Serotype and molecular epidemiology of pneumococci in Iceland before and after pneumococcal vaccination

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Hjúpgerða- og sameindafræðileg faraldsfræði pneumókokka á Íslandi fyrir og eftir pneumókokka bólusetningu

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To my beloved children; Júlía Nótt,
Indíana Líf and
Atli Jökull

“*What the mind can conceive and believe it can achieve*”

− Napoleon Hill

Ágrip

Pneumókokkar eru mikilvægir sýkingavaldar í mönnum. Heilbrigð börn á leikskólaaldri geta borið pneumókokka í nefkoki án nokkurra einkenna en pneumókokkar geta leitt til miðeyrnasýkingar, lungnabólgu og ífarandi sýkinga. Bólusetning með próteintengdum bóluefnum leiðir til fækkunar stofna af hjúpgerðum sem bóluefnið veitir vörn gegn (bóluefnis hjúpgerð, e. vaccine type: VT) en getur auk þess leitt til fjölgunar stofna af hjúpgerðum sem bóluefnið veitir ekki vörn gegn (ekki bóluefnis hjúpgerð, e. non-vaccine type: NVT). Tíu-gilda próteintengda pneumókokka bóluefnið (PHiD-CV, GSK) var innleitt í ungbarnabólusetningar á Íslandi árið 2011.

Markmið rannsóknarinnar var að meta áhrif PHiD-CV á pneumókokka sem ræktaðir voru úr nefkoki heilbrigðra leikskólabarna, miðeyrum (ME) barna með miðeyrnarbólgu og neðri öndunarvegum (LRT) fullorðinna einstaklinga með grun um lungnabólgu. Í rannsókninni var dreifing hjúpgerða, raðgerða (e. sequence types, ST) og sýklalyfjanæmi pneumókokkastofna fundið. Gögn fyrir bólusetningu (2009-2011) voru borin saman við gögn eftir bólusetningu (2012-2017).

Nefkoksstrokum var safnað árlega (í mars, 2009-2017) frá börnum <7 ára frá 15 mismunandi leikskólum á stór-höfuðborgarsvæðinu. Allir pneumókokkastofnar sem ræktuðust frá ME barna <7 ára á Landspítala Háskólasjúkrahúsi árin 2009-2017 voru greindir. Til að leiðrétta fyrir mismunandi fjölda ára fyrir og eftir bólusetningu var meðalfjöldi ME stofna á ári skoðaðir á hverju tímabili fyrir sig. Öll sýni frá LRT fullorðinna ≥18 ára voru með í rannsókninni. Pneumókokkastofnum frá LRT eftir bólusetningu var skipt í tvö tímabil: eftir-I (2012-2014) og eftir-II (2015-2017). Allir stofnar voru hjúpgreindir með latex kekkjun og/eða PCR og/eða hjúpgerðin fengin úr raðgreiningargögnum. Sýklalyfjanæmi stofna var mælt og túlkað samkvæmt EUCAST staðli. Annar hver stofn frá árunum 2009-2014 var heilraðgreindur. ST voru fengnar úr raðgreiningargögnunum og þeim raðað í klónal komplexa (CC). Skyldleikatré voru gerð og lituð með tilliti til hjúpgerða og CC. Tveggja-þátta Fisher´s exact próf var notað til að reikna p-gildi og marktækni var miðuð við p ≤0,05.

Alls voru 5570 stofnar rannsakaðir yfir allt tímabilið: 3020 frá nefkoki, 1330 frá ME, 1047 frá LRT og 173 úr ífarandi sýkingum. Berahlutfall leikskólabarna var óbreytt milli tímabilanna tveggja (fyrir 67,3% og eftir 61,5%; p=0,090). Sýnum frá ME fækkaði eftir bólusetningu og jafnframt fjöldi pneumókokkaræktanna frá ME og LRT. Pneumókokkastofnum af VT

hjúpgerðum fækkaði í öllum sýnaflokkum milli tímabilanna (p<0,001, allir sýnaflokkar) með samhliða fjölgun á pneumókokkastofnum af NVT hjúpgerðum í sýnum frá berum, ME (p<0,001, báðir sýnaflokkar) og LRT frá fullorðnum 18-64 ára (p=0,008). Hjúpgerð 23B tók við af hjúpgerð 23F sem algengasta hjúpgerðin í berasýnum eftir bólusetningu (úr 0,7/1000 sýni fyrir bólusetningu í 49,3 eftir; p<0,001). Stofnar af hjúpgerð 19F voru algengastir bæði tímabilin í ME en fækkaði úr 80,7 á ári fyrir bólusetningu í 11,2 eftir (p<0,001). Stofnar af hjúpgerð 19F voru algengastir fyrir bólusetningu í LRT en fækkaði úr 58,2/100000 fullorðnir fyrir bólusetningu í 10,7 eftir-II (p<0,001). Meirihluti stofna af hjúpgerð 19F var með minnkað næmi fyrir penisillíni (PNSP) og voru fjölónæmir (MDR). Allir PNSP/MDR stofnar af hjúpgerð 19F tilheyrðu CC236/271/320^{19F}, afbrigði af alþjóðlega MDR Taiwan^{19F}-14 klóninum. Hjúpgerð 23B var oftar í nefkoki heilbrigðra leikskólabarna en hjúpgerð 19F sýkti oftar miðeyru barna. Stofnum af hjúpgerð 6C fjölgaði í heildina milli tímabilanna í sýnum frá berum (úr 6,5/1000 sýni fyrir bólusetningu í 49,0 eftir; p<0,001), ME (úr 0,3 á ári fyrir í 6,8 eftir; p<0,001) og LRT (úr 0,6/100000 fullorðnir fyrir í 7,5 eftir-II; p=0,021). Stór hluti stofna af hjúpgerð 6C var PNSP og MDR. Stofnum af öðrum NVT hjúpgerðum sem fjölgaði marktækt eftir bólusetningu í sýnum frá berum og ME meðal barna á mismunandi aldri voru: 15A, 15B/C, 21, 22F, 23A, 35F og 35B. Hjúplausir pneumókokkar voru algengastir eftir bólusetningu í sýnum frá LRT og hjúplausum pneumókokkastofnum fjölgaði í aldurshópi 18-64 ára (úr 4,2/100000 18-64 ára fyrir bólusetningu í 16,2 eftir-II; p=0,028). Meirihluti hjúplausra stofna voru PNSP og MDR.

Niðurstöður rannsóknarinnar sýna að berahlutfall var svipað milli rannsóknartímabilanna. Árlegur fjöldi ME sýna og fjöldi pneumókokkaræktana frá ME og LRT sýnum lækkaði eftir innleiðingu PHiD-CV. Marktækar breytingar urðu á dreifingu hjúpgerða eftir bólusetningu þar sem stofnum af VT hjúpgerðum fækkaði og í staðinn komu stofnar af NVT hjúpgerðum. Hjarðónæmi hjá eldri óbólusettum einstaklingum var sýnilegt innan fjögurra ára frá innleiðingu bóluefnisins. Stofnar af CC236/271/320^{19F} hafa nánast horfið eftir bólusetningu. Hvort að aðrir MDR klónar, eins og CC315^{6C}, komi í staðinn á eftir að koma í ljós.

Lykilorð:

Pneumókokkar, hjúpgerðir, klónal komplex, ónæmi, bólusetning

Abstract

The pneumococcus is a major human pathogen that causes morbidity and mortality worldwide. Pneumococci are normally carried asymptomatically in the nasopharynx of healthy pre-school children. Pneumococcal infections can range from acute otitis media (AOM) and pneumonia to invasive disease (i.e. bacteraemia, meningitis). Vaccination with conjugate pneumococcal vaccines (PCVs) leads to a reduction of serotypes targeted by the vaccines (vaccine-type, VT) but can also lead to replacement by serotypes not targeted by PCVs (nonvaccine-type, NVT). Iceland introduced the 10-valent PCV (PHiD-CV, GSK) into the national immunization program in 2011.

The aim of the study was to assess the impact of PHiD-CV on pneumococci isolated from samples from the nasopharynx of healthy children attending day-care centres (DCCs), middle ear (ME) from children with AOM and lower respiratory tract (LRT) from adults with suspected pneumonia. Serotype distribution, genetic lineages and antimicrobial resistance was analysed by comparing the period before vaccine introduction (PreVac; 2009-2011) to the period post vaccine introduction (PostVac; 2012-2017).

Nasopharyngeal swabs were collected annually (March, 2009-2017), from children <7 years old attending 15 DCCs in the greater Reykjavik area. All pneumococci isolated from ME samples from children <7 years old submitted to Landspitali University Hospital from 2009-2017 were included. ME isolates were analysed as the mean number of isolates detected each year (avg/yr) to correct for the difference in the number of years between the two study periods. All pneumococci from LRT samples from adults ≥18 years old were included in the study. The LRT isolates were analysed as the number of detected isolates according to the population size for the referral area. Two PostVac periods were defined for LRT: PostVac-I; 2012-2014 and PostVac-II; 2015-2017. All isolates were serotyped using latex agglutination and/or PCR and/or sequence based serotyping. Antimicrobial susceptibility testing was according to the methods and criteria of EUCAST. Whole genome sequencing (WGS) was done on selected isolates. Multilocus sequence types (STs) were extracted from the WGS data and STs were assigned to clonal complexes (CCs). Phylogenetic trees were constructed and annotated with serotypes and CCs. A two-sided Fisher´s exact test was used to calculate p-values and the level of significance was set ≤0.05.

A total of 5,570 pneumococcal isolates were analysed: 3,020 from carriage, 1,330 from ME, 1,047 from LRT and 173 from invasive disease.

Carriage rates remained unchanged between the study periods (67.3% vs. 61.5%; p=0.090). The total number of ME samples and pneumococci isolated from ME and LRT samples decreased PostVac. Overall, VTs decreased PostVac in nasopharyngeal, ME and LRT samples (p<0.001, all sample groups), with a concurrent increase in NVTs in nasopharyngeal and ME samples (p<0.001, both) and LRT samples from adults 18-64 years old (p=0.008). Serotype 23B replaced serotype 23F as the most prevalent serotype in carriage PostVac (from 0.7/1,000 samples PreVac to 49.3 PostVac; p<0.001). Serotype 19F was the most prevalent serotype in both study periods in ME but decreased PostVac: ME from 80.7 isolates avg/yr PreVac to 11.2 PostVac (p<0.001). Serotype 19F was the most prevalent serotype PreVac in LRT samples but decreased from 58.2/100,000 adults PreVac to 10.7 PostVac-II (p<0.001). The majority of serotype 19F isolates were penicillin non-susceptible pneumococci (PNSP) and multidrug resistant (MDR). All PNSP/MDR serotype 19F isolates were members of CC236/271/320^{19F}, variants of the international MDR Taiwan^{19F}-14 lineage. Serotype 23B had a predilection for carriage and serotype 19F for the middle ear. Serotype 6C increased overall between the periods in carriage (from 6.5/1,000 samples to 49.0; p<0.001), ME (from 0.3 isolates avg/yr to 6.8; p<0.001) and LRT samples (from 0.6/100,000 adults to 7.5; p=0.021). A large number of serotype 6C were PNSP and MDR. NVT serotypes that increased significantly PostVac in nasopharyngeal and ME samples from children in different age strata were: 15A, 15B/C, 21, 22F, 23A, 35F and 35B. Non-encapsulated isolates were the most prevalent pneumococci overall in LRT samples PostVac and increased in adults 18-64 years of age (from 4.2/100,000 adults 18-64 years PreVac to 16.2 PostVac-II; p=0.028). The majority were PNSP and MDR.

The study revealed that carriage rates were similar in both study periods. The annual number of ME samples and pneumococcal-positive cultures in ME and LRT samples decreased following PHiD-CV implementation. Significant shifts in serotype distribution followed vaccination where VTs decreased and were replaced by NVTs. Herd effect was seen within the older unvaccinated population within four years of vaccine introduction. Isolates of the MDR CC236/271/320^{19F} have nearly been eliminated after vaccine introduction. Whether this lineage will be replaced by other MDR lineages, such as CC315^{6C}, remains to be seen.

Keywords:

Pneumococci, serotypes, clonal complex, resistance, vaccination

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As I began my diploma to acquire the title of Biomedical scientist my goal was never to seek a doctoral degree, it sought me as the pneumococcus “infected” my mind and soul, making it impossible to say goodbye. This has been an adventurous journey in which many have participated along the way and my thanks go out to them all. I would like to especially acknowledge the following:

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Contents

List of abbreviations

AMR	Antimicrobial resistance
AOM	Acute otitis media
Avg/yr	Average per year
BIGSdb	Bacterial Isolate Genome Sequence Database
CC	Clonal complex
cgMLST	Core genome multilocus sequence typing
CRM_{197}	Diphtheria toxoid variant
CPS	Capsular polysaccharide
cps locus	Capsular polysaccharide synthesis locus
CSP	Competence stimulating peptide
DNA	Deoxyribonucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Gal	Galactose
GBD	Global Burden of Disease
Glc	Glucose
GlcA	Glucuronic acid
Hi	*Haemophilus influenzae*
Hyl	Hyaluronidase
IPD	Invasive pneumococcal disease
iTOL	Interactive tree of life
Lps	Less prevalent serotypes
LRT	Lower respiratory tract
LytA	N-acetylmuramoyl-L-alanine amidase
MDR	Multi-drug resistant
MLST	Multilocus sequence typing
mPCR	Multiplex polymerase chain reaction
NanA	Neuraminidase A
Nc	Not calculated
NESp	Non-encapsulated *Streptococcus pneumoniae*
NP	Nasopharyngeal

NT	Non-typeable
NVT	Non-vaccine type
OM	Otitis media
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PCV7	7-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PFGE	Pulsed Field Gel Electrophoresis
PHiD-CV	10-valent Pneumococcal NTHi protein D conjugate vaccine
PHYLOViZ	Phylogenetic inference and data visualization
Ply	Pneumolysin
PMEN	Pneumococcal Molecular Epidemiology Network
Pn	Pneumococcal
PNSP	Penicillin non-susceptible pneumococci
PostVac	Post vaccine implementation
PPV23	23-valent Pneumococcal polysaccharide vaccine
PreVac	Prior to vaccine implementation
PspK	Pneumococcal surface protein K
rMLST	Ribosomal MLST
SDI	Simpson´s Diversity Index
SNPs	Single nucleotide polymorphisms
ST	Sequence type
UDP	Uridine diphosphate
UndPP	Undecaprenyl lipid carrier
VRT	Vaccine-related type
VT	Vaccine type
wgMLST	Whole genome multilocus sequence typing
WGS	Whole genome sequencing

List of figures

List of tables

List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I. Hjálmarsdóttir M.Á.[*], **Quirk S.J.**[*], Haraldsson G., Erlendsdóttir H., Haraldsson Á., Kristinsson K.G. (2017) Comparison of the serotype prevalence of pneumococci isolated from middle ear, lower respiratory tract and invasive disease prior to vaccination in Iceland. ([*]Shared first authorship). PLoS One, 12 (1): e0169210.

II. Sigurdsson S., Erlendsdóttir H., **Quirk S.J.**, Kristjánsson J., Hauksson K., Ingudóttir Andrésardóttir B.D., Jónsson A.J., Halldórsson K.H., Sæmundsson Á., Ólason Ó.H., Hrafnkelsson B., Kristinsson K.G., Haraldsson Á. (2017). Pneumococcal vaccination: Direct and herd effect on carriage of vaccine serotypes and antibiotic resistance in Icelandic children. Vaccine, 35 (39), 5242-5248.

III. **Quirk S.J,** Haraldsson G., Erlendsdóttir H., Hjálmarsdóttir M.Á, van Tonder A.J., Hrafnkelsson B., Sigurdsson S., Bentley S.D., Haraldsson Á., Brueggemann A.B., Kristinsson K.G. (2018) Effect of vaccination on pneumococci isolated from the nasopharynx of healthy children and the middle ears of children with otitis media. J Clin Microbiol. 56 (12): e01046-18.

IV. **Quirk S.J,** Haraldsson G., Hjálmarsdóttir M.Á, van Tonder A.J., Erlendsdóttir H., Hrafnkelsson B., Bentley S.D., Haraldsson Á., Brueggemann A.B., Kristinsson K.G. (2019) Vaccination of Icelandic children with the 10-valent pneumococcal vaccine leads to significant herd effect among Icelandic adults. J Clin Microbiol. 57 (4) e: 01766-18**.**

Other publications by the author on the subject and supporting the findings of the thesis

V. van Tonder A.J., Bray, J. E., Roalfe L., White R., Zancolli M., **Quirk S.J.,** Haraldsson G., Jolley K.A., Maiden M.C., Bentley S.D., Haraldsson Á., Erlendsdóttir H., Kristinsson K.G., Goldblatt D., Brueggemann A.B. (2015). Genomics Reveals the Worldwide Distribution of Multidrug-Resistant Serotype 6E Pneumococci. J Clin Microbiol. 53 (7): 2271-85.

VI. van Tonder A.J., Bray, J. E., **Quirk S.J.,** Haraldsson G., Jolley K.A., Maiden M.C., Hoffmann S., Bentley S.D., Haraldsson Á., Erlendsdóttir H., Kristinsson K.G., Brueggemann A.B. (2016). Putatively novel serotypes and the potential for reduced vaccine effectiveness: capsular locus diversity revealed among 5405 pneumococcal genomes. Microb Genom. 2 (10): 000090.

VII. van Tonder A.J., Bray, J. E., Jolley K.A., Jansen van Rensburg M., **Quirk S.J.,** Haraldsson G., Maiden M.C., Bentley S.D., Haraldsson Á., Erlendsdóttir H., Kristinsson K.G., Brueggemann A.B. (2019). Genomic analyses of <3,100 nasopharyngeal pneumococci revealed significant differences between pneumococci recovered in four different geographic regions. Front Microbiol. 10: 317 doi: 10.3389/fmicb.2019.00317.

Declaration of contribution

Paper I

Biomedical scientists at the Department of Clinical Microbiology Landspítali University Hospital Iceland collected the clinical isolates from middle ear (ME), lower respiratory tract (LRT) and invasive disease samples and did all of the antimicrobial susceptibility testing, serotyped pneumococcal isolates that were non-susceptible to penicillin and those from invasive disease with conventional serotyping methods. Martha Á. Hjálmarsdóttir developed the database, analysed the results and wrote most of the manuscript. Sigríður Júlía Quirk (SJQ) developed the molecular serotyping panel and added additional serotypes, did most of the molecular serotyping and wrote part of the manuscript. All co-authors reviewed the manuscript.

Paper II

Various medical students took the nasopharyngeal samples that were obtained from children in the DCCs over the study period. The students did the antimicrobial susceptibility testing under the supervision of Helga Erlendsdóttir (HE). HE serotyped the pneumococcal isolates with conventional serotyping methods. SJQ developed the molecular serotyping panel and added additional serotypes, did the majority of the molecular serotyping and assisted Samúel Sigurðsson (SS) with a part of molecular serotyping. SS analysed the results and wrote the manuscript, which was reviewed and edited by all co-authors.

Papers III-IV

Nasopharyngeal swabs were obtained and analysed as described above in Paper II. Clinical pneumococcal isolates from ME and LRT samples were collected and analysed as described above in Paper I. SJQ developed the molecular serotyping panel and added additional serotypes, did the majority of the molecular serotyping and assisted with WGS preparations. SJQ set up the VIce data in the REDCap software. SJQ analysed the results, wrote the manuscript, which was reviewed and edited by all co-authors.

1 Introduction

Streptococcus pneumoniae (the pneumococcus) is an important respiratory pathogen that frequently colonises the nasopharynx of healthy children. Although colonisation rarely progresses into pneumococcal disease, it is essential for the establishment of disease (Bogaert et al., 2004; Kadioglu et al., 2008). Pneumococci are a common cause of acute otitis media (AOM), pneumonia and invasive diseases (i.e. bacteraemia, meningitis) (Song et al., 2013; Torres et al., 2014; Yatsyshina et al., 2016). The Global Burden of Disease (GBD) reported over 1.5 million deaths in the year 2015 worldwide due to pneumococcal pneumonia with the majority being children under the age of five and adults over 70 years of age in developing countries (GBD, 2017).

The pathogenicity of pneumococci is mostly attributed to the polysaccharide capsule that surrounds the bacterium and protects it from the host's immune defences (Hyams et al., 2010; Melin et al., 2010). Nearly one hundred serotypes have been described (Geno et al., 2017; McEllistrem, 2009) and each serotype produces a unique capsule that is immunologically different from other capsules (Reijtman et al., 2013).

The first pneumococcal conjugate vaccine (PCV), the 7-valent PCV that targets seven serotypes, was implemented in the United States in 2000. Since then, 10-valent and 13-valent PCVs have been introduced, targeting ten and 13 serotypes, respectively. Countries that have introduced PCVs into their childhood immunisation program have reported a dramatic decrease in pneumococcal disease, mainly due to fewer infections caused by serotypes targeted by PCVs (vaccine type, VT). However, serotypes that were once rare and are not targeted by PCVs (non-vaccine type, NVT) have since become prevalent, some of them belonging to multidrug resistant genetic lineages (Hanage et al., 2010; Linares et al., 2010; van der Linden et al., 2015a). Therefore, global surveillance of pneumococcal serotypes and genotypes detected in nasopharyngeal carriage of healthy children and pneumococcal diseases among all age groups is essential in keeping track of clinically relevant serotypes and antimicrobial resistance, which will benefit future vaccine development.

1.1 History of the pneumococcus

1.1.1 Discovery

The pneumococcus has without a doubt, been the most studied bacterial pathogen from the beginning of microbial investigation since Pasteur (Pasteur, 1881) in France and Sternberg in the United States in 1880 (White, 1938) isolated the bacteria independently. They injected rabbits with human saliva and both reported the same findings of diplococci in the rabbit's blood (Austrian, 1981a; Pasteur, 1881). Pasteur named them *Microbe septicemique du salvia* (Spellerberg & Brandt, 2007) and Sternberg *Micrococcus pasteuri* in honour of Pasteur who was first to publish his findings (Austrian, 1981a; Spellerberg & Brandt, 2007; White, 1938).

In 1886 pneumococci were named *Pneumococcus* by Fraenkel for their tendency to cause pneumonia but the name was changed to *Diplococcus pneumoniae* over thirty years later (Winslow et al., 1920) and in 1974 it was changed to its current name *Streptococcus pneumoniae* (Deibel & Seeley, 1974). Following the discovery of the pneumococcus it was identified as an important pathogen responsible for pneumonia and later found to have had an important role in the mortality during the 1918 influenza pandemic (Morens et al., 2008).

Klemperer and Klemperer (1891) discovered immune factors in the serum of rabbits that had been injected with non-viable pneumococci and that those immune factors protected the rabbits against reinfection with the same pneumococcal strain but not against other pneumococcal strains (Klemperer & Klemperer, 1891). Mosny (1892) began experimenting with immune serum by injecting rabbits with virulent pneumococci and growing pneumococci in both normal and immune serum. He observed a visible change in the immune serum (White, 1938), which was later discovered to be agglutination by Bezançon and Griffon. They also found that pneumococci exhibit serological diversity by reacting differently to anti sera (Bezançon & Griffon, 1897).

Over a decade later, Neufeld and Händel (1910) used a collection of pneumococcal strains isolated from pneumonia patients to immunise rabbits, horses and donkeys to obtain high-potency monovalent sera. They divided pneumococci in two types, type I that belonged to non-invasive pneumococci and type II that belonged to invasive pneumococci. They were the first to propose that pneumonia should be treated with serum developed from all types of pneumococci (Neufeld & Haendel, 1910).

As a result more types of pneumococci were discovered and by 1932, 32 serotypes had been described by Cooper et al. (Cooper et al., 1932). The number of described serotypes had reached 90 in 1995 (Henrichsen, 1995) and to date close to a hundred distinct serotypes have been described (Bentley et al., 2006; Calix & Nahm, 2010; Geno et al., 2017; Oliver et al., 2013; Park et al., 2015).

1.1.2 Assignment of serotypes

As the number of serotypes increased it was clear that they needed to be classified. Two nomenclature systems were established, the Danish system (1940) by Kauffmann et al. (Kauffmann et al., 1940) where antigenic related serotypes were grouped together, and the American system (1944) by Eddy (Eddy, 1944), where serotypes were given a number sequential to their discovery regardless of antigenic relationships between the types.

Closely related serotypes were grouped together according to the Danish system and assigned to a serogroup (e.g. serogroup 7) and subtypes within the serogroup (e.g. serotypes 7F, 7A, 7B and 7C), were the letter “F” represents the first serotype detected within the serogroup (Lund, 1970). However, the Danish system did not receive international recognition until in 1980 (Henrichsen, 1999).

1.2 Pneumococcal cell structure

The pneumococcal cell interacts with the host immune system and antimicrobial agents through its surface, and the components of the cell wall play a considerable part in pneumococcal disease (Riesenfeld-Orn et al., 1989; Tuomanen et al., 1985). The cytoplasm of the bacterium is enclosed by a plasma membrane consisting of lipoteichoic acid, lipid and protein. Located in the plasma membrane is the Forssman antigen, a robust and extremely specific inhibitor of autolysin that is common to all pneumococci (Tomasz, 1981). The cell wall consists of peptidoglycan (murein) and teichoic acid and forms the outmost structure of the bacterium (Tomasz, 1981). A variety of pneumococcal surface proteins and capsular polysaccharides (CPS) are attached to the cell wall through the peptidoglycan, which also plays an important role in the physiology of the bacterium by sustaining the shape and form of the cell (Lovering et al., 2008).

The teichoic acid and lipoteichoic acid are immune catalysts (Schneewind & Missiakas, 2014) that contain phosphorylcholine, which is an essential part of the biological features of pneumococci. Choline residues play a crucial part

in adherence by attaching endothelial cells and pneumocytes (Fischer, 1997), they activate the choline-binding receptors and bind to C-reactive protein (Bruyn et al., 1992) in the host.

1.3 Transformation and competence

The transformation phenomenon was first observed in the pneumococcus in the 1920´s and later led to the discovery of DNA as the genetic material responsible for changes in phenotypes in the 1940´s (Avery et al., 1944; Griffith, 1928). Pneumococci are naturally transformable pathogens that can take up foreign DNA from their environment while in a temporary physiological state called competence. The competence state is initiated by a competence stimulating peptide (CSP) (Havarstein et al., 1995) through quorum sensing and is switched off promptly following DNA uptake (Claverys et al., 2009).

The competence state allows for genetic transformation of external DNA that is homologous to the cells own DNA resulting in homologous recombination. Under stressful conditions pneumococci search for new genetic material, behaving as DNA predators, killing and lysing non-competent sister cells and/or closely related members of streptococci (e.g. *S. mitis*, *S. oralis* and *S. pseudopneumoniae*) (Hanage et al., 2006a; Johnston et al., 2010; Steinmoen et al., 2002) in order to survive, a phenomenon called pneumococcal fratricide (Claverys et al., 2007; Havarstein et al., 2006). Stress induced by antimicrobials can activate the competence state and thereby facilitate the development of multidrug resistant properties (Slager et al., 2014).

The transformation rate can vary considerably among different pneumococcal strains. Pneumococci also have the ability to adjust the transformation rate according to environmental changes (Evans & Rozen, 2013; Joloba et al., 2010). However, natural transformation does not always lead to genotypic changes and if changes occur, they do not always result in phenotypic changes. Natural transformation plays an important role in bacterial fitness, evolution and genomic diversity, and leads to the acquisition of adaptive traits that help in evading clinical interventions and the human host (Claverys et al., 2009; Coffey et al., 1998; Dowson et al., 1989; Fontaine et al., 2015; Hakenbeck, 2000; Lin et al., 2016).

1.4 Pneumococcal polysaccharide capsule

The pneumococcal capsule has been intensively studied since Dochez and Avery discovered the “soluble specific substance” in 1917 (Austrian, 1981b; Dochez & Avery, 1917) and Heidelberger and Avery established that it consisted mainly of polysaccharide (Heidelberger & Avery, 1923, 1924). The characteristics of the capsule make it a major pathogenic (virulence) factor (Jedrzejas, 2004; Mitchell & Mitchell, 2010), protecting the bacterium from the host´s immune system (Hyams et al., 2010; Melin et al., 2010); however, it has also been shown to be important during the initial steps of colonisation (Nelson et al., 2007).

1.4.1 Capsular structure and composition

The capsule is a diverse chemical structure of high molecular mass, approximately 200-400 nm thick (Sørensen et al., 1988). The capsule is made up of layers of repeated oligosaccharides (di- to penta-) units (van Dam et al., 1990) that surrounds the bacterium (Hyams et al., 2010; Melin et al., 2010). Polysaccharides are mainly linear polymers consisting of two or more monosaccharide’s (Paton & Morona, 2000), which determine the serotype. However, a few capsular serotypes (e.g. serotypes of serogroups 7, 11 and 18) possess a rather complex branched structure, consisting of backbones of repeated units with one to six monosaccharide’s and additional side chains (Bruyn et al., 1992; Paton & Morona, 2000). The polysaccharides of most serotypes (apart from serotype 3) are covalently linked to the cell wall peptidoglycan (Sørensen et al., 1990).

The majority of pneumococcal capsules have a negative charge (anionic) with the exception of serotypes 7A, 7F, 14, 33F, 33A and 37, which are uncharged (Kamerling, 2000) and serotype 1, which can be both negatively and positively charged (zwitterionic) (Bentley et al., 2006). Larger capsules tend to have fewer carbons in the polysaccharide repeat unit resulting in the surface displaying a higher negative charge (Li et al., 2013). As a result, more negatively charged capsules are inclined to better resist killing by neutrophils and have a longer duration of carriage (e.g. serotypes 19F and 23F) (Li et al., 2013).

1.4.2 Capsular polysaccharide synthesis (*cps*) locus

The genetic composition of the capsular genes has been a subject of interest since it was established that capsular production was controlled by the capsular locus and presumably all capsular genes were located together at a

specific place in the genome (Austrian et al., 1959). One by one the *cps* loci of different serotypes were sequenced and it was established, that the *cps* genes were grouped together in a single operon on the chromosome (Dillard et al., 1995; Dillard & Yother, 1994; Garcia et al., 1993; Garcia & Lopez, 1997; Guidolin et al., 1994; Iannelli et al., 1999; Kolkman et al., 1997a; Kolkman et al., 1997b; Llull et al., 1999; Morona et al., 1997, 1999; Munoz et al., 1999).

The *cps* locus consists of 10 to over 20 closely linked genes with a varying length of approximately 10-30 kb (Bentley et al., 2006; Paton & Morona, 2007) depending on the capsular serotype. The *cps* genes of all serotypes (apart from serotype 37) are located between the *dexB* and *aliA* genes on the chromosome. The order of the first four genes in most loci is: *cpsA* (*wzg*), *cpsB* (*wzh*), *cpsC* (*wzd*) and *cpsD* (*wze*). This order is rearranged in serotypes 25F, 25A and 38 to *cpsC-D*, *tnp* (transposase) and *cpsA-B* (Yother et al., 2008). These first genes are aligned at the 5´end of the locus.

The first four *cps* genes are relatively conserved (Bentley et al., 2006; Guidolin et al., 1994; Kolkman et al., 1998) and are necessary for capsular regulation and transfer, excluding *cpsA* (Bentley et al., 2006; Yother, 2004). Pneumococci with a deleted *cpsA* gene are able to produce a capsule but at a much lower level (Morona et al., 2004). The serotype specific genes are positioned central in the *cps* locus, downstream of *cpsD* (*cpsB* in serotypes 25F/A and 38), and are essential for capsular synthesis (apart from serotype 3) (Bentley et al., 2006). The first serotype specific gene, *cpsE* (*wchA*), encodes the initiating glycosyltransferase that mediates the linkage of sugar-phosphate to the lipid carrier on the cell membrane. The polysaccharide polymerase, *wzy*, encodes for synthesises of the polysaccharides and the flippase gene, *wzx*, transports the polysaccharides over the cytoplasmic membrane (Bentley et al., 2006; Yother, 2004). Enzymes encoding for the synthesis of activated sugar precursors are located at the 3´end of the locus (Paton & Morona, 2007).

The *cps* locus of serotype 3 is different from the loci of other serotypes as it consists only of three unaltered genes, *cpsC*, *cps3D* (*ugh*) and *cps3S* (*whcE*, synthase gene). They are necessary for capsular synthesis and are transcribed as an operon. The common genes *cpsA*, *cpsB* are shortened and not involved in capsule production (Arrecubieta et al., 1995; Arrecubieta et al., 1996; Dillard et al., 1995; Dillard & Yother, 1994). Serotype 37 has a *cps* locus resembling serotype 33F but does not take part in capsule synthesis as

it consists of numerous mutations and deletions. The single gene necessary for capsule synthesis of serotype 37, the synthase gene (*tts*), is not located within the dexB/aliA region but elsewhere on the chromosome (Bentley et al., 2006; Llull et al., 1999).

1.4.3 Regulation of capsule synthesis

The process of how pneumococcal polysaccharide capsules are synthesizes and regulated has been explored for over two decades. The process is reliant on nucleotide sugar precursors that are synthesized by enzymes encoded by genes within the *cps* locus and outside it (Yother et al., 2008). Pneumococci synthesize CPSs by one of two pathways, the synthase dependent (*tts*) or the wzy dependent pathway.

1.4.3.1 Synthase dependent synthesis

Capsular synthesis by the synthase dependent pathway involves a single integral inner membrane protein that transfers sugar residues straight to the elongating polysaccharide chain and simultaneously extrudes the maturing polysaccharide to the outside of the cell membrane (Cartee et al., 2000; Llull et al., 2001). Serotypes 3 and 37 are the only two pneumococcal serotypes that are synthase dependent (Arrecubieta et al., 1994; Cartee et al., 2001; Dillard et al., 1995; Llull et al., 1999; Waite et al., 2003).

The CPS of serotype 3 consists of simple glucose (Glc) and glucuronic acid (GlcA). The serotype 3 synthase, *cps3S*, is a processive β-glycosyltransferase. This bi-functional synthase transfers both Glc and GlcA to a phosphatidyl glycerol acceptor, elongating the polysaccharide chain and producing serotype 3 CPS of high molecular weight (Cartee et al., 2000).

Cps3S synthase relies on the cells ability to produce the sugar nucleotide precursors, uridine diphosphate (UDP) Glc and UDP-GlcA, for synthesis (Arrecubieta et al., 1996). In the absence of either sugar nucleotide precursor, polymerization comes to a halt and the polysaccharide chain is released from the synthase (Cartee et al., 2001; Forsee et al., 2000; Llull et al., 2001).

The CPS of serotype 37 is a branched homopolymer consisting of Glc and synthesis is driven by the processive membrane bound β-glucosyltransferase, *tts* (Llull et al., 1999). CPSs of serotypes 3 and 37 are exported as the elongating polysaccharide chain is synthesized. The processive transferases are thought to form a pore through the C-terminal

trans-membrane domains, through which the polysaccharide chain is extruded (Keenleyside & Whitfield, 1996).

1.4.3.2 Wzy dependent synthesis

The *cps* loci of the Wzy dependent serotypes have a more complex structure than those of serotypes 3 and 37, which results in a more complicated capsular synthesis system. This synthesis process is related to that of other Gram-positive and Gram-negative bacteria and the classification of this pathway is established on the O antigen synthesis pathway of Gram-negative bacteria, as they are similar (Whitfield, 1995). All pneumococcal serotypes, excluding serotypes 3 and 37, are wzy dependent (Garcia et al., 2000; Kolkman et al., 1998; Yother, 2004).

Capsular synthesis by the Wzy dependent pathway is initiated as a Glc phosphate is transferred to an undecaprenyl lipid carrier (UndPP) by glycosyltransferase (CpsE/WchA), which sequentially assembles the lipid-linked repeat unit inside the cytoplasm. The sugar phosphate is then transferred by the flippase (Wzx) to the outer face of the cytoplasmic membrane where it is polymerised by the polysaccharide polymerase (Wzy) to form a mature CPS. Lastly, the Wzd/Wze complex attaches the lipid-linked CPS to the cell wall peptidoglycan by releasing the UndPP-carrier. A galactose (Gal) phosphate, which is transferred from UDP-Gal, replaces the Glc phosphate in the initial step if the capsule contains Gal and not Glc (Bentley et al., 2006; Paton & Morona, 2007; Sørensen et al., 1990; Yother, 2004).

1.4.4 Capsular diversity

Pneumococcal capsules are highly diverse, which is thought to be the result of selective antigenic diversity imposed by the host defence system (Spratt et al., 2004). Different mechanisms have also contributed to this diversity and different serotypes have arisen as a result of numerous and subtle polymorphisms in the *cps* locus (Bentley et al., 2006).

The *cps* locus is able to undergo different mutations that can affect the antigenicity and biochemical structure of the capsule (Calix et al., 2012). Minor changes in the *cps* genes can alter the structure of the polysaccharide, as in the case of serotypes 6A and 6B, where a single change in the amino acid sequence of *wciP* sets them apart (Mavroidi et al., 2004). Likewise, the *cps* locus can undergo major changes without them resulting in a change in the antigenicity of the capsule. As demonstrated with serotypes 6B and 6Bii

(serotype 6B class two), which are identical in chemical structure and antigenicity but differ markedly in amino acid sequence (Burton et al., 2016; Ko et al., 2013; Mavroidi et al., 2004; van Tonder et al., 2015). Therefore, predicting new serotypes solely by the scale of genetic diversity could be impractical (Burton et al., 2016).

Mutations leading to pseudogenes, the addition or complete deletion of genes, have resulted in the heterogeneousness of serotypes within the same serogroup (Kilian et al., 2014). Recent studies have shown that the capsular cassette contains large genes imported from various commensal species of streptococci, including viridans streptococci and salvarius group (Kilian et al., 2014) attributing to the diversity and evolution of the pneumococcal capsule (Andam & Hanage, 2015). In some cases the whole *cps* locus is replaced, in other, one gene or more is transferred (Kilian et al., 2014).

The ability for a serogroup to produce new serotypes has come to light with increased knowledge of the biochemistry, structure and genetics of the capsule (Geno et al., 2015). Considerable strain-to-stain sequence and structural diversity within the *cps* locus was recently reported in a study of invasive pneumococcal isolates. The level of sequence diversity differed among the isolates and was not limited to the capsular type. These results suggested that in response to selective pressure in humans, pneumococci have both preserved and shared allelic diversity within the c*ps* locus (Wen et al., 2016).

The absences of a capsule and thin capsules have been associated with higher re-combinational rates and transformation in pneumococci (Andam & Hanage, 2015; Chewapreecha et al., 2014; Marks et al., 2012). However, pneumococci with larger capsules tend to be more prevalent and have a greater duration in carriage (Weinberger et al., 2009). This makes pneumococci more exposed to commensal bacteria in the nasopharynx milieu and more suited to undergo recombination (Chaguza et al., 2016).

1.4.5 Capsular switching

The phenomenon of bacteria being able to switch one capsular type for another was initially demonstrated in pneumococci by Griffith in 1928 (Griffith, 1928). The capsular locus can undergo changes through mutations (single base changes, insertions or deletions) or genetic transformation, which result in a change of serotype. Early studies on the biochemistry and genetic function of the capsule indicated that genes encoding capsular synthesis could be transferred as one unit during transformation (Austrian et al., 1959;

Bernheimer et al., 1967). This composition of the *cps* genes has favoured serotype changes in pneumococci (Coffey et al., 1998).

Capsular switching is a natural process of the pneumococcal biology and there is evidence of it having taken place over the last seven decades (Wyres et al., 2013). Capsular switching has been found to be more common between serotypes belonging to the same serogroup (Croucher et al., 2015). Non-vaccine serotypes that come from a genetically related background as those that are included in current vaccines could be better equipped to escape the host defence system (Sabharwal et al., 2014).

Recombination has influenced how serotypes have evolved differently throughout history (Chaguza et al., 2016). The highly diverse nature of the pneumococcal genome and variability within the species enable pneumococci to evade selective pressures such as imposed by vaccination and antibiotics (Andam & Hanage, 2015).

1.4.6 The role of the capsule in pathogenicity

The capsule plays a central role in pneumococcal pathogenicity by shielding the cell from the host defence system and is necessary for survival in the bloodstream (Nelson et al., 2007). Only a small number of serotypes are associated with the majority of invasive disease (Brueggemann et al., 2003; Brueggemann et al., 2004; Sleeman et al., 2006). Different capsules can differ in their shielding ability and protective antibodies generated by the capsule provide the groundwork for current pneumococcal vaccines (Mohale et al., 2016).

Pneumococci expressing the same capsular type can show highly diverse sequence arrangements within the *cps* locus (van Tonder et al., 2016). Furthermore, transcriptional variation in the capsular genes results in different levels of capsular production, which reflects in the cells ability to adhere to epithelia and resist killing by phagocytes (Wen et al., 2016).

1.5 Non-encapsulated pneumococci

The majority of pneumococcal strains are encapsulated but non-encapsulated pneumococci (NESp; non-encapsulated *S. pneumoniae*) are unreactive to antisera and are thus characterised as non-typeable (NT) (Richter et al., 2008; Whatmore et al., 2000). However, the use of antisera to identify NESp is not a preferable identification method as NT pneumococci can express novel capsular types that have yet to be identified or capsular

types belonging to other streptococcal species (Park et al., 2012).

NESp are classified into two groups. Group I NESp are closely related to encapsulated pneumococci but possess a defective *cps* locus as a result of various mutations and are thus unable to produce a capsule. As for group II NESp the *cps* locus is absent and in its place are various novel genes (Hathaway et al., 2004).

Pneumococcal surface protein K (PspK) has been shown to play an important role in colonisation. PspK enables NESp to colonise with the same efficacy as encapsulated pneumococci (Keller et al., 2013; Park et al., 2012) and NESp expressing the *pspK* gene are better equipped to cause otitis media (OM) than those that do not contain the gene (Keller et al., 2014; Keller et al., 2013).

NESp are mostly associated with carriage (Andrade et al., 2010; Sa-Leao et al., 2006) and conjunctivitis (Buck et al., 2006; Porat et al., 2006; Valentino et al., 2014). NESp are seldom found in OM (Hanage et al., 2006b; Xu et al., 2011) and rarely found in invasive diseases (Beall et al., 2006; Park et al., 2014), but following the worldwide implementation of PCVs, countries have reported the increase of NESp in asymptomatic carriage (Keller et al., 2016b; Sa-Leao et al., 2009) and OM (Croney et al., 2013; Hotomi et al., 2016a).

1.6 Pathogenicity other than the capsule

Pneumococcal pathogenicity can be attributed to a variety of enzymes and surface proteins (AlonsoDeVelasco et al., 1995), which interact with host structures or aid in concealing the bacteria from the immune system (Jedrzejas, 2001). Other cell wall factors, such as hyaluronidase and pneumolysin, are mainly involved with inflammation preceding infection. The inflammation process most likely fully develops after the disruption of the bacterial cell with autolysin thus causing the clinical signs of infection (Musher, 1992). These virulence factors may therefore directly contribute to pneumococcal morbidity and mortality (AlonsoDeVelasco et al., 1995).

1.6.1 Major pneumococcal surface proteins

Pneumococcal surface proteins are important for colonisation and survival, interaction between cells, removal of oppositions and interaction with the host. These proteins play a crucial role throughout the course of an infection where adaptation to various host environments is essential (Bergmann & Hammerschmidt, 2006; Jedrzejas, 2004; Perez-Dorado et al., 2012).

1.6.1.1 Hyaluronidase and neuraminidase A

Hyaluronidase (Hyl) and neuraminidase A (NanA) are peptidoglycan bound surface proteins that aid in colonisation and virulence. Hyl assists in pathogen invasion and spread by degrading host tissue, resulting in increased permeability of host tissue (Berry et al., 1994; Jedrzejas, 2001). Hyl is also thought to interact with pro-inflammatory cytokines and chemokines in pneumonia, thereby escalading pulmonary inflammation (Mitchell & Mitchell, 2010).

NanA promotes bacterial adherence in the nasopharynx and lungs, by cleaving sialic acid from cell surface glycoconjugates and revealing surface receptors on host cells. NanA also contributes to survival in biofilms through the release of sialic acid (Mitchell & Mitchell, 2010; Trappetti et al., 2009).

1.6.1.2 Pneumolysin

The cytoplasmic pore-forming toxin pneumolysin (Ply) is a member of cholesterol binding proteins of other pathogenic Gram-positive bacteria. As Ply is missing a typical signal secretion leader sequence, its release from the cell relies on autolysis or lysis by the effect of antibiotics (Mitchell & Dalziel, 2014). It is present in all know encapsulated pneumococci and NESp (Keller et al., 2016a).

Ply plays a major role in the pathogenesis of pneumococcal infections for its cytotoxic (haemolytic) activity and its ability to activate the classical complement pathway (Mitchell & Mitchell, 2010). *ply* negative mutants of NESp have been shown to be less sufficient to cause OM in chinchilla models because *ply* deficient NESp produce significantly less biofilm than NESp that possess the gene (Keller et al., 2016a). Ply is also thought to assist in the first stages of colonisation in the host by damaging the surface of the epithelium and through multiple inhibitory effects on the innate and adaptive defences (Hotomi et al., 2016b).

1.6.1.3 Autolysins

Autolysins belong to a group of various enzymes that break the peptidoglycan backbone of bacteria, an action that leads to lysis of the cell and cell death (Jedrzejas, 2001). The major autolysin N-acetylmuramoyl-L-alanine amidase (LytA) is one of the choline-binding proteins.

LytA is very conserved in pneumococci and can be found in all clinical pneumococcal isolates. It promotes the release of Ply, aids in immune evasion and through the release of extracellular genetic material it

contributes to biofilm formation (Eldholm et al., 2009; Martner et al., 2009; Moscoso & Claverys, 2004; Ramos-Sevillano et al., 2015).

A recent study has shown that in the initial stages of infection when LytA comes in contact with cationic antimicrobial peptides (CAMPs) it promotes capsular shedding and enables bacterial binding to the epithelia. The LytA involved in capsular shedding is bound to choline on the cell wall and is assembled, secreted and dispersed around the cell. This suggests that the LytA involved in capsule shedding is located outside the growth zone, unlike LytA involved in autolysis. Furthermore, the process is highly controlled and likely reversible, without it being fatal for the bacterial cell. Capsular shedding could benefit pathogens in bypassing anti-capsular immune responses, which could therefore have significant meaning for on-going vaccine strategies and treatments (Kietzman et al., 2016).

1.6.1.4 Pneumococcal surface proteins PspA and PspC

The choline-binding pneumococcal surface proteins A and C (PspA and PspC) are key components in protective immunity of the cell by eluding complement-mediated phagocytosis (Janulczyk et al., 2000; Li et al., 2007; Rosenow et al., 1997; Tu et al., 1999). PspA and PspC show great genetic variation (Brooks-Walter et al., 1999; Hollingshead et al., 2000) and might have transformed under a stronger selective pressure than other immunogenic proteins in an attempt to evade host immunity (Croucher et al., 2017). Furthermore, because PspA is present in all clinically relevant pneumococci and for its immunogenic properties, it may be a good candidate for future protein vaccines (Brooks & Mias, 2018; Crain et al., 1990; Ferreira et al., 2009; Khan et al., 2018; Yun et al., 2017).

1.6.1.5 Pneumococcal surface antigen A

Pneumococcal surface antigen A (PsaA) is a metal binding lipoprotein that is covalently linked to the cytoplasmic membrane. PsaA promotes attachment to the host cell and aids in invasion. It is also a part of the ATP binding cassette transporter (ABC transporter), which facilitates uptake of manganese (Dintilhac et al., 1997; Jedrzejas, 2004) enabling pneumococci to resist oxidative stress (Kadioglu et al., 2008; McAllister et al., 2004).

1.7 Pneumococcal nasopharyngeal carriage

Pneumococci frequently colonise the nasopharynx of young healthy children and nasopharyngeal carriage is higher among adults in close contact with

young children than in other adults (Gray et al., 1980). The main risk factors for nasopharyngeal carriage is young age, DCC attendance, having siblings and geographic location (Lindstrand et al., 2016). The duration of colonisation can range from a couple of weeks up to a few months, generally without resulting in disease (Kadioglu et al., 2008) and although colonisation rarely progresses to disease it is essential for disease development, and disease is believed to occur within a short time of colonisation with a newly acquired serotype (Gray et al., 1980; Simell et al., 2012).

Nasopharyngeal carriage can differ widely between countries with carriage rates commonly ranging from 40-60% among ages 0 to <7 years (Bogaert et al., 2004; Tomasson et al., 2005; Usuf et al., 2014). In low income countries pneumococcal carriage rates among healthy children less than 5 years of age can range from 20-93% (Adegbola et al., 2014). However, the serotypes found in nasopharyngeal carriage have changed significantly where PCVs have been introduced but the overall carriage rate has remained similar between the pre and post vaccine era as a result of serotype replacement by NVT serotypes (Azevedo et al., 2016; Devine et al., 2017; Lindstrand et al., 2016).

The NVT serotypes that are frequently detected post PCV implementation, such as serotypes 6C, 11A, 15B/C, 23A, 23B and 35F, have been shown to have a lower invasive disease potential than VT serotypes (Devine et al., 2017; Lindstrand et al., 2016; Neves et al., 2017; Varon et al., 2015; Yildirim et al., 2010; Yildirim et al., 2017).

Simultaneous carriage of multiple serotypes is well known (Brugger et al., 2010; Dhoubhadel et al., 2014; Hjalmarsdottir et al., 2016) and the environment of the nasopharynx of young children is ideal for promoting transformation. As pneumococci are highly transformable organisms, the likelihood for genetic exchange is high, when two or more pneumococcal serotypes co-colonise the nasopharynx at the same time (Shak et al., 2013). Furthermore, it has been found that children, under the age of five, hospitalised with acute respiratory infection were twice as likely to harbour two or more serotypes than healthy children (Dhoubhadel et al., 2014).

1.8 Pneumococcal disease

Pneumococcal transmission occurs when respiratory droplets are passed from one person to another (Yildirim et al., 2015). Young children and adults, mainly ≥65 years, are at greater risk for developing pneumococcal infections. Pneumococcal disease is generally divided into non-invasive pneumococcal

disease (non-IPD; e.g. sinusitis, AOM and pneumonia) and IPD (bacteraemia and meningitis) (Song et al., 2013; Torres et al., 2014; Yatsyshina et al., 2016).

1.8.1 Acute otitis media

Globally, otitis media is one of the most prevalent illnesses in children (GBD, 2015) with pneumococci being one of the leading bacterial pathogens. Almost all children experience at least one episode before the age of 3 years (Vergison et al., 2010). Young children under one year of age with AOM are at a greater risk for experiencing another episode before the age of two years than children are to experience the first episode (Labout et al., 2011). Other risk factors include the use of a pacifier, DCC attendance, history of recurrent OM in the family, having siblings and exposure to smoke (Salah et al., 2013; Vergison et al., 2010). Moreover, simultaneous viral respiratory infection and pneumococcal carriage are strongly associated with AOM (Auranen et al., 2016; Ruohola et al., 2013).

Pneumococci are known to cause more severe AOM and are linked to a larger inflammatory response compared to non-pneumococcal AOM (Ovnat Tamir et al., 2015). Furthermore, pneumococcal AOM is less likely to resolve without antimicrobial treatment (Rodgers et al., 2009) and can lead to serious complications such as permanent hearing loss and delayed speech, which can later affect school performance (Leach & Morris, 2007; Rodgers et al., 2009). Other complications due to AOM can involve facial palsy, vertigo and mastoiditis (Salah et al., 2013), and pneumococcal AOM is the most common cause for acute mastoiditis (Bluestone, 2000; Giannakopoulos et al., 2014; Tawfik et al., 2017).

The disease burden of AOM is highest in low to medium income countries and minority populations (e.g. Australian Aboriginals) (2015; Gunasekera et al., 2009; Leach & Morris, 2007). Serious complications are rarely seen in children in developed countries (Liese et al., 2014) where antimicrobial use is considerably high (Stamboulidis et al., 2011; Vergison et al., 2010). The substantial economic burden (e.g. frequent visits to physicians, antimicrobial prescriptions and loss of work by parents) due to AOM is responsible for some of the highest expenses in health care in developed countries (Alberti, 1999; Kourlaba et al., 2015; Soni, 2014).

1.8.2 Pneumonia

Pneumonia frequently affects children and adults leading to hospitalisation and death, especially among young children and the elderly (Song et al., 2013). Co-morbidities (e.g. asthma, Parkinson´s disease, HIV infection), immunodeficiency, smoking/alcohol abuse and regular contact with young children are some of the risk factors for pneumonia (Feldman & Anderson, 2016; Torres et al., 2015; Torres et al., 2014). Moreover, underlying conditions, such as influenza and other viral infections increase the risk for developing secondary bacterial infections in the lungs, especially with pneumococci (McCullers, 2014; Smith et al., 2013).

Pneumonia among adults is for the most part of pneumococcal origin (Drijkoningen & Rohde, 2014) and is related to poor outcomes, especially in older patients (Palma et al., 2012). Hospitalisation due to pneumococcal pneumonia was found to be five times more prevalent among adults ≥65 years of age in the United States than among younger adults (Jain et al., 2015).

The clinical presentations of pneumonia can range from relatively mild symptoms to serious disease involving respiratory failure and septic shock. Older age combined with co-morbidities is correlated with increased risk of sepsis and respiratory failure and is highly associated with the clinical outcome of the patient (Burgos et al., 2014). Although, only a small proportion of pneumonia leads to bacteraemia (Oggioni et al., 2006; Song et al., 2013), pneumococci were accountable for the highest number of deaths globally, from LRT infections among all age groups in 2015 (GBD, 2017).

Pneumonia due to pneumococci is responsible for a tremendous clinical and economic burden in the ageing population worldwide (GBD, 2017; Huang et al., 2011; Morimoto et al., 2015; Welte et al., 2012) and as the world´s population is expected to continue to age over the years, it will likely result in an enormous strain on both health care resources and the society (Torres et al., 2014; Wroe et al., 2012a). This fact emphasises the need for improved vaccines to prevent adult pneumococcal pneumonia (Benfield et al., 2013; Morimoto et al., 2015).

1.8.3 Serotypes in pneumococcal diseases

Serotype distribution differs over time, by location and age, with antimicrobial resistance and the invasiveness of the infection (Rodgers et al., 2009). Clinical outcome is correlated with the typical features of the serotype (Grabenstein & Musey, 2014) but only a fraction of the almost hundred

serotypes expressed by pneumococci are responsible for the larger part of the IPD burden (Yildirim et al., 2015). Pneumococci of serotypes of a high invasive potential usually act as primary pathogens infecting previously healthy individuals. Serotypes of a low invasive potential, such as those commonly carried by healthy children, act as opportunist pathogens infecting immunodeficient individuals and patient with underlying disease causing high mortality rates (Brueggemann et al., 2003; Sjöström et al., 2006).

Serotype distribution in nasopharyngeal carriage has been shown to be representative of serotypes that cause AOM (Hanage et al., 2004). However, some serotypes seem to be more prone to cause AOM such as serotypes 3, 19A, 19F and 23F and these serotypes, apart from serotype 3, are more frequently associated with antimicrobial resistance (AMR) than other serotypes (Hays et al., 2017; Setchanova et al., 2017).

In pneumonia, the characteristics of the host determine the extent of complications. As for pneumococci, the capsular serotype causing the infection seems to determine the aspects of complications (Grabenstein & Musey, 2014; Herrero et al., 2016; Weinberger et al., 2010). Serotypes 3, 7F and 19A have been found to be linked to respiratory failure and respiratory complications in pneumococcal pneumonia (Herrero et al., 2016) and the use of mechanical ventilation is mostly linked to serotypes 3, 19F and 19A (Burgos et al., 2014). Furthermore, serotype 3 has been shown to be an essential factor for the development of septic shock as a result of pneumococcal pneumonia (Ahl et al., 2013).

Serotype replacement has become more evident with continued use of PCVs and the NVT serotypes that are emerging are often associated with resistance to antimicrobials (Mendes et al., 2015; Richter et al., 2013; Setchanova et al., 2017). Important insight can, therefore, be gained into predicting the long-term effects of PCVs implementation by understanding the mechanism of serotype replacement in both nasopharyngeal carriage and diseases (van Hoek et al., 2014).

1.9 Penicillin non-susceptible pneumococci and multidrug resistance

Penicillin non-susceptible pneumococci (PNSP) are often also resistant to many antimicrobial classes, making them multidrug-resistant (MDR) (Jacobs et al., 1978; McGee et al., 2001). Resistance is, for the larger part, serotype specific (Song et al., 2012) and is mainly associated with serotypes that are commonly carried, such as those of serogroups 6, 9, 14, 19 and 23, with the

majority of PNSP/MDR pneumococci belonging to VT serotypes prior to vaccination (Kawaguchiya et al., 2016; Kempf et al., 2015; Klugman, 2001; Pilishvili et al., 2010; Rodgers et al., 2009; Wroe et al., 2012b). Young children are the main reservoir for AMR through nasopharyngeal carriage (Kawaguchiya et al., 2016) and pneumococcal genetic lineages that are carried for an extended period of time are more exposed to antimicrobials increasing the likelihood of resistance (Henriques-Normark et al., 2008).

The first PNSP in Iceland was isolated in 1988, of serogroup 18 (Kristinsson et al., 1992). A year later the first PNSP/MDR pneumococcus of serotype 6B was detected and rapidly became widespread during the early 1990´s. All isolates belonged to the MDR PMEN Spain^{6B}-2 lineage, which peaked in 1993 and slowly subsided in the following years (Sa-Leao et al., 2002; Soares et al., 1993; Vilhelmsson et al., 2000). PNSP/MDR were also detected in serogroups 9, 14, 19 and 23 but at a much lower rate (Kristinsson, 1995; Vilhelmsson et al., 2000). In 1998, PNSP/MDR of serotype 19F belonging to new genetic lineages related to the PMEN Taiwan^{19F}-14 lineage emerged and expanded, especially after 2004. The increased prevalence of PNSP seen in Iceland the following years was as a result of an increase of PNSP of serotype 19F in respiratory tract isolates (Hjálmarsdóttir & Kristinsson, 2014).

The worldwide use of PCVs has influenced the pneumococcal population in that way that previously susceptible NVT pneumococci have acquired AMR, especially against β-lactams and macrolides. Vaccine escape genotypes have emerged challenging the prevention and treatment of pneumococcal diseases (Croucher et al., 2013; Croucher et al., 2011; Hanage et al., 2010; Kyaw et al., 2006; Porat et al., 2004a; Porat et al., 2004b; Reinert, 2009).

PNSP/MDR serotypes, such as 6C, 15A, 15B/C, 19A, 23A, 23B and 35B have been reported to have replaced VT PNSP/MDR pneumococci in other countries following PCVs implementation (Andam et al., 2017; Beall et al., 2018; Eskola et al., 2001; Horacio et al., 2014; Kawaguchiya et al., 2016; Kempf et al., 2015; McEllistrem et al., 2003; Olarte et al., 2018; Pilishvili et al., 2010).

The increased prevalence of NESp that frequently exhibit non-susceptibility to penicillin and are MDR has also been reported (Keller et al., 2016b; Mendes et al., 2015). NESp have mainly been associated with nasopharyngeal carriage (Andrade et al., 2010; Sa-Leao et al., 2006) and increased prevalence in the nasopharyngeal milieu could easily facilitate the

transfer of resistance genes from NESp to encapsulated pneumococcal strains (Chewapreecha et al., 2014; Langereis & de Jonge, 2017). Although, AMR seems to have remained stable in European countries between the period of 2013-2016, considerable variations in penicillin and macrolide non-susceptibility was seen, with the prevalence of non-susceptibility ranging from 0.4% to 41.1% for penicillin and from 0% to 60% for macrolides (ECDC, 2017).

PCV vaccination has resulted in a variety of NVT PNSP/MDR serotypes post vaccination that have mostly emerged from previously rare genetic lineages and have been associated with considerable disease burden. Continued monitoring of serotype distribution, AMR and genetic lineages is, therefore, imperative for evaluating vaccine impact (Andam et al., 2017; Diamantino-Miranda et al., 2017; Olarte et al., 2017; Sheppard et al., 2016; Shigayeva et al., 2016; van der Linden et al., 2015b).

1.10 Pneumococcal vaccination

Pneumococcal vaccination studies began just over a century ago by Wright and colleagues in 1911 with whole cell pneumococcal vaccination of South African gold miners (Wright et al., 1914). Wrights vaccine lacked serotype specificity, which led to the development of pneumococcal polysaccharide vaccines (PPVs) of different valencies until the 23-valent PPV (PPV23; Pneumovax; Merck) was introduced in 1983 (Grabenstein & Klugman, 2012; Robbins et al., 1983). However, children younger than 2 years of age do not benefit from PPV23 as their immune system is underdeveloped and is not reliable to elicit a strong T-cell independent immune response (Stein, 1992). Young children are at greater risk for IPD and the objective with vaccine development is to meet the needs of all populations, which promoted the development of conjugated pneumococcal vaccines (PCVs) (Black et al., 2000).

1.10.1 Conjugate vaccines

The conjugation of pneumococcal CPSs to a protein carrier has a history back to the 1930´s (Avery & Goebel, 1929) but the clinical significance of this process only gained value several decades later with the development of the *Haemophilus influenzae* type b conjugate vaccine (Hib) (Robbins & Schneerson, 1990). Two decades later the first pneumococcal conjugate vaccine was implemented in the United States. The 7-valent PCV (PCV7; Prevenar; Pfizer) targets seven serotypes, 4, 6B, 9V, 14, 18C, 19F and 23F,

which are conjugated to a non-toxic diphtheria variant carrier protein, CRM_{197}. The carrier protein is highly immunogenic and elicits a T-cell dependent immune response to the conjugated serotypes in young children (Black et al., 2000). Therefore, the vaccine is suitable for infants and young children. The selection of serotypes in the vaccine was affiliated with the most prevalent serotypes that caused IPD in children under the age of 5 years at the time of vaccine development (Pilishvili et al., 2010; Yoshioka et al., 2011).

Nearly a decade later, in 2009, the 10-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV; GlaxoSmithKline Biologicals) was approved by the European Medicines Agency. The vaccine targets three serotypes (1, 5 and 7F) in addition to PCV7. Serotype 19F is conjugated to diphtheria toxoid and serotype 18C to tetanus toxoid but the remaining eight serotypes are conjugated to protein D, a highly conserved lipoprotein of *H. influenzae* (Hi). The inclusion of Hi protein D in the vaccine was thought to possibly prevent AOM caused by Hi (Forsgren et al., 2008; Prymula et al., 2006). Later that year the 13-valent PCV (PCV13; Prevnar; Pfizer) was licensed in Europe and early 2010 in the United States. The vaccine targets three serotypes (3, 6A and 19A) in addition to PHiD-CV and like PCV7 all serotypes are conjugated to CRM_{197} (Scott et al., 2007).

Current PCVs are likely to have a limited lifetime as they are serotype-based and directed against a limited number of serotypes. This has resulted in serotype replacement with serotypes that are not targeted by current PCVs (Lo et al., 2018; Olarte et al., 2017; Setchanova et al., 2017; van der Linden et al., 2015b). PCVs may, however, buy time for further vaccine development that could target proteins that are conserved among all pneumococci. Moreover, future vaccine development might lead us back to the beginning of pneumococcal vaccination with the use of whole cell NESp vaccine (Pichichero et al., 2016).

1.10.2 Herd protection

The global vaccination of infants and young children with PCVs has dramatically changed the epidemiology of pneumococcal disease and has been beneficial in both vaccinated and unvaccinated populations due to herd protection (Tsaban & Ben-Shimol, 2017). Children attending DCCs have been shown to be the main source of pneumococcal transmission in communities through their high nasopharyngeal carriage and herd protection is obtained by hindering acquisition of VT pneumococci among them. The

magnitude of herd protection is associated with high vaccine coverage and uptake within a population. The manifestation of herd protection is delayed in unvaccinated populations compared to direct protection in vaccinated populations. However, the level of protection in the older population is unlikely to be as sufficient as seen in vaccinated children (Miller et al., 2011; Pilishvili et al., 2010; Tsaban & Ben-Shimol, 2017; Weil-Olivier et al., 2012). Nevertheless, the prevalence of VT pneumococcal diseases has decreased substantially among all ages as a result of PCV vaccination and continued implementation of PCVs is warranted to further reduce disease load and the spread of multidrug resistant pneumococci (Kim et al., 2016).

1.11 Pneumococcal identification and typing

Pneumococci are Gram-positive lancet-shaped cocci, normally growing in pairs or short chains. Standard methods for identification of pneumococci are colony morphology, α-haemolysis on blood agar, sensitivity to optochin and bile solubility (Forbes et al., 2007; Spellerberg & Brandt, 2007). Classification at the sub species level is important for monitoring both outbreaks and worldwide expansion of genetic lineages.

1.11.1 Serotyping

Serotyping is becoming increasingly important in monitoring the serotype epidemiology in the PCV era. Vaccine pressure has attributed to increased prevalence of NVTs in pneumococcal diseases and novel serotypes are still being identified (Calix et al., 2012; Geno et al., 2017; Oliver et al., 2013; Park et al., 2015). The gold standard for serotyping pneumococci has been the Quellung reaction in which the serotype-specific antisera react with the corresponding polysaccharide capsule and the capsule appears swollen under the light microscope (Neufeld & Haendel, 1910). This method requires microscopic examination and high expertise to interpret the results (Shutt et al., 2004).

The latex agglutination chessboard typing system (Pneumotest-Latex; Statens Serum Institute) and the use of serotype specific antisera are serotyping methods that were developed later. These tests are able to identify serogroups/serotypes rapidly and are considered quite sensitive and specific (Lalitha et al., 1996; Slotved et al., 2004; Sørensen, 1993).

The sequencing of the *cps* loci of all known serotypes over a decade ago has had a profound impact on the development of serotyping methods (Bentley et al., 2006). Different multiplex PCR (mPCR) methods specific to

serogroups/serotypes have been described (Dias et al., 2007; Dobay et al., 2009; Pai et al., 2006). Genotypic serotyping methods are all based on identifying genetic markers of individual serotypes (Geno et al., 2015). However, some serotypes within the same serogroup are distinguished only by small genetic differences within the *cps* locus of those serotypes (e.g. 7F/7A, 11A/11D, 15B/15C, 22F/22A and 33F/A) and are, therefore, indistinguishable from one another with genetic typing methods (Bentley et al., 2006).

Serotyping with genetic methods has become an important tool for predicting pneumococcal serotypes and has been widely implemented (Geno et al., 2015). However, if single nucleotide changes occur within the *cps* locus they can result in the alteration of serotypes, which go undetected by genotyping methods such as PCR. Furthermore, a single nucleotide change can have a significant effect on the interplay between the bacteria and the host's immune response. Therefore, use of various methods, genetic, serologic and chemical, is crucial when identifying novel serotypes (Geno et al., 2017; Oliver et al., 2013).

1.11.2 Pulsed field gel electrophoresis

Genotyping of pneumococci by pulsed-field gel electrophoresis (PFGE) was for long considered the gold standard for localised epidemiology studies. PFGE is based on the separation of large DNA fragments that have originated from DNA molecules that have been digested with an endonuclease (*Sma*I), in an agarose gel. The electric field in the electrophoresis cell changes periodically and is orientated in different directions to separate a small number of large DNA molecules by size (Elberse, K. E. et al., 2011). This method identifies the bacterial genome with low resolution, yielding limited information. Furthermore, PFGE is not well standardized making data comparison between laboratories of different countries unattainable and is time consuming.

1.11.3 Multilocus sequence typing

The need for standardising the characterisation of bacteria worldwide led to the development of multilocus sequence typing (MLST) two decades ago and was intended for long-term and global epidemiology (Spratt, 2012). The MLST method was initially developed for the bacterium *Neisseria meningitidis* (Maiden et al., 1998) and has since been expanded to include over a hundred other bacterial species (e.g. *S. pneumoniae*, *Streptococcus pyogenes, Staphylococcus aureus* and *Haemophilus influenzae*) (Enright et

al., 2000; Enright & Spratt, 1998; Enright et al., 2001; Meats et al., 2003).

MLST is based on multilocus enzyme electrophoresis (MLEE) (Selander et al., 1986) but instead of analysing the electrophoretic mobility of enzymes the MLST method employs the sequencing of seven highly conserved housekeeping genes (loci; ~450 bp). Furthermore, a unique allele number is assigned to each sequenced locus resulting in an allelic profile (e.g. 8-13-14-4-17-4-14), which is assigned a corresponding sequence type (ST; e.g. ST199) (Enright & Spratt, 1998; Maiden et al., 1998). The STs are assigned to clonal complexes (CCs), which are named after the central genotype; ST (e.g. CC199 designated after ST199) and related STs are grouped with the appropriate CC (Maiden, 2006). Each of the seven loci contains genetic information and every single change that occurs within the allelic profile is counted as one genetic event, without taking in to account the number of single nucleotide polymorphisms (SNPs).

This way the need to classify which changes are a result of recombination or which are due to a recent point mutation are avoided (Maiden et al., 2013). MLST is an effective tool to identify genetic lineages and diversity within bacterial populations (Jolley et al., 2012; Maiden, 2006) and has provided invaluable knowledge regarding studies on both evolution and molecular epidemiology (Perez-Losada et al., 2013). Nevertheless, advances in genome sequencing in recent years provide further insight and resolution, at the highest level and at much lower costs, into the evolution of bacterial populations.

Whole genome sequencing (WGS) of bacterial strains identifies SNPs differences between the strains capturing almost all of the sequence variation whereas the traditional MLST scheme captures only the variation at seven loci (Enright & Spratt, 1998; Geno et al., 2015; Perez-Losada et al., 2013; Spratt, 2012) and with the sequencing of the whole genome, various information can easily be extracted from the WGS data with specialised programs.

The Bacterial Isolate Genome Sequencing Database (BIGSdb) (Jolley & Maiden, 2010) facilitates direct identification of bacteria at the population level from WGS data, linking bacterial origin, phenotype and genotype. The MLST scheme is integrated in the system, which is built on the mlstdbNet software but the method can be adjusted to individual coding sequences in the genome. Isolates can be defined in multiple ways through BIGSdb using genomic data and the database is compatible with other sequence typing schemes. This enables comparison to isolate datasets acquired over time

from various geographic locations and the use for epidemiology, evolutionary or functional studies.

Ribosomal MLST (rMLST) is an extensive MLST method that offers high resolution by sequencing the 53 genes encoding for the ribosomal proteins. Whole genome MLST (wgMLST) and core genome MLST (cgMLST) are a gene-by-gene analyses that apply the same methodology as MLST to analyse genomic diversity. DNA profiles obtained by SNPs analysis and wgMLST/cgMLST contain thousands of loci distributed across the genome that can be determined by various phylogenetic algorithms (Jolley et al., 2012; Jolley & Maiden, 2010, 2014; Maiden et al., 2013; Ribeiro-Goncalves et al., 2016). However, the wgMLST/cgMLST schemes for the pneumococcus are still under development (unpublished data).

1.11.4 Phylogenetic analysis

Phylogenetic analysis has become an essential part of various studies in the field of molecular and evolutionary biology. The increasing amount of available WGS data enables comparative analysis of numerous sequences of a bacterial strain, along with serotype, genotype and more, with high resolution and great genetic detail. Therefore, the relationship and relatedness between bacterial populations can be studied with much accuracy (Anisimova et al., 2013; Croucher et al., 2013; Everett et al., 2012) (Croucher et al., 2011; Liu et al., 2015).

Genomic methods have proven to be of great importance for the analysis of highly recombinant bacterial species such as the pneumococcus by providing better understanding of pneumococcal evolution, how pneumococci are able to evade selective pressures by genetic exchange and how that could possibly affect various pneumococcal lineages (Andam & Hanage, 2015).

2 Aims

The overall aim was to assess the effect of PHiD-CV introduction in Iceland on serotype and molecular epidemiology of pneumococci obtained from healthy children attending DCCs, children with otitis media and adults with suspected pneumonia.

Specific aims were to assess the impact of PHiD-CV vaccination on:

1. Pneumococcal carriage rate, serotype distribution, genetic lineages and the prevalence of antimicrobial resistant pneumococci in the nasopharynx of healthy children attending DCCs (Papers II-III).

2. The distribution of pneumococcal serotypes, genetic lineages and changes in the prevalence of antimicrobial resistant pneumococci from the middle ear of children with otitis media (Paper I and III).

3. The distribution of pneumococcal serotypes, genetic lineages and changes in the prevalence of antimicrobial resistance pneumococci from the lower respiratory tract of adult patients who were not targeted to receive the vaccine (Papers I and IV).

3 Materials and methods

3.1 Study population for clinical samples

Inhabitants of the greater Reykjavik area were included in this study. The Department of Clinical Microbiology, Landspitali University Hospital, Iceland serves the whole country as a reference laboratory. It provides services for individuals from rural areas, serving about 85% of the Icelandic population for primary cultivation of pneumococcal samples.

The main service area for the Landspitali University Hospital was considered to be within 100 km driving distance from the hospital and the population demographic information for this referral region was obtained from Statistics Iceland (www.statice.is).

When two or more pneumococcal isolates were identified, of the same phenotype (serotype, antibiogram and sequence type), within a 30-day period from the same individual, only one isolate was included in these analyses.

3.1.1 Middle ear, lower respiratory tract and invasive disease samples from all age groups (Paper I)

All pneumococci submitted to the Department of Clinical Microbiology between 2007 and 2011, isolated from middle ear (ME; swaps or excretions/pus), lower respiratory tract (LRT; mostly sputum and bronchiolar lavage fluids) and invasive disease (IPD; blood, cerebrospinal fluid and joint fluid) samples from all ages were included in the study. Patients were divided into the following age groups: 0 to <2, 2 to <7, 7 to <18, 18 to 64 and ≥65 years of age.

3.1.2 Middle ear samples from children with acute otitis media (Paper III)

All pneumococci isolated from ME samples submitted to the Department of Clinical Microbiology between, 1st January 2009 and 30th September 2017, from children 0 to <7 years of age with otitis media, were included in this study.

The ME samples in this study were swaps or excretions/pus from the middle ear. The average population size of children 0 to <7 years of age for the referral region during the study period was 23,747 children PreVac and

24,083 children PostVac (approximately 66% of all children in Iceland 0 to <7 years of age). A detailed listing of the population according to age groups can be seen in Table 1.

Table 1. Number of children 0 to <7 years of age within the uptake area according to age group for ME samples. In the PreVac (2009-2011) and PostVac (2012-2017) periods.

Age group	PreVac; 2009-2011	PostVac; 2012-2017
0 to <2 years	7,385	6,621
2 to <4 years	6,818	6,973
4 to <7 years	9,544	10,490
Total 0 to <7 years	23,747	24,083

3.1.3 Lower respiratory tract samples from adults with suspected pneumonia (Paper IV)

All pneumococci submitted to the Department of Clinical Microbiology between 2009 and 2017, isolated from LRT samples from adults ≥18 years of age, were included in the study. The LRT samples were mostly sputum and bronchiolar lavage fluids. The population size for the referral region for adults ≥18 years of age was: PreVac 170,042 adults, PostVac-I 177,490 adults and PostVac-II 186,724 adults. A detailed listing of the population according to age groups can be seen in Table 2.

Table 2. Number of adults ≥18 years of age within the uptake area according to age group for LRT samples. In the PreVac (2009-2011), PostVac-I (2012-2014) and PostVac-II (2015-2017) periods.

Age group	PreVac; 2009-2011	PostVac-I; 2012-2014	PostVac-II; 2015-2017
18 to 64 years	143,507	148,326	154,313
≥65 years	26,535	29,164	32,411
≥18 years	170,042	177,490	186,724

3.2 Nasopharyngeal samples from healthy children (Papers II-III)

Nasopharyngeal swabs were taken in March every year 2009-2017, from healthy children aged 1 to <7 years attending 15 DCCs that were chosen to represent the greater Reykjavík area. The children were divided into age groups; 1 to <2, 2 to <4 and 4 to <7 years of age. Children <1 year of age seldom attend public DCCs and no children of that age were sampled in the study.

3.3 Vaccination

In April 2011, PHiD-CV was introduced into the national childhood immunisation programme. Children born from the 1st of January 2011 and onwards were eligible for vaccination in a 2+1 schedule, without catch-up vaccination for older children. No other pneumococcal vaccine had previously been included thus the population can be considered unvaccinated until 2011.

The period 2009-2011 was defined as the prior to vaccination period (PreVac) for all sample groups. The period 2012-2017 was defined as the post vaccine period (PostVac) for nasopharyngeal carriage and middle ear samples. For lower respiratory tract samples, the post vaccination period was divided into two periods: 2012-2014 period was defined as the PostVac-I period and 2015-2017 period was defined as PostVac-II period.

3.4 Culturing and identification

3.4.1 Patients samples from children and adults (Papers I, III-IV)

The samples were cultured on two 5% horse blood agar plates (Oxoid, Hamshare, UK) overnight. One plate was incubated in 5% CO_2 enhanced atmosphere at 37°C and the other plate anaerobically at 37°C. Pneumococcal identification was done by morphology and susceptibility to optochin. All pneumococcal isolates were stored at -80°C in glycerol broth for further analysis.

3.4.2 Nasopharyngeal samples from healthy children (Papers II-III)

The nasopharyngeal samples were selectively cultured for pneumococci, within 5 hours from obtaining the sample, using blood agar containing 5

µg/mL gentamicin and incubated overnight anaerobically at 37°C (Satzke et al., 2013). Pneumococcal identification was confirmed by morphology, optochin susceptibility and serotyping. All pneumococcal isolates were stored at -80°C in glycerol broth for further analysis.

3.5 Antimicrobial susceptibility testing

3.5.1 Papers I-IV

All pneumococcal isolates were tested for antimicrobial susceptibility using disk diffusion and the methods and criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2012). All pneumococcal isolates were tested for susceptibility to chloramphenicol, erythromycin, tetracycline, trimethoprim-sulfamethoxazole, and clindamycin by disk diffusion tests. Oxacillin disks (1 µg) were used to screen for non-susceptibility to penicillin; isolates sensitive to oxacillin (zone ≥20 mm) were considered to be sensitive to penicillin and other β-lactams. The minimum inhibitory concentration (MIC) of penicillin and ceftriaxone (ME, LRT and IPD isolates only) was measured using the E-test (BioMérieux, France) for isolates not susceptible to oxacillin (Jacobs et al., 1992). Isolates having a MIC of >0.06 mg/ml were defined as PNSP, while isolates having a MIC >2 mg/L were defined as resistant pneumococci. Isolates that were non-susceptible to three or more classes of antimicrobials (regardless of whether PNSP or not) were defined multidrug resistant.

3.6 Pneumococcal typing

Immulex Pool Antisera® (State Serum Institute, Copenhagen, Denmark) was used for conventional serotyping (Slotved et al., 2004) of the pneumococcal isolates and/or PCR and multiplex PCR (mPCR) based on previously published methods (Dobay et al., 2009; Pai et al., 2006; Sourav et al., 2010; Zhou et al., 2007).

3.6.1 DNA extraction and serotyping by PCR

Pneumococcal DNA was extracted by suspending two to three colonies, of newly cultured pneumococci, in a 5% Chelex solution (500 µL) and heated for ten minutes at 100°C. After heating the solution was centrifuged for ten minutes at 14,000 rpm. The supernatant (with the suspended DNA) was extracted and stored at -20°C until further use. The mPCR panel consisted of 78 sets of serogroup/serotype-specific primer pairs and two primer pairs for a

positive internal control: *cpsA* for the capsular locus and *lytA* for autolysin. Serogroup 6 serotypes were determined using previously published PCR methods (Jin et al., 2009a; Jin et al., 2009b; Kawaguchiya et al., 2014). Pneumococcal isolates that were negative for *cpsA* and positive for *lytA*, were tested, with a previously published PCR method, for the presence of the capsular gene *cpsB* and determined non-typeable (non-encapsulated) if it was not detected (Kurola et al., 2010). All PCR primers used in the study are listed in Appendix Table 19.

3.6.2 Whole genome sequencing (Papers III-IV)

Every other pneumococcal isolate from the period 2009-2014 was selected for whole genome sequencing. Pneumococcal isolates were sent to Oxford UK, on transport swabs and the DNA was extracted on the Promega Maxwell® 16 Integrated System. The genomes of each isolate were sequenced on the Illumina platform at the Wellcome Sanger Institute Hinxton, UK.

3.6.2.1 Quality, mapping and assembly (Papers III-IV)

WGS were assembled using Velvet (Zerbino & Birney, 2008) before SSPACE and GapFiller were used to improve the assemblies and close gaps (Boetzer & Pirovano, 2014; Nadalin et al., 2012). The final assembled genomes were uploaded into BIGSdb along with associated metadata (Jolley & Maiden, 2010).

3.6.2.2 Sequence based serotyping (Papers III-IV)

SeqSerotyper was used to extract serotypes from each of the sequenced pneumococcal genomes (van Tonder et al., 2015).

3.6.2.3 *In silico* multilocus sequence typing (Papers III-IV)

BIGSdb and PubMLST database (http://pubmlst.org/spneumoniae/) were used to extract the ST of each pneumococcal genome. Phyloviz was used to assign the STs to CCs (Francisco et al., 2012).

3.6.2.4 Phylogenetic analysis (Papers III-IV)

Prokka was used to predict coding sequences in each genome (Seemann, 2014). The resulting annotation files in gff format were then used as input for Roary and clustered using a sequence identity threshold of 90% (Page et al., 2015). The core genome was estimated using a Bayesian core genome model and a threshold of 99.9% for nasopharyngeal isolates, 99.8% for ME

isolates and 99.6% for LRT isolates (van Tonder et al., 2014). The core genes in all sample groups were extracted and aligned using MAFFT (Katoh et al., 2002). FastTree was used to construct the phylogenetic trees and ClonalFrameML (Didelot & Wilson, 2015) was used to reconstruct the trees to account for recombination. The final phylogenetic trees were then annotated using iTOL (Letunic & Bork, 2011).

3.7 Statistics

In this thesis, two types of tests were required. The first type of test was for comparing two proportions, p_1 and p_2, based on two independent samples. The null hypothesis states that the two proportions are equal, i.e., H_0: $p_1 = p_2$. To execute the test a two-sided Fisher's exact test was used. It was computed by using R, version 3.3.2 or GraphPad QuickCals (www.graphpad.com). This test was used to test the null hypothesis of equal proportions between the PreVac and PostVac periods in nasopharyngeal carriage for a certain age group. This test was also used to test proportions for antimicrobial susceptibility in all sample groups between the two study periods.

The other type of test was for comparing rates between two populations. In particular, the null hypothesis states that the rate (r_1) of a certain serotype, CC or ST in a given age group in the PreVac period is equal to the rate (r_2) of the same serotype, CC or ST in the same age group in the PostVac period, i.e., H_0: $r_1 = r_2$. The test is based on the assumption that the counts of the serotype, CC or ST in the two periods are independent and both follow Poission distributions. It was assumed that the mean of the PreVac counts is equal to the rate r_1 times the total number of individuals in the PreVac age group. The mean of the PostVac counts is equal to the rate r_2 times the total number of individuals in the PostVac age group. A likelihood ratio test was used to test the null hypothesis (Casella. & Berger., 2001). The asymptotic distribution of the likelihood ratio test statistics is a chi-square distribution with one degree of freedom under the null hypothesis. The test statistics and the corresponding p-values were computed by using R, version 3.3.2. This test was used to test the null hypothesis of equal rates and between PreVac and PostVac periods in ME and LRT samples for a certain age group. The level of significance for all tests was set equal to 0.05.

Simpson´s diversity index was calculated to assess the change in ST diversity after vaccine implementation (Simpson, 1949).

3.8 Ethics

The study was approved by The National Bioethics Committee (VSNb2013010015/03.07) and the appropriate authorities at the Landspitali University Hospital, the day-care centres and the appropriate directorates of the day-care centres.

All children attending the day-care centres were invited to participate in this study and signed informed consent forms were obtained from the parents.

4 Results

4.1 Serotype distribution in pneumococcal disease before PHiD-CV introduction (Paper I)

Paper I presents the serotype distribution and non-susceptibility to penicillin in all ages five years prior to PHiD-CV implementation. The three sample groups that were analysed are representative of common types of pneumococcal infections, acute otitis media, pneumonia and invasive disease (bacteraemia and meningitis).

4.1.1 Study population

Overall, 1,711 pneumococcal isolates were cultured from ME, LRT and IPD samples in 2007-2011 from all age groups. Out of the 1,711 isolates, 95 (5.6%) isolates were excluded (not viable or not stored), yielding 1,616 (94.4%) pneumococcal isolates for serotyping. One excluded isolate was from IPD, all other were from ME and LRT samples.

Out of the 1,616 pneumococcal isolates, 879 (54.4%) were collected from ME, 564 (34.9%) from LRT and 173 (10.7%) from IPD samples.

Most of the ME isolates, 72.2% (639/879), were from the youngest age group (0 to <2 years), 292 (51.8%) of the LRT isolates were from adults ≥65 years of age and 129 (74.9%) of the IPD isolates were from adults ≥18 years of age; thereof 60 (34.7%) isolates were from adults ≥65 years of age.

4.1.2 Serotype distribution

A total of 52 different serotypes were detected among the pneumococcal isolates. Overall, VT serotypes were more prevalent than NVT serotypes: VTs 1,052 (65.1%) vs NVTs 564 (34.9%; $p<0.001$). Serotype 19F was the predominant serotype, 36.1% (583/1,616) isolates were of serotype 19F over the study period with 45.5% (400/879) of isolates from ME, 30.5% (172/564) from LRT and 6.4% (11/173) from IPD samples.

Among the 879 ME isolates, 34 serotypes were detected. Among the 564 LRT isolates, 45 serotypes were detected. Serotype 19F was the most prevalent in ME and LRT samples. Among the 173 IPD isolates, 29 serotypes

were detected and serotype 14 was the most prevalent. The most prevalent serotypes are listed in Table 3.

Table 3. Most prevalent serotypes detected in ME, LRT and IPD samples in 2007-2011.

	ME			LRT			IPD	
Serotype	**n**	**%**	**Serotype**	**n**	**%**	**Serotype**	**n**	**%**
19F	400	45.5	19F	172	30.5	14	28	16.2
23F	111	12.6	3	49	8.7	19A	17	9.8
6A	73	8.3	6A	42	7.4	4	14	8.1
14	61	6.9	23F	40	7.1	9V	13	7.5
19A	61	6.9	14	28	5.0	7F	12	6.9
6B	47	5.3	NESp[a]	23	4.1	19F	11	6.4
3	28	3.2	6B	22	3.9	3	9	5.2
9V	14	1.6	19A	21	3.7	6B	8	4.6
Lps[b]	84	9.6	Lps	167	29.6	Lps	61	35.3
Total	**879**	**100**	**Total**	**564**	**100**	**Total**	**173**	**100**
VT[c]	651	44.1	VT	300	53.2	VT	101	58.4
NVT[d]	228	25.9	NVT	264	46.8	NVT	72	41.6

[a]NESp: Non-encapsulated *S. pneumoniae*.

[b]Lps: Less prevalent serotypes.

[c]VT: Serotypes detected in the study that are targeted by PHiD-CV (4, 6B, 7F, 9V, 14, 18C, 19F and 23F).

[d]NVT: Serotypes not targeted by PHiD-CV.

4.1.3 Penicillin non-susceptible pneumococci

Overall, 40.3% (651/1,616) of the pneumococcal isolates were PNSP, thereof 93.9% (611/651) were of VT serotypes. Serotype 19F was the most prevalent PNSP serotype in all sample groups but 82.2% (535/651) of serotype 19F were PNSP (Table 4). Pneumococci of VT serotypes were more prevalent in ME samples compared to LRT samples ($p<0.001$) and pneumococci of VT serotypes were more common in LRT samples compared to IPD samples ($p=0.003$). PNSP of VT serotypes were more prevalent in all sample groups than PNSP of NVT serotypes.

Table 4. Most prevalent PNSP serotypes in ME, LRT and IPD in 2007-2011.

PNSP	ME		LRT			IPD		
Serotype	n	%	Serotype	n	%	Serotype	n	%
19F	378	88.3	19F	150	73.2	19F	7	38.9
14	12	2.8	NESp[a]	13	6.3	9V	2	11.1
6B	19	4.4	6B	12	5.9	14	2	11.1
19A	7	1.6	9V	10	4.9	23F	2	11.1
Lps[b]	12	2.8	Lps	20	9.8	Lps	5	27.8
Total	**428**	**100**	**Total**	**205**	**100**	**Total**	**18**	**100**
VT[c]	416	97.2	VT	181	88.3	VT	14	77.8
NVT[d]	10	2.3	NVT	24	11.7	NVT	4	22.2

[a]NESp: Non-encapsulated *S. pneumoniae*.

[b]Lps: Less prevalent serotypes.

[c]VT: Serotypes detected in the study that are targeted by PHiD-CV (4, 6B, 7F, 9V, 14, 18C, 19F and 23F).

[d]NVT: Serotypes not targeted by PHiD-CV.

4.2 Pneumococci from the nasopharynx of healthy children (Papers II-III)

Papers II-III represent results from healthy children 1 to <7 years of age attending DCCs before and after PHiD-CV implementation. Paper III also includes children 0 to <7 years of age with AOM. These papers demonstrate the serotype distribution, genetic lineages and antimicrobial resistance between the two study periods in different age strata.

4.2.1 Carriage study population and bacterial isolates

A total of 4,461 nasopharyngeal swabs were obtained (450-550 samples annually; 1,380 samples prior to vaccination and 3,081 post vaccination). The number of nasopharyngeal swabs taken from each age group is listed in Table 5. The median age of the sampled children was 4.2 years and the mean age was 4.1 years (age range 1.1-6.3 years). The bacterial cultures yielded 3,029 pneumococcal isolates and 250 children carried two pneumococcal strains. Nine isolates were excluded (not viable/not stored), leaving 3,020 isolates (991 isolates PreVac and 2,029 PostVac) for further analysis.

Of the pneumococcal isolates collected, 51.8% (1,563/3,020) were from the older children (4 to <7 years) and 3.5% (105/3,020), from the youngest children (1 to <2 years). The overall pneumococcal carriage rate was 67.3%

in the PreVac period and 61.5% in the PostVac period (p=0.090). The carriage rate in different age strata was: Children 1 to <2 years 71.7% PreVac and 66.9% PostVac (p=1.000), children 2 to <4 years 69.5% PreVac and 66.1% PostVac (p=0.107), children 4 to <7 years 60.6% PreVac and 53.9% PostVac (p=0.007).

Table 5. Number of nasopharyngeal swabs obtained from children 1 to <7 years of age attending DCCs. According to age group in the PreVac (2009-2011) and PostVac (2012-2017) periods.

Age group	PreVac; 2009-2011	PostVac; 2012-2017
1 to <2 years	81	73
2 to <4 years	567	1,262
4 to <7 years	732	1,746
Total 1 to <7 years	1,380	3,081

4.2.2 Distribution of serotypes in carriage

Overall, 36 distinct serotypes were detected: 27 serotypes in the PreVac period and 35 in the PostVac period. VT serotypes decreased from 446 (323.2/1,000 samples) isolates to 304 (98.7/1,000 samples; p<0.001) isolates between the periods. NVT serotypes increased from 545 (394.9/1,000 samples) isolates to 1,725 (559.9/1,000 samples; p<0.001) isolates between the periods.

Serotype 23B was the most prevalent serotype in the PostVac period, increasing from 0.7 to 49.3/1,000 samples (p<0.001), followed by serotype 6C which increased from 6.5 to 49.0/1,000 samples (p<0.001). Serotypes 15A and 35F were only detected in the PostVac period (Table 6).

Table 6**.** Most prevalent serotypes detected in nasopharyngeal carriage in the PreVac (2009-2011) and PostVac (2012-2017) periods.

DCC	PreVac		PostVac		
Serotype	**n**	**/1000 samples[a]**	**n**	**/1000 samples**	**p-value**
3	68	49.3	113	36.7	0.052
6A	87	63.0	150	48.7	0.051
6B	109	79.0	71	23.0	<0.001
6C	9	6.5	151	49.0	<0.001
11A	58	42.0	136	44.1	0.756
14	74	53.6	29	9.4	<0.001
15A	0	0	35	11.4	<0.001
15B/C	37	26.8	146	47.4	<0.001
16F	24	17.4	25	8.1	0.008
19F	87	63.0	87	28.2	<0.001
19A	89	64.5	145	47.1	0.016
21	5	3.6	86	27.9	<0.001
22F	7	5.1	69	22.4	<0.001
23F	125	90.6	97	31.5	<0.001
23A	29	21.0	109	35.4	0.009
23B	1	0.7	152	49.3	<0.001
35F	0	0	56	18.2	<0.001
35B	6	4.3	57	18.5	<0.001
Lps[b]	108	78.3	199	64.6	Nc[c]
NESp[d]	68	49.3	116	37.7	0.075
Total	**991**	**718.1**	**2,029**	**658.6**	**<0.001**
VT[e]	446	323.2	304	98.7	<0.001
NVT[f]	545	394.9	1,725	559.9	<0.001
All NP[g] samples	1,380	-	3,081	-	-

[a]Per nasopharyngeal samples.

[b]Lps: Less prevalent serotypes.

[c]Nc: Not calculated.

[d]NESp: Non-encapsulated *S. pneumoniae*.

[e]VT: Serotypes detected in the study that are targeted by PHiD-CV (4, 6B, 9V, 14, 18C, 19F and 23F).

[f]NVT: Serotypes not targeted by PHiD-CV.

[g]NP: Nasopharyngeal samples.

4.2.3 Genetic lineages in carriage

Every other pneumococcal isolate was sequenced from the period 2009-2014, resulting in 987 (49.2%; 987/1,872) pneumococcal genomes: 482 genomes PreVac and 505 PostVac. Out of the 987 sequenced genomes, 47 CCs (35 CCs in the PreVac period and 41 CCs in the PostVac period) were detected and 104 STs (66 STs in the PreVac period and 83 STs in the PostVac period).

The Simpson diversity index was 0.97 for the STs in both study periods, implying that there were no changes in the diversity between the periods.

A phylogenetic tree was created with the concatenated sequences of 1,066 full-length coding loci found in 99.9% of the genomes from nasopharyngeal carriage. The tree was annotated with CC and serotype designations (Figure 1).

$CC439^{23F/A/B}$ was the most prevalent in both study periods; 77.6% (45/58) were of serotype 23F in the PreVac period while 40.6% (28/69) were of serotype 23B in the PostVac period. Only one isolate of serotype 23B was detected before vaccination. $CC433^{22F}$ was rare prior to vaccination but increased from two to 30 isolates ($p<0.001$) between the periods (Figure 1 and Table 7).

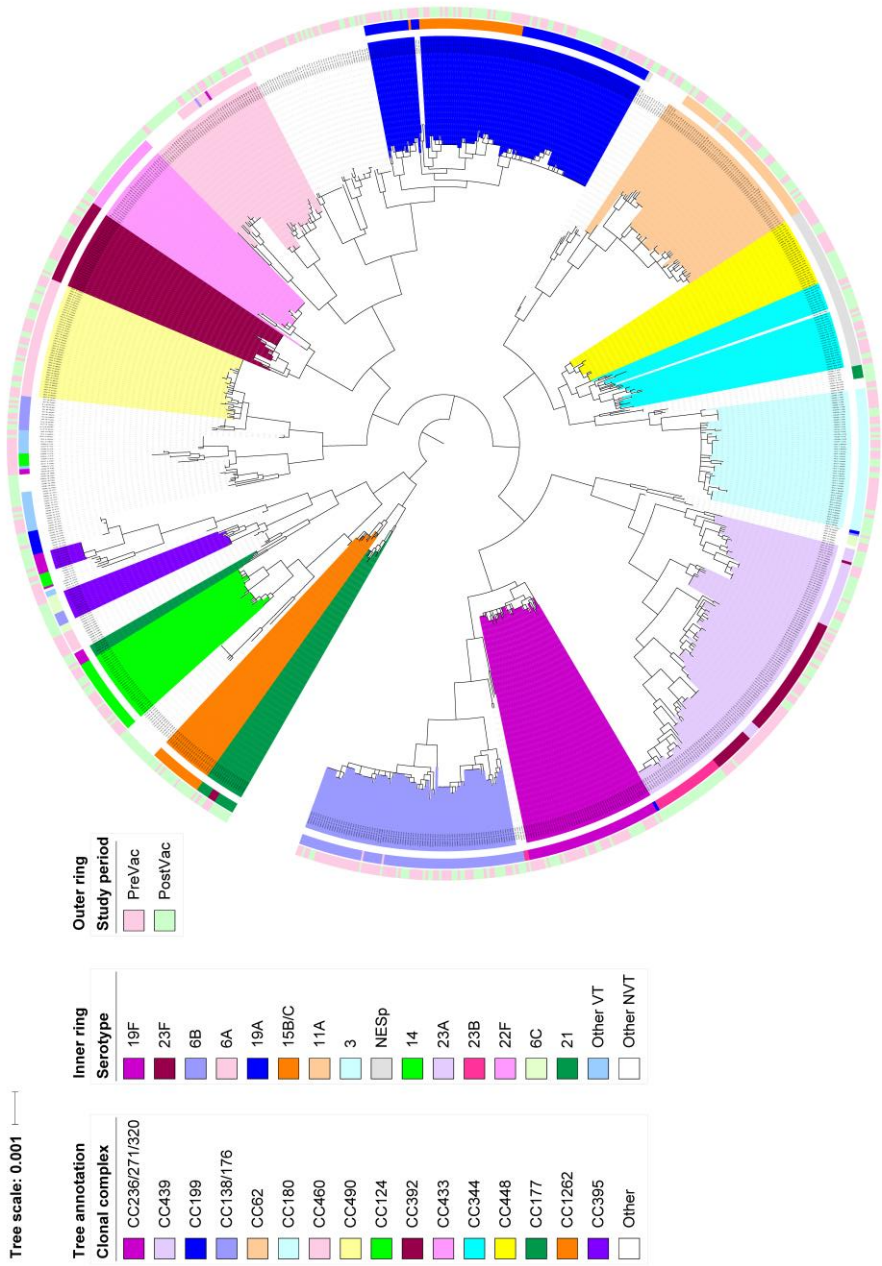

Figure 1. Mid rooted phylogenetic tree, created from 1,066 full-length loci found in 99.9% of 987 genomes from carriage. Annotated with CC designations. Serotypes (inner circle) are presented using the same colours as the appropriate CC, where possible. Study periods (outer circle) are also presented

Table 7. Serotypes, CCs, STs and PMEN lineages detected in nasopharyngeal samples.

Nasopharyngeal samples from carriage PreVac; 2009-11		
Serotype (n)	**CC (n)**	**ST (n: PMEN[a])**
23F (64)	439 (45)	311 (18: DLV[b] Tennessee^{23F}-4), 36 (9), 507 (9: DLV Tennessee^{23F}-4)
		37 (6: Tennessee^{23F}-4), 442 (1), 10353 (1) 10359 (1)
	392 (16), 177 (3)	440 (16), 1877 (3: DLV Greece^{21}-30)
6B (65)	138/176 (57), 90 (5)	176 (39: DLV Poland^{23F}-16), 138 (16), 90 (5: Spain^{6B}-2)
	315 (1)	315 (1: Poland^{6B}-20)
	396 (1), 460 (1)	1716 (1), 460 (1)
19A (45)	199 (42)	667 (19: SLV[e] Netherlands^{15B}-37), 199 (11: Netherlands^{15B}-37)
		10360 (12: DLV Netherlands^{15B}-37)
	3017 (2), Sing1801[f] (1)	3017 (2), 1801 (1)
19F (41)	236/271/320 (35)	3014 (22: DLV Taiwan^{19F}-14), 9165 (7: DLV Taiwan^{19F}-14)
		9458 (4: DLV Taiwan^{19F}-14), 271 (2: SLV Taiwan^{19F}-14)
	156/162 (2), 15 (1)	162 (2: SLV Spain^{9V}-3), 3016 (1)
	177 (1)	12986 (1: SLV Portugal^{19F}-21)
	395 (1), 460 (1)	425 (1), 460 (1)
6A (41)	490 (24), 460 (9)	2221 (24), 460 (6), 65 (2), 2340 (1)
	15 (8)	3981 (5)
3 (34)	180 (34)	180 (27: Netherlands^{3}-31), 505 (7: DLV Netherlands^{3}-31)
14 (30)	124 (21), 15 (4),	124 (21: Netherlands^{14}-35), 9 (4: England^{14}-9)
	143 (3), 156/162 (2)	143 (3), 2306 (2: SLV Spain^{9V}-3)
11A (24)	62 (24)	62 (24: DLV Netherlands^{8}-33)
15B/C (18)	199 (15), 1262 (3)	199 (15: Netherlands^{15B}-37), 1262 (3)
16F (14)	30 (14)	30 (14)
38 (13)	393 (13)	393 (13)
23A (13)	439 (13)	42 (7: DLV Tennessee^{23F}-4), 436 (4: DLV Tennessee^{23F}-4)
		190 (1), 10349 (1: DLV Tennessee^{23F}-4)
18C (10)	113 (8)	113 (5: Netherlands^{18C}-36), 116 (2: SLV Netherlands^{18C}-36) 110 (1: SLV Netherlands^{18C}-36)
	102 (2)	1071 (2)
9V (9)	156/162 (9)	162 (9: SLV Spain^{9V}-3)
33F (7)	100 (7)	100 (7)
9N (5)	66 (5)	66 (5: SLV Tennessee^{14}-18)
6C (4)	315 (2), 395 (2)	386 (2: DLV Poland^{6B}-20), 1692 (2: DLV Portugal^{6A}-41)
10A (3)	460 (2)	461 (2)
	5825/11845 (1)	11845 (1)
21 (2)	177 (1)	1877 (2: DLV Greece^{21}-30)
22F (2)	433 (2)	433 (2)
35B (2)	1816 (2)	1967 (2)
4 (1)	205 (1)	205 (1: Sweden^{4}-38)
31 (1)	568 (1)	1601 (1)
23B (1)	338 (1)	2372 (1)
10B (0)		
15A (0)		
33_Hybrid (0)		
35F (0)		
NESp[g] (33)	344 (17)	10371 (12: SLV Norway^{NT}-42), 344 (4: Norway^{NT}-42)
		4149 (1: SLV Norway^{NT}-42)
	448 (15), 62 (1)	448 (15: USA^{NT}-43), 62 (1: DLV Netherlands^{8}-33)

Nasopharyngeal samples from carriage PostVac; 2012-14		
Serotype (n)	**CC (n)**	**ST (n: PMEN)**
23F (36)	439 (19)	311 (11: DLV Tennessee^{23F}-4), 36 (4), 42 (1: DLV Tennessee^{23F}-4)
		507 (1: DLV Tennessee^{23F}-4), 442 (1) 13126 (1)
	392 (16), 433 (1)	440 (16), 10368 (1)
6B (40)	138/176[d] (26)	138 (16), 176[d] (10: DLV Poland^{23F}-16),
	395 (5), 90 (4)	395 (5: SLV Portugal^{6A}-41), 90 (4: Spain^{6B}-2)
	396 (3), 171 (2)	1716 (3), 639 (2)
19A (37)	199 (27)	10360 (11: DLV Netherlands^{15B}-37), 199 (8: Netherlands^{15B}-37), 667 (8: SLV Netherlands^{15B}-37)
	Sing1801 (8), 346 (1)	1801 (8), 10365 (1)
	236/271/320 (1)	320 (1: DLV Taiwan^{19F}-14)
19F (28)	236/271/320 (17)	3014 (10: DLV Taiwan^{19F}-14), 9165 (4: DLV Taiwan^{19F}-14)
		9828 (2), 271 (1: SLV Taiwan^{19F}-14)
	395 (7)	10358 (6), 425 (1)
	177 (4)	179 (3: SLV Portugal^{19F}-21), 51 (1: SLV Portugal^{19F}-21)
6A (45)	490 (22), 460 (21)	2221 (22), 460 (13), 65 (6), 2340 (2)
	138/176 (2)	176 (2: DLV Poland^{23F}-16)
3 (21)	180 (21)	180 (20: Netherlands^{3}-31), 505 (1: DLV Netherlands^{3}-31)
14 (10)	124 (9),	124 (8: Netherlands^{14}-35), 13127 (1: SLV Netherlands^{14}-35)
	15 (1)	9 (1: England^{14}-9)
11A (37)	62 (37)	62 (35: DLV Netherlands^{8}-33), 10345 (2)
15B/C (44)	199 (26), 1262[c] (18)	199 (26: Netherlands^{15B}-37), 1262[c] (18)
16F (6)	30 (6)	2340 (2), 2042 (1)
38 (3)	393 (3)	393 (3)
23A (22)	439 (22)	42 (13: DLV Tennessee^{23F}-4), 190 (4: DLV Tennessee^{23F}-4)
		438 (2), 2404 (2) 992 (1)
18C (7)	113 (7)	113 (7: Netherlands^{18C}-36)
9V (1)	156/162 (1)	162 (1: SLV Spain^{9V}-3)
33F (4)	100 (3), 717 (1)	100 (3), 717 (1)
9N (5)	66 (5)	66 (5: SLV Tennessee^{14}-18)
6C (10)	395 (5)	1692 (4: DLV Portugal^{6A}-41), 1714 (1: DLV Portugal^{6A}-41)
10A (2)	460 (2)	461 (1), 816 (1)
21 (16)	177 (11), 432 (5)	1877 (11: DLV Greece^{21}-30), 432 (5)
22F (29)	433[c] (29)	433[c] (28), 13129 (1)
35B (16)	198 (11), 452 (2)	4346 (10), 198 (1), 452 (2)
	1816 (2)	1967 (2)
	Sing2690 (1)	2690 (1)
4 (0)		
31 (0)		
23B (29)	439 (28), 338 (1)	439 (28: SLV Tennessee^{23F}-4), 1349 (1: DLV Colombia^{23F}-26)
10B (7)	Sing6524 (7)	6524 (7)
15A (1)	473 (1)	473 (1)
33_Hybrid (4)	62 (4)	673 (4)
35F (12)	460 (12)	1635 (10), 446 (2)
NESp (33)	344 (17)	344 (8: Norway^{NT}-42), 10371 (6: SLV Norway^{NT}-42), 4145 (1: SLV Norway^{NT}-42)
		4149 (1: SLV Norway^{NT}-42), 13128 (1: SLV Norway^{NT}-42)
	448 (13)	448 (12: USA^{NT}-43), 10373 (1: SLV USA^{NT}-43)
	Sing10375 (2)	10375 (2)
	Sing10346 (1)	10346 (1)

[a]PMEN: Pneumococcal molecular epidemiology network.
[b]DLV: Double locus variant.
[c]P-value=0.001.
[d]P-value <0.001.
[e]SLV: Single locus variant.
[f]Sing: Singleton.
[g]NESp: Non-encapsulated *S. pneumoniae*.

4.2.4 Prevalence of antimicrobial resistance in pneumococci from carriage

The non-susceptibility to penicillin did not differ significantly between the periods in nasopharyngeal carriage samples (15.0% vs 16.7%; p=0.268) but non-susceptibility to erythromycin and trimethoprim-sulfamethoxazole decreased from 17.6% to 13.7% (p=0.007) and 27.5% to 20.8% (p<0.001, respectively; Table 8). The overall prevalence of MDR pneumococci decreased also from 15.2% to 12.4% (p=0.030).

PNSP that were also MDR prior to PHiD-CV implementation decreased from 85.2% to 70.1% post vaccination (p<0.001). Serotype 19F was the most prevalent PNSP/MDR detected in the PreVac period and NESp in the PostVac period (Table 9). The prevalence of NESp isolates that were MDR increased between the two periods (p=0.025). Out of the PNSP isolates of serotype 19F, 92.5% were members of CC236/271/320^{19F} (Figure 1 and Table 4). PNSP/MDR isolates of serotype 6C increased from 2.0% to 13.6% (p<0.001) and were members of CC315$^{6B/C}$ (Table 9, Figure 1 and Table 7).

Table 8. Pneumococcal isolates from nasopharyngeal carriage: non-susceptible to standard antimicrobials. In the PreVac (2009-2011) and PostVac (2012-2017) periods.

DCC	PreVac		PostVac		
Non-susceptibility to	**n**	**%**	**n**	**%**	**p-value**
Penicillin	149	15.0	338	16.7	0.268
Erythromycin	174	17.6	278	14.7	0.007
Chloramphenicol	5	0.5	15	0.7	0.077
Tetracycline	152	15.3	259	12.8	0.055
Clindamycin	99	10.0	189	9.3	0.553
Trimethoprim-sulfamethoxazole	273	27.5	423	20.8	<0.001
Total DCC isolates	**991**		**2,029**		

Table 9. Most prevalent serotypes from nasopharyngeal carriage that were PNSP and PNSP that were MDR. In the PreVac (2009-2011) and PostVac (2012-2017) periods.

	PNSP					PNSP that were MDR				
DCC	PreVac		PostVac			PreVac		PostVac		
Serotype	n	%	n	%	p-value	n	%	II	%	p-value
19F	74	49.7	59	17.5	<0.001	72	48.3	59	17.5	<0.001
NESp[a]	34	22.8	85	25.1	0.648	32	21.5	83	24.6	0.059
6B	12	8.1	4	1.2	<0.001	11	7.4	4	1.2	<0.001
14	11	7.4	1	0.3	<0.001	3	2.0	1	0.3	0.087
19A	6	4.0	35	10.4	0.021	2	1.3	9	2.7	0.516
6C	3	2.0	46	13.6	<0.001	3	2.0	46	13.6	<0.001
15A	0	0	29	8.6	<0.001	0	0	29	8.6	<0.001
23B	0	0	32	9.5	<0.001	0	0	0	0	Nc[b]
35B	0	0	25	7.1	<0.001	0	0	0	0	Nc
Lps[c]	9	6.0	22	6.5	1.000	4	2.7	6	1.8	0.503
Total	**149**	**100**	**338**	**100**	**0.269**	**127**	**85.2**	**237**	**70.1**	**<0.001**

[a]NESp: Non-encapsulated *S. pneumoniae*.

[b]Nc: Not calculated.

[c]Lps: Less prevalent PNSP serotypes and PNSP that were also MDR.

4.3 Pneumococci from middle ear samples from children (Paper III)

The results for ME samples are presented as the average number of isolates per year (avg/yr). This was done to give a clearer picture of the serotype distribution between the PreVac period, which consisted of three years and the PostVac period, which consisted of six years.

4.3.1 Middle ear study population and bacterial isolates

During the study period, the Department of Clinical Microbiology received 6,651 samples from the ME. The highest number of ME samples were received in 2009, 966 samples which reduced to 452 samples in 2016 and 421 samples by the end of September 2017. A total of 994 of the 6,651 samples were culture positive for pneumococci and 18 isolates, that were not stored or not viable were excluded yielding 976 isolates for further examination.

The median age of the children from which the ME isolates were obtained from was 1.5 years and the mean age was 1.8 years (age range 0.1-6.8 years). Most of the isolates, 69.1% (674/976), were obtained from the youngest age group (0 to <2 years of age) and the fewest isolates, 5.3% (52/976) from the oldest age group (4 to <7 years of age). A total of 197 (8.3/1,000 children) pneumococcal isolates were detected in ME in 2009, this decreased to 44 (1.8/1,000 children) isolates in 2016 and to 23 (1.0/1,000 children) by the end of September 2017.

4.3.2 Distribution of serotypes isolated from the middle ear

Overall, 91.6% (894/976) pneumococcal isolates were successfully serotyped and 8.4% (82/976) were of serotypes that the mPCR scheme did not include. In both study periods 28 serotypes were detected: 23 serotypes in the PreVac period and 22 in the PostVac period. VT serotypes decreased from 398 (132.7 avg/yr) isolates to 96 (16.0 avg/yr) isolates between the periods ($p<0.001$) and NVT serotypes increased from 145 (48.3 avg/yr) isolates to 337 (56.2 avg/yr; $p<0.001$).

Serotype 19F was the most prevalent serotype in children 0 to <7 years of age in the PostVac period but decreased ($p<0.001$) and serotypes 15B/C and 6C were the most prevalent NVT serotypes in children 0 to <2 years of age (Table 10).

Table 10. Most prevalent NVTs detected in ME samples in age groups 0 to <2 years and 2 to <4 years. In the PreVac (2009-2011) and PostVac (2012-2017) periods.

	0 to < 2 years			2 to < 4 years		
ME NVT Serotype	PreVac Avg/yr[a] (n)	PostVac Avg/yr (n)	p-value	PreVac Avg/yr (n)	PostVac Av/yr (n)	p-value
3	1.0 (3)	0.7 (4)	0.730	2.0 (6)	1.3 (8)	0.745
6A	10.7 (32)	2.5 (15)	0.159	5.0 (15)	2.2 (13)	0.773
6C	0.3 (1)	4.8 (29)	<0.001	0 (0)	2.0 (12)	Nc[b]
11A	1.3 (4)	1.5 (9)	0.292	1.0 (3)	1.0 (7)	0.411
15B/C	0.3 (1)	6.7 (40)	<0.001	0.3 (1)	1.8 (11)	0.042
15A	0 (0)	0.7 (4)	Nc	0 (0)	0.3 (2)	Nc
19A	8.7 (26)	2.3 (14)	0.302	3.0 (9)	1.0 (6)	0.589
21	0 (0)	1.5 (9)	Nc	0 (0)	0.5 (3)	Nc
23A	0.7 (2)	3.0 (18)	0.007	0 (0)	0.5 (3)	Nc
23B	0.3 (1)	1.7 (10)	0.042	0 (0)	0.7 (4)	Nc
33F	1.0 (3)	1.7 (10)	0.150	0.3 (1)	0.2 (1)	0.992
35B	0 (0)	1.0 (6)	Nc	0 (0)	0.5 (3)	Nc
Other NVTs	6.0 (18)	7.3 (44)	Nc	3.0 (9)	3.5 (21)	Nc
NESp[c]	0 (0)	0.2 (1)	Nc	0.3 (1)	0 (0)	Nc
Total NVTs[d]	**30.3 (91)**	**35.5 (213)**	**<0.001**	14.3 (43)	15.7 (94)	0.005
Total pn[e] isolates	133.7 (401)	45.5 (273)	0.020	40.3 (121)	21.5 (129)	0.829

[a]Avg/yr: Average/year.

[b]Nc: Not calculated.

[c]NESp: Non-encapsulated *S. pneumoniae*.

[d]NVTs: Serotypes not targeted by PHiD-CV.

[e]Pn: Pneumococcal.

4.3.3 Genetic lineages in middle ear

Every other pneumococcal isolate was sequenced from the period 2009-2014, resulting in 441 (50.6%; 441/871) pneumococcal genomes: 275 genomes PreVac and 166 PostVac. Out of the 441 sequenced genomes, 41 CCs (29 CCs in the PreVac period and 21 CCs in the PostVac period) and 86 STs (55 STs in the PreVac period and 52 STs in the PostVac period) were detected in ME isolates.

The Simpson diversity index of the STs was 0.91 in the PreVac period and 0.96 in the PostVac period, indicating that there were no changes in the diversity between the study periods.

A mid rooted phylogenetic tree was created with the concatenated sequences of 1,250 full-length coding loci found in 99.8% of the ME pneumococcal genomes. The tree was annotated with CC designations and serotypes (Figure 2).

CC236/271/320^{19F} was the most prevalent CC in both study periods but decreased in prevalence from 41.8% (115/275) to 22.3% (37/166; p<0.001). One isolate of serotype 23B in the PostVac period was a member of a VT lineage, CC156/162^{9V}. CC315$^{6B/C}$ increased between the periods from one isolate of serotype 6B to six isolates of serotype 6C, five of which belonged to ST386 (p=0.05; Figure 2 and Table 11).

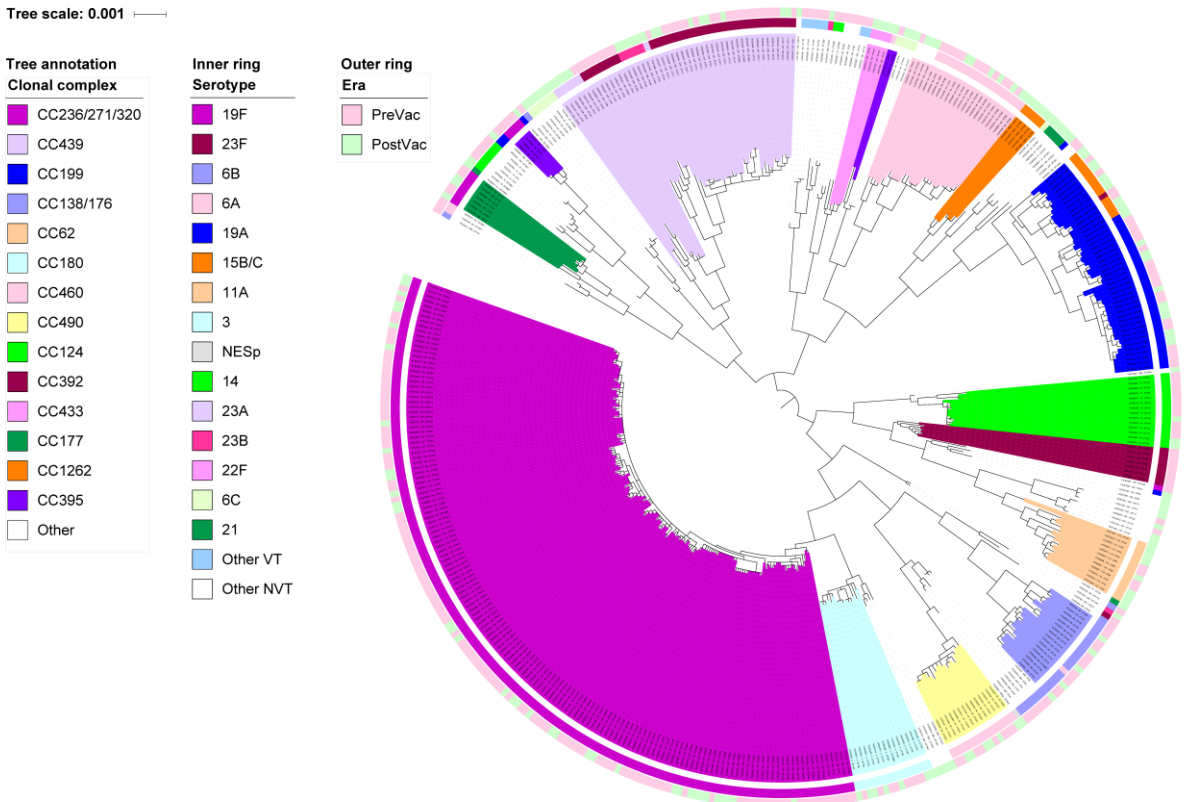

Figure 2. Mid rooted phylogenetic tree, created from 1,250 full-length coding loci found in 99.8% of the 441 genomes from ME. Annotated with CC designations. Serotypes (inner circle) are presented using the same colours as the appropriate CC, where possible. Study periods (outer circle) are also presented.

Table 11. Serotypes, CCs, STs and PMEN lineages detected in ME samples.

	ME samples PreVac; 2009-2011	
Serotype (n)	**CC (n)**	**ST (n: PMEN[a])**
19F (124)	236/271/320 (115)	3014 (76: DLV[b] Taiwan^{19F}-14), 9165 (15: DLV Taiwan1^{9F}-14) 9458 (13: SLV[d] Taiwan^{19F}-14), 271 (4: SLV Taiwan^{19F}-14) 1968 (2: DLV Taiwan^{19F}-14), 9828 (2) 10385 (2: DLV Taiwan^{19F}-14), 13094 (1)
	177 (4), 395 (4)	179 (3: SLV Portugal^{19F}-21), 9827 (1: SLV Portugal^{19F}-21), 425 (4)
	63 (1)	1545 (1: DLV Sweden^{15A}-25)
23F (37)	439 (28)	311 (16: DLV Tennessee^{23F}-4), 36 (6), 9829 (3), 37 (1: Tennessee^{23F}-4) 442 (1), 507 (1: DLV Tennessee^{23F}-4)
	392 (7), 199 (1)	440 (7), 199 (1: Netherlands^{15B}-37)
	361 (1)	277 (1)
6A (23)	460 (12), 490 (8)	460 (6), 65 (5), 2340 (1), 2221 (8)
	15 (2), 176 (1)	3981 (2), 176 (1: DLV Poland^{23F}-16)
19A (20)	199 (19)	667 (8: SLV Netherlands^{15B}-37) 199 (7: Netherlands^{15B}-37), 10360 (4: DLV Netherlands^{15B}-37)
	Sing1801[e] (1)	1801 (1)
6B (21)	138/176 (11)	176 (6: DLV Poland^{23F}-16), 138 (5)
	171 (1) 396 (1)	639 (1), 1716 (1)
	90 (6), 135 (1)	90 (6: Spain^{6B}-2), 135 (1)
	315 (1)	315 (1: Poland^{6B}-20)
14 (18)	124 (11)	124 (11: Netherlands^{14}-35),
	15 (5) 156/162	9 (4: England^{14}-9), 1964 (1: DLV England^{14}-9), 2306 (2: SLV Spain^{9V}-3)
3 (12)	180 (12)	180 (12: Netherlands^{3}-31)
11A (4)	62 (4)	62 (4: DLV Netherlands^{8}-33)
9V (5)	156/162 (5)	162 (4: SLV: Spain^{9V} -3), 1269 (1: DLV Spain^{9V}-3)
15B/C (3)	199 (3)	199 (2: Netherlands^{15B}-37), 2220 (1: SLV Netherlands^{15B}-37)
16F (2)	30 (2)	30 (2)
18C (2)	113 (2)	113 (1: Netherlands^{18C}-36), 116 (1: SLV Netherlands^{18C}-36)
33F (2)	100 (1), 717 (1)	100 (1), 717 (1)
38 (1)	393 (1)	393 (1)
23A (1)	439 (1)	42 (1: DLV Tennessee^{23F}-4)
23B (0)		
24F (1)	230 (1)	230 (1: Denmark^{14}-32)
35F (1)	460 (1)	446 (1)
6C (1)	395 (1)	1692 (1: DLV Portugal^{6A}-41)
9N (1)	66 (1)	10344 (1: DLV Tennessee^{14}-18)
21 (0)		
22F (0)		
35B (0)		
10B (0)		
15A (0)		
NESp[h] (0)		

	ME samples PostVac; 2012-2014	
Serotype (n)	**CC (n)**	**ST (n: PMEN)**
19F (40)	236/271/320[c] (37)	3014 (23: DLV Taiwan[19F]-14), 9828 (10)
		9165 (3: SLV Taiwan[19F]-14), 9458 (1: DLV Taiwan[19F]-14)
	177 (3)	177 (1: Portugal[19F]-21), 179 (2: SLV Portugal[19F]-21
23F (8)	439 (8)	311 (8: DLV Tennessee[23F]-4)
6A (13)	460 (7), 490 (6)	460 (5), 2340 (2), 2221 (5), 10029 (1)
	395 (1)	395 (1: SLV Portugal[6A]-41)
19A (15)	199 (11)	667 (4: Netherlands[15B]-37), 199 (4: Netherlands[15B]-37)
		10360 (3: DLV Netherlands[15B]-37)
	63 (1), 230 (1)	63 (1: Sweden[15A]-25), 2013 (1: DLV Denmark[14]-32)
	346 (1) Sing1801 (1)	10365 (1), 1801 (1)
6B (5)	138/176 (5)	176 (2: Poland[23F]-16), 10393 (1: DLV Poland[23F]-16)
		138 (2)
14 (4)	124[f] (3)	124[f] (3: Netherlands[14]-35)
	15 (1)	1964 (1: DLV England[14]-9)
3 (7)	180 (7)	180 (6: Netherlands[3]-31), 13099 (1)
11A (8)	62 (8)	62 (7: DLV Netherlands[8]-33), 1367 (1)
9V (0)		
15B/C (15)	199 (10)	199 (10: Netherlands[15B]-37)
	1262 (5)	1262 (4), 8711 (1)
16F (0)		
18C (0)		
33F (5)	100 (3), 717 (2)	100 (3), 717 (2)
38 (0)		
23A (6)	439 (6)	42 (3: DLV Tennessee[23F]-4), 190 (1)
		436 (1: DLV Tennessee[23F]-4), 438 (1)
23B (7)	439 (5), 156/162 (1)	439 (5: SLV Tennessee[23F]-4), 162 (1: SLV Spain[9V]-3)
	338 (1)	1349 (1: DLV Colombia[23F]-26)
24F (0)		
35F (3)	460 (3)	1635 (3)
6C (10)	315[g] (6)	386 (5: DLV Poland[6B]-20), 10362 (1)
	1379 (3), 177 (1)	1379 (3), 1533 (1: SLV Greece[21]-30)
9N (0)		
21 (6)	193 (4), 432 (1)	1877 (4: DLV Greece[21]-30), 432 (1)
	Sing10356 (1)	10356 (1)
22F (4)	433 (4)	433 (4)
35B (5)	1816 (4), 198 (1)	1967 (3), 10361 (1), 4346 (1)
10B (1)	Sing6524 (1)	6524 (1)
15A (2)	63 (2)	63 (2: Sweden[15A]-25)
NESp (1)	1182 (1)	3691 (1)

[a]PMEN: Pneumococcal molecular epidemiology network.
[b]DLV: Double locus variant.
[c]P-value<0.001).
[d]SLV: Single locus variant.
[e]Sing: Singleton. [f]P-value=0.024.
[g]P-value=0.05.
[h]NESp: Non-encapsulated *S. pneumoniae*.

4.3.4 Prevalence of antimicrobial resistance in pneumococci from middle ear samples

Non-susceptibility to penicillin decreased between the study periods from 48.1% to 23.4% (p<0.001) and non-susceptibility to all the antimicrobials tested, except for chloramphenicol, decreased also (Table 12).

Table 12. Pneumococcal isolates from ME samples: non-susceptibility to standard antimicrobials. In the PreVac (2009-2011) and PostVac (2012-2017) periods.

ME samples	PreVac		PostVac		
Non-susceptibility to	**n**	**%**	**n**	**%**	**p-value**
Penicillin	261	48.1	123	28.4	<0.001
Erythromycin	270	49.7	129	29.8	<0.001
Chloramphenicol	6	1.1	1	0.2	0.140
Tetracycline	252	46.4	118	27.3	<0.001
Clindamycin	245	45.1	121	27.9	<0.001
Trimethoprim-sulfamethoxazole	324	59.7	136	31.4	<0.001
Total ME isolates	**543**		**433**		

The prevalence of MDR pneumococci in ME samples decreased from 49.2% to 29.8% (p<0.001). The proportion of PNSP that were also MDR in the PreVac period was 98.1%, which decreased to 91.9% (p<0.001; Table 13). Serotype 19F was the most prevalent PNSP and MDR serotype in both study periods and 89.2% of PNSP of serotype 19F were members of CC236/271/320^{19F} (Figure 2 and Table 11).

PNSP of serotype 6C were not detected in ME samples before vaccine implementation but serotype 6C was the second most prevalent PNSP and MDR serotype after vaccination (Table 13). The sequenced PNSP/MDR serotype 6C isolates were members of CC315$^{6B/C}$ (Figure 2 and Table 11).

Table 13. Most prevalent serotypes from ME samples that were PNSP and PNSP that were MDR. In the PreVac (2009-2011) and the PostVac (2012-2017) periods.

	PNSP					PNSP that were MDR				
ME	PreVac		PostVac			PreVac		PostVac		
Serotype	n	%	n	%	p-value	n	%	n	%	p-value
19F	231	88.6	66	53.7	<0.001	231	88.5	65	52.8	<0.001
6B	9	3.4	3	2.4	0.759	9	3.4	3	2.4	0.759
14	6	2.3	2	1.6	1.000	5	1.9	2	1.6	1.000
19A	4	1.5	9	7.3	0.006	2	0.8	9	7.3	<0.001
23F	4	1.5	0	0	0.311	4	1.5	0	0	0.311
6C	0	0	19	15.4	<0.001	0	0	19	15.4	<0.001
15A	0	0	6	4.9	0.001	0	0	6	4.9	0.001
23B	1	0.4	5	4.1	0.014	1	0.4	1	0.8	0.539
Other PNSP/MDR[a]	6	2.3	13	10.6	0.001	4	1.5	8	6.5	0.120
Total	**261**		**123**		**<0.001**	**256**		**113**		**<0.001**

[a]Other less prevalent PNSP serotypes and PNSP that were also MDR.

PNSP of serotype 19A increased from 1.5% to 7.3% (p=0.006) in the PostVac period, all isolates were also MDR and were members of different CCs with CC199^{19A} being the most prevalent (Table 13, Figure 2 and Table 11). PNSP/MDR serotype 15A was only detected in the PostVac period in ME samples and the sequenced isolates were members of CC63^{15A} (Table 13, Figure 2 and Table 11). Other PNSP/MDR pneumococci were single isolates of various serotypes.

4.4 Comparison of pneumococcal serotypes from nasopharyngeal carriage and acute otitis media (Paper III)

4.4.1 Study population and bacterial isolates

A total of 2,291 pneumococcal isolates were obtained from both nasopharyngeal samples from carriage (1,457 isolates; PreVac: 493 and PostVac: 964) and ME samples from AOM (834 isolates; PreVac: 467 and PostVac: 367; Table 14) from children 1 to <4 years of age. Overall median and mean age of children 1 to <4 years was 2.5 years (age range 1.0-3.9 years).

4.4.2 Comparison of serotypes from carriage and ME samples from children 1 to <4 years of age

The serotype distribution in both sample groups was similar prior to vaccination and the serotypes that replaced VT serotypes post vaccination were alike. No VT pneumococci were detected after 2016 in children with

AOM, but one VT isolate of serotype 23F was detected in nasopharyngeal carriage in 2017 (Figure 3). Serotypes 6C, 23B and 23A increased in both sample groups post vaccination and serotype 6C was only detected post vaccination in children 1 to <4 years of age with AOM (Table 14 and Figure 4).

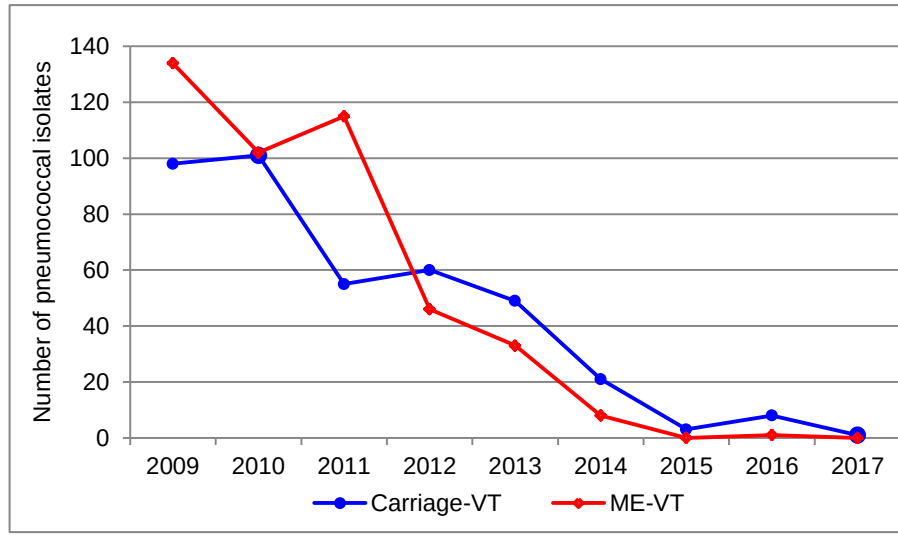

Figure 3. Number of VT serotypes each year of the study in children 1 to <4 years of age in samples from nasopharyngeal carriage and ME.

Table 14. Most prevalent serotypes detected in nasopharyngeal carriage and AOM in children 1 to <4 years of age. In the PreVac (2009-2011) and PostVac (2012-2017) periods.

	Carriage: Age 1 to <4 years			AOM: Age 1 to <4 years		
Serotype	PreVac Avg/yr[a] (n)	PostVac Avg/yr (n)	p-value	PreVac Avg/yr (n)	PostVac Avg/yr (n)	p-value
6A	16.0 (48)	12.7 (76)	0.118	14.3 (43)	4.7 (28)	0.050
6B	21.0 (63)	12.3 (37)	<0.001	11.0 (33)	1.7 (10)	0.030
6C	2.0 (6)	15.0 (90)	<0.001	0 (0)	6.0 (36)	Nc[b]
11A	8.3 (25)	10.7 (64)	0.352	2.0 (6)	2.5 (15)	0.368
15A	0 (0)	2.3 (14)	0.009	0 (0)	1.0 (6)	Nc
15B/C	4.3 (13)	14.7 (88)	<0.001	0.7 (2)	7.8 (47)	<0.001
19F	20.7 (63)	7.2 (43)	<0.001	71.7 (215)	8.8 (53)	<0.001
19A	14.7 (44)	11.2 (67)	0.104	9.0 (27)	2.8 (17)	0.107
21	0.3 (1)	5.5 (33)	<0.001	0 (0)	1.7 (10)	Nc
22F	0.7 (2)	4.3 (26)	0.004	0 (0)	0.5 (3)	Nc
23F	22.0 (67)	7.5 (45)	<0.001	21.7 (65)	2.8 (17)	<0.001
23A	5.3 (16)	11.7 (70)	0.002	0.3 (1)	3.2 (19)	0.010
23B	0.3 (1)	12.0 (72)	<0.001	0.3 (1)	2.2 (13)	0.047
35F	0 (0)	3.3 (20)	0.002	1.0 (3)	1.7 (10)	0.322
35B	1.0 (3)	4.8 (29)	0.005	0 (0)	1.2 (7)	Nc
Lps[c]	35.0 (105)	21.8 (131)	Nc	23.3 (70)	12.5 (75)	Nc
NESp[d]	12.0 (36)	9.8 (59)	0.376	0.3 (1)	0.2 (1)	0.896
Total	**164.3 (493)**	**160.7 (964)**	**0.054**	**155.7 (467)**	**61.2 (367)**	**<0.001**
VT[e]	85.3 (256)	23.5 (141)	<0.001	117.0 (351)	12.8 (77)	<0.001
NVT[f]	79.0 (237)	137.2 (823)	<0.001	38.7 (116)	48.3 (290)	<0.001

[a]Avg/yr: Average/year.
[b]Nc: Not calculated.
[c]Lps: Less prevalent serotypes.
[d]NESp: Non-encapsulated *S. pneumoniae*.
[e]VT: Serotypes detected in the study that are targeted by PHiD-CV (4, 6B, 9V, 14, 18C, 19F and 23F).
[f]NVT: Serotypes not targeted by PHiD-CV.

Serotype 19F was more common in children with AOM than in children carrying pneumococci in both study periods: 46.0% (215/367) of ME isolates compared to 12.8% (63/964) of nasopharyngeal isolates in the PreVac period (p<0.001) and 14.4 % of ME isolates compared to 4.5% of nasopharyngeal isolates in the PostVac period (p<0.001; Figure 4).

Serotype 23B was more common in nasopharyngeal carriage than in AOM after vaccination: 7.5% (72/964) of nasopharyngeal isolates compared to 3.5% (13/367) of ME isolates (p<0.001) and also serotype 22F: 2.7% (26/964) of nasopharyngeal isolates compared to 0.8% (3/367) of ME isolates (p=0.036). Serotype 6C was prevalent in both sample groups but MDR

pneumococci of serotype 6C were more frequently detected in AOM than in nasopharyngeal carriage (p<0.001).

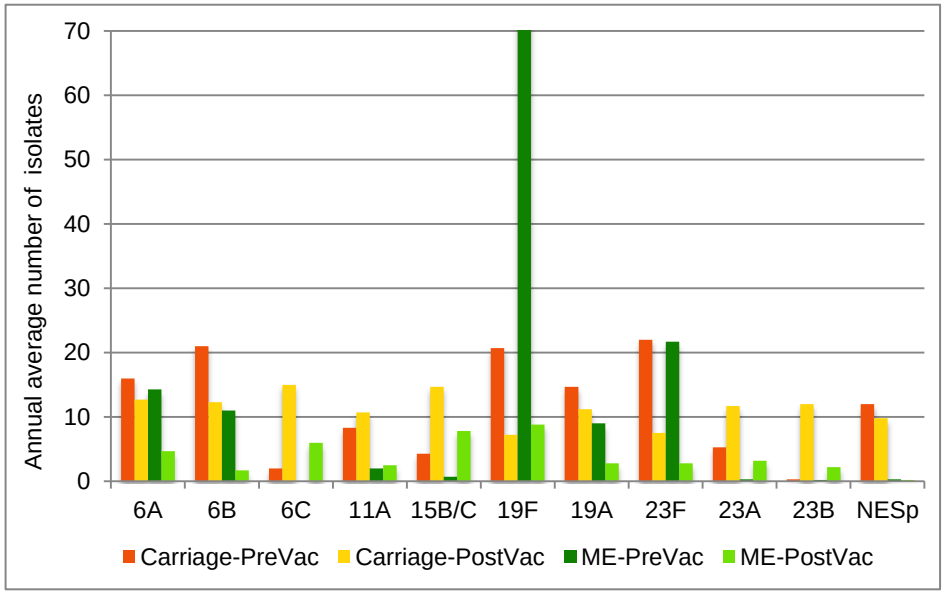

Figure 4. The most prevalent serotypes detected in nasopharyngeal and ME samples from children 1 to <4 years of age. In the PreVac (2009-2011) and PostVac (2012-2017) periods. NESp: Non-encapsulated *S. Pneumoniae.*

NESp isolates were prevalent in nasopharyngeal carriage in both study periods and were very rare in AOM: 7.3% (36/493) of nasopharyngeal isolates compared to 0.2% (1/467) of ME isolates (p<0.001) in the PreVac period and 6.1% (n=95/964) of nasopharyngeal isolates compared to 0.3% (n=1/367) of ME isolates in the PostVac period (p>0.001; Table 14 and Figure 4).

4.5 Pneumococci from lower respiratory tract samples from adults (Paper IV)

Paper IV presents results from the adult population with suspected pneumonia. Studies have shown that a herd effect among the adult population becomes more profound the more time has passed by from PCV implementation (Tsaban & Ben-Shimol, 2017). Therefore, the study was divided into three periods, PreVac (2009-2011), PostVac-I (2012-2014) and PostVac-II (2015-2017). The PreVac period was compared to each PostVac period in order to give a definite picture of vaccine effect on the adult population.

4.5.1 Lower respiratory tract study population and bacterial isolates

During the study period, the Department of Clinical Microbiology received 17,762 samples from the LRT (Figure 5). Thereof, pneumococci were cultured from 814 samples. Isolates that were either not viable or not stored were excluded (n=17), yielding 797 pneumococcal isolates for further examination. The median age of the patients from which the LRT samples were obtained from was 75.2 years and the mean age was 64.9 years (age range 18.4-96.0 years). Over half of the isolates obtained were from adults ≥65 years of age, 54.0% (430/797).

The number of pneumococcal isolates detected prior to vaccination was 314 (184.7/100,000 adults), which decreased to 230 (123.2/100,000 adults) in the later post vaccination period (p=0.002).

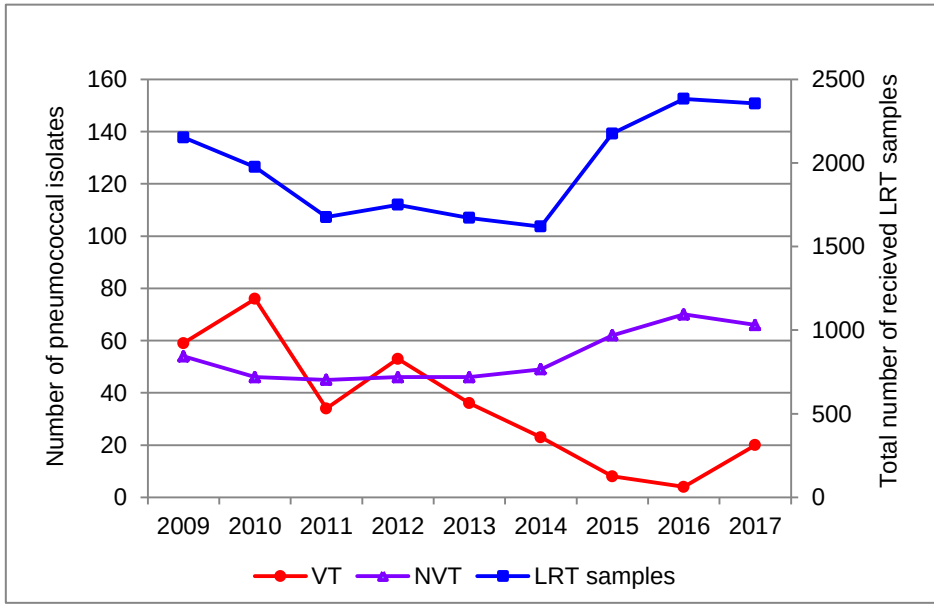

Figure 5. The annual number of LRT isolates of VT and NVT serotypes and LRT samples received by the laboratory in 2009-2017. *Seven isolates of serotype 19F were detected from the same patient in 2017.

4.5.2 Distribution of serotypes isolated from the lower respiratory tract

Over the study, 99.0% (789/797) of the pneumococcal isolates were successfully serotyped and 1.0% (8/797) were of other serotypes that the mPCR scheme did not include. Over the study periods, 31 serotypes were detected: 28 serotypes in the PreVac period and 24 in both post vaccination periods. VT serotypes decreased from 170 (100.0/100,000 adults) isolates in the PreVac period to 33 (17.7/100,000 adults) in the PostVac-II period ($p<0.001$; Table 15). Overall, serotype 6A decreased from 18 (10.6/100,000 adults) isolates in the PreVac period to three (1.6/100,000 adults; $p=0.016$) in the PostVac-II period. Serotype 6C increased from one (0.6/100,000 adults) isolate to 14 (7.5/100,000 adults; $p=0.021$; Table 15) in the PostVac-II period.

NESp isolates increased in adults 18-64 years of age from six (4.2/100,000 adults 18-64 years) isolates in the PreVac period to 25 (16.2/100,000 adults 18-64 years) in the PostVac-II period ($p=0.028$; Table 15).

Table 15. Most prevalent serotypes from LRT samples. Detected in the PreVac (2009-2011), PostVac-I (2012-2014) and PostVac-II (2015-2017) periods

	PreVac		PostVac-I		Pre- vs	PostVac-II		Pre- vs
LRT	2009-2011		2012-2014		PostVac-I	2015-2017		PostVac-II
Serotype	n	/100,000	n	/100,000	p-value	n	/100,000	p-value
3	25	14.7	20	11.3	0.558	20	10.7	0.485
6A	18	10.6	9	5.1	0.221	3	1.6	0.016
6B	21	12.3	21	11.8	0.927	7	3.7	0.052
6C	1	0.6	2	1.1	0.719	14	7.5	0.021
11A	14	8.2	11	6.2	0.641	20	10.7	0.617
14	15	8.8	7	3.9	0.229	1	0.5	0.008
15B/C	8	4.7	11	6.2	0.694	11	5.9	0.749
19F	99	58.2	67	37.7	0.068	20[a]	10.7	<0.001
19A	10	5.9	13	7.3	0.730	12	6.4	0.891
22F	12	7.1	9	5.1	0.619	10	5.4	0.670
23F	26	15.3	15	8.5	0.219	2	1.1	0.001
23A	3	1.8	4	2.3	0.832	13	7.0	0.111
23B	4	2.4	5	2.8	0.859	10	5.4	0.337
35B	6	3.5	7	3.9	0.895	14	7.5	0.289
Lps[b]	39	22.9	33	18.6	Nc[c]	58	31.1	Nc
NESp[d]	13	7.6	19	10.7	0.534	35	18.7	0.054
Total	**314**	**184.7**	**253**	**142.5**	**0.043**	**230**	**123.2**	**0.002**
VT[e]	170	100.0	113	63.7	0.013	33	17.7	<0.001
NVT[f]	144	84.7	140	78.9	0.693	197	105.5	0.184
All LRT[g]	5,805	3413.9	5,041	2840.2	<0.001	6,916	3703.9	0.003

[a]Seven isolates of serotype 19F were detected from the same patient in 2017.
[b]Lps: Less prevalent serotypes.
[c]Nc: Not calculated.
[d]NESp: Non-encapsulated *S. pneumoniae*.
[e]VT: Serotypes detected in the study that are targeted by PHiD-CV (4, 6B,7F, 9V, 14, 18C, 19F and 23F).
[f]NVT: Serotypes not targeted by PHiD-CV.
[g]All LRT: Number of LRT samples received by the Department of Clinical Microbiology.

4.5.3 Genetic lineages in lower respiratory tract

In the years 2009-2014, 567 pneumococcal isolates were obtained. Every other isolate was sequenced from the period 2009-2014, resulting in 275 (48.5%; 275/567) pneumococcal genomes: 157 genomes PreVac and 118 PostVac-I. Out of the 275 sequenced genomes, 41 CCs (31 CCs in the PreVac period and 32 CCs in the PostVac-I period) and 73 STs (56 STs in the PreVac period and 47 STs in the PostVac-I period) were detected among LRT isolates.

Simpson diversity index was 0.97 PreVac and 0.96 PostVac-I, implying that there were no changes in the diversity between the periods.

A mid rooted phylogenetic tree was created with intergraded sequences of 1,130 full-length coding loci found in 99.6% of the pneumococcal genomes. The tree was annotated with CC designations and serotypes (Figure 6).

CC236/271/320^{19F} was predominant in both study periods but decreased from 31.2% (49/157) isolates in the PreVac period to 24.6% (29/118) in the PostVac-I period (p=0.010; Figure 6 and Table 16).

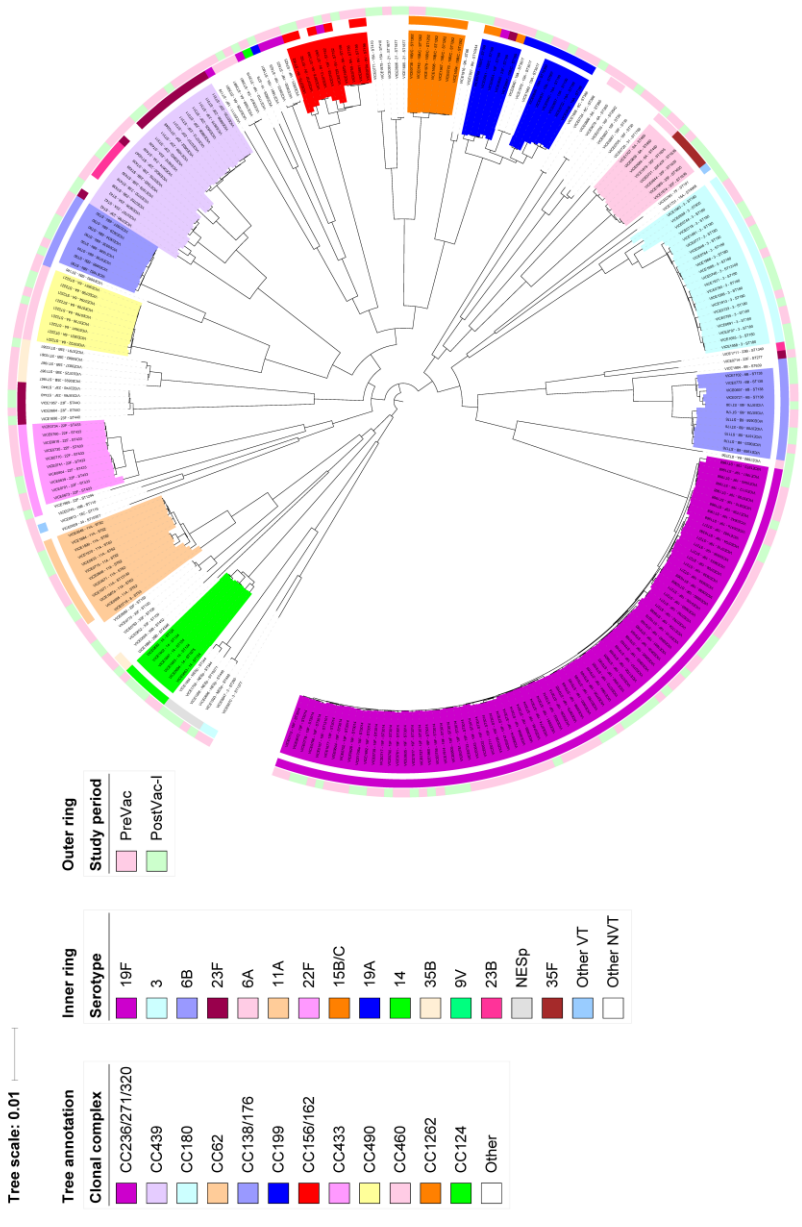

Figure 6. Mid rooted phylogenetic tree, created from 1,221 full-length loci found in 99.6% of 275 genomes from LRT. Annotated with CC designations. Serotypes (inner circle) are presented using the same colours as the appropriate CC, where possible. Study periods (outer circle) are also presented.

Table 16. Serotypes, CCs, STs and PMEN lineages detected in LRT samples.

	LRT samples PreVac (2009-2011)	
Serotype (n)	**CC (n)**	**ST (n: PMEN[a])**
19F (54)	236/271/320 (49)	3014 (24: DLV[b] Taiwan^{19F}-14), 271 (8: DLV Taiwan^{19F}-14), 9165 (7: DLV Taiwan^{19F}-14)
		1968 (6: DLV Taiwan^{19F}-14), 10369 (2: DLV Taiwan^{19F}-14), 10376 (1), 13101 (1)
	395 (3), 199 (1)	425 (3), 199 (1: Netherlands^{15B}-37)
	177 (1)	179 (1: SLV[e] Portugal^{19F}-21)
23F (12)	439 (8)	311 (6: DLV Tennessee^{23F}-4), 10348 (1: DLV Tennessee^{23F}-4), 10347 (1)
	392 (2), 199 (1)	440 (2), 199 (1: Netherlands^{15B}-37)
	361 (1)	277 (1)
6A (12)	490 (6), 460 (2)	2221 (6), 460 (2)
	15 (2), 395 (2)	3981 (2), 395 (2: SLV Portugal^{6A}-41)
3 (10)	180 (8)	180 (6: Netherlands^{3}-31), 13100 (1: SLV Netherlands^{3}-31)
		505 (1: DLV Netherlands^{3}-31), 13100 (1)
	260 (1), 378 (1)	260 (1), 1377 (1)
6B (10)	138/176 (5)	176 (3: DLV Poland^{23F}-16), 138 (2)
	90 (4), 146 (1)	90 (4: Spain^{6B}-2), 146 (1)
22F (8)	433 (8)	433 (8)
11A (6)	62 (6)	62 (6: DLV Netherlands^{8}-33)
19A (6)	199 (5)	199 (3: Netherlands^{15B}-37), 667 (1: SLV Netherlands^{15B}-37) 10360 (1: DLV Netherlands^{15B}-37)
	3017 (1)	3017 (1)
14 (4)	124 (3), 15 (1)	124 (2: Netherlands^{14}-35), 1975 (1: SLV Netherlands^{14}-35), 9 (1: England^{14}-9)
35B (5)	1816 (4), 452 (1)	1967 (3), 10361 (1), 452 (1)
9V (4)	156/162 (4)	156 (2: Spain^{9V}-3), 162 (1: SLV Spain^{9V}-3), 1269 (1: DLV Spain^{9V}-3)
15B/C (4)	199 (2), 1262 (2)	199 (2: Netherlands^{15B}-37), 1262 (2)
23B (3)	439 (3)	439 (3: SLV Tennessee^{23F}-4)
23A (3)	439 (3)	436 (2: DLV Tennessee^{23F}-4), 42 (1: DLV Tennessee^{23F}-4)
16F (3)	30 (3)	30 (2), 2042 (1)
33F (2)	100 (2)	100 (2)
9A (2)	156/162 (2)	156 (1: Spain^{9V}-3), 1269 (1: DLV Spain^{9V}-3)
8 (1)	62 (1)	53 (1: Netherlands^{8}-33)
21 (1)	193 (1)	1877 (1: DLV Greece^{21}-30)
31 (1)	113 (1)	1766 (1)
34 (1)	4878 (1)	10357 (1)
18B (1)	113 (1)	110 (1: SLV Netherlands^{18C}-36)
18C (1)	113 (1)	116 (1: SLV Netherlands^{18C}-36)
35F (1)	460 (1)	1635 (1)
6C (1)	315 (1)	386 (1: DLV Poland^{6B}-20)
15A (0)		
9N (0)		
7F (0)		
NESp[h] (1)	448 (1)	448 (1: USA^{NT}-43)

LRT samples PostVac-I (2012-2014)		
Serotype (n)	**CC (n)**	**ST (n: PMEN)**
19F (31)	236/271/320[c] (29)	3014[d] (12: DLV Taiwan[19F]-14), 9165 (8: DLV Taiwan[19F]-14), 271 (3: SLV Taiwan[19F]-14)
		1968 (3: DLV Taiwan[19F]-14), 9458 (1: DLV Taiwan[19F]-14)
		9828 (1), 10390 (1: DLV Taiwan[19F]-14)
	15 (1), 156/162 (1)	3016 (1), 162 (1: SLV Spain[9V]-3)
23F (7)	439 (4)	311 (3: DLV Tennessee[23F]-4)
		42 (1: DLV Tennessee[23F]-4)
	392 (3)	440 (3)
6A (6)	490 (3), 15 (1), 460 (1)	2221 (3), 3981 (1), 460 (1)
	9789 (1)	2756 (1)
3 (13)	180 (13)	180 (13: Netherlands[3]-31)
6B (11)	138/176 (6)	38 (3), 176 (3: DLV Poland[23F]-16)
	90 (4), 171 (1)	90 (4: Spain[6B]-2), 1639 (1)
22F (3)	433[f] (2), 1294/4522 (1)	433[f] (2), 1294 (1)
11A (5)	62 (5)	62 (4: DLV Netherlands[8]-33), 13130 (1)
19A (4)	3017 (2), 199 (1)	3017 (2), 199 (1: Netherlands[15B]-37)
	Sing[g]1801 (1)	1801 (1)
14 (3)	124 (3)	124 (3: Netherlands[14]-35)
35B (2)	198 (1), 1816 (1)	4346 (1), 10361 (1)
9V (2)	156/162 (2)	162 (2: SLV Spain[9V]-3)
15B/C (6)	1262 (5), 199 (1)	1262 (5), 199 (1: Netherlands[15B]-37)
23B (3)	439 (2), 338 (1)	439 (2: SLV Tennessee[23F]-4), 1349 (1: DLV Colombia[23F]-26)
23A (1)	439 (1)	42 (1: DLV Tennessee[23F]-4)
16F (1)	30 (1)	30 (1)
33F (2)	100 (2)	100 (2)
9A (1)	156/162 (1)	156 (1: Spain[9V] -3)
8 (0)		
21 (2)	193 (2)	1877 (2: DLV Greece[21]-30)
31 (0)		
34 (0)		
18B (0)		
18C (0)		
35F (4)	460 (4)	1635 (4)
6C (1)	315 (1)	386 (1: DLV Poland[6B]-20)
15A (3)	193 (2), 3058 (1)	410 (2: SLV Greece[21]-30), 4965 (1)
9N (2)	66 (2)	66 (1: SLV Tennessee[14]-18), 10344 (1: DLV Tennessee[14]-18)
7F (1)	191 (1)	191 (1: Netherlands[7F]-39)
NESp (4)	344 (3), 448 (1)	344 (2: Norway[NT]-42), 10371 (1:SLV Norway[NT]-42), 448 (1: USA[NT]-43)

[a]PMEN: Pneumococcal molecular epidemiology network.
[b]DLV: Double locus variant.[c]P-value=0.001.
[d]P-value=0.030.
[e]SLV: Single locus variant.
[f]P-value=0.040.
[g]Sing: Singleton.
[h]NESp: Non-encapsulated *S. pneumoniae.*

4.5.4 Prevalence of antimicrobial resistance in lower respiratory tract

Non-susceptibility to penicillin and erythromycin was similar between the PreVac and PostVac-II periods (Table 17). Non-susceptibility to clindamycin and trimethoprim-sulfamethoxazole decreased from 32.5% to 24.3% (p=0.045) and 42.7% to 21.3% (p<0.001, respectively; Table 17). The prevalence of MDR pneumococci decreased also from 34.4% to 23.9% (p=0.010).

Table 17. Pneumococcal isolates from LRT samples: non-susceptibility to standard antimicrobials. In the PreVac (2009-2011) and PostVac-II (2015-2017) periods.

LRT	PreVac		PostVac-II		
Non-susceptibility to	**n**	**%**	**n**	**%**	**p-value**
Penicillin	116	36.9	70	30.4	0.121
Erythromycin	118	37.6	77	33.5	0.366
Chloramphenicol	5	1.6	5	2.2	0.750
Tetracycline	109	34.7	62	27.0	0.062
Clindamycin	102	32.5	56	24.3	0.045
Trimethoprim-sulfamethoxazole	134	42.7	49	21.3	<0.001
Total LRT isolates	**314**		**230**		

The proportion of PNSP that were also MDR before vaccination was 87.9%, which reduced to 72.7% in the PostVac-II period (p=0.010; Table 18). Serotype 19F was the predominant PNSP/MDR serotype in LRT samples and 91.8% of PNSP serotype 19F were members of CC236/271/320^{19F} (Table 18, Figure 6 and Table 16). In the PostVac-II period, 8.6% of PNSP were of serotype 35B (one MDR) and 7.1% of serotype 6C and 15A isolates were PNSP (also MDR). MDR pneumococci of serotypes 6C and 15A were 16.4% and 9.1% in the PostVac-II period (Table 18). The sequenced PNSP/MDR serotype 6C isolates were members of CC315$^{6B/C}$ (Figure 6 and Table 16). PNSP of serotypes 6C, 15A and 35B were not detected before vaccination in LRT samples.

NESp isolates that were PNSP increased from 4.3% in the PreVac period to 24.3%, thereof 21.8% also MDR, in the PostVac-II period (p<0.001; Table 18). The sequenced NESp isolates that were PNSP were members of CC344^{NT} (Figure 6 and Table 16).

Table 18. Most prevalent serotypes from LRT samples that were PNSP and PNSP serotypes that were MDR. In the PreVac (2009-2011) and PostVac-II (2015-2017) periods.

	PNSP					PNSP that were MDR				
LRT	PreVac		PostVac-II			PreVac		PostVac-II		
Serotype	n	%	n	%	p-value	n	%	n	%	p-value
6B	8	6.9	5	7.1	1.000	8	6.9	5	7.1	1.000
6C	0	0	5	7.1	0.007	0	0	5	7.1	0.007
14	3	2.6	1	1.4	1.000	2	1.7	1	1.4	1.000
15A	0	0	5	7.1	0.007	0	0	5	7.1	0.007
19F	91	78.4	20[a]	28.6	<0.001	87	75.0	20[a]	28.6	<0.001
35B	0	0	6	8.6	0.003	0	0	1	1.4	0.376
Lsp[b]	9	7.8	11	15.7	Nc[c]	1	0.9	2	2.9	Nc
NESp[d]	5	4.3	17	24.3	<0.001	4	3.4	12	17.1	0.002
Total PNSP/MDR	**116**	**100**	**70**	**100**	**0.121**	**102**	**87.9**	**51**	**72.9**	**0.016**

[a]:Seven isolates of serotype 19F were detected in the same patient in 2017.

[b]Lsp: Less prevalent PNSP.

[c]Nc: Not calculated.

[d]NESp: Non-encapsulated *S.pneumoniae*.

5 Discussion

The main findings of this study are the significant decrease in serotypes targeted by the PHiD-CV vaccine within six years of vaccine introduction in Iceland in healthy children (1 to <7 years of age), children with AOM (0 to <7 years of age) and adults (≥18 years of age) with suspected pneumonia. The total number of cultured pneumococcal isolates from ME and LRT samples decreased after vaccine implementation. This was a consequence of the decrease in VT serotypes, especially serotype 19F, which equally resulted in the decreased burden of pneumococcal disease. Furthermore, the total number of ME samples received by the Department of Clinical Microbiology also decreased after vaccination. The ME samples are usually obtained from children with ruptured tympanic membranes as a result of AOM or from children who have received tympanostomy tube insertion with an effusion from the middle ear due to inflammation. Therefore, it is likely that the decrease in the number of ME samples mirrors a reduction in the burden of AOM (Sigurdsson et al., 2018).

Nasopharyngeal carriage rates did not change significantly overall between the two periods due to serotype replacement with NVT serotypes. However, nasopharyngeal carriage rates decreased significantly in children 4 to <7 years of age in the PostVac period. This decrease is most likely due to significantly lower nasopharyngeal carriage rates in 2012 and 2017 compared to the years 2013 to 2016. Different personnel obtained the nasopharyngeal swabs each year, except for 2014 to 2016 were the same person obtained all samples. Therefore the low carriage rates seen in 2012 and 2017 could be a result of different sampling techniques as nasopharyngeal carriage rates in 2018 were comparable to the years 2013 to 2016 (unpublished data).

The proportion between VT and NVT serotypes in nasopharyngeal carriage before vaccine implementation was quite similar but NVT serotypes increased with time and VT serotypes were extremely rare in the final two years of the study. In ME samples, VT pneumococci had decreased by half already the year after vaccine implementation, however, it must be kept in mind that the majority of ME isolates were obtained from the youngest age group, which would have been eligible for vaccination. The rapid decrease of VT pneumococci in the following year after vaccine implementation highlights the brisk effect of PHiD-CV vaccination on pneumococcal epidemiology. Also,

no VT pneumococci were detected in ME samples in 2015 and 2017 and only two isolates of serotype 19F were detected in 2016.

A significant decrease of VT serotypes was also apparent in LRT samples from adults with suspected pneumonia due to a herd effect within four years of vaccine implementation. High vaccine uptake (Eyþórsson et al., 2017) and good vaccine acceptance (Óskarsson et al., 2015) in Iceland could to some extent explain this prompt decline in VT serotypes in adults following vaccine implementation. High vaccine coverage (>70–80%) within a population has been shown to lead to considerable herd protection (Tsaban & Ben-Shimol, 2017).

5.1 Vaccine serotypes

5.1.1 Serotype 19F

Serotype 19F was the predominant serotype in ME and LRT samples in Iceland before vaccine implementation. Although serotype 19F decreased significantly, it was still the most prevalent serotype in ME samples in the PostVac period. This would be expected for a dominant serotype but the prevalence of serotype 19F decreased constantly with time. Serotype 19F was also found to be more persistent in circulation after vaccination than other VT serotypes. This might be because of the very high prevalence of serotype 19F six years prior to vaccine implementation in both ME and LRT samples, prolonging the time for serotype 19F to be eliminated from the general population. In addition, serotype 19F did not decrease significantly in nasopharyngeal carriage or ME samples in older children, i.e. not vaccinated (4 to <7 years) who probably serve as a latent reservoir for serotype 19F after vaccination. Moreover, increased carriage rates in older unvaccinated children and adults were reported after PCV implementation (van Hoek et al., 2014).

A particularly high number of serotype 19F isolates were found in LRT samples in 2017 compared to the two previous years. This can be explained to some extent by the fact that seven of the 18 isolates in 2017 came from a single 77-year old immunodeficient patient. However, this could also partly be due to fluctuations as has already been documented for serotype 19F in the years following PCV implementation (Hays et al., 2017; Rodrigo et al., 2015; Steens et al., 2015) and increased carriage rates of serotype 19F in unvaccinated children and adults as mentioned above (van Hoek et al., 2014).

The majority of serotype 19F pneumococci from the three sample groups were non-susceptible to penicillin, MDR and were members of CC236/271/320^{19F}, variants of the multidrug resistant PMEN Taiwan^{19F}-14 lineage. The vast decrease of serotype 19F in ME samples resulted in an overall decrease in the prevalence of isolates that were non-susceptible to penicillin and MDR after vaccination. Serotype 19F had a propensity for the middle ear, it was found in almost half of the ME samples in children 1 to <4 years of age before vaccination and was four times more prevalent in children with AOM than in nasopharyngeal carriage. CC236/270/320^{19F} is one of many lineages that contain genes encoding for pili (pilus 1 and pilus 2), which are associated with adherence and likely aid in the attachment to the middle ear mucosa (Bagnoli et al., 2008; Hjalmarsdottir et al., 2015; Sjostrom et al., 2007).

5.1.2 Serotype 6B

Serotype 6B decreased significantly after vaccination in nasopharyngeal samples from healthy children, ME samples from children and LRT samples from adults. The significant decrease of serotype 6B in ME samples was due to the youngest age group (0 to <2 years), which had the highest amount of samples, and was eligible for vaccination. Furthermore, serotype 6B was not detected after 2014 in ME samples from children 0 to <7 years of age.

Even though the number of serotype 6B isolates decreased significantly following vaccination, isolates of serotype 6B that were non-susceptible to penicillin and multidrug resistant did not change in ME and LRT samples. However, it must be noted that there was only a small number of PNSP/MDR serotype 6B isolates detected after vaccination and when taken together with other PNSP/MDR, they represent a relative proportion of the overall PNSP/MDR detected in each sample group in the PostVac period.

The first multidrug resistant pneumococci were detected in the late 1980´s in Iceland. They belonged to the widespread multidrug resistant PMEN Spain^{6B}-2 lineage, CC90^{6B}. This lineage became predominant in the country and accounted for the majority of PNSP/MDR pneumococci in the early 1990s (Kristinsson, 1995; Soares et al., 1993; Vilhelmsson et al., 2000). Almost all of the PNSP/MDR serotype 6B isolates detected in nasopharyngeal carriage and ME samples in this study belonged to CC90^{6B}. However, PNSP/MDR serotype 6B isolates were not detected in healthy children in the last three years of the study and not in ME samples from children after 2012.

All of the PNSP/MDR serotype 6B isolates in LRT samples belonged to CC90^{6B}. PNSP/MDR serotype 6B was rarely detected in the second PostVac period in LRT samples from adults. The five PNSP/MDR serotype 6B recovered all shared identical antimicrobial patterns as the isolates of CC90^{6B} detected in the study. Thus, it is highly likely that the PNSP/MDR serotype 6B isolates detected in the second PostVac period also belonged to CC90^{6B}.

Serotype 6B can be grouped into two classes depending on the sequence content of the *cps* locus, which have been called serotype 6B of class I and class II. The latter class, which was believed to be of a novel serotype at the time of discovery, was designated serotype 6E (Bratcher et al., 2010; Elberse, K. et al., 2011; Mavroidi et al., 2004). However, pneumococci of serotype 6E generate capsular polysaccharides that are indistinguishable from serotype 6B capsular polysaccharides (Burton et al., 2016).

Recently, our group analysed a diverse global dataset of over 1,000 serogroup 6 genomes, which included serogroup 6 isolates from this study, and found that the *cps* locus of the multidrug resistant PMEN Spain^{6B}-2 and PMEN Poland^{6B}-20 lineages consisted of a serotype 6E sequence and had in fact been in circulation worldwide for over three decades (van Tonder et al., 2015).

5.2 Non-vaccine serotypes

Serotypes belonging to the same serogroup as VT serotypes could possibly be affected by the PCVs through cross-protection of VT serotypes. While this is true to some extent, many serotypes belonging to the same serogroup are genetically diverse and can be more related to other serotypes of different serogroups (Bentley et al., 2006).

5.2.1 Serotypes 6A and 19A

Serotype 6A showed a trend towards decreasing in nasopharyngeal carriage in healthy children 1 to <7 years of age while serotype 19A decreased significantly after vaccination. No significant changes were seen in the prevalence of nasopharyngeal carriage of serotypes 6A and 19A when different age strata were analysed, probably due to the small sample size within each age group. Moreover, the prevalence of these two serotypes did not change in children 0 to <7 years of age in ME samples. However, in LRT samples from adults ≥18 years of age, serotype 6A decreased significantly after vaccine implementation but no changes were seen in serotype 19A. Vaccination with PHiD-CV has been shown to reduce the prevalence of

serotype 6A in IPD in children and adults (Naucler et al., 2017) but studies seem to disagree on the efficacy of PHiD-CV against serotype 19A in nasopharyngeal carriage and non-invasive diseases (Best et al., 2016; Brandileone et al., 2016; Setchanova et al., 2017; Vesikari et al., 2016). Nonetheless, temporal and geographical fluctuations in serotype distribution can occur in the absence of vaccine pressure (Harboe et al., 2010) and the high prevalence of serotype 19A seen in the United States following PCV7 vaccination was also noted in countries with no vaccination schedule (Choi et al., 2008; Dagan et al., 2009; Moore et al., 2008; Pai et al., 2005).

PNSP of serotype 19A increased significantly in children in nasopharyngeal carriage and ME samples post vaccination and all of the PNSP of serotype 19A in ME samples were also MDR. This raises the question whether PNSP of serotype 19A will further increase in the next years but PNSP were extremely rare prior to vaccination in adults in LRT samples and were not detected after vaccination. Furthermore, four of the penicillin non-susceptible and MDR serotype 19A isolates detected after vaccine introduction in children were members of CC236/271/320 and ST320^{19A}, related to the multidrug resistant PMEN Taiwan^{19F}-14 lineage. ST320^{19A} has also been shown to contain genes that encode for both pilus 1 and pilus 2 (Metcalf et al., 2016) and could possibly be the result of the PNSP/MDR serotype 19F lineage co-transforming to serotype 19A (Pillai et al., 2009). Furthermore, the four ST320^{19A} isolates showed identical drug resistance patterns as the major MDR serotype 19F isolates in this study, which were members of CC236/271/320^{19F}.

ST320^{19A} emerged following PCV7 implementation in the United States and Spain (Ardanuy et al., 2009; Moore et al., 2008; Pelton et al., 2007) but was present in South Korea before PCV implementation (Choi et al., 2008). The increased prevalence in the PostVac period of PNSP/MDR isolates of serotype 19A in ME samples and PNSP of serotype 19A in nasopharyngeal carriage is alarming as serotype 19A is equally capable to colonise the nasopharynx and cause invasive disease (Shouval et al., 2006; Sleeman et al., 2006), although ST320^{19A} was not detected in IPD cases in Iceland in 2012-2015 (unpublished data). Whether, the increase of PNSP/MDR isolates of serotype 19A is due to ST320^{19A} will require the sequencing of all PNSP/MDR isolates of serotype 19A detected in 2015-2017.

5.2.2 Serotypes 6C, 23B and 23A

The prevalence of serotypes 6C, 23B and 23A increased significantly with time in the PostVac period in nasopharyngeal carriage and ME samples. These findings are in line with findings from other countries (Allemann et al., 2017; Choe et al., 2016; Devine et al., 2017; Hadjipanayis et al., 2016; Kawaguchiya et al., 2016; Millar et al., 2010; Neves et al., 2017; Wouters et al., 2018).

Serotype 6C became highly prevalent in the two older age groups (age 2 to <7 years) in nasopharyngeal carriage and in the two younger age groups (age 0 to <4 years) in ME samples. Although, serotype 6C increased in adults ≥18 years of age, the increase was not significant when different age groups were analysed, which is likely due to the small sample size. Increased prevalence of serotype 6C following vaccination has been especially associated with PHiD-CV implementation into routine childhood vaccination programs as the vaccine does not specifically target serotype 6A, which is thought to provide some cross-protection against serotype 6C (Cooper et al., 2011).

The serotype 6C isolates detected in the present study were mainly of two genetic lineages, CC395^{6C}, that was the major lineage in nasopharyngeal carriage and CC315^{6C}, that was the major lineage in ME and LRT samples. All serotype 6C isolates of CC395^{6C} were fully susceptible to the antimicrobials tested and most of the CC315^{6C} isolates were non-susceptible to penicillin and all were MDR. These findings are consistent with previous reports from other European countries following PCV implementation (Janoir et al., 2014; Lambertsen & Kerrn, 2010; Nahm et al., 2009; Nunes et al., 2009; Rolo et al., 2011; Tocheva et al., 2010). Pneumococcal isolates of CC315^{6C} are closely related to the multidrug resistant PMEN Poland^{6B}-20 lineage, and have evolved through capsular switching, as confirmed by our group (van Tonder et al., 2015).

The MDR serotype 6C isolates in the study displayed the same antimicrobial resistance patterns as those that were sequenced in both sample groups. Although, the number of serotype 6C isolates sequenced was low due to the small sample size at the time, it can be speculated that the MDR serotype 6C isolates detected in the sample groups between 2015-2017 were possibly also members of CC315^{6C}. Whether CC315^{6C} will replace CC236/271/320^{19F} as the dominant MDR genotype in Iceland in the forthcoming years remains to be seen.

Serotype 23F was replaced as the most prevalent serotype in nasopharyngeal carriage by serotype 23B following vaccination. Isolates of serotype 23B increased significantly in children in nasopharyngeal carriage and ME samples between the periods. The majority of serotype 23B isolates in the PostVac period were variants of CC439^{23B}, related to the PMEN Tennessee^{23F}-4 lineage. CC439^{23B} was not detected among serotype 23B isolates before vaccination in nasopharyngeal carriage and ME samples from children and was extremely rare in LRT samples from adults. It is unclear why serotype 23B increased in children and not in adults. However, serotype 23B, is thought to be of a low invasive disease potential and is mostly associated with nasopharyngeal carriage (Varon et al., 2015; Yildirim et al., 2017) but serotype 23B was twice more common in nasopharyngeal carriage than in children with AOM in this study.

Interestingly, one isolate of serotype 23B from ME samples belonged to a VT lineage in the PostVac period, CC156/162, which is closely related to the multidrug resistant PMEN Spain^{9V}-3 lineage that circulated widely in the pre PCV7 era (Gertz et al., 2003). All isolates of CC156/162 detected in ME samples before vaccine introduction were of serotype 9V, which was not detected after vaccination in ME samples. The CC156/162^{9V} isolates were all non-susceptible to penicillin and MDR; in contrast, CC156/162^{23B} was susceptible to all the antimicrobials tested. Furthermore, in this study, CC156/162 expressed several serotypes, 9V, 14, 19F and 23B, which could imply past capsular switching events within this lineage. This is in agreement with other studies that found variants of the PMEN Spain^{9V}-3 lineage to also express these serotypes in the past, along with serotypes 9A, 11A, 15B/C, 19A and 35B (Beall et al., 2006; Beall et al., 2011; Metcalf et al., 2016; Olarte et al., 2017; Porat et al., 2004a). A more recent study from Norway reported CC156/162 mainly to be of serotype 24F post PCV13 introduction in nasopharyngeal carriage (Steens et al., 2015), but PCV introduction appears to favour the spread of previously uncommon genetic lineages and their variants, such as CC315$^{6B/C}$ and CC439$^{23F/B/A}$, as opposed to the emergence of new ones (van der Linden et al., 2015b).

Serotype 23A increased significantly overall in nasopharyngeal carriage and ME samples. This increase was due to the increase in healthy children 1 to<4 years of age and children 0 to<2 years of age with AOM. Although serotype 23A did not increase significantly in LRT samples from adults, an increase in the incidence of serotype 23A in adults ≥65 years of age was clearly noticeable between the PreVac and the second PostVac period.

This is in line with reports of serotype 23A being prevalent in non-bacteraemic pneumonia in older adults (Akata et al., 2017; Shigayeva et al., 2016). The majority of serotype 23A isolates detected in ME and LRT samples and more than half of the samples from nasopharyngeal carriage were detected in the last three study years, therefore the genomic data was scarce. However, all of the sequenced isolates of serotype 23A in the study from all sample groups were variants of CC439^{23A}, related to the PMEN Tennessee^{23F}-4 lineage and over half of CC439^{23A} were of ST42^{23A}.

Non-susceptibility to penicillin was uncommon but the few isolates that were PNSP were detected in 2016 and 2017 in nasopharyngeal carriage and LRT samples, thereof one isolate from LRT was also MDR. Two isolates of serotype 23A were MDR but both were detected in 2013 in ME samples and were of ST42^{23A}. Increased prevalence of MDR serotype 23A has recently been reported following vaccination with PHiD-CV (Setchanova et al., 2017). Furthermore, another study reported the increase of PNSP serotype 23A, which belonged to CC338^{23A}, of the PMEN Colombia^{23F}-26 lineage following vaccination (Su et al., 2015). In contrast, the two penicillin non-susceptible isolates that belonged to CC338 detected in this study in nasopharyngeal carriage and ME samples after vaccination were of serotype 23B. The one CC338^{23B} isolate detected in LRT samples was susceptible to penicillin. However, almost all of the PNSP serotype 23B isolates in all sample groups were detected in the last three study years. Therefore, it would be difficult to guess which CCs these PNSP of serotype 23B would belong to.

5.2.3 Serotype 22F

The serotype 22F isolates detected in the study were members of a single genetic lineage, CC433/ST433^{22F}, regardless of the sample group. Serotype 22F increased significantly in nasopharyngeal samples and was five times more prevalent in nasopharyngeal carriage than in AOM after vaccination but this serotype was not detected in ME samples in the PreVac period. No significant changes in serotype 22F were observed in LRT samples. However, serotype 22F replaced serotype 14 in adult IPD as the most prevalent serotype in Icelandic adults (>40 years of age) from 2012-2015 and all of these isolates were also members of CC433/ST433^{22F} (unpublished data). Thus, the prevalence of serotype 22F isolates in LRT samples from adults could quite possibly increase in the coming years, as a state of serotype equilibrium has probably not yet been reached in the adult population this shortly after the start of childhood vaccination. Further supporting this theory is that serotype 22F has increased in prevalence in

nasopharyngeal carriage and pneumococcal diseases following PCV implementation in countries worldwide (Deng et al., 2016; Kendall et al., 2016; Metcalf et al., 2016; Pichon et al., 2013; van der Linden et al., 2015a).

Serotype 22F seems to be both a successful coloniser of the nasopharynx in children and a frequent cause of IPD in adults. Furthermore, serotype 22F has been shown be of a higher invasive disease potential than other colonising NVT serotypes, such as serotypes 6C, 11A, 15B/C, 23A and 35B (Yildirim et al., 2010).

5.2.4 Serotype 35B

Serotype 35B increased significantly after vaccination in nasopharyngeal carriage but the significance between the two periods for ME samples could not be calculated as serotype 35B was not detected before vaccination. Serotype 35B was seldom detected prior to PCV implementation in the United States but expanded in the PCV7 era and increased significantly in the post PCV13 era in healthy pre-school children and non-invasive disease (Kaplan et al., 2015; Kaur et al., 2016; Martin et al., 2014; Richter et al., 2014). Serotype 35B was found to be almost three times more common in nasopharyngeal carriage in healthy children than in children with AOM in this study and although it did not increase significantly in the PostVac period in adults, it was found to be more common in LRT samples than in ME samples. Serotype 35B is not considered to be of a high invasive disease potential (Croney et al., 2013) but recent work has suggested that serotype 35B may be more prone to cause IPD in adults over 40 years of age than in children under five years of age (Geno et al., 2018). Furthermore, serotype 35B has been associated with substantial biofilm formation in vitro (Domenech et al., 2015). This can potentially facilitate colonisation and mucosal disease, explaining the increased prevalence in nasopharyngeal carriage among healthy children.

PNSP of serotype 35B were reported before PCV implementation in the United States (Beall et al., 2002) and their prevalence has since increased following PCV13 implementation, but the majority of PNSP of serotype 35B were detected in the late PCV13 era (Richter et al., 2013). Isolates of serotype 35B that were non-susceptible to penicillin were not detected in this study in nasopharyngeal carriage and LRT samples in the PreVac period. However, serotype 35B was one of the most common PNSP in both sample groups after vaccination. Almost all PNSP of serotype 35B were detected in

the last three study years in healthy children and all were detected in the last two study years in LRT samples.

The increasing non-susceptibility to penicillin due to serotype 35B in the post PCV era, as seen in this study, has been described (Cohen et al., 2015; Martin et al., 2014; Mendes et al., 2015; Metcalf et al., 2016; Miyazaki et al., 2017; Nakano et al., 2016; Varon et al., 2015; Yildirim et al., 2017) and was mainly associated with CC558^{35B} (Chochua et al., 2017; Metcalf et al., 2016; Nakano et al., 2016; Olarte et al., 2017). There was very limited data regarding the genotype of the PNSP serotype 35B isolates in this study as the majority were detected in the last three study years when no sequencing was done. One of the four PNSP serotype 35B isolates detected in nasopharyngeal samples in 2014 was sequenced and was a member of CC198^{35B} but the distribution of genotypes is likely to vary with geographic location (Olarte et al., 2017). CC198^{35B} was the major lineage among the remaining sequenced serotype 35B isolates, which were all susceptible to penicillin. One isolate of serotype 35B in LRT samples from 2016 was MDR but the emergence of MDR pneumococci of serotype 35B has been reported previously (Metcalf et al., 2016; Olarte et al., 2017; Richter et al., 2014). Whether CC558^{35B} has already emerged among PNSP of serotype 35B in the present study, or if the prevalence of non-susceptibility to penicillin will continue to increase in the future and develop multidrug resistant properties warrants further monitoring.

5.2.5 Capsular switching

Capsular switching is a relatively rare event that occurs naturally between serotypes (Wyres et al., 2013). However, pressure from antimicrobials and vaccination has been shown to influence capsular switching (Beall et al., 2011; Brueggemann et al., 2007). The switching of capsules between genetically related serotypes seems to be more favourable and more common than capsular switching between distant related serotypes (Croucher et al., 2015). This would be beneficial in both evading current PCVs and antimicrobial drugs as resistance genes are commonly transferred with the capsular locus (Chewapreecha et al., 2014; Sabharwal et al., 2014). The increased prevalence of serotypes related to VT serotypes in this study, such as serotypes 6C, 23B and 23A, as mentioned above, is not surprising. The majority of these serotypes after vaccination were members of VT lineages and shared identical antimicrobial resistance patterns, which could be indicative of capsular switching having taken place (Pai et al., 2005). However, if this were to be confirmed, the genetic background of all these isolates would have to be analysed in more detail.

5.2.6 Summary of serotypes

Highly invasive serotypes are more prone to cause invasive disease and are normally not carried for a prolonged time in the nasopharynx of healthy pre-school children (Brueggemann et al., 2003; Yildirim et al., 2010; Yildirim et al., 2017). In contrast, serotypes carried for longer time periods are less invasive but commonly act as opportunistic pathogens causing invasive infections in immunocompromised patients, young children and the elderly (Sjöström et al., 2006).

The replacement serotypes in nasopharyngeal carriage of young children following PCV introduction have been found to be of a relatively lower invasive disease potential when compared to VT serotypes (Yildirim et al., 2017). The serotypes and genetic lineages detected in nasopharyngeal carriage in this study were reflective of serotypes detected in ME samples from children and in LRT samples from adults, although the prevalence differed between the sample groups.

5.3 Non-encapsulated *S. pneumoniae*

NESp isolates were associated with nasopharyngeal carriage in healthy children in both study periods and with LRT samples from adults post vaccination. However, NESp isolates were almost never detected in ME samples from children. The NESp isolates were mostly members of two PMEN lineages, CC448/ST448^{NT}, USA^{NT}-43 and CC344/ST344^{NT}, Norway^{NT}-42. These lineages lack the capsular biosynthetic locus and might be especially well apt for nasopharyngeal colonisation (Hilty et al., 2014). Non-susceptibility to penicillin was solely associated with CC344^{NT}; in contrast, all NESp isolates of CC448^{NT} were susceptible to penicillin. These findings correlate with a large-scale study of NESp isolates collected from 17 geographic locations worldwide (Hilty et al., 2014). One penicillin non-susceptible and MDR NESp isolate from nasopharyngeal carriage was a member of CC62, a variant of the PMEN Netherlands^{8}-33 lineage. All other pneumococcal isolates within CC62 in the study were of serotype 11A, which indicates that this CC62^{NT} isolate was previously an encapsulated strain that lost the capsular locus (Hanage et al., 2006b).

The prevalence of NESp isolates remained similar between the periods in nasopharyngeal carriage but the proportion of MDR NESp isolates increased significantly after vaccination. An increasing trend in the prevalence of NESp isolates was seen in LRT samples but NESp isolates were the most prevalent pneumococci detected in the last three study years. This was mostly

attributed to the significant increase in adults 18 to <64 years of age. In contrast, NESp isolates decreased in adults between the ages of 50-64 years of age in the United States with an equivalent increase in adults ≥65 years of age (Mendes et al., 2015). Furthermore, the prevalence of non-susceptibility to penicillin and MDR also increased significantly in LRT samples.

The increasing drug resistance among NESp isolates is a cause of concern, as NESp are highly recombinant organisms that have the ability to both donate and receive genetic material (Chewapreecha et al., 2014). The likelihood for genetic exchange in the nasopharyngeal niche is high and simultaneous carriage of two or more pneumococcal strains is well known (Dhoubhadel et al., 2014; Hjalmarsdottir et al., 2016; Shak et al., 2013). Therefore, MDR NESp isolates could promote transmission of drug resistance to capsulated pneumococci and become a future burden in pneumococcal diseases, as they are not targeted by current PCVs.

5.4 Strengths and limitations

This was a large study where a total of 5,570 pneumococcal isolates were analysed to assess the effect of PHiD-CV vaccination on serotypes, genetic lineages and antimicrobial susceptibility. This was assessed in nasopharyngeal carriage and disease in children (middle ear) and disease in adults (lower respiratory tract) from a representative part of the Icelandic population before and after vaccine introduction. PCVs were not part of the routine infant immunisation program before PHiD-CV implementation in 2011.

Iceland is a geographically isolated island with a well-defined homogenous population of <350,000 individuals that can reflect other Western European countries, providing favourable circumstances for vaccine impact studies. The Department of Clinical Microbiology is the primary laboratory for the country, serving about 85% of the nation for culture and identification of samples cultured for pneumococci. All pneumococcal isolates are preserved frozen at -80°C, with isolate information (e.g. serotype, antimicrobial susceptibility, genotype) and patient demographic information. Furthermore, repeated carriage studies have been conducted in the same communities, using the same methodology that provide long-term data resulting in a precise evaluation of vaccine effect over time.

The majority of the LRT samples in the study were sputum samples but a fraction could represent colonisation. This is not considered to be a significant problem since pneumococcal colonisation among adults is very uncommon (Hamaluba et al., 2015) and sputum samples are normally not

obtained from asymptomatic healthy individuals but from patients suspected to have pneumonia or have confirmed pneumonia.

Fewer LRT samples were sent to the laboratory in 2010-2014 as physicians were advised to reduce the number of laboratory tests at the Landspitali University Hospital following the financial crisis in 2008. When the effect of the crisis diminished in the subsequent years there was a parallel increase in the number of LRT samples. Regardless, pneumococcal isolates progressively decreased, which was most apparent in adults ≥65 years of age. Older adults (the elderly) are known to benefit greatly from PCV infant immunisation (Rodrigo et al., 2015; Tsaban & Ben-Shimol, 2017) because the immune system weakens with higher age and as a result older adults are more vulnerable to infectious diseases (Nikolich-Zugich, 2018; Simon et al., 2015).

Monitoring serotype distribution and susceptibilities to antimicrobials is crucial for assessing vaccination programs and therapeutic recommendations. Studying vaccine effect at the genetic level provides a deeper understanding on pneumococcal epidemiology, enabling detection of changes that occur between serotype and genotype (Metcalf et al., 2016). Nearly all (almost 95.0%) of the pneumococcal isolates in the study were successfully serotyped, providing an overall serotype distribution in the country. Over 1,700 pneumococcal isolates were subjected to WGS from 2009-2014, from which serotypes and MLSTs were derived. Despite a high number of sequenced pneumococcal isolates, the number of upcoming NVT serotypes sequenced within ME and LRT samples in 2012-2014 was in some cases limited. There is a need to sequence pneumococci isolated in 2015-2017, in order to draw any tangible conclusions on which successive resistant genetic lineages could become prevalent in the future. However, due to financial reasons this was not attainable.

Enhanced surveillance in pneumococcal diseases and serotype distribution on a global scale will not only promote the development of future vaccines, but also help in keeping track with resistance patterns. By applying WGS on a large number of samples within the same bacterial species can offer numerous possibilities for future research with high resolution at the population level. Deepening our understanding on vaccine effect regarding pneumococcal ecology, serotype replacement, antimicrobial resistance, vaccine escape lineages, disease and more.

6 Summary and conclusions

- The total nasopharyngeal carriage rate of pneumococci did not change from the PreVac to PostVac period. This is presumably due to serotype replacement by NVT serotypes.
- Serotype 23B was the most prevalent serotype after vaccination in nasopharyngeal carriage.
- The total number of pneumococcal isolates from ME samples decreased in children following vaccine implementation. The total number of ME samples sent for analysis to the Microbiological laboratory decreased substantially. This is considered to reflect fewer OM cases in children.
- Despite the increasing number of LRT samples with improved economy, the total number of pneumococcal isolates decreased. This herd effect on pneumococcal infections in the LRT became evident three to four years after PHiD-CV implementation in adults.
- VT serotypes decreased after vaccination in all sample groups and NVT serotypes increased in nasopharyngeal carriage and ME samples.
- Serotype 19F was the most prevalent serotype detected in ME samples from children and LRT samples from adults prior to vaccination. Serotype 19F decreased after vaccination in all sample groups but was still the most prevalent serotype in ME samples after vaccination.
- Serotype 19F was the most prevalent PNSP and MDR serotype and all isolates were members of CC236/271/320^{19F}, variants of the PMEN Taiwan^{19F}-14 lineage. Furthermore, multidrug resistance was mainly attributed to isolates of serotype 19F in both study periods.
- Serotypes 22F, 23B, 35B and NESp had a preference for nasopharyngeal carriage but serotype 19F had preference for AOM in children 1 to <4 years of age.
- Serotype 6C was the most prevalent NVT serotype that was PNSP and MDR in all sample groups after vaccination and was a member of CC315^{6C} related to the PMEN Poland^{6B}-20 lineage.
- NESp were the most prevalent pneumococci detected in LRT samples from adults ≥18 years of age in the second PostVac period.

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Original publications

Paper I

Hjálmarsdóttir M.Á.[*], **Quirk S.J.**[*], Haraldsson G., Erlendsdóttir H., Haraldsson Á., Kristinsson K.G. (2017) Comparison of the serotype prevalence of pneumococci isolated from middle ear, lower respiratory tract and invasive disease prior to vaccination in Iceland. ([*]Shared first authorship). PLoS One, 12 (1): e0169210.

PLOS ONE

RESEARCH ARTICLE

Comparison of Serotype Prevalence of Pneumococci Isolated from Middle Ear, Lower Respiratory Tract and Invasive Disease Prior to Vaccination in Iceland

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Abstract

Background

Information on pneumococcal serotype distribution before vaccination is a prerequisite for evaluation of vaccine effect. The aim was to investigate the prevalence of pneumococcal serotypes isolated from middle ear (ME), lower respiratory tract (LRT) and from invasive disease (IPD) in Iceland prior to implementation of ten-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV-10) into the infant vaccination program (April 2011).

Methods and findings

All isolates cultured 2007–2011 from ME, LRT and IPD identified as pneumococci were serotyped and tested for susceptibility at the Clinical Microbiology Department, Landspitali University Hospital that serves approximately 85% of the Icelandic population. Pneumococcal isolates were 1711 and 1616 (94.4%) were available for serotyping and included. Isolates belonging to PHiD-CV10 serotypes (VTs) were 1052 (65.1%). Isolates from ME were 879 (54.4%), with 639 (72.7%) from 0–1 year old patients and 651 of VTs (74%). Isolates from LRT were 564 (34.9%), with 292 (51.8%) from ≥65 years old patients, and 300 (53.2%) of VTs. IPD isolates were 173 (10.7%), although more evenly distributed according to age than isolates from the other sites most were from adults and the youngest age group,101 (58.4%) isolates were of VTs. The most common serotype was 19F, 583 (36.1%). Its prevalence was highest in ME, 400 (45.5%), 172 (30.5%) in LRT and 11 isolates (6.4%), in IPD. Penicillin non-susceptible isolates were 651 (40.3%), mainly belonging to VTs, 611 (93.9%), including 535 (82.2%) of 19F.

Conclusions

Multiresistant isolates of serotype 19F were highly prevalent, especially from ME of young children but also from LRT of adults. Serotype 14 was the most common serotype in IPD.

Competing Interests: Some of the authors (HE, ÁH and KGK) have received a research grant from GSK, to study the effectiveness of pneumococcal vaccination in Iceland. That study is an investigator initiated study with no conflicting interests related to the current study. GSK did not fund this specific study. There are no patents, products in development or marketed products to declare. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

The rate of VTs was high and almost all PNSP were of VTs. There was great difference in vaccine coverage between sampling sites, also reflecting difference in vaccine coverage by age groups.

Introduction

Streptococcus pneumoniae (pneumococcus) causes a range of diseases from mild localized infections like acute otitis media to more severe infections like pneumonia, bacteraemia and meningitis [1]. Pneumococci are also commonly carried in the nasopharynx, especially by young children and spread from there to other parts of the body, or to other individuals [2, 3].

Serotype prevalence is dynamic and depends on many variables, which can be related to pneumococcal properties, host factors, antimicrobial use and vaccination in the community. Furthermore, prevalence may differ according to geographic area and time [4–7]. Serotypes with low invasive potential e.g. 6A, 6B, 14 and 19F most commonly cause non-bacteraemic pneumonia and acute otitis media and are frequently found in the nasopharynx of healthy children [8–10]. Serotypes that are considered to be of high invasive potential, e.g. 1, 4, 7F and 9V, cause invasive disease, but are infrequently found in milder infections and very rarely in carriage [9, 11]. However, serotypes of low invasive potential can be a relatively common cause of invasive disease in high risk individuals, such as young children, the elderly and immunocompromised patients [12].

Pneumococcal disease, both in children and older non-vaccinated individuals, has decreased following childhood vaccinations with protein conjugated vaccines (PCV) and replacement from vaccine to non-vaccine serotypes has been documented [8, 13–15]. At the same time the overall levels of pneumococcal carriage in children remain virtually unchanged, but the types carried are non-vaccine serotypes. [16, 17].

It is important to study serotype prevalence prior to implementation of pneumococcal vaccination programs and to monitor post vaccination changes to evaluate the effect of the vaccine on disease and carriage and changes in the serotype prevalence. The aim was to investigate the prevalence of pneumococcal serotypes isolated from middle ear, lower respiratory tract and from invasive disease in Iceland prior to implementation of ten-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV-10) into the infant vaccination program.

Materials and Methods

Study population

The Department of Microbiology, Landspitali University Hospital, Iceland, serves as the primary microbiology laboratory for the Reykjavik metropolitan area. Furthermore, the laboratory serves as a reference laboratory for the whole country. Inhabitants from other areas of the country often seek health services, both general and specialist services, in the capital and are included in the study. The population of Iceland was 312,872 in 2007 and 318,452 in 2011. In 2011 202,339 inhabitants lived in the metropolitan area. It is estimated that the departments serves 60% of the countryside, thus in that year it served 272,009 (85.4%) inhabitants. Children younger than 7 years old were then 10.1% of the total population and in the metropolitan area 10.7%. (www.statice.is).

The study was approved by the National Bioethics Committee (license no: 12.010.S1).

Vaccination with PHiD-CV10 was initiated in April 2011 thus only part of the children born in that year had received the first vaccinations.

When analysing the data, the patients were divided into the age groups: 0–1, 2–6, 7–17, 18–64 and ≥65 years old.

Bacterial isolates

The study included all clinical isolates from middle ear (ME), lower respiratory tract (LRT) and invasive diseases (blood, cerebrospinal fluid and joint fluid) (IPD) identified as pneumococci during a five year period, 2007–2011, at the Department of Clinical Microbiology, Landspitali University Hospital. Only one isolate of the same phenotype (defined as identical serotype and antibiogram) from the same patient within 30 days was used. When possible, the first isolate indicating the most severe infection was chosen (IPD, LRT and ME respectively). The isolates were stored in tryptose-glycerol broth at -80˚C.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was performed on all isolates by disk diffusion, against oxacillin, chloramphenicol, erythromycin, trimethoprim-sulfamethoxazole, tetracycline and clindamycin, according to the methods of the Clinical and Laboratory Standards Institute (CLSI) [18, 19] and also interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [20]. Screening for penicillin non-susceptibility was done using 1 μg oxacillin disks. All oxacillin sensitive isolates were defined as sensitive to penicillin, but all resistant isolates were tested for minimum inhibitory concentration to penicillin and ceftriaxone using the E-test (AB-Biodisk) [21]. Isolates with penicillin MIC >0.06mg/L were defined as penicillin non-susceptible.

Serotyping

All available isolates were serotyped by conventional methods using the Pneumotest kit and/or latex or coagglutination solutions for single antisera (State Serum Institute, Copenhagen) [22, 23] and /or multiplex PCR [10].

A multiplex PCR panel was designed based on previously published methods [24–27]. The serotypes were selected to include the vaccine serotypes that are included in the PHiD-CV10 (VT), the additional serotypes in the 13-valent vaccine and the most common non-vaccine serotypes (NVT) in Iceland, according to our previous studies. The panel consisted of seven multiplex PCR reactions, each with three to four serotype specific primers pairs, in total 27 serogroups/serotypes, and primers for *cpsA*, for the *cps* locus, and *lytA*, for autolysin, that were used for internal positive control. Furthermore, a series of PCR reactions previously described was used to detect serotypes 6A, B,C, D and to separate 6B and its variant 6Bii in all isolates of serogroup 6 [28, 29].

Statistical analyses

To study association between groups Pearson´s Chi-square and Fisher's exact test were used. P value <0.05 was considered significant.

Results

In total, 1711 pneumococcal isolates fulfilled the criteria of the study and of those 1616 (94.4%) were available for serotyping and were included. Distribution according to specimen site and age group did not differ between the available and the 95 (5.6%) non-available (not stored, or non-viable) except for the yield of available isolates from IPD that was higher (99.4%) than for the other sampling sites.

Table 1. Distribution of all isolates and PNSP isolates according to sampling site and age of the patient and their proportions (%) within age group and sampling site.

Age	ME		LRT		IPD		Total	
group	All n (%)	PNSP n (%)	All n (%)	PNSP n (%)	All n (%)	PNSP n (%)	All n (%)	PNSP n (%)
0–1	639 (72.7)	353 (55.2)	8 (1.4)	3 (37.5)	26 (15.0)	5 (19.2)	**673 (42.7)**	**361 (53.6)**
2–6	209(23.8)	68 (32.5)	25 (4.4)	11 (44.0)	14 (8.1)	0	**248 (23.8)**	**79 (31.9)**
7–17	14 (1.6)	1 (7.1)	13 (2.3)	6 (46.2)	4 (2.3)	0	**31 (1.9)**	**7 (22.6)**
18–64	14 (1.6)	5 (35.7)	226 (40.1)	76 (33.6)	69 (39.9)	5 (7.2)	**309 (19.1)**	**86 (27.8)**
≥65	3 (0.3)	1 (33.3)	292 (51.8)	109 (37.3)	60 (34.7)	8 (13.3)	**355 (22.1)**	**118 (33.2)**
Total	**879 (54.4)**	**428 (48.7)**	**564 (34.9)**	**205 (36.3)**	**173 (10.7)**	**18 (10.4)**	**1616**	**651 (40.3)**

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The isolates from ME specimens were 879/1616 (54.4%) and most were from young children. Isolates from 0–1 year old children more often originated from ME than other specimens ($p<0.0001$). The ME isolates were also more often from 0–1 than 2–6 year old children ($p<0.0001$). LRT isolates were 564/1616 (34.9%) and most were from adults. Isolates from ≥65 year old patients were more commonly from LRT than the other sampling sites ($p<0.0001$). The isolates from IPD specimens were 173/1616 (10.7%), although more evenly distributed according to age than isolates from the other sites most were from adults and the youngest age group (Table 1).

Temporal changes in number of pneumococcal isolates were noted, the highest number of isolates was in 2007, 357 (22.1%) and gradually declined to 283 in 2011 (17.5%).

Prevalence of serotypes

The isolates were of 52 serotypes. The most common was serotype 19F with 583/1616 (36.1%) isolates, followed by 23F with 157/1616 (9.7%) (Table 2). Of all isolates, 1052/1616 (65.1%)

Table 2. Ranking of the eight most common serotypes according to infection site and their proportions (%) and of VTs and NVTs according to sampling site.

ME		LRT		IPD		Total	
Type	n (%)	Type	n (%)	Type	n (%)	Type	n (%)
19F	400 (45.5)	19F	172 (30.5)	14	28 (16.2)	19F	583 (36.1)
23F	111 (12.6)	3	49 (8.7)	19A	17 (9.8)	23F	157 (9.7)
6A	73 (8.3)	6A	42 (7.4)	4	14 (8.1)	6A	122 (7.5)
14	61 (6.9)	23F	40 (7.1)	9V	13 (7.5)	14	117 (7.2)
19A	61 (6.9)	6B	32 (5.7)	7F	12 (6.9)	6B	101 (6.3)
6B	60 (6.8)	14	28 (5.0)	19F	11 (6.4)	19A	99 (6.1)
3	28 (3.2)	NT	23 (4.1)	3	9 (5.2)	3	86 (5.3)
9V	14 (1.6)	19A	21 (3.7)	6B	9 (5.2)	9V	47 (2.9)
Other	71 (8.1)	Other	157 (27.9)	Other	60 (34.7)	Other	304 (18.8)
Total	**879 (54.4)**	**Total**	**564 (34.9)**	**Total**	**173 (10.7)**	**Total**	**1616**
VT-10	651 (74.1)	VT	300 (53.2)	VT	101 (58.4)	VT	1052 (65.1)
NVT-10	228 (25.9)	NVT	264 (46.8)	NVT	72 (41.6)	NVT	564 (34.9)
VT-13	813 (92.5)	VT-13	412 (73.0)	VT-13	134 (77.5)	VT-13	1359 (84.1)
NVT-13	66 (7.5)	NVT-13	152 (27.0)	NVT-13	39 (22.5)	NVT-13	257 (34.9)

n = the number of isolates.

NT = unencapsulated isolates, negative in PCR reactions using *cpsA* and serotype specific primers, positive in *lyt*A reactions. Other possibilities might be serotypes 25F/A, or 38, known for changes in the *cps*A gene causing negative reactions.

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belonged to VT-10 and were significantly more common than non-vaccine serotypes (NVT-10) (P<0.0001).

The ME isolates were of 34 serotypes and the most common was serotype 19F with 400/879 (45.5%) isolates and significantly more common than in LRT and IPD (P<0.0001). The prevalence of the 8 most common serotypes ranged from 45.5–1.6% (Table 2). The proportion of isolates belonging to VTs was highest in ME, 651/879 (74.1%). The LRT isolates were of 45 serotypes and the most common was 19F with 172/564 (30.5%) isolates. The prevalence of the 8 most common serotypes ranged from 30.5–3.7%. The proportion of isolates belonging to VT-10 was lowest in LRT, 300/564 (53.2%). The IPD isolates were of 29 serotypes and the most common serotype was serotype 14 with 28/173 (16.2%) isolates. The prevalence of the 8 most common serotypes ranged from 16.2–4.6% (Table 2). The isolates belonging to VTs were 101/173 (58.4%) (Table 2). Overall, the most common NVTs were 11A, 41 isolates (2.5%) and 33D, 18 (1.1%) isolates.

Antimicrobial susceptibility

Penicillin non-susceptible pneumococcal isolates (PNSP) were 651/1616 (40.3%) (Table 1). The number of PNSP isolates remained stable during the study period, 128–138 isolates each year, while the number of all isolates gradually decreased from 357–283. Accordingly, the PNSP proportions gradually increased from 36.7% in 2007 to 44.9% in 2011, resulting in significant difference between the first and last year (p = 0.035). PNSP isolates of VT-10 were 611/651 (93.9%). Similar temporal changes in the rates of VTs of PNSP were seen but with more fluctuations between years.

PNSP isolates were 428/879 (48.7%) of ME, 205/564 (36.3%) of LRT and 18/173 (10.4%) of IPD isolates. PNSP of VTs were significantly more common in ME than in LRT (p = 0.0001) and also more common than in IPD (p = 0.0025). The VTs were significantly more common than the NVTs among PNSP in all instances (Table 3).

The PNSP were of 18 serotypes with serotype 19F being the most common, 535/651 (82.2%) isolates. Serotype 19F was the most common PNSP serotype in all specimen groups (p<0.0001), most commonly found in ME with 378/428 (88.3%), in LRT 150/205 (73.2%) and in IPD 7/18 (38.9%) of PNSP isolates (Table 3). The majority of the serotype 19F isolates, or 501/535 (93.6%) isolates had identical antibiograms and were multi-resistant. Their penicillin MIC was close to the breakpoint between intermediate and resistant (median MIC 1.0 μg/mL), thus either defined as intermediate or resistant, resistant to erythromycin, tetracycline and trimethoprim-sulfamethoxazole and sensitive to chloramphenicol and clindamycin as previously

Table 3. Ranking of the 4 most common PNSP serotypes according to sampling site.

ME		LRT		IPD		Total	
Type	**n (%)**	**Type**	**n (%)**	**Type**	**n (%)**	**Type**	**n (%)**
19F	378 (88.3)	19F	150 (73.2)	19F	7 (38.9)	19F	535 (82.2)
14	12 (2.8)	NT	13 (6.3)	9V	2 (11.1)	6B	32 (4.9)
6B	19 (4.4)	6B	12 (5.9)	14	2 (11.1)	14	20 (3.1)
19A	7 (1.6)	9V	10 (4.9)	23F	2 (11.1)	9V	14 (2.2)
Other	12 (2.8)	Other	20 (9.8)	Other	5 (27.8)	Other	50 (7.7)
Total	**428 (65.7)**	**Total**	**205 (31.5)**	**Total**	**18 (2.8)**	**Total**	**651**
VT	416 (97.2)	VT	181 (88.3)	VT	14 (77.8)	VT	611 (93.9)
NVT	10 (2.8)	NVT	24 (11.7)	NVT	4 (22.2)	NVT	40 (6.1)

n = the number of isolates

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described [7]. The remaining 34 (6.4%) isolates of PNSP serotype 19F had diverse antibiograms. Isolates of 6B sharing the antibiograms of the previously dominating clone in the country, of the variant 6Bii of serotype 6B [30] were 24 (1.5%).

Discussion

Serotype 19F was the dominant serotype, with more than a third of all isolates and more than four of every five PNSP isolates belonging to 19F. It had a predilection for middle ear where most of the samples originated from young children. The serotype was also the dominant PNSP type and almost all the isolates had identical antibiogram. The vast majority of isolates of PNSP belonged to VTs.

The ME isolates were from patients with perforated tympanic membranes or with tympanic tubes, therefore mainly representing acute otitis media which is most common in children. Accordingly, it must be taken into account that when we compare isolates from the different sites, it not only highlights differences between different diseases but also different age groups. Almost half of the isolates belonged to serotype 19F that was the most common and two thirds of all isolates of serotype 19F originated from ME. The vast majority of the 19F isolates were multi-resistant and shared identical antibiograms with the previously described single and double locus variant of the Taiwan^{19F}-14 clone [7]. Other isolates belonged to serotypes commonly found in otitis media and carriage and isolates with high invasive potential were rarely seen [11, 12]. Besides the exceptionally high rate of 19F our findings were in concordance with other studies [9, 31–36]. The reason for this high rate might be related to a relatively high antimicrobial usage in the country, making the environment favourable for this multiresistant piliated clone [37]. The high prevalence of VTs and PNSP in ME compared to LRT and IPD can mostly be explained with the exceptionally high rate of 19F in ME samples.

The LRT isolates were mainly isolated from sputum samples and bronchoalveolar lavage fluids, specimens that are rarely obtained from children. Therefore, these isolates are mainly from adults, especially the elderly. The age distribution reflects high risk groups for pneumococcal pneumonia except for young children. Close to a third of the isolates belonged to serotype 19F that was the most common, but isolates of high invasive potential were rarely seen. The rate of isolates of VTs was lower in LRT than in the other specimen groups. Most likely this reflects the high diversity of the serotypes found in LRT isolates, lower rate of 19F than in ME and compared to IPD low rate of isolates of high invasive serotypes that are VTs. More than a third of the isolates were PNSP, most of serotype 19F.

About half the IPD isolates were from the youngest and the oldest age groups. The two most common serotypes were 14 and 19A. This is of interest as these serotypes are generally considered to be of a low or moderate invasive potential [12, 38]. The reason for this remains unclear. It may be related to clonal properties as different clones of serotype 14 have been reported to be more prevalent in IPD than carriage and vice versa [12]. Following vaccination with the 7-valent conjugative vaccine serotype 19A was commonly reported in IPD [39, 40]. It is possible that the effect of vaccination in neighbouring countries may have had an indirect effect on serotype distribution in IPD in this country. Another possible explanation for the relatively common IPD by these less invasive serotypes is the patient's age. Young children and older individuals have less competent immune system. In addition, older people may more often be immunocompromised due to co-morbidity or medical therapy. This weak immune system may certainly render the individuals more prone to infections, even with less invasive serotypes [41]. The next three serotypes in the rank, serotypes 4, 7F and 9V are considered highly invasive [4, 11, 42, 43]. The age distribution of the patients diagnosed with the highly invasive serotypes tended to be more evenly distributed which is in concordance with our

explanations and other studies [5, 12]. Serotypes that are commonly carried by children, or known for causing milder disease, were a relatively common cause for IPD [8–10].

The PNSP rates were high and mostly seen in serotypes that are frequent colonizers and more often in ME than in other infections. This is in concordance with other studies showing that antimicrobial resistance is more likely to be found in milder infections than in invasive disease, with the exception of individuals at high risk [44]. The outpatient usage of antimicrobials in Iceland is relatively high and brings selective pressure for resistant clones that along with the properties of successful clones can influence the rates of serotypes [7, 37]. However, the vast majority of PNSP were of VTs and vaccination is therefore likely to reduce the PNSP rates substantially.

In summary, the rates of serotype 19F were very high, especially in isolates from ME that were mostly from young children. 19F was also the dominant serotype in isolates from LRT that were mainly from adults and the elderly. The vast majority of the 19F isolates were multi-resistant, with identical antibiograms. The most common serotype in IPD was serotype 14. The rate of vaccine serotypes was highest in isolates from middle ear and lowest in isolates from lower respiratory tract, where serotype diversity was the most. Penicillin non-susceptible isolates were almost solely of vaccine serotypes. There was great difference in vaccine coverage between sampling sites, also reflecting difference in vaccine coverage by age groups. These results provide important information on serotype distribution and antibiotic resistance prior to immunization and will serve as a basis for evaluation of the effect of infant pneumococcal vaccination.

Supporting Information

S1 Table. This is the full supporting information that includes impersonalized relevant information of specimen aquisition and results.
(XLSX)

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Paper II

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Pneumococcal vaccination: Direct and herd effect on carriage of vaccine types and antibiotic resistance in Icelandic children

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ABSTRACT

Background: Since the introduction of pneumococcal conjugate vaccines, vaccine type pneumococcal carriage and disease has decreased world-wide. The aim was to monitor changes in the nasopharyngeal carriage of pneumococci, the distribution of serotypes and antimicrobial resistance in children before and after initiation of the 10-valent pneumococcal vaccination in 2011, in a previously unvaccinated population.

Methods: Repeated cross-sectional study at 15 day-care centres in greater Reykjavik area. Nasopharyngeal swabs were collected yearly in March from 2009 to 2015. The swabs were selectively cultured for pneumococci, which were serotyped using latex agglutination and/or PCR and antimicrobial susceptibility determined. Two independent studies were conducted.
In study 1, on total impact, isolates from children aged <4 years were included. The vaccine-eligible-cohort (birth-years: 2011–2013, sampled in 2013–2015) was compared with children at the same age born in 2005–2010 and sampled in 2009–2012. In study 2 on herd effect, isolates from older non-vaccine-eligible children (3.5–6.3 years) were compared for the periods before and after the vaccination (2009–2011 vs 2013–2015. Vaccine impact was determined using 1-odds-ratio.

Results: Following vaccination, the vaccine impact on vaccine type acquisition was 94% (95% CI: 91–96%) in study 1 and 56% (95% CI: 44–65%) in study 2. The impact on serotype 6 A was 33% (95% CI: −9%; 59%) in study 1 and 42% (95% CI: 10–63%) in study 2 with minimal effect on 19A. The non-vaccine serotypes/groups 6C, 11, 15 and 23B were the most common serotypes/groups after vaccination. Isolates from the vaccine-eligible-cohort had lower penicillin MICs, less resistance to erythromycin and co-trimoxazole and less multi resistance than isolates from the control-group.

Conclusions: The efficacy of the vaccination on vaccine serotypes was high, and a milder effect on vaccine-associated-serotype 6A was observed for the vaccine-eligible-cohort. There was a significant herd effect on vaccine types in older non-vaccine-eligible children. Overall antimicrobial non-susceptibility was reduced.

1. Introduction

Streptococcus pneumoniae, is an important pathogen in respiratory tract and invasive pneumococcal infections (IPD), especially in children [1,2]. Acute otitis media (AOM) is among the most common reasons for health care visits and the most common reason for antibiotic prescriptions [3] in young children, contributing to antimicrobial resistance [4]. Major risk factors for pneumococcal carriage and antibiotic resistance include young age, crowding, recent upper respiratory tract infection, day-care centre (DCC) attendance, larger family size, passive smoking and low socioeconomic status [5–9]. In addition, recent or current antimicrobial usage temporarily reduces the risk of carriage [7,8]. Nasopharyngeal (NP) carriage of pneumococci is a prerequisite for pneumococcal infections [10]. Therefore, it is important to monitor the

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prevalence of serotypes and antibiotic-resistance in the nasopharynx of children.

Penicillin non-susceptible pneumococci (PNSP) are of worldwide concern and the main risk factors for PNSP infections are young age and recent antibiotic usage [4,5,7,8]. In recent years, serotype 19F has been the most common PNSP and multi-resistant serotype in Iceland [11].

Vaccinations with pneumococcal conjugate vaccines (PCVs) have resulted in a lower incidence of IPD [2,12–16], AOM [2,12,17–19] and pneumonia [2,12,18,20], especially in children. Decrease in nasopharyngeal carriage of vaccine types and replacement by non-vaccine types after implementation of vaccination in childhood vaccinations schedules has been widely reported both in vaccinated children [2,9,12,19,21–26] and children not vaccinated [2,27].

The 10-valent protein conjugated pneumococcal vaccine (PHiD-CV) was added to the childhood vaccination schedule in Iceland in 2011 for all children born that year without catch-up (at 3, 5 and 12 months of age). This population had previously not been routinely vaccinated against pneumococci. In this investigation, we report the results from two analyses (referred to as study 1 and 2). The aim of the first one was to compare pneumococcal carriage rate, serotype distribution and prevalence of antibiotic resistant pneumococci in the nasopharynx of healthy children attending DCC before and after the initiation of the vaccination. The second analysis evaluated the herd effect on unvaccinated children in DCC.

2. Material and methods

The study is an ongoing, repeated cross-sectional study where nasopharyngeal samples are collected annually (in March) from children attending 15 DCCs in the Reykjavik capital area, from 2009 to 2015. The DCCs were selected so they would be representative both geographically and socially for the Reykjavik capital area. The same 15 DCCs participated during the whole study period with only two exceptions, where they were changed for neighbouring DCCs due to scheduling difficulties.

The children attending the DCCs participating in this study are aged from 1.1 years (earliest time of entering DCC) to 6.3 years of age (when leaving DCC and starting primary school). All children attending the DCCs were invited to participate in this study. Signed informed consent forms were obtained and parents were asked to fill in questionnaires including questions on current and previous 30-day usage of antimicrobials and whether the child had been diagnosed with acute otitis media, sinusitis or pneumonia by a physician in the previous six months.

Single nasopharyngeal sample was obtained (COPAN transport medium swabs, Copan, Italy) from each child attending the DCCs on the day of sampling for which informed consent had been obtained. Children could participate more than once if they were still attending the DCC in the later years, at the day of sampling. Although many children participated more than once in the study, these events were considered independent in the main analysis. In an analysis of carriage of 19F, individual data was examined to search for repeated carriage of 19F. The swabs were inoculated within six hours of sampling and selectively cultured for pneumococci on blood agar containing 5 μg/mL gentamicin. The blood agar plates were incubated anaerobically at 35 °C for 18–20 h using anaerobic jars with gaspak envelopes to create a reduction in O_2 and an increase in CO_2 concentrations [28]. Pneumococci were identified by morphology and susceptibility to optochin. Normally, when all colonies appeared identical two to four colonies were selected for antimicrobial susceptibility testing and serotyping. If there were colonies with different morphology [28], additional two to four colonies were selected from each type for susceptibility testing as well as serotyping.

All pneumococcal isolates were tested for antimicrobial susceptibilities using disc diffusion and the EUCAST methods and criteria (www.eucast.org; to erythromycin, clindamycin, tetracycline and trimethoprim/sulfamethoxazole). The isolates were screened for penicillin non-susceptibility with oxacillin discs and penicillin MIC measured for all oxacillin resistant isolates using the E-test (BioMérieux, France). Isolates defined as PNSP were divided into low MIC (0.094–0.5) and high MIC (>0.5) groups. Resistant and intermediate resistant isolates were termed non-susceptible. Multi-resistance was defined as non-susceptibility to at least three different antimicrobial classes.

Serotyping was done as previously described [29] using pneumococcal Latex antisera [30] (Statens Serum Institute, Denmark) [28]. The same senior biomedical scientist did all the agglutinations. When the latex agglutination test did not detect a specific serotype, mono and multiplex PCR was used. The vaccine serotypes (VT) were defined as serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. The Vaccine associated serotypes (VaT) were defined as 6A and 19A. Non-vaccine serotypes (NVT) are those not belonging to VT or VaT.

Information on pneumococcal vaccinations was acquired from The National Vaccination Registry, and the percentage of children from each birth-cohort receiving at least two PCV doses before the age of two, were the following: For; 2003–2007: <1%, 2008: 2.4%, 2009: 7.4%, 2010: 19.2%, 2011: 97.7%, 2012: 98.4%, 2013: 98.7%.

2.1. Statistics

Statistics were done using the statistical software R version 3.3.2. Differences between characteristics of the children, overall carriage prevalence of pneumococci, antimicrobial resistant pneumococci and answers to questionnaires between study groups were tested using two-sided Fisher's exact test and z-test for categorical outcomes and *t*-test for continuous outcomes, using $\alpha = 0.05$ as significance level. Odds ratios and 95% confidence intervals were calculated for the risk of carrying individual and pooled serotypes in the vaccine and non-vaccine groups. The estimation of vaccine impact on acquisition (VEacq) was determined by 1 – OR as elucidated by Rinta-Kokko et al. [31]. Large sample theory was used to construct approximate confidence intervals for VEacq and a hypothesis test for the difference between two VEacq coefficients. When testing the null hypothesis of the VEacq difference being equal to zero the distribution of the test statistic is approximated with a standard normal distribution. For serotypes with zero observations in either of the groups a conservative one-sided 95% confidence interval for vaccine impact was found by replacing the observed zero with one. As some children carried two serotypes the denominator for these analyses were number of children in that group + number of children with dual carriage in that group.

Two independent studies were done (Table 1).

Study 1. For the estimation of the total impact of the vaccination on children eligible for the vaccination, the vaccine eligible cohorts (VEC, children born 2011 and later, sampled in 2013–2015) were compared to the control group (CG, children born 2010 and earlier, sampled in 2009–2012). To attain comparable age distribution, only children <4 years of age were included. To minimize the potential bias caused by the herd effect, CG children sampled in 2013 and later were not included.

Study 2. For the estimation of the herd effect on children not eligible for the vaccination, children born 2010 and earlier were compared before (sampled 2009–2011, PreVac period) and after

Table 1
Study design.

Study	Study groups	Age inclusion criteria	Birth cohort inclusion criteria	Sampling year inclusion criteria	Mean age	Median age (range)	Sample size
Study 1: total impact	CG	<4 years	2005–2010	2009–2012	2.9	2.9 (1.2–4.0)	853
	VEC	<4 years	2011–2013	2013–2015	2.8	2.8 (1.1–4.0)	371
Study 2: herd effect	PreVac	3.5–6.3 years	2003–2007	2009–2011	5.0	5.0 (3.6–6.3)	847
	PostVac	3.5–6.3 years	2007–2010	2013–2015	5.0	5.0 (3.6–6.2)	949

Two independent studies were conducted.
In Study 1 (Total impact), only children less than 4 years of age were included. Children born before the vaccine initiation (2010 and earlier, Control Group, CG), sampled in 2009–2012 were compared to children born after the vaccine initiation (2011 and later, Vaccine Eligible Cohort, VEC).
In Study 2 (Herd effect), only children between the ages 3.5 years and 6.3 years, born before the vaccination (birth-cohort 2010 and earlier) were included. Children sampled in 2009–2012 were compared to those sampled in 2013–2015.

(sampled 2013–2015, PostVac period) the vaccination. To attain comparable age distribution only children >3.5 and <6.5 years of age were included. Isolates sampled in 2012 were excluded as they belonged to the transition year. Sixteen children, born in 2007 were sampled both in 2011 and 2013, were excluded from the analysis.

In analysis of antibiotic non-susceptibility, non-typeable pneumococci were excluded, as they are not considered an important cause of disease in immunocompetent hosts.

The study was approved by The National Bioethics Committee (VSNb2013010015/03.07), The National Data Protection Authority (2013010100VEL/–), The University Hospital medical director and the appropriate directorates of the DCC's. The study is a part of a larger study on vaccinations in Iceland (The VIce study).

3. Results

3.1. Study on the impact on the vaccine eligible cohorts

The number of children was 853 in the CG and 371 in the VEC. The average age in the CG was 2.89 and 2.80 in the VEC ($p < 0.05$), the median age and ranges were 2.90 (1.15–4.00) and 2.80 (1.10–4.00) (Table 1). Males were 51.4% and 53.7% ($p = 0.45$) of children in CG and VEC respectively. Dual carriage was 7.5% in the CG and 4.3% in the VEC ($p = 0.051$). No difference was found in overall carriage prevalence of pneumococcus (69.5% vs 70.1%, $p = 0.84$). Parent-reported recent antibiotic use was lower in the VEC group than in the CG (17.8% vs 23.2%, $p < 0.05$), with no difference in parent-reported respiratory tract infections (RTIs) in the previous 6 months (43.8% vs 41.8%, for the CG and VEC respectively, $p = 0.54$).

The vaccine impact for VT carriage was 94% (95% CI: 91–96%), with reduction of individual serotypes ranging from 84% to 100%. The combined impact against the VaT (6A and 19A) was 33% (95% CI: 1–55%), with non-significant individual impact being 33% (95% CI: −9%; 59%) and 29% (95% CI: −31%; 61%) for 6A and 19A respectively (Tables 2 and 5). Serotype replacement was noted with non-vaccine serotypes being more common in the VEC than CG (53% vs 18%, respectively). Serogroup 15 was the most prevalent isolate in the VEC, 9.6% compared to 3.9% in the CG. The greatest difference was noted for Serotype 23B (0.8% vs 7.8% for CG and VEC, respectively) (Table 2).

The prevalence of PNSP isolates in CG and VEC was similar. When comparing these groups after stratifying the MIC into low vs high MIC a significant difference was found between the CG and VEC, where the isolates from the VEC were mainly in the low MIC range and the CG mainly in the high MIC range (Table 3).

The prevalence of co-trimoxazole and erythromycin resistance was higher in the CG than VEC, 22.1% and 13.1% vs 12.1% ($p < 0.001$) and 9.0% ($p < 0.05$) respectively. The prevalence of clindamycin, tetracycline, erythromycin and penicillin non-susceptible strains did not differ between the two cohorts. Resistance to ≥ 3 antibiotic classes were more common in the CG than the VEC 9.4% vs 1.6% respectively ($p = 0.003$). Isolates that showed non-susceptibility to all antibiotics tested (penicillin, erythromycin, co-trimoxazole, tetracycline and clindamycin) were also more common in the CG, 4.4% vs 0.3% ($p < 0.001$) respectively (Table 3). Serotypes/groups non-susceptible to ≥ 3 antibiotic classes belonged to serotypes 19F (n = 73), 6B (n = 6), 14 (n = 2) and 6C (n = 1) in the CG and 15 (n = 8), 19F (n = 5) and 6C (n = 3) in the VEC. Isolates non-susceptible to all five antibiotics tested belonged to serotypes 19F (n = 30), 6B (n = 6) and 14 (n = 2) in the CG and 19F (n = 1) in the VEC.

3.2. Study on the herd effect

There were 831 children in the pre-vaccination (PreVac) period and 933 in the post vaccination (PostVac) period. The average age in the PreVac period was 4.97 and 4.98 in the PostVac period ($p = 0.83$) and median ages (with ranges) were 4.96 (3.59–6.33) and 5.00 (3.59–6.22) respectively (Table 1). Males were 49.4% and 51.2% of participants in PreVac and PostVac respectively. Dual carriage was seen in 5.8% and 5.0% respectively ($p = 0.56$). No difference was found in overall carriage (62.6% vs 64.4%, $p = 0.42$ for PreVac period and PostVac period, respectively).

For the PostVac the vaccine impact against acquisition of VT was 56% (95% CI: 44–65%) and 33% (95% CI: 7–51%) for 6A and 19A. A significant VEacq was found for 6A and VTs 6B, 9V, 14, 18C and 23F. (Tables 4 and 5). Further analysis of the 19F carriage prevalence showed stable prevalence over the study-period of study 2 with 4.2%, 2.7%, 4.7%, 4.2%, 4.5% and 3.1% of isolates being 19F for the study years 2009, 2010, 2011, 2013, 2014 and 2015 respectively. Of the 39 isolates of serotype 19F identified in the PostVac period, seven were sampled from children that carried 19F more than once, and up to 3 years in-a-row. Vaccine serotypes 1, 5 and 7F were not isolated. Serotype replacement was noted, but no change in total NP carriage. Non-vaccine serotypes increased from 24.5% of all isolates in the PreVac to 42.6% in the PostVac. Serotypes 6C, 10, 21, 22, 23A, 23B, 35B and 35F/47F all differed significantly between the periods and were more common in the PostVac period. Serotype 23B differed the most between the periods, 0% vs 4.5% in the PreVac and PostVac, respectively (Table 4).

The prevalence of PNSP was 5.0% in both study periods ($p > 0.99$). The prevalence of erythromycin, clindamycin, tetracycline, combined penicillin and erythromycin and multi resistance did not differ significantly between the PreVac and PostVac periods (6.2% vs 4.4%, $p = 0.12$, 3.6% vs 2.5%, $p = 0.26$, 5.2% vs 5.1%, $p = 0.97$, 4.2% vs 3.7%, $p = 0.70$ and 5.0% vs 3.3%, $p = 0.09$, respectively). The prevalence of co-trimoxazole non-susceptibility was significantly higher in the PreVac than in the PostVac (13.4% vs 8.3%, $p < 0.001$).

19F was the dominant serotype causing penicillin non-susceptibility and multi-resistance in both periods, causing 66.7%

Table 2
PHiD-CV vaccine impact estimates by serotype, for vaccine eligible cohort compared to control cohort.

Serotype		Number of CG isolates (%)	Number of VEC isolates (%)	VEacq (95% CI)	p-value
VT	VT total	322 (35.1)	12 (3.1)	0.94 (0.91; 0.96)	<0.001
	6B	77 (8.4)	2 (0.5)	0.94 (0.84; 0.98)	<0.001
	9V	3 (0.3)	0 (0)	1.00 (−6.57; 1.00)λ	0.26
	14	57 (6.2)	0 (0)	1.00 (0.85; 1.00)λ	<0.001
	18C	6 (0.7)	0 (0)	1.00 (−2.04; 1.00)λ	0.11
	19F	84 (9.2)	6 (1.6)	0.84 (0.67; 0.93)	<0.001
	23F	95 (10.4)	4 (1)	0.91 (0.80; 0.96)	<0.001
VAT	VAT total	122 (13.3)	36 (9.3)	0.33 (0.01; 0.55)	0.04
	6A	76 (8.3)	22 (5.7)	0.33 (−0.09; 0.59)	0.1
	19A	46 (5)	14 (3.6)	0.29 (−0.31; 0.61)	0.27
NVT	NVT total	169 (18.4)	207 (53.5)	−4.09 (−5.53; −2.97)	<0.001
	3	14 (1.5)	11 (2.8)	−0.89 (−3.14; 0.14)	0.11
	6C	8 (0.9)	25 (6.5)	−6.85 (−14.6; −2.94)	<0.001
	10	0 (0)	8 (2.1)	Not calculated	
	11	32 (3.5)	32 (8.3)	−1.49 (−3.07; −0.53)	<0.001
	15	36 (3.9)	37 (9.6)	−1.59 (−3.10; −0.63)	<0.001
	16F	9 (1)	2 (0.5)	0.48 (−1.37; 0.88)	0.4
	21	4 (0.4)	10 (2.6)	−5.05 (−15.9; −1.17)	<0.001
	22	2 (0.2)	5 (1.3)	−4.99 (−24.5; −0.41)	0.02
	23A	19 (2.1)	18 (4.7)	−1.31 (−3.37; −0.22)	0.01
	23B	7 (0.8)	30 (7.8)	−9.92 (−20.5; −4.56)	<0.001
	29/35B	5 (0.6)	9 (2.3)	−3.34 (−10.9; −0.58)	0.004
	33	13 (1.4)	2 (0.5)	0.64 (−0.51; 0.91)	0.16
	35F/47F	0 (0)	10 (2.6)	Not calculated	
	38	8 (0.9)	1 (0.3)	0.71 (−1.09; 0.96)	0.22
	Other	7 (0.8)	6 (1.6)	−1.05 (−5.00; 0.30)	0.19
	NT	47 (5.1)	22 (5.7)	−0.12 (−0.88; 0.34)	0.68
	NONE	261 (28.5)	111 (28.7)	−0.01 (−0.32; 0.22)	0.94
Total		917 (100)	387 (100)		

Direct Vaccine impact on carriage prevalence. NVEC: Non-Vaccine eligible cohort, CG: Control group. Children in the CG sampled 2013 and later were excluded to prevent possible bias caused by herd immunity. λ Conservative one-sided 95% confidence interval for vaccine impact. The total number is higher than the number of children because some children carried more than one serotype.

Table 3
Antibiotic non-susceptibility for vaccine eligible cohort compared to non-vaccine eligible cohort.

Non-susceptibility to:	Number CG isolates (%)	Number VEC isolates (%)	OR (95% CI)	p value
PNSP (all isolates)	97 (11.2%)	38 (10.4%)	0.92 (0.62; 1.38)	0.76
Penicillin (Low MIC)	9 (1.0%)	33 (9.0%)	9.48 (4.54; 20.3)	<0.001
Penicillin (High MIC)	88 (10.1%)	5 (1.4%)	0.12 (0.047; 0.31)	<0.001
Erythromycin	114 (13.1%)	33 (9.0%)	0.66 (0.44; 1.00)	0.05
Tetracycline	95 (10.9%)	31 (8.5%)	0.76 (0.48; 1.16)	0.22
Clindamycin	54 (6.2%)	26 (7.1%)	1.15 (0.70; 1.16)	0.61
Co-trimoxazole	192 (22.1%)	44 (12.1%)	0.48 (0.34; 0.69)	<0.001
Penicillin and Erythromycin	82 (9.4%)	30 (8.2%)	0.86 (0.55; 1.33)	0.59
≥3 antibiotic classes	82 (9.4%)	16 (4.4%)	0.44 (0.25; 0.77)	0.003
All 5 tested antibiotics	38 (4.4%)	1 (0.27%)	0.06 (0.003; 0.37)	<0.001
Number of isolates	869	365		

Antimicrobial non-susceptibility in the CG and the VEC groups. For isolates with penicillin non-susceptibility non-typeable pneumococci were excluded. *PNSP penicillin non-susceptible pneumococci. The denominator signifies the number of isolates (number of children sampled + number of children with dual carriage). CG: Control Group. VEC: Vaccine Eligible Cohort.

(28/42) of penicillin non-susceptibility and 64.3% (27/42) of multi-resistance in the PreVac period and 45.8% (22/48) of PNSP and 71.0% (22/31) of multi-resistance in the PostVac period.

The PreVac period reported 6.2% higher RTIs in the last 6 months (27.6 vs 21.4%, p = 0.003) and 3.3% more antibiotic usage in the last 3 months than the PostVac (12.2 vs 8.9%, p = 0.02).

4. Discussion

The vaccine impact, determined as a reduction of VT pneumococci in the nasopharynx was very high and some VT serotypes were not detected in the VEC and thus arguably eliminated from the nasopharynx by the vaccination. This was also seen for unvaccinated children after the initiation of the vaccination – although with lower impact, indicating an important herd effect. This is in line with other studies on the PCVs which have showed reduction in VT pneumococci [9,15,19,21–24,26,32]. Interestingly, despite strong direct effect on carriage of 19F, no obvious herd effect was observed. As 19F is the most antibiotic resistant serotype in this study its persistence in older, unvaccinated children is of concern. Of the 40 children carrying 19F in the PostVac period, seven carried the serotype, at one to three sampling-occasions. Persistent, continuous low level carriage of 19F has been described in older children after initiation of PCV [33].Waning immunity in older children [34] and longer duration of carriage may be important factors for this persistent or repeated carriage of 19F [35–37].

Carriage was similar as in earlier studies conducted in Iceland [5,7] but higher than most other studies [9,21,22]. This high carriage can partly be due to the study design, as all isolates were plated within six hours and cultured on selective medium under

Table 4
Indirect PHiD-CV vaccine impact estimates by serotype for PreVac compared to PostVac.

Serotype		PreVac period (%)	PostVac period (%)	VEacq (95% CI)	p value
VT	VT total	219 (24.91)	125 (12.76)	0.56 (0.44; 0.65)	<0.001
	4	2 (0.23)	0 (0)	1.00 (−3.65; 1.00)λ	0.14
	6B	51 (5.8)	27 (2.76)	0.54 (0.27; 0.71)	0.001
	9V	13 (1.48)	0 (0)	1.00 (0.68; 1.00)λ	<0.001
	14	26 (2.96)	14 (1.43)	0.53 (0.10; 0.75)	0.02
	18C	28 (3.19)	10 (1.02)	0.69 (0.38; 0.84)	<0.001
	19F	34 (3.87)	39 (3.98)	−0.03 (−0.65; 0.36)	0.90
	23F	65 (7.39)	35 (3.57)	0.54 (0.30; 0.69)	<0.001
VAT	VaT total	95 (10.81)	74 (7.55)	0.33 (0.07; 0.51)	0.01
	6A	50 (5.69)	32 (3.26)	0.44 (0.12; 0.64)	0.01
	19A	45 (5.12)	42 (4.29)	0.17 (−0.28; 0.46)	0.40
NVT	NVT total	215 (24.46)	417 (42.55)	−1.29 (−1.79; −0.88)	<0.001
	3	58 (6.60)	46 (4.69)	0.30(−0.04; 0.53)	0.07
	6C	4 (0.46)	18 (1.84)	−3.09 (−10.17; −0.50)	0.006
	9A/N/L	9 (1.02)	13 (1.33)	−0.30 (−2.05; 0.45)	0.55
	10	4 (0.46)	13 (1.33)	−1.94 (−7.60; −0.01)	0.05
	11	42 (4.78)	42 (4.28)	0.11 (−0.38; 0.42)	0.61
	15	27 (3.07)	45 (4.60)	−0.52 (−1.46; 0.06)	0.09
	16F	14 (1.59)	8 (0.82)	0.49 (−0.20; 0.78)	0.12
	21	5 (0.57)	32 (3.27)	−4.90 (−12.6; −1.56)	<0.001
	22	5 (0.57)	39 (3.98)	−6.25 (−15.2; −2.25)	<0.001
	23A	16 (1.82)	33 (3.37)	−0.88 (−2.41; −0.04)	0.04
	23B	0 (0)	44 (4.49)	Not calculated	
	33	5 (0.57)	10 (1.02)	−0.80 (−4.21; 0.38)	0.28
	35B	3 (0.34)	26 (2.65)	−6.96 (−20.1; −1.89)	<0.001
	35F/47F	0 (0)	31 (3.16)	Not calculated	
	38	12 (1.37)	5 (0.51)	0.63 (−0.01; 0.87)	0.05
	Other	11 (1.25)	12 (1.2)	0.02 (−1.23; 0.57)	0.96
	NT	38 (4.32)	33 (3.37)	0.23 (−0.24; 0.52)	0.28
	NONE	312 (35.50)	331 (33.78)	0.07 (−0.12; 0.24)	0.44
Total		879 (100)	980 (100)		

Indirect impact of the vaccination. PreVac period vs the PostVac period (study years: 2009–2011 vs 2013–2015). Only isolates from children born 2010 and earlier & >3.5 years of age were included. λ Conservative one-sided 95% confidence interval for vaccine impact. The total number is higher than the number of children due to some children carrying more than one serotype.

Table 5
Vaccine impact of study 1 and study 2.

Study	Impact on overall pneumococcal carriage (95% CI)	Impact against vaccine types (95% CI)	Impact against vaccine associated types (95% CI)		
			Overall	6A	19A
Study 1	1% (−32; 22%)	94% (91; 96%)	33% (1; 55%)	33% (−9; 59%)	29% (−31; 61%)
Study 2	7% (−12; 24%)	56% (44; 65%)	33% (8; 51%)	44% (12; 64%)	17% (−28; 46%)

Impact on carriage prevalence in study 1 and in study 2. In study 1 Vaccine Eligible Children (birth-year 2011 and later) were compared to the Control Group (birth-year 2010 and earlier) and in study 2 older, Non-Vaccine-Eligible children (born 2010 and earlier) were compared before (calendar year: 2009–2011) and after the vaccination (calendar year: 2013–2015).

anaerobic conditions. In addition, the isolates were sampled in March every year when carriage may be higher during and shortly after the frequent winter infections in this age group [8,38].

The total nasopharyngeal carriage remained high throughout our study despite the reduction of VT. This can be attributed to serotype replacement with NVTs. Serotype replacement has been widely recognized after introduction of the pneumococcal vaccines [9,12,21–26]. In the current study, non-vaccine serotypes/groups most prevalent in the post vaccination years were 15, 11, 23B, 23A, 6C all with large increases in prevalence. This is similar to other studies on PHiD-CV and PCV-13 [9,19,21,25,32], although studies on PCV-13 generally show no difference or reduction in the prevalence of 6C. This indicates that replacing serotypes in Iceland are similar to others using the higher valent vaccines.

For the VEC, a non-significant reduction was found for serotypes 6A and 19A, when considering each serotype individually, but with a significant reduction in the combined prevalence. This is interesting primarily due to the magnitude of serotype replacement witnessed with other serotypes. Similar results were seen for the PostVac period, which had significant herd effect on the carriage of 6A but not 19A. Various studies have shown an increase in the prevalence of 19A after the implementation of the PCV-7 vaccine, due to serotype replacement [15,21,23,24,32,39]. The PHiD-CV direct effectiveness against 19A IPD has been reported in recent years [2], with data suggesting similar direct effectiveness as the PCV-13 vaccine [40]. Similar findings, albeit milder have been reported for 6A IPD [13,19] and carriage of both 6A and 19A [2,19], although other studies show less or no effectiveness against acquisition [39,41,42]. This apparent discrepancy, where more consistent data on 19A IPD effectiveness is available than for carriage, could be explained by the fact that the protection against IPD is more complicated than can be explained by acquisition alone [10].

This study shows replacement of serotypes non-susceptible to penicillin, with the replacing serotypes exhibiting lower MIC. This has been reported after the introduction of PCV-7 [23].

Pneumococcal isolates cultured from the VEC had less erythromycin and co-trimoxazole resistance and were less often multi-resistant as compared to CG. The reduction noted for the VEC was mainly driven by the reduction in serotype 19F which was the main PNSP and multi-resistant serotype in the pre-vaccination period.

Due to a lack of herd effect on serotype 19F in the PostVac cohort, as discussed above, no change in PNSP or multi resistance was noted, but there was a reduction in co-trimoxazole resistance.

The resistant and multi-resistant NVTs, mainly of serogroup 15, but also serotypes 6C and 23B, are emerging in the post vaccination period. Reports on serogroup 15 have shown it to be one of the most prevalent resistant serogroups in many parts of the world [32,43]. Other studies have shown a decrease in non-susceptible pneumococci after vaccination [9,22,44] or even no effect on antibiotic resistance [45]. Many factors may influence this, the most important being the level of antibiotic resistance in both the vaccine serotypes and the replacing serotypes, the selective pressure from antibiotic usage, the level of day-care attendance, vaccine uptake in the study population in addition to study size and design [5–9].

Different capsular types have varying invasive potential [10,46]. Replacing invasive [46], antibiotic resistant vaccine types with more benign ones may result in a reduction in pneumococcal infections and treatment failures. Parent-reported antibiotic usage by children in the VEC was lower than by children in the CG, yet with no difference in RTIs. In the herd effect analysis, children in the PostVac period had less antibiotic consumption and fewer recent RTIs than those in the PreVac period. As this is parent-reported and thus prone to recall bias, conclusions on vaccine impact on RTI must be drawn with caution. The reduction noted here is however in line with other studies that show fewer infections after PCVs [12–18,20].

The strength of this study is its size, its well-defined groups and high vaccine uptake. All culturing and serotyping was done at the same laboratory, using exactly the same methods and protocols throughout the study period. The weakness is that it is a pre-post analysis where the groups compared are sampled in different years, and subject to different external factors, such as fluctuations in serotype prevalence and incidence of viral infections that influence both pneumococcal carriage and disease. This cannot be easily controlled in a study such as this and must be acknowledged as a source of potential bias. However, the study periods compared are directly adjacent to each other minimizing such differences.

Studies like this one, with monitoring of serotype prevalence, serotype replacement and antibiotic resistance are important for public health policies and treatment guidelines. Moreover, they add to the important knowledge base needed for deciding which serotypes should be included in the next generation of pneumococcal vaccines. Importantly, it shows that the vaccination has great impact against all VT in vaccinated children in addition to herd effect on unvaccinated children contributing to lowering antimicrobial resistance rates.

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Paper III

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EPIDEMIOLOGY

Effect of Vaccination on Pneumococci Isolated from the Nasopharynx of Healthy Children and the Middle Ear of Children with Otitis Media in Iceland

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ABSTRACT Vaccination with pneumococcal conjugate vaccines (PCVs) disrupts the pneumococcal population. Our aim was to determine the impact of the 10-valent PCV on the serotypes, genetic lineages, and antimicrobial susceptibility of pneumococci isolated from children in Iceland. Pneumococci were collected between 2009 and 2017 from the nasopharynges of healthy children attending 15 day care centers and from the middle ears (MEs) of children with acute otitis media from the greater Reykjavik capital area. Isolates were serotyped and tested for antimicrobial susceptibility. Whole-genome sequencing (WGS) was performed on alternate isolates from 2009 to 2014, and serotypes and multilocus sequence types (STs) were extracted from the WGS data. Two study periods were defined: 2009 to 2011 (PreVac) and 2012 to 2017 (PostVac). The overall nasopharyngeal carriage rate was similar between the two periods (67.3% PreVac and 61.5% PostVac, $P = 0.090$). Vaccine-type (VT) pneumococci decreased and nonvaccine-type (NVT) pneumococci (serotypes 6C, 15A, 15B/C, 21, 22F, 23A, 23B, 35F, and 35B) significantly increased in different age strata post-PCV introduction. The total number of pneumococci recovered from ME samples significantly decreased as did the proportion that were VTs, although NVT pneumococci (6C, 15B/C, 23A, and 23B) increased significantly. Most serotype 6C pneumococci were multidrug resistant (MDR). Serotype 19F was the predominant serotype associated with MEs, and it significantly decreased post-PCV introduction: these isolates were predominantly MDR and of the Taiwan^{19F}-14 PMEN lineage. Overall, the nasopharyngeal carriage rate remained constant and the number of ME-associated pneumococci decreased significantly post-PCV introduction; however, there was a concomitant and statistically significant shift from VTs to NVTs in both collections of pneumococci.

KEYWORDS Iceland, *Streptococcus pneumoniae*, carriage, epidemiology, molecular epidemiology, otitis media, pneumococcus, vaccination, vaccine

Streptococcus pneumoniae is an important human pathogen that can cause relatively mild upper respiratory tract infections, such as acute otitis media (AOM), or more severe infections, such as pneumonia and invasive pneumococcal disease (IPD) (1). Pneumococci frequently colonize the nasopharynges of humans, especially children

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attending day care centers (DCCs) (2, 3), and asymptomatic carriage occurs at least once prior to the age of 2 years (4). Nasopharyngeal colonization precedes pneumococcal disease, is a major factor for horizontal transmission within the community, especially among young children (5), and reflects the pneumococcal strains circulating in the community (6). Pneumococci are among the most frequent causes of bacterial AOM (7–9), which is the most common bacterial infection in children under 3 years of age (10). Pneumococcal conjugate vaccines (PCVs) interrupt the transmission of antibiotic-resistant pneumococci and thus decrease the burden of disease caused by antibiotic-resistant isolates in immunized children. Therefore, studies of the impact of these vaccines on antibiotic resistance and serotype distribution should focus both on pneumococci from nasopharyngeal carriage and from middle ears of children of similar ages with AOM (11).

Current PCVs target only a limited number of serotypes, especially those commonly causing IPD in young children (12–14). PCVs have been implemented in the infant vaccine immunization program in over 100 countries (15), which has resulted in a decrease of IPD caused by vaccine serotypes (VTs) in vaccinated children and other age groups due to herd immunity (16–18). Among healthy children, PCV implementation has also resulted in serotype replacement in carriage where VTs have been replaced with nonvaccine serotypes, meaning that the total carriage rate has remained constant (19–21). Pneumococcal carriage has been monitored and recorded in Iceland for the past 2 decades (22–24). The carriage rates have been consistent over time, ranging from 50% to 70% in healthy preschool children 1 to <7 years of age (25). The 10-valent PCV (PHiD-CV [Synflorix; GSK]), which directly targets 10 serotypes, was introduced into the national pediatric immunization program in Iceland in April 2011 in a 2-plus-1 schedule without catch-up. No other pneumococcal vaccine was previously included. The aim of this study was to assess the impact of PHiD-CV on the distribution of pneumococcal serotypes and genetic lineages among pneumococci from the nasopharynges of healthy children and the middle ears of children with otitis media and assess any changes in antimicrobial resistance rates after vaccine implementation.

MATERIALS AND METHODS

DCC study and bacterial isolates. Nasopharyngeal swabs were taken from healthy children 1 to <7 years of age in March every year from 2009 to 2017 after informed consent was obtained from the parents. The children were attending 15 DCCs, chosen to be representative of the greater Reykjavík area. The period 2009 to 2011 was defined as the prevaccination period (PreVac), and the years 2012 to 2017 were the postvaccine implementation period (PostVac). The number of nasopharyngeal swabs taken from each age group is listed in Table S1 in the supplemental material. The samples were selectively cultured for pneumococci on blood agar containing 5 μg/ml gentamicin and incubated anaerobically (26). Very few children attended public DCCs before the age of 12 months, and no children <1 year of age were sampled in this study.

Middle ear study and bacterial isolates. All pneumococci isolated from middle ear (ME) samples from children 0 to <7 years of age with otitis media submitted to the Department of Clinical Microbiology, Landspitali University Hospital, between 1 January 2009 and 30 September 2017 were included in this study. This is the primary microbiology laboratory for the greater Reykjavík capital area. It provides services for individuals from the rural area and visiting specialists in the capital, and it is the reference laboratory for the whole country (approximately 85% of the population for ME samples).

The primary service area for the Landspitali University Hospital was considered to be within 100 km driving distance from the hospital, and the population demographic information for this referral region was obtained from Statistics Iceland (www.statice.is). The average population sizes for children 0 to <7 years of age in the referral region during the study period were 23,747 children PreVac and 24,083 children PostVac (approximately 66% of all children in Iceland 0 to <7 years of age). A detailed listing of the populations according to age groups can be seen in Table S2. When two or more pneumococcal isolates of the same phenotype were identified from the same patient within 30 days, only one isolate was included in the analyses.

Serotyping. Serotypes were determined for all available isolates with the Immulex pool antisera (State Serum Institute, Copenhagen, Denmark) and/or by multiplex PCR (mPCR), according to previously published methods (27–30). The mPCR scheme included 78 sets of serogroup/serotype-specific primer pairs and two primer pairs for a positive internal control: *cpsA* for the capsular locus and *lytA* for autolysin. Serotypes of serogroup 6 were identified using previously described PCR methods (31–33). Nontypeable (nonencapsulated *S. pneumoniae* [NESp]) isolates, i.e., those that were negative for *cpsA* and positive for *lytA*, were tested for the *cpsB* gene, which is essential for capsulation (34), according to a previously published PCR method (35). For those pneumococcal genomes that were sequenced, seqSerotyper

(https://github.com/avantonder/seqSerotyper) was used to extract the serotypes from sequence data (36).

DNA extraction and whole-genome sequencing. Every other pneumococcal isolate from nasopharyngeal and ME samples from the period 2009 to 2014 was selected for whole-genome sequencing (WGS). DNA was extracted using the Promega Maxwell 16 platform, and the DNA extracts were sequenced on the Illumina HiSeq 2000. WGS data were assembled using Velvet (37) before SSPACE and GapFiller were used to improve the assemblies and close gaps (38, 39). The final assembled genomes were uploaded into a Bacterial Isolate Genome Sequence Database (BIGSdb) along with associated metadata (40). The multilocus sequence type (ST) of each isolate was extracted from the WGS data using BIGSdb and the PubMLST database (http://pubmlst.org/spneumoniae/). STs were assigned to clonal complexes (CCs) via Phyloviz (41).

Genomic analyses. Prokka was used to predict the coding sequences in each genome (42). The resulting annotation files in gff format were then used as the input for Roary and clustered using a sequence identity threshold of 90% (43). The core genome was estimated using a Bayesian core genome model and a threshold of 99.9% for nasopharyngeal isolates and 99.8% for ME isolates (44). The core genes in both sample groups were extracted and aligned using MAFFT (45). FastTree was used to construct phylogenetic trees, and ClonalFrameML (46) was used to reconstruct the trees to account for recombination. The final phylogenetic trees were then annotated using iTOL (47).

Antibiotic susceptibility testing. All isolates were tested for antimicrobial susceptibility to chloramphenicol, erythromycin, tetracycline, trimethoprim-sulfamethoxazole, and clindamycin by disk diffusion tests. Oxacillin disks (1 μg) were used to screen for penicillin resistance; isolates susceptible to oxacillin were considered to be susceptible to penicillin. For oxacillin-resistant isolates, the MICs to penicillin and ceftriaxone (ME isolates only) were measured using the Etest (bioMérieux, France) (48). Penicillin-nonsusceptible pneumococci (PNSP) were defined as isolates having a MIC of $>$0.06 mg/liter, while resistant pneumococci were isolates having a MIC of $>$2 mg/liter. Multidrug resistance (MDR) was defined as nonsusceptibility to three or more classes of antimicrobials (regardless of penicillin nonsusceptibility). The susceptibility testing was performed according to the methods and criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (49).

Statistical analyses. A likelihood ratio test (50) was used to test the null hypothesis of equal rates when comparing the rate (r_1) of a certain serotype, CC, or ST in a given age group PreVac to the rate (r_2) of the same serotype, CC, or ST in the same age group PostVac. The test is based on the assumption that the counts of the serotype, CC, or ST in the two periods are independent and both follow Poisson distributions. It is assumed that the mean of the counts PreVac is equal to the rate r_1 times the total number of individuals in the age group PreVac. The mean of the counts PostVac is equal to the rate r_2 times the total number of individuals in the age group PostVac. The asymptotic distribution of the likelihood ratio test statistics is a chi-square distribution with one degree of freedom under the null hypothesis. Microsoft Excel was used to calculate the test statistics and the corresponding *P* values.

The two-sided Fisher's exact test was used to calculate the *P* values for antimicrobial resistance by using the statistical software R, version 3.3.2. The level of significance for all tests was $\leq$0.05. The Simpson diversity index was used to calculate the diversity of STs (51).

Ethics. The study was approved by The National Bioethics Committee (VSNb2013010015/03.07) and the appropriate authorities at the Landspitali University Hospital and the day care centers.

RESULTS

Nasopharyngeal samples from children attending DCCs. A total of 4,461 nasopharyngeal swabs were collected (450 to 550 samples each year): 1,380 PreVac and 3,081 PostVac (Table 1). The median age of the children who were sampled was 4.1 years. The cultures yielded 3,029 pneumococcal isolates, and 250 children carried two pneumococcal strains. Nine isolates were excluded (not viable/not stored), yielding 3,020 isolates: 991 (32.8%) PreVac and 2,029 (67.2%) PostVac. The carriage rates were 67.3% PreVac and 61.5% PostVac ($P = 0.090$). Overall, 51.8% ($n = 1,563$) of the isolates were collected from children 4 to $<$7 years of age, and the fewest isolates, 3.5% ($n = 103$), were from the children 1 to $<$2 years of age (see Table S3 in the supplemental material). The genomes of 987 (49.2%) pneumococcal isolates from 2009 to 2014 were sequenced.

Serotypes. A total of 36 different serotypes were detected: 27 PreVac and 35 PostVac. Overall, the numbers of isolates of serotypes included in PHiD-CV (vaccine type [VT]) decreased between the two periods ($P < 0.001$) (Table 1). VT pneumococci were most common in 2010 (171/324 [52.8%]) and least common in 2016 (5/354 [1.4%]) (Table 1). The numbers of isolates of serotypes not included in PHiD-CV (nonvaccine type [NVT]) increased from PreVac ($n = 545$, 395.2/1,000 samples) to PostVac ($n = 1,725$, 559.9/1,000 samples; $P < 0.001$) (Table 1). NVT pneumococci were most common in 2016 (349/354 [98.6%]) and least common in 2010 (153/324 [47.2%]) (Table 1).

In children 1 to $<$2 years of age, only one NVT, serotype 23A, increased from PreVac ($n = 0$, 0/1,000 samples) to PostVac ($n = 7$, 95.9/1,000 samples; $P = 0.005$). The NVTs

TABLE 1 Serotype distribution each study year in nasopharyngeal samples among children 1 to <7 years old PreVac (2009 to 2011) and PostVac (2012 to 2017)

Serotype or sample type	No. of isolates										2009 to 2011 (*n*/1,000 samples)	No. of isolates PostVac	2012 to 2017 (*n*/1,000 samples)	*P* value
	2009	2010	2011	2012	2013	2014	2015	2016	2017	PreVac				
3	18	26	24	11	13	20	32	25	12	68	49.3	113	36.7	0.052
4	2	0	0	0	0	0	0	0	0	2	1.4	0	0	0.096
6A	38	24	25	56	23	16	21	23	11	87	63.0	150	48.7	0.051
6B	50	45	14	17	16	26	9	1	2	109	79.0	71	23.0	<0.001
6C	3	4	2	0	11	7	31	49	53	9	6.5	151	49.0	<0.001
9V	12	3	3	1	0	0	0	0	0	18	13.0	1	0.3	<0.001
9A	0	0	1	0	1	0	0	0	0	1	0.7	1	0.3	0.619
9N	6	0	3	3	4	6	5	1	0	9	6.5	19	6.2	0.873
10	0	0	0	0	0	0	3	5	0	0	0	8	2.6	0.052
10A	5	0	0	2	1	8	3	1	8	5	3.6	23	7.5	0.131
10B	0	0	0	0	0	7	0	0	0	0	0	7	2.3	0.075
11A	18	19	21	22	10	38	37	24	5	58	42.0	136	44.1	0.756
13	0	0	0	0	1	0	0	0	0	0	0	1	0.3	0.691
14	26	36	12	8	9	9	3	0	0	74	53.6	29	9.4	<0.0(
15	0	0	1	0	0	0	1	1	1	1	0.7	4	1.3	0.385
15A	0	0	0	0	0	1	14	11	9	0	0	35	11.4	<0.0(
15B/C	21	6	10	29	27	29	24	22	15	37	26.8	146	47.4	<0.0(
16F	3	8	13	3	2	4	5	5	6	24	17.4	25	8.1	0.008
17	0	0	0	0	0	0	1	0	0	0	0	1	0.3	0.691
18C	0	0	0	0	0	0	2	0	0	0	0	2	0.6	0.477
19	15	12	4	7	6	3	3	0	0	31	22.5	19	6.2	<0.0(
19F	30	28	29	22	28	21	13	3	0	87	63.0	87	28.2	<0.0(
19A	47	16	26	12	26	29	22	31	25	89	64.5	145	47.1	0.020
21	2	1	2	4	15	17	14	23	13	5	3.6	86	27.9	<0.0(
22F	3	2	2	3	17	31	4	9	5	7	5.1	69	22.4	<0.0(
23	0	0	0	0	0	0	1	2	1	0	0	4	1.3	0.227
23F	50	47	28	39	30	7	17	1	3	125	90.6	97	31.5	<0.0(
23A	12	10	7	6	22	24	16	35	6	29	21.0	109	35.4	0.009
23B	1	0	0	9	15	25	41	34	28	1	0.7	152	49.3	<0.0(
24F	0	0	0	0	0	0	0	0	4	0	0	4	1.3	0.227
29	3	0	0	2	0	0	0	0	0	3	2.2	2	0.6	0.210
31	2	0	0	0	0	0	0	1	5	2	1.4	6	1.9	0.765
33	2	2	1	4	2	4	0	1	0	5	3.6	11	3.6	0.954
33F	3	2	5	1	1	2	4	8	1	10	7.2	17	5.5	0.492
33_Hybrid	0	0	0	0	0	4	0	0	0	0	0	4	1.3	0.227
35F	0	0	0	3	15	18	16	4	0	0	0	56	18.2	<0.0(
35B	1	1	4	0	17	12	13	2	13	6	4.3	57	18.5	<0.0(
38	0	9	10	2	5	3	3	1	3	19	12.8	17	5.5	0.007
Other serotypes[a]	0	1	1	0	1	1	11	17	19	2	1.4	49	15.9	<0.0(
NESp[b]	21	22	25	18	16	22	22	14	24	68	49.3	116	37.7	0.075
Total	394	324	273	284	334	394	391	354	272	991	718.1	2,029	658.6	<0.0(
VT[c]	185	171	90	94	89	66	45	5	5	446	323.2	304	98.7	<0.0(
NVT[d]	209	153	183	190	245	328	346	349	267	545	394.9	1,725	559.9	<0.0(
All NP[e] samples	516	444	420	465	471	566	533	540	506	1380		3,081		
Samples of positive Pn[f] (%)	76.4	73.0	65.0	61.1	70.9	69.6	73.4	65.6	53.8	71.8		65.9		

[a]Serotypes other than those included in the multiplex PCR panel of the study.
[b]NESp, nonencapsulated *S. pneumoniae*.
[c]Serotypes detected in the study that are included in PHiD-CV (4, 6B, 9V, 14, 18C, 19F, and 23F).
[d]Serotypes that are not included in PHiD-CV.
[e]NP, nasopharyngeal samples.
[f]Pn, pneumococci.

that increased PostVac and were the most prevalent in children 2 to <4 years of age were of serotypes 6C ($n = 82$, 65.0/1,000 samples; $P < 0.001$), 15B/C ($n = 81$, 64.2/1,000 samples; $P < 0.001$), and 23B ($n = 69$, 54.7/1,000 samples; $P < 0.001$) (Table S3).

The NVTs that increased PostVac in children 4 to <7 years of age were serotypes 23B ($n = 80$, 45.8/1,000 samples; $P < 0.001$), 6C ($n = 56$, 32.1/1,000 samples; $P < 0.001$), and 21 ($n = 53$, 30.4/1,000 samples; $P < 0.001$). Serotype 23B was only detected PostVac among children 4 to <7 years of age (Table S3).

MLST/CC. Among the 987 sequenced isolates, 47 CCs (35 CCs PreVac and 41 CCs PostVac) and 104 STs (66 STs PreVac and 83 STs PostVac) were detected, and 12 CCs and 43 STs were unique to nasopharyngeal isolates. The Simpson diversity index of the STs was 0.97 for both periods. A phylogenetic tree was created with the concatenated sequences of 1,066 full-length coding loci found in 99.9% of the nasopharyngeal

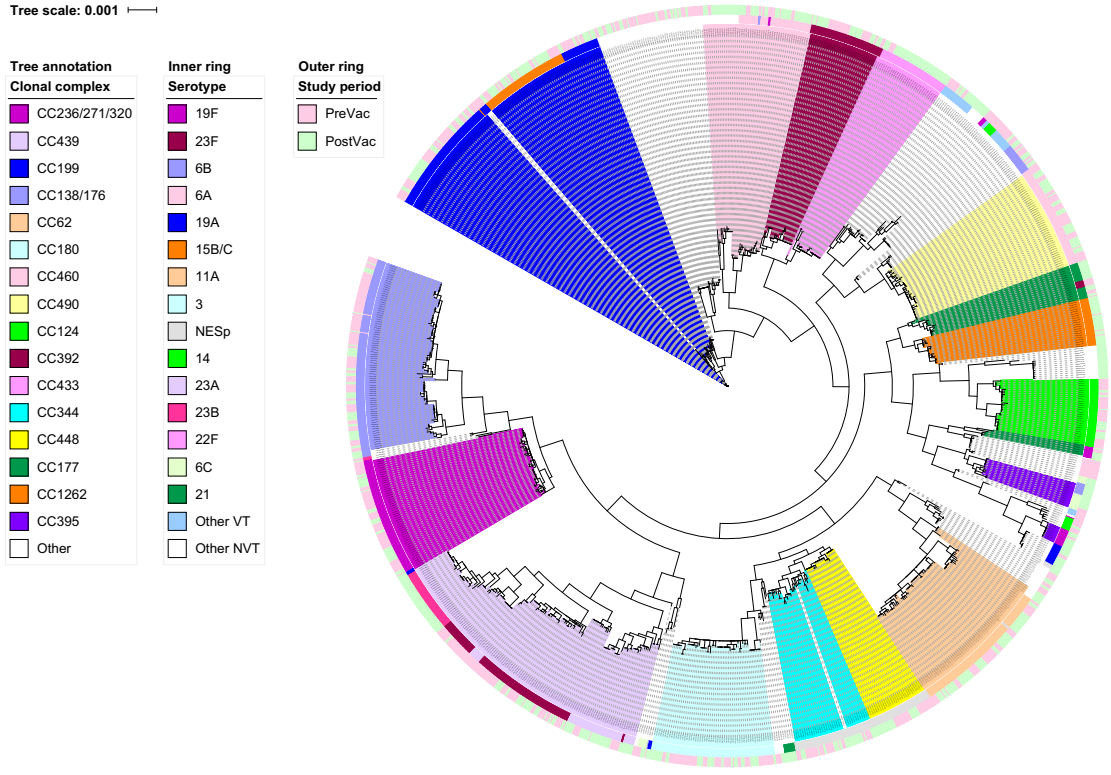

FIG 1 Phylogenetic tree created from 1,066 full-length coding loci found in 99.9% of 987 genomes from carriage samples and annotated with CC designations. Serotypes (inner circle) and study periods (outer circle) are also presented.

carriage pneumococcal genomes. The tree was annotated with CC designations and serotypes (Fig. 1).

CC439$^{23F/A/B}$ was the most common CC in both study periods. Initially, 77.6% of the isolates of CC439$^{23F/A/B}$ were VT serotype 23F, but PostVac, 42.0% were NVT serotype 23B; however, serotype 23B was rare prior to vaccine introduction (Fig. 1 and Table S4). Between the two periods, the prevalence of CC433^{22F} increased from two isolates (1.5/1,000 samples) to 30 (20.0/1,000 samples; $P < 0.001$) (Fig. 1 and Table S4), and similarly, CC1262$^{15B/C}$ increased from three isolates (2.2/1,000 samples) to 18 (12.0/1,000 samples; $P = 0.032$) (Fig. 1 and Table S4).

Antimicrobial resistance. The overall prevalence of PNSP among nasopharyngeal isolates did not change significantly between the two study periods: 15.0% (n = 149/991) versus 16.7% (338/2,029; $P = 0.268$) (Table 2). Erythromycin-resistant pneumococci decreased from 17.6% to 13.7% ($P = 0.007$). The overall prevalence of MDR pneumococci decreased slightly but significantly between the periods, from 15.2% (n = 151/991) to 12.4% (n = 251/2,029; $P = 0.030$) (Table 2). Before PHiD-CV was introduced, 85.2% of PNSP pneumococci were also MDR, and this was reduced to 70.1% ($P < 0.001$). The most prevalent PNSP and MDR isolates were of serotype 19F PreVac and NESp PostVac (Table 2).

Among the PNSP serotype 19F isolates, 92.5% belonged to CC236/271/320^{19F} (Fig. 1 and Table S4). Serotype 19A PNSP increased from 4.0% to 10.4% of isolates ($P = 0.021$) (Table 2), and the isolates were predominantly members of CC199$^{19A,15B/C}$ (Fig. 1 and Table S4). PNSP with serotypes 6C (also MDR) and 35B were also detected.

TABLE 2 Most common PNSP and MDR serotypes in nasopharyngeal samples PreVac (2009 to 2011) and PostVac (2012 to 2017)

	PNSP					MDR Pn[a]				
	PreVac		PostVac			PreVac		PostVac		
Serotype or sample type	*n*	%	*n*	%	*P* value	*n*	%	*n*	%	*P* value
19F	74	49.7	59	17.5	<0.001	73	48.3	60	23.9	<0.001
NESp[b]	34	22.8	85	25.1	0.657	35	23.2	85	33.9	0.025
6B	12	8.1	4	1.2	<0.001	16	10.6	5	2.0	<0.001
14	11	7.4	1	0.3	<0.001	3	2.0	1	0.4	0.087
19A	6	4.0	35	10.4	0.021	3	2.0	9	3.6	0.516
6C	3	2.0	46	13.6	<0.001	4	2.6	47	18.7	<0.001
15A	0	0	29	8.6	<0.001	0	0	29	11.6	<0.001
23B	0	0	32	9.5	<0.001	0	0	1	0.4	1.000
35B	0	0	25	7.1	<0.001	0	0	0	0	NC[c]
Other PNSP/MDR[d]	9	6.0	22	6.5	1.000	4	2.6	6	2.4	1.000
Total	149		338		0.269	151		251		0.030

[a]Pn, pneumococci.
[b]NESp, nonencapsulated *S. pneumoniae*.
[c]NC, not calculated.
[d]Other less prevalent serotypes of PNSP and MDR pneumococci.

Serotype 6C belonged to $CC315^{6B/C}$. Serotype 35B PNSP were only detected after vaccine introduction, and they were members of $CC198^{35B}$, which was not previously detected in carriage (Table 2, Fig. 1, and Table S4). The proportion of MDR NESp isolates increased between the two study periods from 23.2% to 33.9% ($P = 0.025$) (Table 2), and 82.4% of MDR NESp PostVac isolates were members of $CC344^{NT}$ (Fig. 1 and Table S4).

Middle ear samples from AOM. The Department of Clinical Microbiology received 6,651 ME samples during the study period. The total annual number of ME samples decreased from 966 samples in 2009 to 421 by the end of September 2017 (Table 3). Among all 6,651 samples, 994 were positive for pneumococci and 18 isolates were excluded, as they were not stored or not viable, leaving a total of 976 isolates for further analysis. The annual number of pneumococcal isolates from ME decreased from 197 (8.5/1,000 children aged 0 to <7 years) in 2009 to 44 (1.9/1,000 children) in 2016, and by the end of September 2017, the number of isolates was 23 (1.0/1,000 children) (Table 3). The median age of the children from which the ME isolates were obtained was 1.5 years, and 69.1% ($n = 674$) of the isolates were collected from the youngest age group (0 to <2 years of age). The average annual number of isolates in the youngest age group (0 to <2 years) decreased from 133.7 (18.1/1,000 children aged 0 to <2 years per year) PreVac to 45.5 (6.9/1,000 children aged 0 to <2 years per year) PostVac ($P = 0.020$) (see Table S5). The genomes of 441 (50.6%) pneumococcal isolates from 2009 to 2014 were sequenced.

Serotypes. Overall, 894/976 (91.6%) pneumococcal isolates from ME samples were successfully serotyped, but 82 isolates (8.4%) were of serotypes other than those included in the mPCR scheme. Twenty-eight serotypes were detected overall: 23 PreVac and 22 PostVac. The numbers of VT pneumococci decreased significantly between the two periods ($P < 0.001$) (Table 3). The numbers of VT pneumococci decreased significantly in the two younger age groups (children 0 to <2 years, $P < 0.001$; and 2 to <4 years of age, $P = 0.005$), while there was no change between the periods for the oldest age group (children 4 to <7 years of age; $P = 0.450$) (Table S5).

The NVT pneumococci that increased PostVac in children aged 0 to <2 years were serotypes 15B/C ($n = 1$ to $n = 40$; $P < 0.001$), 6C ($n = 1$ to $n = 29$; $P < 0.001$), 23A ($n = 2$ to $n = 18$; $P = 0.007$), and 23B ($n = 1$ to $n = 10$; $P = 0.042$). Serotype 15B/C was the only NVT that increased in children 2 to <4 years of age ($n = 1$ to $n = 11$; $P = 0.042$), and serotype 6C ($n = 12$) was only detected PostVac within that age group (Table S5).

MLST/CC. Among the 441 sequenced isolates, 41 CCs (29 CCs PreVac and 31 CCs PostVac) and 86 STs (55 STs PreVac and 52 STs PostVac) were detected, and 7 CCs and

TABLE 3 Serotype distribution each study year in ME samples among children 0 to <7 years old PreVac (2009 to 2011) and PostVac (2012 to 2017)

Serotype or sample type	No. of isolates										2009 to 2011 (avg/yr)	No. of isolates PostVac	2012 to 2017 (avg/yr)	*P* value
	2009	2010	2011	2012	2013	2014	2015	2016	2017[a]	PreVac				
3	3	2	7	3	9	4	0	0	0	12	4.0	16	2.7	0.635
4	1	0	0	0	0	0	0	0	0	1	0.3	0	0	NC[b]
6A	14	22	11	13	12	2	1	1	0	47	15.7	29	4.8	0.159
6B	17	14	6	8	2	1	0	0	0	37	12.3	11	1.8	0.010
6C	0	0	1	2	10	10	7	8	4	1	0.3	41	6.8	<0.001
9N	0	0	1	0	0	0	0	0	0	1	0.3	0	0	NC
9V	4	2	1	0	0	0	0	0	0	7	2.3	0	0	NC
10B	0	0	0	0	1	0	0	0	0	0	0	1	0.2	NC
11A	1	4	3	6	3	4	1	2	1	8	2.7	17	2.8	0.239
14	14	13	10	1	4	1	0	0	0	37	12.3	6	1.0	<0.001
15A	0	0	0	0	1	1	0	1	3	0	0	6	1.0	NC
15B/C	2	2	1	15	22	2	5	8	1	5	1.7	53	8.8	<0.001
16F	1	1	0	0	0	0	0	1	0	2	0.7	1	0.2	0.695
17	0	1	0	0	0	0	0	0	0	1	0.3	0	0	NC
18C	1	1	0	1	0	0	0	0	0	2	0.7	1	0.2	0.695
19F	85	69	88	33	27	5	0	2	0	242	80.7	67	11.2	<0.001
19A	17	13	5	8	6	5	1	1	1	35	11.7	22	3.7	0.240
19C	1	0	0	0	0	0	0	0	0	1	0.3	0	0	NC
21	0	0	0	3	5	1	1	2	1	0	0	13	2.2	NC
22F	0	0	0	1	1	2	0	0	0	0	0	4	0.7	NC
23F	29	21	23	9	6	3	0	0	0	73	24.3	18	3.0	<0.001
23A	1	1	0	2	5	1	8	6	1	2	0.7	23	3.8	0.003
23B	0	0	1	5	3	2	1	2	2	1	0.3	15	2.5	0.012
24F	0	0	1	0	0	0	0	2	0	1	0.3	2	0.3	0.707
33F	0	0	4	3	4	3	0	1	0	4	1.3	11	1.8	0.231
35F	0	1	0	0	3	0	1	0	0	1	0.3	4	0.7	0.365
35B	0	0	0	3	2	2	1	1	0	0	0	9	1.5	NC
38	1	0	1	0	0	0	0	0	0	2	0.7	0	0	NC
Other serotypes[c]	4	10	5	13	12	11	11	6	9	19	6.3	62	10.3	0.001
NESp[d]	1	0	0	1	0	0	0	0	0	1	0.3	1	0.2	0.995
Total	197	177	169	130	138	60	38	44	23	543	181	433	72.2	0.014
VT[e]	151	120	128	52	39	10	0	2	0	399	133.0	96	16.0	<0.001
NVT[f]	46	57	41	78	99	50	38	42	23	144	49.0	337	56.2	<0.001
All ME samples	966	926	951	849	894	687	505	452	421	2,843	947.7	3,808	634.7	
Samples positive for Pn[g] (%)	20.4	19.1	17.8	15.3	15.4	8.7	7.5	9.7	5.5	19.1		11.4		

[a]From 1 January to 30 September 2017.
[b]NC, not calculated.
[c]Serotypes other than those included in the multiplex PCR panel of the study.
[d]NESp, nonencapsulated *S. pneumoniae*.
[e]Serotypes detected in the study that are included in PHiD-CV (4, 6B, 9V, 14, 18C, 19F, and 23F).
[f]Serotypes detected in the study that are not included in PHiD-CV.
[g]Pn, pneumococci.

24 STs were unique to ME isolates. The STs of two isolates could not be determined as a full-length allele was missing from the WGS data, but these isolates were members of CC180 and CC236/271/320. The Simpson diversity indices of the STs were 0.91 PreVac and 0.96 PostVac. A phylogenetic tree was created with the concatenated sequences of 1,250 full-length coding loci found in 99.8% of the ME pneumococcal genomes. The tree was annotated with CC designations and serotypes (Fig. 2).

Fewer VT CCs were detected PostVac (6 CCs) than PreVac (18 CCs), and fewer STs were detected within the CCs. Six STs of VT isolates were detected within CC439^{23F} PreVac, compared to only one ST PostVac (Fig. 2 and Table S6). CC236/271/320^{19F} was the most common CC in both study periods: 41.8% (115/275, 4.8/1,000 children) PreVac, but it decreased in prevalence to 22.3% (37/166, 1.5/1,000 children) PostVac ($P < 0.001$) (Fig. 2 and Table S6). Between the periods, CC315$^{6B/C}$ increased from one serotype 6B isolate to six serotype 6C isolates ($P = 0.05$). CC1262$^{15B/C}$, CC193^{21}, and CC1816^{35B}, which contained NVTs, were only detected PostVac in ME samples, but CC1262$^{15B/C}$ was detected in low numbers in nasopharyngeal carriage PreVac. One isolate of NVT serotype 23B PostVac belonged to CC156/162^{9V}, a typically VT lineage (Fig. 2 and Table S6).

Antimicrobial resistance. The overall prevalence of PNSP among ME isolates decreased between the two study periods (48.1% versus 28.4%; $P < 0.001$). The

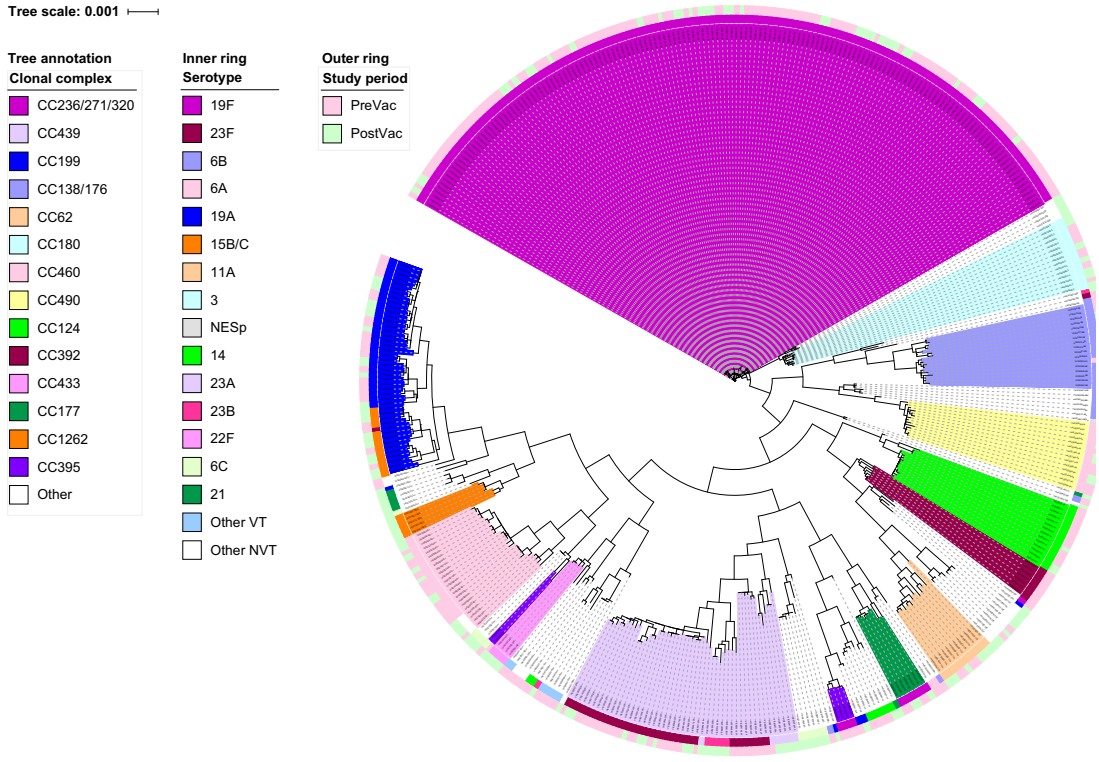

FIG 2 Phylogenetic tree created from 1,250 full-length coding loci found in 99.8% of the 441 genomes from ME samples and annotated with CC designatior Serotypes (inner circle) and the study periods (outer circle) are also presented.

resistance to erythromycin decreased between the two periods (49.7% versus 29.8% of isolates; $P < 0.001$), and the prevalence of MDR pneumococci decreased also (49.2% versus 29.8%; $P < 0.001$). Before the introduction of PHiD-CV, 98.1% (56/261) of PNSP were also MDR, and this was reduced to 91.9% (113/123; $P < 0.001$). PNSP and MDR isolates were mainly serotype 19F in both study periods (Table 4). Among the PNSP serotype 19F isolates, 89.2% belonged to CC236/271/320^{19F} (Fig. 2 and Table S6). NVT

TABLE 4 Most common PNSP and MDR serotypes in ME samples PreVac (2009 to 2011) and PostVac (2012 to 2017)

	PNSP					MDR Pn[a]				
	PreVac		PostVac		*P*	PreVac		PostVac		*P*
Serotype	*n*	%	*n*	%	value	*n*	%	*n*	%	value
19F	231	88.6	66	53.7	<0.001	232	86.9	65	50.4	<0.001
6B	9	3.4	3	2.4	0.759	16	6.0	3	2.3	0.017
14	6	2.3	2	1.6	1.000	5	1.9	2	1.6	0.472
19A	4	1.5	9	7.3	0.006	2	0.7	9	7.0	0.014
23F	4	1.5	0	0	0.311	4	1.5	0	0	0.134
6C	0	0	19	15.4	<0.001	0	0	30	23.3	<0.001
15A	0	0	6	4.9	0.001	0	0	6	4.7	0.007
23B	1	0.4	5	4.1	0.014	1	0.4	1	0.8	1.000
Other PNSP/MDR Pn[b]	6	2.3	13	10.6	0.001	7	2.6	13	10.1	0.040
Total	261		123		<0.001	267		129		<0.001

[a]Pn, pneumococci.
[b]Other less prevalent serotypes of PNSP and MDR pneumococci.

TABLE 5 The most common VTs and NVTs in carriage and ME samples from children 1 to <4 years old PreVac (2009 to 2011) and PostVac (2012 to 2017)

	PreVac					PostVac				
	Carriage		ME			Carriage		ME		
Serotype or sample type	No. of isolates	% positive cultures	No. of isolates	% positive cultures	*P* value	No. of isolates	% positive cultures	No. of isolates	% positive cultures	*P* value
6A	48	9.7	43	9.2	0.766	76	7.9	28	7.6	0.889
6B	63	12.8	33	7.1	0.003	37	3.8	10	2.7	0.332
6C	6	1.2	0	0	0.018	90	9.3	36	9.8	0.696
11A	25	5.1	6	1.3	<0.001	64	6.6	15	4.1	0.074
15B/C	13	2.6	2	0.4	0.005	88	9.1	47	12.8	0.051
19F	63	12.8	215	46.0	<0.001	43	4.5	53	14.4	<0.001
19A	44	8.9	27	5.8	0.061	67	7.0	17	4.6	0.117
23F	67	13.6	65	13.9	0.830	45	4.7	17	4.6	0.993
23A	16	3.2	1	0.2	<0.001	70	7.3	19	5.2	0.172
23B	1	0.2	1	0.2	0.975	72	7.5	13	3.5	0.006
Less prevalent serotypes	111	22.5	73	15.6	NC[a]	255	26.5	111	30.2	NC
NESp[b]	36	7.3	1	0.2	<0.001	59	6.1	1	0.3	<0.001
Total	493	100	467	100	NC	964	100	367	100	NC
VT[c]	256	51.9	353	75.6	<0.001	141	14.6	87	23.7	<0.001
NVT[d]	237	48.1	114	24.4	<0.001	823	85.4	280	76.3	<0.001

[a]NC, not calculated.
[b]NESp, nonencapsulated *S. pneumoniae*.
[c]Serotypes detected in the study that are included in PHiD-CV (4, 6B, 9V, 14, 18C, 19F, and 23F).
[d]Serotypes detected in the study that are not included in PHiD-CV.

serotype 6C amounted to 15.4% of PNSP ($P < 0.001$) and 23.3% of MDR pneumococci ($P < 0.001$) detected PostVac (Table 4). The serotype 6C PNSP/MDR isolates were not detected before vaccine implementation and were members of $CC315^{6B,6C}$.

Serotype 19A PNSP increased from 1.5% to 7.3% ($P = 0.006$), and MDR serotype 19A increased from 0.7% to 7.0% ($P = 0.014$) (Table 4). Serotype 19A PNSP/MDR pneumococci were members of various CCs (Fig. 2 and Table S6). NVT PNSP with serotypes 15A and 23B also increased after vaccine introduction, and they were members of $CC63^{15A}$ and $CC338^{23B}$, respectively. NVT serotype 15A PNSP (also MDR) was only detected after vaccination in ME isolates (Table 4, Fig. 2, and Table S6).

Comparison of serotypes in nasopharyngeal isolates from carriage and ME isolates from children with AOM 1 to <4 years of age. Overall, 2,291 pneumococcal isolates were obtained from children 1 to <4 years of age: 1,457 isolates from nasopharyngeal samples from carriage ($n = 493$ PreVac and $n = 964$ PostVac) and 834 isolates from AOM ME samples ($n = 467$ PreVac and $n = 367$ PostVac).

The same serotypes were among the most prevalent serotypes PreVac in both sample groups, and the levels of serotype replacement PostVac were similar in both groups (Table 5, Fig. 3). One isolate of VT serotype 23F was detected in carriage among children 1 to <4 years of age in 2017, and no VTs were detected in children with AOM within the same age group after 2016. Serotype 6A decreased slightly PostVac among children with AOM but not in carriage ($P = 0.050$) (Fig. 3). Serotypes 6C, 15B/C, 23A, and 23B increased (Fig. 3).

VT serotype 19F was more frequently found in AOM than in carriage in children 1 to <4 years of age in both study periods: 32.1% (268/834) of ME isolates were serotype 19F compared to 7.2% (105/1,457) of carriage isolates ($P < 0.001$). The NVT serotype 23B was more frequently found in carriage than in AOM in children 1 to <4 years of age: 5.0% (73/1,457) of carriage isolates were serotype 23B compared to only 1.7% (14/834) of the ME isolates ($P = 0.006$). Serotype 6C was prevalent in both carriage and AOM among children 1 to <4 years of age, but MDR was more common in ME isolates ($P < 0.001$). NESp isolates were more frequently found in carriage than among children with AOM in both study periods ($P < 0.001$) (Table 5).

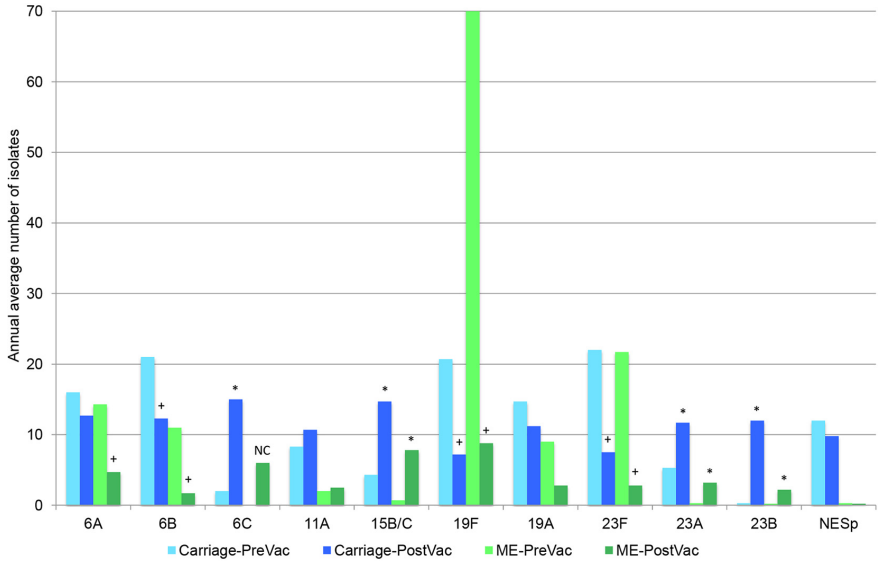

FIG 3 The annual average numbers of the most prevalent serotypes and NESp isolates detected in carriage versus ME samples among children 1 <4 years of age PreVac (2009 to 2011) and PostVac (2012 to 2017). +, decreased significantly between the two study periods; *, increased significantly between the two study periods; Nc, not calculated; NESp, nonencapsulated *S. pneumoniae*.

DISCUSSION

This study shows a significant reduction of VTs in nasopharyngeal carriage of healthy children and in ME samples in children <7 years old 6 years after PHiD-CV implementation in Iceland. The total number of pneumococci isolated from the nasopharynges of children remained unchanged after vaccination due to serotype replacement by NVTs; however, at the same time, the total number of ME isolates decreased significantly. The ME samples in our study were most often from children with ruptured tympanic membranes as a consequence of AOM or from children with tympanic tubes and discharge from the middle ear as a consequence of inflammation in the middle ear. Consequently, we postulate that the decrease in the number of samples from the ME most likely reflects a decrease in the burden of AOM (52). Certain serotypes are associated with nasopharyngeal carriage in healthy children, while others are more prone to cause disease (53, 54); however, the NVTs that have replaced the VTs following routine vaccination may, to a great extent, express a low invasive disease potential (55).

It was confirmed in our study that the distribution of serotypes and genetic lineages in nasopharyngeal carriage in children reflected those identified from the discharges from ears of children with AOM (56). Furthermore, the diversity of the pneumococcal STs did not change between the periods.

The same serotypes and genetic lineages were often found in nasopharyngeal and ME samples in children 1 to <4 years of age, both PreVac and PostVac but often in different proportions. There was a slight but significant decrease in serotype 6A among children with AOM, which has been described in IPD among all ages following PHiD-CV vaccination (57). Serotypes 6C, 23A, and 23B were not affected by the vaccination, as these serotypes increased significantly in both sample groups, which has also been documented elsewhere (21, 58–62).

Serotype 19F was particularly "otogenic," as it was four times more prevalent in ME samples than in nasopharyngeal samples. The main serotype 19F in CC236/270/320^{19F} harbors genes for pili (both PI-1 and PI-2), which likely contributed to the adherence to the mucosa of the middle ear (63). Serotype 6B had a preference for nasopharyngeal

colonization PreVac, while the same was true for serotype 23B PostVac. This finding is in concordance with other reports where serotype 23B was associated with persistent carriage and posed a lower risk for IPD (55, 64).

The NESp isolates were largely associated with carriage in both study periods but extremely rare in AOM. However, while the prevalence of NESp did not change significantly after vaccination in carriage, MDR among NESp isolates increased significantly.

In our study, only one VT isolate was detected between 2014 and 2016 in ME samples among children 1 to <4 years of age, whereas 11 VT isolates were detected between 2014 and 2017 in the nasopharynges of healthy children of the same ages. This indicates that PHiD-CV may be more effective in preventing AOM (measured as fewer positive ME samples) than in preventing nasopharyngeal carriage. This is important, as AOM caused by pneumococci is more severe than that caused by other common pathogens (65, 66).

The increase of the MDR serotype 6C isolates PostVac in both nasopharynx and ME samples is of concern. Other researchers have also described an increase in serotype 6C following the introduction of PCV7, especially in non-IPD among children (67). We rarely detected isolates of serotype 6C PreVac; however, in a recent study, serotype 6C was only detected among vaccinated children with non-IPD after PHiD-CV implementation (68). Serotype 6C isolates detected PostVac most often belonged to CC315^{6B} and ST386^{6C} (a double locus variant [DLV] of PMEN Poland^{6B}-20). The MDR ST386^{6C} was detected in Spain 6 years after PCV7 implementation (69), and other countries have also reported the emergence of this lineage in nasopharyngeal carriage and IPD following the implementation of PHiD-CV (62). This lineage might be derived from a capsular switch from serotype 6B to serotype 6C, as has previously been reported by our group (36).

The majority of serotype 23B isolates belonged to CC439/ST439^{23B} (single locus variant [SLV] of PMEN Tennessee^{23F}-4); however, this lineage was only detected PostVac in both sample groups in our study. CC439/ST439^{23B} was present, although uncommon, in Germany prior to the implementation of vaccinations but increased after vaccine implementation. This lineage has also been documented in other countries worldwide (70).

One ME isolate of serotype 23B belonged to CC156/162 and ST162 (an SLV of PMEN Spain^{9V}-3). This was the only isolate of this lineage detected PostVac in ME isolates. Serotype switch variants of the related ST156 expressing serotypes 9V, 9A, 14, 19F, and 11A have also been reported (71). Pneumococci have the ability to change their capsular serotype by exchanging the capsular locus genes (72). The expansion of preexisting lineages and their variants, such as CC315$^{6B/C}$ and CC439$^{23F/B}$, may be more likely following PCV vaccination than the emergence of new lineages (70).

Iceland offers a unique opportunity for researching vaccine effects for several reasons. The reference laboratory at the Department of Clinical Microbiology, Landspitali University Hospital, serves approximately 85% of the country for pneumococcus-positive samples and stores all isolates (at −80°C). Furthermore, carriage studies have been conducted within the same DCCs using the same methodology throughout the study period. PCVs were not part of the routine infant immunization program before PHiD-CV implementation in 2011. Since then, vaccine acceptance and the uptake of PCVs have been high (73, 74). In our study, we analyzed a large number of pneumococcal isolates representative of the Icelandic population. Furthermore, one third of the pneumococcal isolates were subjected to WGS, which gives a good overview of the composition of genetic lineages in the country. However, fluctuations in serotypes and genotypes are known among pneumococci, even without the selective pressure of PCVs (75, 76).

In conclusion, PHiD-CV implementation eliminated VTs in the MEs of children with otitis media within 5 years. The carriage rate of pneumococci in healthy children remained constant between the periods due to serotype replacement of NVTs, but the total number of ME isolates decreased significantly PostVac. Serotype 23B and NESp

had a preference for nasopharyngeal carriage among children 1 to <4 years of age. Multidrug resistance among serotype 6C was more common in ME samples among children 1 to <4 years of age than in nasopharyngeal carriage samples PostVac.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01046-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 4, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 5, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 6, PDF file, 0.1 MB.

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Paper IV

Quirk S.J, Haraldsson G., Hjálmarsdóttir M.Á, van Tonder A.J., Erlendsdóttir H., Hrafnkelsson B., Bentley S.D., Haraldsson Á., Brueggemann A.B., Kristinsson K.G. (2019) Vaccination of Icelandic children with the 10-valent pneumococcal vaccine leads to significant herd effect among Icelandic adults. J Clin Microbiol. 57 (4) e: 01766-18**.**

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EPIDEMIOLOGY

Vaccination of Icelandic Children with the 10-Valent Pneumococcal Vaccine Leads to a Significant Herd Effect among Adults in Iceland

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ABSTRACT The introduction of pneumococcal conjugate vaccines (PCVs) into childhood vaccination programs has reduced carriage of vaccine serotypes and pneumococcal disease. The 10-valent PCV was introduced in Iceland in 2011. The aim of this study was to determine PCV impact on the prevalence of serotypes, genetic lineages, and antimicrobial-resistant pneumococci isolated from the lower respiratory tract (LRT) of adults. Pneumococci isolated between 2009 and 2017 at the Landspitali University Hospital were included ($n = 797$). The hospital serves almost three-quarters of the Icelandic population. Isolates were serotyped and tested for antimicrobial susceptibility, and the genome of every other isolate collected between 2009 and 2014 was sequenced ($n = 275$). Serotypes and multilocus sequence types (STs) were extracted from the genome data. Three study periods were defined, 2009 to 2011 (PreVac), 2012 to 2014 (PostVac-I), and 2015 to 2017 (PostVac-II). The total number of isolates and vaccine-type (VT) pneumococci decreased from PreVac to PostVac-II ($n = 314$ versus $n = 230$ [$p = 0.002$] and $n = 170$ versus $n = 33$ [$p < 0.001$], respectively), but non-vaccine-type (NVT) pneumococci increased among adults 18 to 64 years old ($n = 56$ versus $n = 114$ [$p = 0.008$]). Serotype 19F decreased in the PostVac-II period; these isolates were all multidrug resistant (MDR) and were members of the Taiwan^{19F}-14 PMEN lineage. Serotype 6A decreased among adults ≥65 years old in the PostVac-II period ($p = 0.037$), while serotype 6C increased ($p = 0.021$) and most serotype 6C isolates were MDR. Nonencapsulated *Streptococcus pneumoniae* (NESp) isolates increased among adults 18 to 64 years old in the PostVac-II period, and the majority were MDR ($p = 0.028$). An overall reduction in the number of LRT samples and pneumococcus-positive cultures and significant changes in the serotype distribution became evident within 4 years, thereby demonstrating a significant herd effect.

KEYWORDS Iceland, *Streptococcus pneumoniae*, adults, epidemiology, lower respiratory tract, molecular epidemiology, pneumococcus, pneumonia, vaccination

Pneumococcus is an important human pathogen that causes significant morbidity and mortality worldwide (1). It is one of the most important human pathogens in community-acquired and nosocomial pneumonia, causing an estimated two-thirds of

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all cases of bacterial pneumonia and resulting in hospitalization and death among older adults (2–6). One of its main virulence factors is a polysaccharide capsule (serotype), and nearly 100 different serotypes have been identified to date (7, 8).

Pneumococcal conjugate vaccines (PCVs) have been included in the infant vaccination program of more than 100 countries (9), which has resulted in a significant reduction in disease and circulating vaccine serotypes within those countries. Protection of the unvaccinated adult population through herd immunity is generally observed later than vaccine-induced immunity among the pediatric population (10–12). Importantly, serotype replacement and the circulation of antimicrobial-resistant pneumococcal lineages expressing non-vaccine serotypes can also occur (13–16).

In April 2011, the 10-valent PCV (PHiD-CV, Synflorix; GSK) was introduced into the national infant immunization program in a 2 + 1 vaccine schedule, without catch-up vaccination. The vaccine directly targets serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F, but the potential cross-protection against vaccine-related serotypes 6A and 19A seems to vary among studies (17–21). Pneumococcal vaccines had not previously been a part of the routine infant immunization program in Iceland. In this study, we analyzed the impact of PHiD-CV implementation among pneumococci recovered from the lower respiratory tract (LRT) of adult patients. The distribution of pneumococcal serotypes and genetic lineages along with changes in antimicrobial resistance rates were assessed before and after vaccine implementation.

MATERIALS AND METHODS

Study population and bacterial isolates. All pneumococci isolated from lower respiratory tract (LRT) samples taken from adults ≥18 years of age with suspected pneumonia and submitted to the Department of Clinical Microbiology at Landspitali University Hospital in Iceland between 2009 and 2017 were included in the study. When two or more pneumococcal isolates of the same phenotype (i.e., the same serotype and antimicrobial susceptibility pattern) were identified from the same patient within 30 days, they were considered to be from the same infection, and only the first isolate was included in the subsequent analyses.

The Department of Clinical Microbiology serves as the reference laboratory for the whole country and is the primary microbiology laboratory for the greater Reykjavík capital area. The study was divided into three different periods for the analyses, 3 years prior to vaccination (2009 to 2011, PreVac), 1 to 3 years postvaccination (2012 to 2014, PostVac-I), and 4 to 6 years postvaccination (2015 to 2017, PostVac-II).

The primary catchment area for the Landspitali University Hospital was considered to be within a 100-km driving distance from the hospital, and the population demographic information for this referral region was obtained from Statistics Iceland (www.statice.is). The population sizes of the referral region for adults ≥18 years of age (which includes over 70% of Icelandic adults ≥18 years old) were 170,042 (PreVac), 177,490 (PostVac-I), and 186,724 (PostVac-II). The prevalences of the pneumococcal isolates were calculated using the population size of the referral region as the denominator. The population sizes stratified by age group (18 to 64 and ≥65 years old) are shown in Table S1 in the supplemental material.

Serotyping. Serotypes were determined for all available isolates with ImmuLex pool antisera (State Serum Institute, Copenhagen, Denmark) and/or by a multiplex PCR (mPCR) method, which included 78 sets of serogroup/serotype-specific primer pairs. Serotypes of serogroup 6 were identified as previously described (22). Nonencapsulated *Streptococcus pneumoniae* (NESp) isolates (i.e., those that were negative for *cpsA* and positive for *lytA*) were tested for the *cpsB* gene, which is essential for encapsulation (23), using a previously published PCR method (24).

DNA extraction, whole-genome sequencing, and phylogenetic analysis. DNA extraction and whole-genome sequencing (WGS) were performed as previously described on every other pneumococcal isolate from the years 2009 to 2014 (22). Multilocus sequence types (STs) and clonal complexes (CCs) were defined in the standard manner. Genome annotation, gene clustering, and sequence alignments were performed using Prokka and Roary. FastTree and ClonalFrameML were used to reconstruct the phylogenetic tree as described in our previous paper (22). Note that a core genome threshold of 99.6% was calculated for this data set.

Antibiotic susceptibility testing. All isolates were tested for antimicrobial susceptibility to chloramphenicol, erythromycin, tetracycline, trimethoprim-sulfamethoxazole, and clindamycin using disk diffusion tests. Oxacillin disks (1 μg) were used to screen for penicillin resistance. E-tests were used to measure the MIC (bioMérieux, France) of penicillin and ceftriaxone on oxacillin-resistant isolates. Multidrug resistance (MDR) was defined as nonsusceptibility to three or more classes of antimicrobials (regardless of penicillin susceptibility). Susceptibility testing was performed according to the methods and criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (25).

Statistical analyses. A likelihood ratio test (26) was used to test the null hypothesis of equality when comparing the rate (r_1) of a certain serotype, CC, or ST in a given age group PreVac to the rate (r_2) of the same serotype, CC, or ST in the same age group in PostVac-II (for serotypes) and PostVac-I (for CC/ST). For the CCs and STs, PreVac was compared to PostVac-I (2012 to 2014), as there was no genome sequencing done on pneumococcal isolates after 2014. The two-sided Fisher's exact test was used to

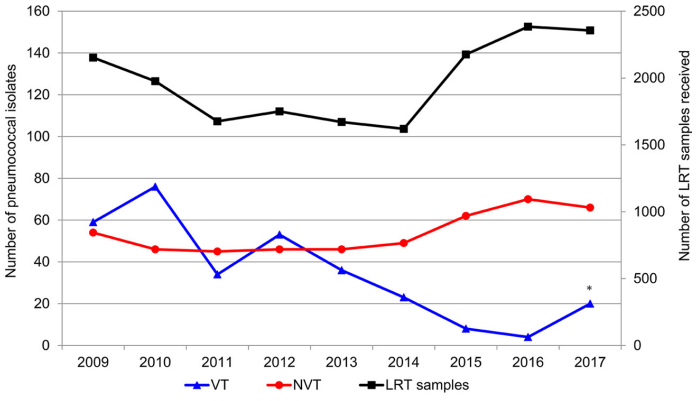

FIG 1 Annual number of isolates belonging to serotypes targeted by PHiD-CV (vaccine-type [VT]), serotypes not targeted by PHiD-CV (non-vaccine-type [NVT]), and lower respiratory tract (LRT) samples received by the laboratory. *, Seven isolates of serotype 19F were detected in the same patient in 2017.

calculate the *p* values for antimicrobial resistance by using R version 3.3.2. The level of significance for all tests was ≤0.05. Simpson's diversity index was calculated to assess the change in ST diversity after vaccine implementation (27).

Ethics. This study was approved by The National Bioethics Committee (VSNb2013010015/03.07) and the appropriate authorities at Landspitali University Hospital in Iceland.

RESULTS

Demographics. The laboratory received 17,762 samples during the study period (Fig. 1). This yielded 814 pneumococcal isolates, of which 17 isolates were not stored or were nonviable, leaving 797 isolates for further analyses. About 10% of the LRT samples originated from adults residing outside of the primary catchment area. The total number of pneumococcal isolates decreased significantly from 314 (184.7/100,000 adults) PreVac to 230 (123.2/100,000 adults) PostVac-II ($p = 0.002$; Table 1). The median age of the patients with confirmed pneumonia was 75.2 years. More than half (430/797, 54.0%) of all isolates obtained were from patients ≥65 years of age, and in this age group, the total number of isolates decreased from 191 (719.8/100,000 adults aged ≥65 years) in the PreVac period to 102 (314.7/100,000 adults aged ≥65 years) in the PostVac-II period ($p < 0.001$; Table 2).

Serotyping. Among all 797 pneumococcal isolates, 789 (99.0%) were successfully serotyped, but 8 isolates were of serotypes other than those included in the mPCR scheme and were not characterized further. Overall, 31 serotypes were detected, 28 in the PreVac period and 24 each in the PostVac-I and PostVac-II periods. Overall, isolates of serotypes 4, 6B, 7F, 9V, 14, 18C, 19F, and 23F, which are targeted by PHiD-CV (vaccine type [VT]), decreased significantly between the PreVac and PostVac-II periods ($p < 0.001$; Table 1). VT serotypes 1 and 5 were not detected in the study. The prevalence of isolates with serotypes not targeted by PHiD-CV (non-vaccine type [NVT]) did not change significantly ($p = 0.184$; Table 1). VTs were most prevalent in 2010 (77/122; 63.1%) and least prevalent in 2016 (4/74; 5.4%) (Table 1 and Fig. 1).

Overall, among vaccine-related serotypes 6A and 19A, the frequency of serotype 19A did not change during the study period (PreVac, $n = 10$; PostVac-I, $n = 13$; and PostVac-II, $n = 12$), but serotype 6A decreased significantly from the PreVac to PostVac-II period ($n = 18$ versus $n = 3$, respectively; $p = 0.016$; Table 1). Notably, serotype 6C increased significantly from PreVac to PostVac-II ($n = 1$ versus $n = 14$, respectively; $p = 0.021$; Table 1).

Analyses by age group revealed that while the prevalence of VT pneumococci decreased significantly in both age groups, the prevalence of NVT and NESp isolates increased significantly in adults aged 18 to 64 years ($p = 0.008$ and 0.028, respectively).

TABLE 1 Serotype distribution for each study year, 2009 to 2017, in adults ≥18 years of age

Serotype	No. of isolates per yr									PreVac isolates (2009–2011)		PostVac-I isolates (2012–2014)		PreVac vs PostVac-I *p* value	PostVac-II isolates (2015–2017)		PreVac vs PostVac-II *p* value
	2009	2010	2011	2012	2013	2014	2015	2016	2017	No.	No. per 100,000	No.	No. per 100,000		No.	No. per 100,000	
3	11	9	5	9	3	8	6	7	7	25	14.7	20	11.3	0.558	20	10.7	0.485
4	0	0	1	0	0	0	1	0	0	1	0.6	0	0	Nc[a]	1	0.5	0.965
6A	10	4	4	5	4	0	1	1	1	18	10.6	9	5.1	0.221	3	1.6	0.016
6B	9	10	2	11	7	3	3	3	1	21	12.3	21	11.8	0.927	7	3.7	0.052
6C	0	1	0	1	1	0	2	7	5	1	0.6	2	1.1	0.719	14	7.5	0.021
7F	0	2	0	1	0	0	2	0	0	2	1.2	1	0.6	0.683	2	1.1	0.951
8	0	1	0	0	0	0	0	0	0	1	0.6	0	0	Nc	0	0	Nc
9V	3	4	2	2	0	0	0	0	0	9	5.3	2	1.1	0.136	0	0	Nc
9A	1	0	1	0	1	1	0	0	0	2	1.2	2	1.1	0.977	0	0	Nc
9N	0	1	0	0	0	2	1	1	2	1	0.6	2	1.1	0.136	4	2.1	Nc
10	0	0	1	0	0	0	0	0	0	1	0.6	0	0	Nc	0	0	Nc
11A	5	6	3	2	6	3	7	4	9	14	8.2	11	6.2	0.641	20	10.7	0.617
14	8	7	0	3	3	1	0	0	1	15	8.8	7	3.9	0.229	1	0.5	0.008
15A	1	1	0	1	3	1	2	2	4	2	1.2	5	2.8	0.470	8	4.3	0.230
15B/C	3	0	5	3	5	3	4	6	1	8	4.7	11	6.2	0.694	11	5.9	0.749
16F	2	3	0	1	1	0	0	1	0	5	2.9	2	1.1	0.426	1	0.5	0.231
17F	0	0	1	0	0	1	0	0	0	1	0.6	1	0.6	0.984	0	0	Nc
18B	0	0	1	0	0	0	0	0	0	1	0.6	0	0	Nc	0	0	Nc
18C	2	0	0	0	0	0	0	0	0	2	1.2	0	0	Nc	0	0	Nc
19F	30	45	24	29	23	15	1	1	18[b]	99	58.2	67	37.7	0.068	20[b]	10.7	<0.001
19A	5	3	2	3	4	6	4	5	3	10	5.9	13	7.3	0.730	12	6.4	0.891
20	0	0	1	0	0	0	0	0	0	1	0.6	0	0	Nc	0	0	Nc
21	1	0	0	1	1	0	1	1	4	1	0.6	2	1.1	0.719	6	3.2	0.217
22F	4	3	5	5	1	3	5	4	1	12	7.1	9	5.1	0.619	10	5.4	0.670
23F	8	11	7	7	4	4	2	0	0	26	15.3	15	8.5	0.219	2	1.1	0.001
23A	3	0	0	1	1	2	5	2	6	3	1.8	4	2.3	0.832	13	7.0	0.111
23B	0	1	3	0	2	3	5	4	1	4	2.4	5	2.8	0.859	10	5.4	0.337
24F	0	0	0	0	0	0	1	1	0	0	0	0	0	Nc	2	1.1	Nc
31	0	1	0	0	0	0	0	0	0	1	0.6	0	0	Nc	0	0	Nc
33F	2	1	1	3	0	0	1	1	0	4	2.4	3	1.7	0.774	2	1.1	0.536
34	1	0	0	0	0	0	1	0	0	1	0.6	0	0	Nc	1	0.5	0.965
35F	0	1	1	3	3	4	1	3	3	2	1.2	10	5.6	0.123	7	3.7	0.298
35B	2	1	3	4	1	2	3	7	4	6	3.5	7	3.9	0.895	14	7.5	0.289
Not determined[c]	0	1	0	0	1	2	0	0	4	1	0.6	3	1.7	0.518	4	2.1	0.396
NESp[d]	2	5	6	4	7	8	11	13	11	13	7.6	19	10.7	0.534	35	18.7	0.054
Total	113	122	79	99	82	72	70	74	86	314	184.7	253	142.5	0.043	230	123.2	0.002
VT[e]	59	77	34	53	37	23	9	4	20	170	100.0	113	63.7	0.013	33	17.7	<0.001
NVT[f]	54	45	45	46	45	49	61	70	66	144	84.7	140	78.9	0.693	197	105.5	0.184
All LRT samples	2,153	1,976	1,676	1,750	1,671	1,620	2,176	2,384	2,356	5,805	3,413.9	5,041	2,840.2	<0.001	6,916	3,703.9	0.003
Percentage of LRT samples positive for pneumococci	5.2	6.2	4.7	5.7	4.9	4.4	3.2	3.1	3.7	5.4		5.0			3.3		

[a]Nc, not calculated.
[b]Seven isolates of serotype 19F were detected in the same patient in 2017.
[c]Serotypes other than those included in the multiplex PCR panel of the study.
[d]NESp, nonencapsulated *S. pneumoniae*.
[e]Serotypes detected in the study that are targeted by PHiD-CV (4, 6B, 7F, 9V, 14, 18C, 19F, and 23F).
[f]Serotypes detected in the study that are not targeted by PHiD-CV.

TABLE 2 Serotype distribution within each age group during the PreVac (2009 to 2011), PostVac-I (2012 to 2014), and PostVac-II (2015 to 2017) periods in LRT samples

Serotype	18–64 yrs								≥65 yrs							
	PreVac isolates (2009–2011)		PostVac-I isolates (2012–2014)		PreVac vs PostVac-I *p* value	PostVac-II isolates (2015–2017)		PreVac vs PostVac-II *p* value	PreVac isolates (2009–2011)		PostVac-I isolates (2012–2014)		PreVac vs PostVac-I *p* value	PostVac-II isolates (2015–2017)		PreVac vs PostVac-II *p* value
	No.	No. per 100,000	No.	No. per 100,000		No.	No. per 100,000		No.	No. per 100,000	No.	No. per 100,000		No.	No. per 100,000	
3	9	6.3	9	6.1	0.963	11	7.1	0.851	16	60.3	11	37.7	0.425	9	27.8	0.209
4	1	0.7	0	0	Nc[a]	1	0.6	0.973	0	0	0	0	Nc	0	0	Nc
6A	6	4.2	2	1.3	0.325	1	0.6	0.170	12	45.2	7	24.0	0.371	2	6.2	0.037
6B	3	2.1	13	8.8	0.095	5	3.2	0.688	18	67.8	8	27.4	0.143	2	6.2	0.005
6C	0	0	2	1.3	Nc	7	4.5	Nc	1	3.8	0	0	Nc	7	21.6	0.191
7F	1	0.7	1	0.7	0.988	1	0.6	0.973	1	3.8	0	0	Nc	1	3.1	0.926
8	0	0	0	0	Nc	0	0	Nc	1	3.8	0	0	Nc	0	0	Nc
9V	2	1.4	2	1.3	0.983	0	0	Nc	7	26.4	0	0	Nc	0	0	Nc
9A	0	0	0	0	Nc	0	0	Nc	2	7.5	2	6.9	0.950	0	0	Nc
9N	0	0	1	0.7	Nc	3	1.9	Nc	1	3.8	1	3.4	0.965	1	3.1	0.926
11A	7	4.9	6	4.0	0.824	11	7.1	0.601	7	26.4	5	17.1	0.665	9	27.8	0.946
14	7	4.9	3	2.0	0.379	0	0	Nc	8	30.1	4	13.7	0.381	1	3.1	0.068
15A	0	0	4	2.7	Nc	6	3.9	Nc	2	7.5	1	3.4	0.662	2	6.2	0.895
15B/C	4	2.8	3	2.0	0.781	9	5.8	0.400	4	15.1	8	27.4	0.508	2	6.2	0.482
16F	0	0	0	0	Nc	1	0.6	Nc	5	18.8	2	6.9	0.401	0	0	Nc
17F	1	0.7	1	0.7	0.988	0	0	Nc	0	0	0	0	Nc	0	0	Nc
18B	0	0	0	0	Nc	0	0	Nc	1	3.8	0	0	Nc	0	0	Nc
18C	1	0.7	0	0	Nc	0	0	Nc	1	3.8	0	0	Nc	0	0	Nc
19F	39	27.2	22	14.8	0.126	7	4.5	0.001	60	226.1	45	154.3	0.199	13[b]	40.1	<0.001
19A	5	3.5	6	4.0	0.973	7	4.5	0.765	5	18.8	7	24.0	0.784	5	15.4	0.835
20	0	0	0	0	Nc	0	0	Nc	1	3.8	0	0	Nc	0	0	Nc
21	0	0	2	1.3	Nc	4	2.6	Nc	1	3.8	0	0	Nc	2	6.2	0.786
22F	8	5.6	7	4.7	0.525	5	3.2	0.524	4	15.1	2	6.9	0.536	5	15.4	0.982
23F	14	9.8	6	4.0	0.264	0	0	Nc	12	45.2	9	30.9	0.566	2	6.2	0.037
23A	2	1.4	3	2.0	0.983	6	3.9	0.375	1	3.8	1	3.4	0.965	7	21.6	0.191
23B	1	0.7	3	2.0	0.715	4	2.6	0.388	3	11.3	2	6.9	0.715	6	18.5	0.638
24F	0	0	0	0	Nc	1	0.6	Nc	0	0	0	0	Nc	1	3.1	Nc
31	1	0.7	0	0	Nc	0	0	Nc	0	0	0	0	Nc	0	0	Nc
33F	0	0	3	2.0	Nc	2	1.3	Nc	4	15.1	0	0	Nc	0	0	Nc
34	0	0	0	0	Nc	1	0.6	Nc	1	3.8	0	0	Nc	0	0	Nc
35F	1	2.1	5	3.4	0.272	2	1.3	0.731	1	3.8	5	17.1	0.293	5	15.4	0.332
35B	3	2.1	2	1.3	0.749	7	4.5	0.441	3	11.3	5	17.1	0.703	7	21.6	0.522
Not determined[c]	1	2.1	3	2.0	0.514	1	0.6	0.973	1	3.8	0	0	NC	3	9.0	0.585
NESp[d]	6	4.2	7	4.7	0.886	25	16.2	0.028	7	26.4	12	41.1	0.532	10	30.9	0.833
Total	123	85.7	116	78.2	0.641	128	82.9	0.864	191	719.8	137	469.8	0.011	102	314.7	<0.001
VT[e]	67	46.7	47	31.7	0.176	14	9.1	<0.001	107	403.2	65	222.9	0.022	19	61.7	<0.001
NVT[f]	56	39.0	69	46.5	0.519	114	73.9	0.008	84	316.6	72	246.9	0.201	83	253.0	0.367

[a]Nc, not calculated.
[b]Seven isolates of serotype 19F were detected in the same patient in 2017.
[c]Serotypes other than those included in the multiplex PCR panel of the study.
[d]NESp, nonencapsulated *S. pneumoniae*.
[e]Serotypes detected in the study that are targeted by PHiD-CV (4, 6B, 7F, 9V, 14, 18C, 19F, and 23F).
[f]Serotypes detected in the study that are not targeted by PHiD-CV.

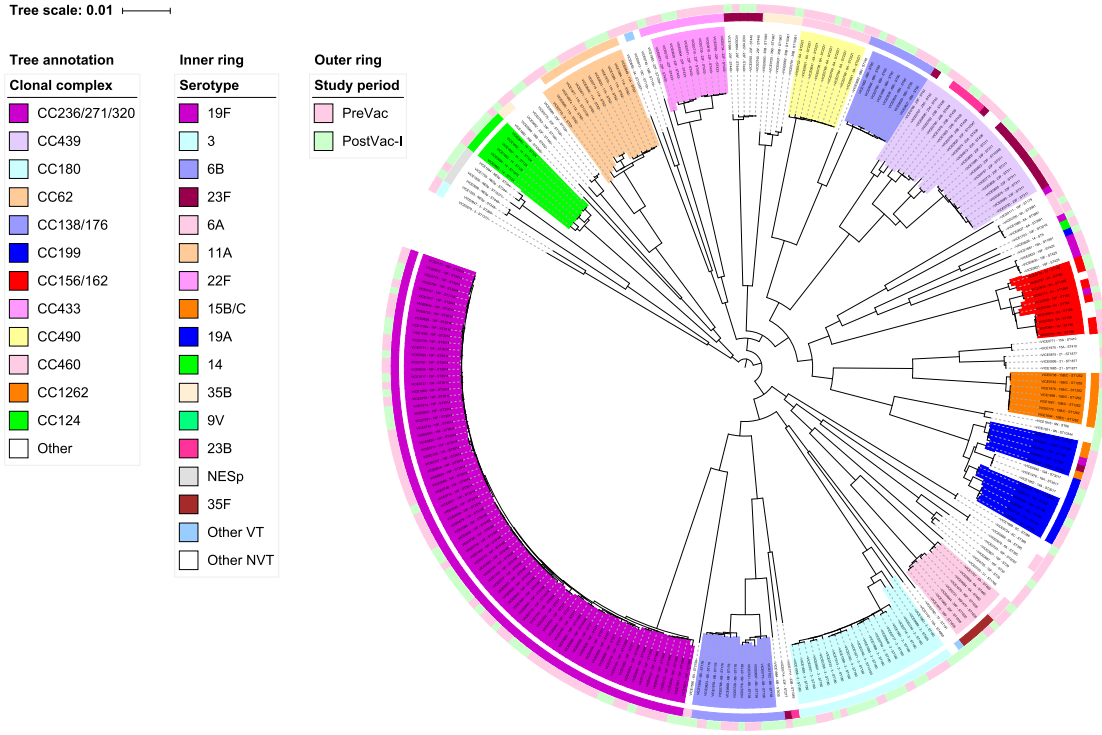

FIG 2 Midrooted phylogenetic tree, created from 1,221 full-length coding loci found in 99.6% of 275 genomes from LRT samples, annotated with CC designations. Serotypes (inner circle) are presented using the same colors as the appropriate CC where possible. Study periods (outer circle) are also presented

There was a nonsignificant decrease in the incidence of serotype 3 in the ≥65-year age group (from 60.3/100,000 adults in the PreVac period to 27.8/100,000 adults in the PostVac-II period). There was no significant change in the overall prevalence of NVT or NESp isolates in the ≥65-year age group (Table 2).

CC/MLST. Between 2009 and 2014, 567 pneumococcal isolates were recovered and 275 (48.5%) of these isolates were sequenced, among which 41 different CCs (31 CCs PreVac and 32 CCs PostVac-I) and 73 STs (56 STs PreVac and 47 STs PostVac-I) were detected. There was no difference in the ST diversity between the two periods (Simpson's diversity indexes of the STs were 0.97 PreVac and 0.96 PostVac-I).

A phylogenetic tree was created with concatenated sequences of 1,130 full-length coding loci found in 99.6% of the pneumococcal genomes. The tree was annotated with CC designations and serotypes (Fig. 2).

The most prevalent CC in both study periods was CC236/271/320^{19F}, which comprised 31.2% (49/157 [28.8/100,000 adults]) of the PreVac pneumococci, but this CC decreased to 24.6% (29/118 [16.3/100,000 adults]) in the PostVac-I period ($p = 0.010$) (Fig. 2; see also Table S3 in the supplemental material). Minimal and insignificant changes in the prevalence of other CCs were observed, although the prevalence of CC433^{22F} decreased in the PostVac-I period ($p = 0.042$) (Fig. 2; Table S3).

Antimicrobial resistance. Approximately one-third of pneumococci were resistant to penicillin and/or erythromycin in all three study periods, and there was no significant change in those resistance rates over time (Table 3; see also Table S2 in the supplemental material). MDR pneumococci decreased from 34.4% in the PreVac period to 23.9% in the PostVac-II period ($p = 0.010$; Table 4). Prior to PHiD-CV implementation, 87.9% (102/116) of isolates that were penicillin nonsusceptible were also MDR, but

TABLE 3 PNSP serotypes during the PreVac (2009 to 2011) and PostVac-II (2015 to 2017) periods

	PreVac isolates		PostVac-II isolates		
PNSP serotype	No.	%	No.	%	*p* value
6A	0	0	1	1.4	0.458
6B	8	6.9	5	7.1	1.000
6C	0	0	5	7.1	0.007
9V	4	3.4	0	0	Nc[a]
9A	1	0.9	0	0	1.000
11A	0	0	1	1.4	0.376
14	3	2.6	1	1.4	1.000
15A	0	0	5	7.1	0.007
15B/C	0	0	1	1.4	0.376
19F	91	78.4	20[b]	28.6	<0.001
19A	2	1.7	0	0	Nc
22F	0	0	2	2.9	0.209
23F	1	0.9	0	0	1.000
23A	0	0	3	4.3	0.094
23B	0	0	3	4.3	0.094
35B	0	0	6	8.6	0.003
Not determined[c]	1	0.9	0	0	1.000
NESp[d]	5	4.3	17	24.3	<0.001
Total PNSP	116	100	70	100	0.121
Total pneumococcal isolates	314	36.9	230	30.4	0.002

[a]Nc, not calculated.
[b]Seven isolates of serotype 19F were detected in the same patient in 2017.
[c]Serotypes other than those included in the multiplex PCR panel of the study.
[d]NESp, nonencapsulated *S. pneumoniae*.

this was reduced to 72.9% (51/70; $p = 0.016$) in the PostVac-II period (Table 5). This reduction was related mainly to the decrease in MDR serotype 19F pneumococci.

Serotype 19F isolates were the most prevalent penicillin-nonsusceptible pneumococci (PNSP) and MDR pneumococci in all three study periods. A total of 78.4% of PNSP and 80.6% of MDR pneumococci recovered in the PreVac period were serotype 19F, but this decreased to 28.6% and 36.4%, respectively, in the PostVac-II period ($p < 0.001$ for both; Tables 3 and 4). Nearly all (91.8%) of the sequenced PNSP serotype 19F isolates were members of the internationally distributed MDR lineage CC236/271/320^{19F} (Taiwan^{19F}-14; Table 3 and Fig. 2; see also Table S3 in the supplemental material).

TABLE 4 MDR pneumococcal serotypes during the PreVac (2009 to 2011) and PostVac-II (2015 to 2017) periods

	PreVac isolates		PostVac-II isolates		
MDR serotype	No.	%	No.	%	*p* value
6A	1	0.9	0	0	1.000
6B	9	8.3	5	9.1	1.000
6C	1	0.9	9	16.4	<0.001
14	2	1.9	1	1.8	1.000
15A	1	0.9	5	9.1	0.017
15B/C	0	0	1	1.8	0.337
19F	87	80.6	20[a]	36.4	<0.001
23F	1	0.9	0	0	1.000
23A	0	0	1	1.8	0.337
35B	0	0	1	1.8	0.337
Not determined[b]	1	0.9	0	0	1.000
NESp[c]	5	4.6	12	21.8	<0.001
Total MDR serotypes	108	100	55	100	0.010
Total pneumococcal isolates	314	34.4	230	23.9	0.002

[a]Seven isolates of serotype 19F were detected in the same patient in 2017.
[b]Serotypes other than those included in the multiplex PCR panel of the study.
[c]NESp, nonencapsulated *S. pneumoniae*.

TABLE 5 PNSP serotypes that were also MDR PreVaC (2009 to 2011) and PostVac-II (2015 to 2017)

PNSP/MDR serotype	PreVac isolates		PostVac-II isolates		*p* value
	No.	%	No.	%	
6B	8	6.9	5	7.1	1.000
6C	0	0	5	7.1	0.007
14	2	1.7	1	1.4	1.000
15A	0	0	5	7.1	0.007
15B/C	0	0	1	1.4	0.376
19F	87	75.0	20[a]	28.6	<0.001
23A	0	0	1	1.4	0.376
35B	0	0	1	1.4	0.376
Not determined[b]	1	0.9	0	0	1.000
NESp[c]	4	3.4	12	17.1	0.002
Total PNSP/MDR	102	87.9	51	72.9	0.016
Total PNSP	116	100	70	100	0.121

[a]Seven isolates of serotype 19F were detected in the same patient in 2017.
[b]Serotypes other than those included in the multiplex PCR panel of the study.
[c]NESp, nonencapsulated *S. pneumoniae*.

There were three serotypes, 6C, 15A, and 35B, that were not associated with penicillin resistance in the PreVac period, but each serotype described 7 to 9% of the PNSP by the PostVac-II period (Table 3). Similarly, there were changes among the MDR pneumococci of all three of these serotypes; in particular, there were significant increases in MDR serotype 6C and 15A pneumococci (16.4% [$n = 9$] and 9.1% [$n = 5$] PostVac-II, respectively) (Tables 3 to 5). Among all PNSP isolates, NESp isolates increased from 4.3% to 24.3% from the PreVac period to the PostVac-II period ($p < 0.001$), of which 17.1% were also MDR ($p = 0.002$; Tables 3 to 5). The overall number of isolates of serotypes 6C, 15A, and 35B and that of the NESp isolates was relatively low, and only every other isolate from 2009 to 2014 was chosen for genome sequencing. Therefore, only a few of each of these pneumococci were selected for genome sequencing, and it is difficult to draw any major conclusions about the genetic lineages associated with these PNSP or MDR pneumococci, except to say that the characterized STs corresponded to widely distributed genetic lineages such as ST344^{NT} and ST315^{6B} (Fig. 2; Table S3).

DISCUSSION

The results of this study demonstrated an indirect (herd) effect of PHiD-CV among adults by decreasing the overall number of LRT samples and the proportion of those samples that were positive for pneumococci and by causing a reduction in the proportion of pneumococci that were of vaccine serotypes. The vaccine-induced herd effect leading to a reduction in the incidence of pneumococcal serotypes in unvaccinated children and adults has been widely studied for invasive pneumococcal disease (IPD) (10, 28–31), but few studies have documented a herd effect on vaccine serotypes in adults with pneumonia (11, 32).

High vaccine coverage (>70 to 80%) leads to extensive herd protection in a population (33), which becomes evident in the adult population within a few years following vaccine implementation (10, 12). At the beginning of the PostVac-II period, over 97% of Icelandic children <5 years of age were fully vaccinated (34). We assessed the differences in the prevalences of pneumococcal serotypes between two postimplementation periods by comparing the PreVac period (2009 to 2011) to both the PostVac-I (2012 to 2014) and PostVac-II (2015 to 2017) periods. The relatively rapid establishment of herd protection and the decline of VTs in adults (29, 30, 33) could partly be explained by the high vaccine uptake in Iceland, a country where vaccines are generally well accepted (35). However, serotype replacement of NVTs has been observed where PCVs have been implemented (36–38), and this was also the case in our study, although serotype replacement was significant only in adults aged 18 to 64 years.

The penicillin-nonsusceptible and multidrug-resistant serotype 19F isolates were all members of the globally distributed CC236/271/320^{19F} lineage. Serotype 19F was the most prevalent PNSP/MDR serotype in all study periods, although it decreased significantly in the PostVac-II period. The prevalence of PNSP/MDR serotype 19F was unusually high in 2017 compared to that in the two previous years, but this was partly because one 77-year-old immunodeficient patient contributed 7 of the 18 isolates detected that year. However, fluctuations in the prevalence of serotype 19F are also known to occur following PCV introduction, and this will need to be monitored in Iceland going forward (11, 38). Adult infections are frequently a reflection of nasopharyngeal carriage among young children (31), but interestingly, serotype 19F was not detected in 2017 among healthy Icelandic children or in Icelandic children with acute otitis media (22). Therefore, it is possible that older children and/or older adults can serve as a reservoir for serotype 19F, and this maintains serotype 19F in the unvaccinated population after vaccine introduction. Serotypes 1 and 5 were not detected in this study. These serotypes are rare in Iceland. Serotype 1 was last detected in 2012, serotype 5 was last detected in 1996, and both serotypes were recovered from patients with IPD (S. J. Quirk, G. Haraldsson, M. Á. Hjálmarssdóttir, H. Erlendsdóttir, and K. G. Kristinsson, unpublished data).

Among vaccine-related serotypes, the prevalence of serotype 6A decreased significantly in the second PostVac period even though it is not a direct target of the vaccine, and other countries that have implemented PHiD-CV have also described similar results (18, 20). Furthermore, in our previous study, the prevalence of serotype 6A decreased in Icelandic children 1 to $<$4 years of age with acute otitis media (22). The possible cross-protection against serotype 19A through the serotype 19F conjugate has been widely debated (17–21); however, the Icelandic adult population did not appear to benefit from the childhood vaccinations against serotype 19A, since the incidence of serotype 19A did not change between the study periods. Before vaccine implementation in Iceland, serotype 19A was more commonly found in IPD and nasopharyngeal carriage than in non-IPD (acute otitis media and pneumonia), and serotype 19F was predominant in non-IPD (22, 39).

Serotypes 6C and 15A have been reported as upcoming PNSP and MDR serotypes following PCV introduction (13, 40, 41), but among adults in Iceland, these serotypes were detected only in low numbers in the PostVac periods of the study. Our group has also detected MDR isolates of serotype 6C that were members of CC315$^{6B/6C}$ and ST386^{6C} (a double locus variant of PMEN Poland^{6B}-20) among children (22, 39), but it remains to be seen whether CC315^{6C} will replace CC236/271/320^{19F} as a major MDR lineage in Iceland.

Notably, the prevalence of NESp isolates increased significantly, and they were the most frequently detected pneumococci in the PostVac-II period in adults aged 18 to 64 years. The opposite was seen in the United States, where NESp isolates decreased in adults between the ages of 50 and 64 years, with a parallel increase among adults $\geq$65 years of age (32).

Increased vaccine pressure caused by PCVs might open an environmental niche that NESp is adept to employ. This could explain the increased prevalence of NESp isolates in the PostVac-II period, or the increase might simply be a natural trend that reflects longer-term variation (42, 43). Our data support the need for continued surveillance of pneumococci in Iceland. It should be noted that some serotypes were found only in low numbers; therefore, the statistical power for detecting a difference in the frequency between the PreVac and PostVac periods among these serotypes was low.

Following the financial crisis in Iceland in 2008, physicians were advised to reduce test samples at the Landspitali University Hospital, and as a result, fewer LRT samples were received from 2010 to 2014. In the following years, when the effect of the crisis subsided, the number of LRT samples gradually increased again, but at the same time, the number of pneumococcal isolates continued to decrease. This decrease was particularly evident among patients aged $\geq$65 years, and PCV introduction into child-

hood immunization programs has been shown to be very beneficial for older adults (11, 33).

Most of the LRT samples in this study were sputum samples; thus, some pneumococcal isolates may represent colonization, although colonization among adults is rare (44), and sputum samples should have been collected only from patients with suspected pneumonia and not healthy adults. Therefore, using the number of positive pneumococcal isolates as a proxy for pneumococcal pneumonia could be considered a weakness. In general, the financial crisis mentioned above was the only known factor influencing sampling. Vaccine uptake area, guidelines, the health care system, and microbiological methods remained the same during the study period.

The herd effect became evident in our study 3 to 4 years after PHiD-CV implementation and was associated with significant changes in both the serotype distribution and the number of pneumococcal isolates cultured from the lower respiratory tract samples of adults. Pneumococcus was the most frequent pathogen recovered from adults with pneumonia in Iceland prior to vaccination (45); hence, the protection of older adults through pneumococcal vaccination and herd immunity is of the utmost importance.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01766-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.01 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

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Appendix

Table 19. PCR primers used for serotyping pneumococal isolates in the study.

Primer	Amplicon size (bp)	Sequence 5´ to 3´	Reference
1-f 1-r	280	CTCTATAGAATGGAGTATATAAACTATGGTTA CCAAAGAAAATACTAACATTATCACAATATTGGC	(Pai et al., 2006)
3-f 3-r	371	ATGGTGTGATTTCTCCTAGATTGGAAAGTAG CTTCTCCAATTGCTTACCAAGTGCAATAACG	(Pai et al., 2006)
4-f 4-r	430	CTGTTACTTGTTCTGGACTCTCGATAATTGG GCCCACTCCTGTTAAAATCCTACCCGCATTG	(Pai et al., 2006)
6-f 6-r	250	AATTTGTATTTTATTCATGCCTATATCTGG TTAGCGGAGATAATTTAAAATGATGACTA	(Pai et al., 2006)
7F/A-f 7F/A-r	826	CCTACGGGAGGATATAAAATTATTTTTGAG CAAATACACCACTATAGGCTGTTGAGACTAAC	(Pai et al., 2006)
9V/A-f 9V/A-r	753	CTTCGTTAGTTAAAATTCTAAATTTTTCTAAG GTCCCAATACCAGTCCTTGCAACACAAG	(Pai et al., 2006)
9N/L-f 9N/L-r	501	TCAGCATTTTCTCATTAACGATTT CAAGATCTGACGGGCTAATCA	Based on (Dias et al., 2007)
10A-f 10A-r	517	CAAATAAAGTGTATGAGCGTGT TCTTTAAGATTCGGATATTTCTC	New Based on (Pai et al., 2006)
10F/C-f 10F/C-r	248	GGAGTTTATCGGTAGTGCTCAT CTAACAAATTCGCAACACGAG	Based on (Zhou et al., 2007)
11A/D-f 11A/D-r	463	GGACATGTTCAGGTGATTTCCCAATATAGTG GATTATGAGTGTAATTTATTCCAACTTCTCCC	(Pai et al., 2006)
12F/A/B-f 12F/A/B-r	375	CAACAAACGGCGTGAAAGT CAAGATGAATATCACTACCAATAACAAAAC	New (Pai et al., 2006)
14-f 14-r	208	GAAATGTTACTTGGCGCAGGTGTCAGAATT GCCAATACTTCTTAGTCTCTCAGATGAAT	(Pai et al., 2006)
15A-f 15A-r	436	ATTAGTACAGCTGCT ATATCTCTTC GATCTA GTG ACCGTACTATTCCAA AC	(Pai et al., 2006)
15B/C-f 15B/C-r	496	TTGGAATTTTTTAATTAGTGGCTTACCTA CATCCGCTTATTAATTGAAGTAATCTGAACC	(Pai et al., 2006)
16F-f 16F-r	311	GTTCAGATAGGCCATTTACAG TCCAGCTAAATTAGGAGCAG	Based on (Pai et al., 2006) New
17F-f 17F-r	677	TTCGTGATGATAATTCCAAG AGCGACTAAGGTCTGCTTGT	Based on (Pai et al., 2006) New
18-f 18-r	285	GCCGTGGGAAGCTTATTTTT CCTGCCTAAAGGCAACAATG	(Dobay et al., 2009)
19F-f 19F-r	304	GTTAAGATTGCTGATCGATTAATTGATATCC GTAATATGTCTTTAGGGCGTTTATGGCGATAG	(Pai et al., 2006)
19A-f 19A-r	478	GTTAGTCCTGTTTTAGATTTATTTGGTGATGT GAGCAGTCAATAAGATGAGACGATAGTTAG	(Pai et al., 2006)
19B/C-f 19B/C-r	354	AGAATTCGGAGATTTGTGGA AATCCCAGATCAAATGTTCC	(Zhou et al., 2007)
20A/B-f 20A/B-r	453	TTCACCTGACAGCGAGAAG TCTGAAAATGCAAACGTCCT	Based on (Pai et al., 2006) New
21-f 21-r	629	CAATTCTACTGAGTCCATATTATG CTCCTAAAGTTGCCAATAAGA	Based on (Zhou et al., 2007) New
22F/A-f 22F/A-r	643	GAGTATAGCCAGATTATGGCAGTTTTATTGTC CTCCAGCACTTGCGCTGGAAACAACAGACAAC	(Pai et al., 2006)
23F-f 23F-r	384	GTAACAGTTGCTGTAGAGGGAATTGGCTTTTC CACAACACCTAACACTCGATGGCTATATGATTC	(Pai et al., 2006)
23A-f 23A-r	853	GATTTGGAGCGGATCGATTA AATGGGTAATGGAGGGGAGT	(Dobay et al., 2009)
23B-f 23B-r	268	GTGGGTTGACGCATAAGAAT GATAATAAAGAAATTACTAACCATGTCGT	New (Zhou et al., 2007)
24F/A/B-f 24F/A/B-r	100	GGCTCCCTGCTATTGTAATCTTTA GTGTCTTTTATTGACTTTATCATAATAGGTC	Based on (Zhou et al., 2007)

Primer	Amplicon size (bp)	Sequence 5´ to 3´	Reference
29-f	654	CCGAAAATTGTTCACAGGATAC	(Zhou et al., 2007)
29-r		AAAAGAATTGTTTGATCCGAGA	New
31-f	711	GGAAGTTTTCAAGGATATGATAGTG	Based on (Pai et al., 2006)
31-r		GAAACCATTACCGAATAATATATTCAA	
33F/A-f	338	GAAGGCAATCAATGTGATTGTGTCGCG	(Pai et al., 2006)
33F/A-r		CTTCAAAATGAAGATTATAGTACCCTTCTAC	
33B/D-f	264	TCGTTGGAGACAAAACTCTTAC	(Zhou et al., 2007)
33B/D-r		CCTCCCTGAGCCAAAATAAC	
34-f	409	CTTTTGTAAGAGGAGATTATTTTCAC	Based on (Pai et al., 2006)
34-r		CCCAATCCGACTAAGTCTTC	
35F/47F-f	521	CCCATCTATCTTGATGATGAAC	New
35F/47F-r		TTCCTAGAGCGAGTAAACCAA	(Pai et al., 2006)
35B-f	688	TTGGATAAGTCTGTTGTGGAGA	Based on (Pai et al., 2006)
35B-r		CGCAGCTTCTTTCCAGATAA	
38 (25F)-f	568	CGTTCTTTTATCTCACTGTATAGTATCT	Based on (Pai et al., 2006)
38 (25F)-r		GAATTAAAGCTAACGTAACAATCC	
42 (35A/C)-f	492	TCCCTTTTTCAGACGTAGC	Based on (Dobay et al., 2009)
42 (35A/C)-r		CAAGAAATTGATCCGCTTG	
47-f	789	TGCCATAACGGACTCTAGAAC	Based on (Zhou et al., 2007)
47-r		CTGTCCCTTAGCTCTGTCCA	New
cpsA-f	160	GCAGTACAGCAGTTTGTTGGACTGACC	(Pai et al., 2006)
cpsA-r		GAATATTTTCATTATCAGTCCCAGTC	
lytA-f	318	CAACCGTACAGAATGAAGCGG	Based on (Sourav et al., 2010)
lytA-r		TTATTCGTGCAATACTCGTGCG	
wzh-f (*cpsB*)	117	TAGATGACGGTCCCAAGTCA	(Kurola et al., 2010)
wzh-r (*cpsB*)		GAGTTTCAAACATGCCCTTG	
wzh-r2 (*cpsB*)	160	ATTTCCCGAACCTGAAGAAAG	(Kurola et al., 2010)
wzh2-f (*cpsB*)	216	GTTGGCTTGTCCATGTCCTT	(Kurola et al., 2010)
wzh2-r (*cpsB*)		ATTCGGTGAAACGACTCCTG	
wciP-6A/C-f	149	ATTTATATATAGAAAAACTGGCTCATGATAG	(Jin et al., 2009b)
wciP-6A/C-r		GCGGAGATAATTTAAAATGATGACTAGTTG	
wciP-6B/D-f	155	AAGATTATTTATATATAG AAA AAC TGT CTC ATG ATA A	(Jin et al., 2009b)
wciP-6B/D-r		GCGGAGATAATTTAAAATGATGACTAGTTG	
wciN β-6C/D-f	359	ATCTCTAAATCTGAATATGAAGCGGCTCAATC	(Jin et al., 2009b)
wciN β-6C/D-r		GAACTGAGCTAAATAATCCTCTGGATTATCCACC	