Importantly, serotype 3 remains a significant burden in adult disease causing, for example, 57% of pneumococcal community acquired pneumonias between 2013 and 2018 (13) and 65% of IPD in those aged >65 years old (10). In the UK this group is offered the Pneumococcal Polysaccharide Vaccine (PPV). This lower vaccine efficacy has been shown to derive from extensive capsule release, a by-product of the way in which the capsular polysaccharide is not covalently anchored to the cell surface, which prevents antibody-mediated opsonophagocytosis (14).

Whilst clonal complex 180 (CC180) represents the major clone, recent studies have refined the population structure of serotype 3 pneumococci into three clades: Iα, Iβ and II, with the latter being a recent divergent and characterised as more antibiotic resistant (15, 16). However, there is no evidence to link this changing epidemiology to PCV13 introduction (15). In the present study, we aimed to build upon these data and present the genomic analysis of serotype 3 isolates from a serial, cross-sectional paediatric carriage study between 2005 and 2017 in Southampton, UK, in addition to temporally and geographically concomitant isolates from all-age invasive disease.

Materials and Methods

Isolate Collection: Carriage isolates were obtained using nasopharyngeal swabs collected from children aged 4 years or under each year commencing in the winter (October to March) of 2006/07 and for each consecutive year until 2017/18. Parents/guardians were approached for informed consent either prior to or following their child’s appointment in an outpatient department of Southampton General Hospital. Aside from age, the only other exclusion criterium was that only one child per family was swabbed. Nasopharyngeal Rayon tipped Transwabs (Medical Wire, Corsham, UK) in charcoal Amies media were used for swabbing and then plated onto Columbia Colistin Naladixic Acid agar (CNA; Oxoid, Basingstoke, UK) within 9h of swabbing. IPD isolates from blood or CSF specimens were isolated in the Public Health England (PHE) laboratory at University Hospital Southampton NHS Foundation Trust between July 2005 and June 2017.

Pneumococcal growth and confirmation: Confirmation of presumptive *S. pneumoniae* was done on Columbia Blood Agar (CBA; Oxoid, Basingstoke, UK) using optochin sensitivity indicated by a ⩾14 mm diameter inhibition zone around the disc (Thermo Scientificä, Loughborough, UK). Only one colony of *S. pneumoniae* per participant was selected for further analysis.

Whole Genome Sequencing: Isolates from STGG stocks were cultured on CNA plates and incubated o/n at 37oC in 5% CO2. Genomic DNA extraction was carried out using QIAamp® DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA extracts were sent to the Wellcome Sanger Institute (WSI) for whole genome sequencing (WGS) using Illumina HiSeq or 10X platforms generating initially 2 ´ 75 bp, 2 ´ 100bp and later 2 ´ 150 bp paired-end reads from libraries prepared using TruSeq chemistry.

Bioinformatic Analysis: The processing of WGS data has been previously described (17) where serotype is derived using SeroBA (18) and sequence type using MLST (19). Global Pneumococcal Sequence Cluster (GPSC) for each isolate was done using Kmer-based clustering in popPUNK (20). Likewise antibiotic resistance profiles were generated using previously described methods (21-23), including penicillin (encoded by the genes *pbp1A*, *pbp2B*, *pbp2A*), chloramphenicol (*cat*), cotrimoxazole (*folA* and *folP*), erythromycin (*ermB* and *mefA*), fluoroquinolones (*gyrA* and *parC*), tetracycline [*tet*(M), *tet*(O) and *tet*(S/M)], and vancomycin (*vanA* and *vanB)*.Phylogenies were made using nextflow v21.04.1 nf-core/bactmap v1.0.0 (24). Briefly, reads were mapped to a serotype 3 ST180 strain OXC141 (accession: NC\_017592.1) with bwa mem (25), alignments indexed and sorted using samtools v1.10 (26) with variants called and filtered using bcftools v1.11 (27). Recombinogenic regions were then identified and removed using Gubbins v2.4.1 (28) and non-variable sites removed using snp-sites v2.5.1 (29). Finally, a maximum likelihood tree was built using RAxML-NG v1.0.2 with the GTR + Gamma model of nucleotide substitution (30). Recombination regions were visualised using Phandango (31). Clade designation was determined by placement of isolates within the phylogeny previously generated by Azarian *et al.*, (2018) using the method described above. Temporal analysis of the CC180 isolates was done using BactDating v1.1.0 (32) using the *aligned\_pseuedogenomes* output from Gubbins with the number of MCMC iterations set to 10,000 (nbIts=1e4). Protein antigen detection as previously described (15) was done using both ABRicate v1.0.1 (33) and SRST2 v0.2.0 (34) with a reference sequence database kindly donated by Dr Taj Azarian. The database contains 61 alleles of thirteen antigens (*pspC* n=18, *pspA* n=12, *nanA* n=3, *phtD* n=2, *ply* n=2, *zmpA* n=7, *rrgA* n=3, *rrgB* n=3, *rrgC* n=3, *stkP* n=2, *strH* n=2, SP0609 n=2 and SP2194 n=2). Default parameters were used throughout, apart from for SRST2 and both *pspC* and *pspA* where the coverage threshold was reduced to 80% and maximum divergence increased from 10 (default) to 20% to account for the greater diversity in UK collections as described by Groves *et al.*, (2019).

Compute Resources: Nextflow nf-core/bactmap was implemented on the Cloud Infrastructure for Microbial Bioinformatics (CLIMB) resource (35). The Iridis HPC at University Southampton was used for all other compute requirements.

Statistics and Data Visualisation: All statistical analysis and data visualisation was done in R v4.2.1 (36) using RStudio v2021.09.2 (37) with graphics built using the grammar of graphics package, ggplot v3.3.5 (37) and the phylogenetic tree extension, ggtree v3.2.1 (38).

Data Availability: All sequencing data (fastqs) have been deposited in the European Nucleotide Archive under study accession PRJEB2417 (Whole genome sequencing of carried *Streptococcus pneumoniae* during the implementation of pneumococcal conjugate vaccines in the UK) and PRJEB6332 (Identifying pneumococcal genetic determinants for progression from colonisation to serious disease).

Results and Discussion

Following the global emergence of Clade II CC180 serotype 3 (15) there is a pressing need to examine the epidemiology of this serotype, particularly given that it continues to be a burden of invasive disease. Notwithstanding the important focus on disease, these analyses must also include the use of isolates derived from episodes of carriage (39). To this end we gathered isolates collected from the Southampton area in the UK, collected over a 12-year period to undertake a genomic analysis of population structure, antimicrobial resistance, and the distribution of important virulence-associated antigens.

Between 2005 and 2017, n=18 isolates of serotype 3 pneumococci were isolated from healthy children under the age of five years. No carriage isolates were identified in 2012/13 or 2014/15 (the absence in 2005/06 is a consequence of this predating the start of the paediatric carriage study which began in October 2006 i.e., the 2006/07 winter period) (Figure 1A). A similar number of IPD isolates (from children and adults) were available (n=23) (Table 1; Figure 1A). No serotype 3s were isolated from IPD cases in 2008/09, 2009/10, 2010/11 or 2011/12 (Figure 1). The highest number of isolates from IPD was seen in 2012/13 when six cases of serotype 3 associated disease were recorded. All age groups of <5-year-olds had at least one episode of carriage. In contrast, of the 21.7% (n=5/23) of IPD cases that occurred in children under 5-years old, three cases were between the ages of 0 and 11 months (average age: 3 months) with the remaining two being from a 3 and a 4-year-old (Table 1; Figure 1B). On average the proportion of serotype 3 observed in carriage has remained relatively steady at 1.4%, ranging from 0% to 3.9% which was observed in the 2011/12 winter period (Figure 1C). This contrasts with other PCV7 and PCV13 serotypes that have decreased over the same period (Cleary et al Sci Rep). For IPD the proportion of serotype 3 ranged from 0% (all periods between 2008/09 and 2011/12) to a maximum of 16.7 (average: 6.6%) (Figure 1C). Five of the six highest serotype 3 related IPD periods have occurred since the introduction of PCV13. The majority of IPD was in the >65-year-old group (39.1%; n=9/23; average age: 83) followed by the 50-64-year-olds (26.1%; n=6/23; average age: 59). However, proportionally serotype 3 accounted for 12.5% of IPD in children <5 years (n=5/40) compared to 6.7% of adults >18 (n=18/274) (Figure 1D).

The increase in proportion of serotype 3 associated IPD between 2012/13 and 2016/17 matches generally that observed across the UK (16), although 2012/13 was notably higher. It is tempting to consider a link between the higher carriage observed in the previous winter period (2011/12) with this high level of adult disease. Although such a relationship between paediatric carriage and adult IPD has been noted elsewhere the model was shown not to account for the disease burden associated with serotype 3 (40), particularly in adults over 40 which accounts for 94% of the adult IPD presented in this study. Additional caution should also come from considering the small numbers involved and that stochastic fluctuations due to this being a local convenience collection may better explain this phenomenon.

Genomically, all carriage isolates were CC180 (ST180), GPSC12, with 82.6% (n=19/23) of IPD isolates also belonging to this same clonal complex and sequence cluster. The remaining four IPD isolates were split between GPSC83 (n=3; ST 1377 and 260) and GPSC3 (n=1, ST17176). GPSC3 serotype 3 is not a common combination, representing only 1.1% (n=4/358) of previously described isolates in this sequence cluster (17); ST 17176 had also not been described previously. All four of these previously described were isolated from cases of disease, with two from South Africa (isolated in 2005 and 2014 respectively) and one each from Trinidad and Tobago (1997) and Qatar (2014) (17). It is worth noting however that GPSC3 does include invasive serotypes 8 and 33F and has been flagged as a lineage that causes significant non-VT disease since the introduction of PCV13 (41). In contrast, all GPSC83 described so far have been serotype 3 (87.0%, n=20/23; the remaining three having inconclusive serotype designations), with all but two from carriage and found globally in Africa, Asia, Europe and both North and South America (17). ST 260 is also the most common sequence type of GPSC83 (34.8%, n=8/23) with ST 1377 only found twice previously (17). The former has though been found sporadically within other serotypes (14, 6B, 7F and 9V) but these are single incidences and represent <5% of publicly available data (42).

No phylogenetic distinction between carriage and IPD isolates was seen for CC180 GPSC12 isolates in which n=17/18 carriage and n=18/19 IPD were assigned to Clade Iα (Figure 2A). The absence of Iβ is unsurprising and in keeping with previous data given that it is the most infrequently observed both globally (15) and in England and Wales (16). Despite the apparent close phylogenetic relatedness of the Clade Iα isolates (Figure 2B), there was no evidence for transmission between study participants nor for a temporal signal (Figure 2C). Previously, Clade II had been shown to be rapidly increasing in IPD in England and Wales (16) although it appears not be a consequence of narrow vaccine efficacy (15, 43). That said we note that 3 of the 4 isolates that could not be assigned to a clade were recovered post-PCV13 and were all from cases of invasive disease which is suggestive perhaps of an increase in diversity of serotype 3 post-PCV13. Regardless, antigenic variation and antimicrobial resistance within this diverging Clade II population has been raised as a potential cause of its emergence. In contrast, only one disease isolate of this Clade II was observed in this study, having been recovered from a 49-year-old in 2015. We could not see a significant shift in carriage as has been seen elsewhere (43) with only one isolate recovered from a 34-month-old in 2017. The dates of isolation (post-PCV13) are in keeping with the emergence of Clade II however with limited numbers it is difficult to determine the extent of this replacement. What is interesting is that higher levels of serotype 3 IPD observed in Southampton is despite the absence of Clade II. Examining the diversity of certain pneumococcal antigens however does support the supposition of diversity being a factor in emergence of Clade II. Some antigens consistently belonged to the same variants regardless of Clade such as *pspA* (pneumococcal surface protein A; with one exception as described below) where all Clade Iα and II isolates had Family 2 variants (Figure 3). Other examples included SP0609 (an amino acid ABC transporter), SP2194 (ATP-dependent Clp protease), *ply* (pneumolysin) and *strH* (Beta-N-acetylhexosaminidase) (Figure 3). Conversely, there were antigens absent as expected in serotype 3 isolates (i.e., *rrgABC*; pilus subunit). Nevertheless, diversity was seen for *pspC* where 32/34 Clade Iα isolates were Group 6 and both Clade II were Group 8. This is entirely in keeping with that described before (16). The two Clade Iα isolates which possessed a Group 8 *pspC* are perhaps more unexpected. Both were isolated in 2013 from adult invasive disease. Isolate UOS\_IPD\_423 was also variant for *pspA* (Family 1) and *nanA* which had, unlike the other Clade Iα isolates, a variant of Var-III - the type found in both Clade II isolates. The *pspC* and *nanA* antigen designations suggest this isolate is, antigenically at least, more akin to a Clade II isolate than Clade Iα. Looking at recombination within the clade and on the terminal nodes representing this isolate however did not shed any light on these differences (Figure 4). As previously described Clade II was more recombinogenic at the internal, ancestral node compared to Clade Iα. Here the relative impacts of recombination to mutation (*r/m*) were 20.0 and 8.7, and the relative rates of recombination to mutation (*ρ/θ*)were 0.181 and 0.112 respectively. No terminal node recombination was detected for the Clade II isolates, which is not surprising given there are only two. However, terminal node recombination statistics for Clade Iα were consistent with that observed before (*r/m* = 0.07 and *ρ/θ*  = 0.001 (15)) with those in the present study being 0.02 and 0.003.

All isolates were shown to be genotypically susceptible to penicillin, erythromycin, tetracycline, co-trimoxazole and chloramphenicol. Two isolates (one each from carriage and IPD; both CC180 GPSC12) were phenotypically resistant to erythromycin and tetracycline; the IPD isolate, which was isolated in 2014, was also resistance to oxacillin.

Whilst this study provides useful data on the epidemiology and population structure of an important pneumococcal serotype there are limitations. From a carriage perspective, the population represents a convenience sample from a study that was not powered to specifically detect serotype 3. The paediatric carriage study was powered to enable the detection of an estimated 50% relative reduction in carriage following PCV7 introduction with 80% power at a 5% significance level. This meant a minimum of 100 pneumococcal isolates collected each year; with the infrequency of colonisation this snapshot collection is not an accurate picture of serotype 3 carriage in this community. Further, no carriage was undertaken in an adult population. This is a much-needed addition to the current study is as much as it might reveal a serotype 3 expansion across demographics which could explain the increase in adult IPD in the absence of a notably increased carriage in children. The IPD isolates also represent a convenience sample taken from one hospital, albeit a large regional centre, and are therefore may not be representative of the national picture.

Conclusion

Serotype 3 carriage and invasive disease episodes were identified throughout the study period, in both infants and adults. This adds further support to the notion of lower PCV13 effectiveness against this serotype. The serotype 3 epidemiology around Southampton is driven by a closely related Clade Iα CC180 GPSC12 pneumococcal population. Although we did not see a large transition between Clade Iα and Clade II, the fact we did recover the latter may reflect a shift and continued surveillance would determine if this is an expansion as seen elsewhere.

Funding

This work was supported by Pfizer as an investigator-led research grant. Funding for whole genome sequencing was provided by the Wellcome Sanger Institute.

Acknowledgments

We would like to acknowledge the support of our technical colleagues, Rebecca Anderson, Denise E. Morris, Stephen Gomer and Emily J. Dineen in the collection and processing of samples for the study. We are grateful to staff associated with the Global Pneumococcal Sequencing Project at the WSI for their support in genome sequencing of collected isolates. We are indebted to staff at the NIHR Southampton Clinical Research Facility for their assistance in the collection of samples and to staff at Public Health England for swab processing between 2006/07 and 2011/12. Computational analyses were made possible through access to CLIMB-computing servers. We also acknowledge the use of the IRIDIS High Performance Computing Facility, and associated support services at the University of Southampton, in the completion of this work. Finally, we acknowledge the patients, guardians, and participants without whom this study would not have been possible.

Authors and Contributors

DWC – Investigation, Formal Analysis, Writing – Original Draft Preparation, and Visualisation; SNF - Conceptualisation and Funding, Writing – Review and Editing; SDB – Funding, Resources, Writing – Review and Editing; SWL – Formal Analysis, Data Curation, Writing – Review and Editing; NK - Formal Analysis, Data Curation, Writing – Review and Editing; SCC - Conceptualisation and Funding, Writing – Review and Editing.

Conflicts of Interest

DWC was a post-doctoral researcher on GSK funded projects in 2014/15, and currently receives grant support from Pfizer and the National Institute for Health via the NIHR Southampton Biomedical Research Centre. SNF is an NIHR Senior Investigator and receives support from the National Institute for Health Research funding via the NIHR Southampton Wellcome Trust Clinical Research Facility and the NIHR Southampton Biomedical Research Centre. SNF and SCC act as principal investigators for clinical trials and other studies conducted on behalf of University Hospital Southampton NHS Foundation Trust/University of Southampton that are sponsored by vaccine manufacturers. No personal payments are received from them. SNF and SCC have participated in advisory boards for vaccine manufacturers including Pfizer but receive no personal payments for this work. DWC, SNF, SCC have received financial assistance from vaccine manufacturers to attend conferences. All grants and honoraria are paid into accounts within the respective NHS Trusts or Universities, or to independent charities. All other authors have no conflicts of interest.

Ethical Approval: The paediatric carriage study was approved by UK NHS Research Ethics Service (06/Q1704/105 and 14/NS/1064). The collection of IPD isolates was approved by UK NHS Research Ethics (08/H0504/28). All methods were performed in accordance with relevant regulations. Informed consent was secured from the legal guardians of all participants of the paediatric carriage study.

Figure Legends

Figure 1: Isolation period of *S. pneumoniae s*erotype 3 isolates (A) with carriage isolates shown in purple and IPD in yellow. The split between age groups is shown in B where all carriage isolates (n=18) were from children <5-years old, compared to just 21.7% (n=5/23) of IPD isolates. Serotype 3 as a proportion of all *S. pneumoniae* sampled each period is shown in C., with the proportion that accounted for IPD by age group in D.

Figure 2: A: Maximum-likelihood phylogeny of all Serotype 3 pneumococci generated using RAxML-NG on a recombination corrected alignment done using Gubbins. Tree tips are are colored according to various study groups: carriage (green circle), IPD (purple triangle) and the reference (OXC141; NC\_017592.1). Associated metadata are shown as coloured rings depicting ST (inner), GPSC (middle) and Clade (outer). B. The same phylogeny with CC180 isolates highlighted by the blue box from which a time-corrected phylogeny (C) was constructed using BactDating where blue tips show earliest isolate collections and red the most recent.

Figure 3: Maximum-likelihood phylogeny Serotype 3 pneumococci with distribution of variable antigens. Tree tips are are colored according to various study groups: carriage (green circle), IPD (purple triangle) and the reference (OXC141; NC\_017592.1).

Figure 4: Maximum likelihood phylogeny of all Serotype 3 isolates generated using RAxML-NG with clade Iα and II shown (left) with linear genome map of the reference *S. pneumoniae* OXC141 (top). Recombination events are shown as red and blue blocks, with red indicating a recombination event that has occurred on an ancestral branch and blue showing recombination blocks unique to that terminal node i.e., isolate. The bottom plot shows the cumulative frequency of recombination at positions along the reference.

References

1. Wasserman M, Chapman R, Lapidot R, Sutton K, Dillon-Murphy D, Patel S, et al. Twenty-Year Public Health Impact of 7- and 13-Valent Pneumococcal Conjugate Vaccines in US Children. Emerging infectious diseases. 2021;27(6):1627-36.

2. Mackenzie GA, Hill PC, Jeffries DJ, Hossain I, Uchendu U, Ameh D, et al. Effect of the introduction of pneumococcal conjugate vaccination on invasive pneumococcal disease in The Gambia: a population-based surveillance study. The Lancet Infectious Diseases. 2017;17(9):965-73.

3. Galanis I, Lindstrand A, Darenberg J, Browall S, Nannapaneni P, Sjöström K, et al. Effects of PCV7 and PCV13 on invasive pneumococcal disease and carriage in Stockholm, Sweden. European Respiratory Journal. 2016.

4. Waight PA, Andrews NJ, Ladhani SN, Sheppard CL, Slack MPE, Miller E. Effect of the 13-valent pneumococcal conjugate vaccine on invasive pneumococcal disease in England and Wales 4 years after its introduction: an observational cohort study. The Lancet Infectious Diseases. 2015;15(5):535-43.

5. Moore MR, Link-Gelles R, Schaffner W, Lynfield R, Lexau C, Bennett NM, et al. Effect of use of 13-valent pneumococcal conjugate vaccine in children on invasive pneumococcal disease in children and adults in the USA: analysis of multisite, population-based surveillance. The Lancet Infectious Diseases. 2015;15(3):301-9.

6. Løchen A, Croucher NJ, Anderson RM. Divergent serotype replacement trends and increasing diversity in pneumococcal disease in high income settings reduce the benefit of expanding vaccine valency. Scientific Reports. 2020;10(1):18977.

7. Kandasamy R, Voysey M, Collins S, Berbers G, Robinson H, Noel I, et al. Persistent Circulation of Vaccine Serotypes and Serotype Replacement After 5 Years of Infant Immunization With 13-Valent Pneumococcal Conjugate Vaccine in the United Kingdom. J Infect Dis. 2020;221(8):1361-70.

8. Gladstone RA, Devine V, Jones J, Cleary D, Jefferies JM, Bentley SD, et al. Pre-vaccine serotype composition within a lineage signposts its serotype replacement – a carriage study over 7 years following pneumococcal conjugate vaccine use in the UK. Microbial Genomics. 2017;3(6).

9. Devine VT, Cleary DW, Jefferies JMC, Anderson R, Morris DE, Tuck AC, et al. The rise and fall of pneumococcal serotypes carried in the PCV era. Vaccine. 2017;35(9):1293-8.

10. Ladhani SN, Collins S, Djennad A, Sheppard CL, Borrow R, Fry NK, et al. Rapid increase in non-vaccine serotypes causing invasive pneumococcal disease in England and Wales, 2000–17: a prospective national observational cohort study. The Lancet Infectious Diseases. 2018;18(4):441-51.

11. Sings HL, De Wals P, Gessner BD, Isturiz R, Laferriere C, McLaughlin JM, et al. Effectiveness of 13-Valent Pneumococcal Conjugate Vaccine Against Invasive Disease Caused by Serotype 3 in Children: A Systematic Review and Meta-analysis of Observational Studies. Clin Infect Dis. 2019;68(12):2135-43.

12. Southern J, Andrews N, Sandu P, Sheppard CL, Waight PA, Fry NK, et al. Pneumococcal carriage in children and their household contacts six years after introduction of the 13-valent pneumococcal conjugate vaccine in England. PloS one. 2018;13(5):e0195799.

13. Pick H, Daniel P, Rodrigo C, Bewick T, Ashton D, Lawrence H, et al. Pneumococcal serotype trends, surveillance and risk factors in UK adult pneumonia, 2013-18. Thorax. 2020;75(1):38-49.

14. Choi EH, Zhang F, Lu YJ, Malley R. Capsular Polysaccharide (CPS) Release by Serotype 3 Pneumococcal Strains Reduces the Protective Effect of Anti-Type 3 CPS Antibodies. Clin Vaccine Immunol. 2016;23(2):162-7.

15. Azarian T, Mitchell PK, Georgieva M, Thompson CM, Ghouila A, Pollard AJ, et al. Global emergence and population dynamics of divergent serotype 3 CC180 pneumococci. PLoS Pathog. 2018;14(11):e1007438.

16. Groves N, Sheppard CL, Litt D, Rose S, Silva A, Njoku N, et al. Evolution of Streptococcus pneumoniae Serotype 3 in England and Wales: A Major Vaccine Evader. Genes. 2019;10(11).

17. Gladstone RA, Lo SW, Lees JA, Croucher NJ, van Tonder AJ, Corander J, et al. International genomic definition of pneumococcal lineages, to contextualise disease, antibiotic resistance and vaccine impact. EBioMedicine. 2019;43:338-46.

18. Epping L, van Tonder AJ, Gladstone RA, Consortium TGPS, Bentley SD, Page AJ, et al. SeroBA: rapid high-throughput serotyping of Streptococcus pneumoniae from whole genome sequence data. Microbial Genomics. 2018;4(7).

19. Page AJT, B.; Keane, J. A. . Multilocus sequence typing by blast from de novo assemblies against PubMLST. Journal of Open Source Software. 2016;1(8).

20. Lees JA, Harris SR, Tonkin-Hill G, Gladstone RA, Lo SW, Weiser JN, et al. Fast and flexible bacterial genomic epidemiology with PopPUNK. Genome Research. 2019;29(2):304-16.

21. Metcalf BJ, Gertz RE, Jr., Gladstone RA, Walker H, Sherwood LK, Jackson D, et al. Strain features and distributions in pneumococci from children with invasive disease before and after 13-valent conjugate vaccine implementation in the USA. Clin Microbiol Infect. 2016;22(1):60.e9-.e29.

22. Li Y, Metcalf BJ, Chochua S, Li Z, Gertz RE, Walker H, et al. Validation of β-lactam minimum inhibitory concentration predictions for pneumococcal isolates with newly encountered penicillin binding protein (PBP) sequences. BMC Genomics. 2017;18(1):621.

23. Li Y, Metcalf BJ, Chochua S, Li Z, Gertz RE, Jr., Walker H, et al. Penicillin-Binding Protein Transpeptidase Signatures for Tracking and Predicting β-Lactam Resistance Levels in Streptococcus pneumoniae. mBio. 2016;7(3).

24. Ewels PA, Peltzer A, Fillinger S, Patel H, Alneberg J, Wilm A, et al. The nf-core framework for community-curated bioinformatics pipelines. Nature Biotechnology. 2020;38(3):276-8.

25. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25.

26. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25.

27. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics. 2011;27(21):2987-93.

28. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, et al. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Research. 2014;43(3):e15-e.

29. Page AJ, Taylor B, Delaney AJ, Soares J, Seemann T, Keane JA, et al. SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. Microb Genom. 2016;2(4):e000056.

30. Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics. 2019;35(21):4453-5.

31. Hadfield J, Croucher NJ, Goater RJ, Abudahab K, Aanensen DM, Harris SR. Phandango: an interactive viewer for bacterial population genomics. Bioinformatics. 2017;34(2):292-3.

32. Didelot X, Croucher NJ, Bentley SD, Harris SR, Wilson DJ. Bayesian inference of ancestral dates on bacterial phylogenetic trees. Nucleic Acids Res. 2018;46(22):e134.

33. Seemann T. Abricate Github [Available from: <https://github.com/tseemann/abricate>.

34. Inouye M, Dashnow H, Raven L-A, Schultz MB, Pope BJ, Tomita T, et al. SRST2: Rapid genomic surveillance for public health and hospital microbiology labs. Genome Medicine. 2014;6(11):90.

35. Connor TR, Loman NJ, Thompson S, Smith A, Southgate J, Poplawski R, et al. CLIMB (the Cloud Infrastructure for Microbial Bioinformatics): an online resource for the medical microbiology community. Microbial Genomics. 2016;2(9).

36. R Core Team. R: A language and environment for statistical computing. [Available from: <https://www.R-project.org/>.

37. Wickham H. ggplot2: Elegant Graphics for Data Analysis: Springer-Verlag New York; 2016.

38. Yu G. Using ggtree to Visualize Data on Tree-Like Structures. Current Protocols in Bioinformatics. 2020;69(1):e96.

39. Coughtrie AL, Jefferies JM, Cleary DW, Doncaster CP, Faust SN, Kraaijeveld AR, et al. Microbial epidemiology and carriage studies for the evaluation of vaccines. Journal of Medical Microbiology. 2019;68(10):1408-18.

40. Weinberger DM, Grant LR, Weatherholtz RC, Warren JL, O'Brien KL, Hammitt LL. Relating Pneumococcal Carriage Among Children to Disease Rates Among Adults Before and After the Introduction of Conjugate Vaccines. American Journal of Epidemiology. 2016;183(11):1055-62.

41. Lo SW, Gladstone RA, van Tonder AJ, Lees JA, du Plessis M, Benisty R, et al. Pneumococcal lineages associated with serotype replacement and antibiotic resistance in childhood invasive pneumococcal disease in the post-PCV13 era: an international whole-genome sequencing study. The Lancet Infectious Diseases. 2019;19(7):759-69.

42. Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res. 2018;3:124.

43. Mitchell PK, Azarian T, Croucher NJ, Callendrello A, Thompson CM, Pelton SI, et al. Population genomics of pneumococcal carriage in Massachusetts children following introduction of PCV-13. Microb Genom. 2019;5(2).