The epidemiology of penicillin non-susceptible pneumococci in Iceland

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Thesis for the degree of Philosophiae Doctor

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Faraldsfræði pneumókokka með minnkað næmi fyrir penisillíni á Íslandi

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Ágrip

Streptococcus pneumoniae, pneumókokkar, sem ekki voru næmir fyrir penisillíni (PÓP) fundust fyrst á Íslandi 1988 og urðu á fjórum árum 20% allra pneumókokka stofna sem greindust hjá sjúklingum. Flestir voru þeir af hjúpgerð 6Bii/E, CC90. Árið 2011 hófust bólusetningar barna með bóluefni með próteintengdum fjölsykrum af 10 hjúpgerðum pneumókokka.

Markmið rannsóknarinnar voru að rannsaka algengi PÓP í stofnum frá sjúklingum; dreifingu hjúpgerða í sjúklingastofnum áður en bólusetningar hófust; hvort heilbrigð börn bæru fleiri stofna af fleiri en einni hjúpgerð í senn og hvort þeir bæru gen fyrir festiþræði. Í öllum tilvikum var algengi hjúpgerða í pneumókkabóluefni fyrir börn og sýklalyfjanæmi rannsakað.

Notaðir voru allir PÓP stofnar sem voru greindir á Sýklafræðideild Landspítalans frá öllum sjúklingasýnum, 1995-2015; allir pneumókokka-stofnar, frá miðeyra, neðri öndunarvegum og ífarandi pneumókokkasjúkdómi 2007-2011; nefkoksstrok frá börnum sem safnað var á 15 leikskólum á höfuðborgarsvæðinu vorið 2009. Stofnar af sömu svipgerð og áður hafði ræktast frá sjúklingi innan 30 daga voru útilokaðir.

Næmispróf voru gerð samkvæmt CLSI og EUCAST stöðlum, hjúpgerð greind með hefðbundnum aðferðum og/eða PCR, erfðaskyldleiki kannaður með PFGE og MLST og gen festiþráða, PI-1 og PI- 2, með PCR.

Árin 1995-2010 ræktuðust 13.937 pneumókokkastofnar sem uppfylltu skilyrði rannsóknarinnar. Yfir tímabilið voru 27,7% þeirra ónæmir fyrir penisillíni og 89,8% af bóluefnis hjúpgerðum. Árið 1996 voru 25,9% PÓP, flestir af hjúpgerð 6Bii/E, CC90, sem var hæsta hlutfallið á fyrri hluta rannsóknarinnar. Samfara hnignun CC90 sem síðan hófst varð hlutfall PÓP lægst, 13,6%, árið 2001. Eftir það var hröð fjölgun aftur orsökuð af 19F, CC320 og varð hlutfall PÓP 42,7% árið 2011. Árið 2015 voru PÓP stofnar sexfalt færri en 2011 og kom fækkunin fyrst fram hjá yngstu börnunum.

Árin 2007-2011 ræktuðust 1.616 pneumókokkastofnar frá miðeyra, neðri öndunarvegum og ífarandi sýkingum. Þar af voru 54,4% frá miðeyra, 34,9% frá neðri öndunarvegum og 10,7% frá ífarandi sýkingum. Algengasta hjúpgerðin var 19F, 36,4% og var hún ríkjandi hjúpgerð í eyrum og öndunavegum, en hjúpgerð 14 var algengust í ífarandi sýkingum. Megnið af stofnunum frá miðeyra voru frá börnum, 65,7% voru PÓP og 74,1% af

bóluefnishjúpgerðum. Flestir stofnanna frá öndunarvegum voru frá fullorðnum, 31,5% PÓP og 53,2% af bóluefnishjúpgerðum. Ífarandi stofnar dreifðust jafnar í aldurshópa, 2,8% PÓP og 58,4% af bóluefnishjúpgerðum.

Nefkoksstrok fengust frá 514 leikskólabörnum árið 2009. Af þeim báru 76,6% pneumókokka. Hlutfall þeirra sam báru fleiri en eina hjúpgerð í einu var 23,5% og greindist það mun betur með PCR. Engin hjúpgerð var ríkjandi gagnstætt því sem var þá hjá veikum börnum.

Til leitar að genum fyrir festiþræði, PI-1 og PI-2, fengust 398 stofnar frá leikskólabörnunum. PI-1 gen greindust í 33,7% stofnanna og PI-2 í 9,5%. PI-1 gen voru algengust í stofnum af hjúpgerð 6B og voru af flokki II og 19F af flokki I. Algengast var að 19F bæri gen fyrir PI-2, ásamt PI-1 genunum.

Algengi ónæmra stofna var sérlega hátt miðað við önnur lönd í norðanverðri Evrópu og var það knúið áfram af tveim fjölónæmum alþjóðlegum klónum, fyrst 6Bii/E, CC90 og síðan 19F,CC320. Við sáum CC90 ná hámarki og dala síðan smám saman í öllum aldurshópum, líklegast á náttúrulegan hátt. Síðan tók CC320 sér bólfestu, dreifðist hratt og náði hámarki árið sem bólusetningar hófust, en hnignaði hratt eftir það og fyrst í bólusettum börnum.

Dreifing hjúpgerða í sjúklingasýnum 2007-2011 mótaðist af hjúpgerð 19F sem var ríkjandi í öllum aldurshópum. Hún var sækin í miðeyra og megin ástæðan fyrir því að þar voru stofnar af bóluefnishjúpgerðum algengastir. 19F var einnig ríkjandi í neðri öndunarvegum, en sjaldgæfari í ífarandi sýkingum.

Hlutfall heilbrigðra barna sem báru pneumókokka árið 2009 var mjög hátt og greindust fleiri en ein hjúpgerð í senn hjá fjórða hverju barni sem bar þá. Þetta endurspeglar möguleika á erfðabreytingum og vali á ónæmum stofnum með röngu vali sýklalyfja til meðferðar eða ófullnægjandi sýklalyfjanotkun.

Gen sem kóða fyrir festiþráðum voru algengust í hjúpgerðum 6B og 19F. Hæfileikinn til að mynda festiþræði fylgir klónum og er það ásamt sýklalyfja-ónæmi mögulegur hluti af ástæðunni fyrir velgengni CC320 og CC90.

Rannsóknin sýndi hve mikil áhrif einstakir klónar geta haft á útbreiðslu ónæmis. Þegar bólusetningar barna hófust var ónæmi, sem þá var orsakað af 19F klóninum, meira en nokkurn tíma fyrr. Með bólusetningunum fækkaði ónæmum pneumókokkum verulega, sérstaklega í miðeyrnasýnum frá börnum þar sem klónninn er nú nánast horfinn.

Lykilorð: Pneumókokkar, ónæmi, bóluefnis hjúpgerðir, festiþræðir

Abstract

Penicillin non-susceptible pneumococci (PNSP) were first found in Iceland in 1988 and became 20% of all pneumococci in four years, most belonging to serotype 6Bii/E, CC90. Infant vaccination with the ten-valent *Haemophilus influenza* protein D conjugated vaccine (PHiD-CV10) was started in 2011.

The aims of the study were to investigate the prevalence of PNSP in isolates from patients, serotypes in pneumococcal diseases prior to vaccination, co-colonization of serotypes in healthy unvaccinated children, pilus genes (PI-1 and PI-2) in pneumococci and in all instances to relate the findings to antimicrobial susceptibility and vaccine serotypes (VT).

The pneumococcal isolates used were all identified at the Department of Clinical Microbiology, Landspitalinn, and included all clinical PNSP isolates during 1995-2015, all isolates from the middle ear (ME), lower respiratory tract (LRT) and invasive disease (IPD) in 2007-2011Isolates of same phenotype from any patient within 30 days were excluded. Furthenrmore all isolates and nasopharyngeal swabs obtained in carriage study from children attending 15 daycare centres in Reykjavik area 2009.

Susceptibility testing was done according to CLSI and EUCAST standards and serotyping using conventional methods and/or PCR. Genetic relatedness was studied using PFGE and MLST. Pili genes were detected using PCR.

For the prevalence of PNSP in clinical isolates in the pre-vaccination time 1995-2010, 13.937 isolates met the study criteria, thereof 24.7% PNSP and of those 89.8% belonged to VT. In 1996, when PNSP peaked in the beginning of the study, the prevalence of PNSP was 25.9%, mostly of serotype 6Bii/E, CC90. A gradual decrease was seen to 13.6% in 2001, due to a decline of CC90. A rapid increase followed to become 42.7% during 2011, the year when vaccination was implemented. PNSP isolates were six fold fewer in 2015 than in 2011, a decline first noted in the youngest children.

For serotype prevalence and comparison between different diseases 1616 isolates from 2007-2011 met the study criteria, 54.4% for ME, 34.9% for LRT and 10.7% for IPD. The most common serotype was 19F, seen in 36.4% isolates, ranking highest in ME and LRT. Serotype 14 was the most common serotype in IPD. The majority of the ME isolates were from children and the rate of PNSP was 65.7% and of VTs 74.1%. The majority of LRT isolates

were from adults, 31.5% PNSP and 53.2% VTs. The IPD isolates were more equally distributed in age groups, 2.8% were PNSP and 58.4% VTs.

To investigate rates of co-colonization, samples from 514 children were available. Carriage rate was very high, 76.6% and co-colonization detected using molecular methods in 23.5% of those children, 2-4 serotypes. No serotype was dominating in contrast with clinical isolates from children.

For detection of pilus genes, PI-1 and PI-2, 398 isolates from the same children were used and 33.7% carried genes for PI-1 and 9.5% for PI-2. PI-1 genes were most commonly carried by serotypes 6B and were of clade II and by 19F, clade I. Dominant clones were thus of different clades. PI-2 genes were most commonly carried by 19F, along with genes for PI-1.

The rates of PNSP were exceptionally high compared to other countries in northern Europe and driven by two dominant multi-resistant international clones, first CC90 of serotype 6Bii/E and then CC320 of 19F. We saw the former peak and gradually decline and the latter emerge and rapidly expand. CC320 peaked in the year infant pneumococcal vaccination started and then rapidly declined, first in the youngest vaccinated children.

Serotype distribution in patient samples from 2007-2011 reflected the development of 19F, that was the dominant serotype in all age groups. It had a predilection for ME and was the main cause for the highest rates of VTs in ME. It was also very common in LRT, but ranked low in IPD.

Pneumococcal carriage in healthy children in 2009 was among the highest reported. One in four of the carriers carried more than one serotype, reflecting opportunities for genetic exchange and selection of resistant strains by wrong use of antimicrobials.

Genes encoding for pili were most commonly found in isolates of serotype 6B and 19F. Possibly the pili genes in susceptible 6B originate from CC90. Carriage of pili is a clonal property and along with their multi-resistance likely to be a part of the success of CC320 and CC90 in Iceland.

The impact of the two dominant clones on pneumococcal epidemiology and antimicrobial resistance in Iceland was remarkable. When vaccination started resistance was higher than ever because of the 19F clone. Following vaccination it declined markedly, especially in children, where the clone has almost disappeared.

Keywords: Pneumococci, resistance, vaccine serotypes, co-colonization, pili

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Working on this study has been a long journey where many good people have paved my way.

First I would like thank my supervisor, Karl G. Kristinsson, for giving me the opportunity to do the study and for all his support. I would also like to thank the other scientists on the doctoral committee, Gunnsteinn Haraldsson for work and supervison with the molecular analyses and in the writing tasks, - and for generosity with his time. I am grateful to Ásgeir Haraldsson for his constant encouragement, comments and thought-provoking questions, Þórólfur Guðnason and María Heimisdóttir for sharing their knowledge and experience.

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Contents

List of abbreviations

AOM	Acute otitis media
ATC	Anatomical therapeutical chemical classification system
CBP	Choline binding protein
CC	Clonal complex
CC90	The Spain^{6B}-2, CC90
CC320	Taiwan19F-14, CC236/271/320
CLSI	Clinical and Laboratory Standards Institute
CPS	Capsular polysaccharide
DDC	Daycare centre
DID	Defined daily doses/1000 inhabitants/year
DNA	Deoxyribionucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
IPD	Invasive pneumococcal disease
LRT	Lower respiratory tract
MIC	Minimal inhibitory concentration
MLST	Multi-locus sequence typing
NPH	Nasopharyngeal swab
NVT	Non-vaccine serotype, unspecified
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PCV	Protein-conjugated vaccine
PHiD-CV10	Ten-valent *Haemophilus influenza* protein D conjugated vaccine
PI	Pilus genomic islet
PNSP	Penicillin non-susceptible pneumococci
PSP	Penicillin susceptible pneumococci
PCV	Protein-conjugated vaccine
ST	Sequence type
VT	Vaccine type, unspecified
VT-7	PCV 7-valent: 4, 6B, 9V, 14, 18C, 19F and 23F
VT-10	PCV 10-valent: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F
VT-13	PCV 10-valent: 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F
VT-23	Polysaccharide vaccine 23-valent: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F

List of Figures

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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.A., Kristinsson K.G. (2014). Epidemiology of penicillin-non-susceptible pneumococci in Iceland, 1995-2010. *J Antimicrob Chemother*, 2014,69(4), 940-946.

II. Hjalmarsdóttir M.A.*, Quirk S.J.*, Haraldsson G., Erlendsdóttir H., Haraldsson A., Kristinsson K.G. Comparison of serotype prevalence of pneumococci isolated from middle ear, lower respiratory tract and invasive disease prior to vaccination in Iceland.
(*shared first authorship). Manuscript submitted.

III. Hjalmarsdóttir M.A., Gumundsdóttir P.F., Erlendsdóttir H., Kristinsson K.G., Haraldsson G. (2016). Co-Colonization of Pneumococcal Serotypes in Healthy Children Attending Day Care Centres: Molecular Versus Conventional Methods. *Pediatr Infect Dis J*, 35(5), 477-480.

IV. Hjalmarsdóttir M.A., Pétursdóttir B., Erlendsdóttir H., Haraldsson G., Kristinsson K.G. (2015). Prevalence of pilus genes in pneumococci isolated from healthy preschool children in Iceland: association with vaccine serotypes and antibiotic resistance. *J Antimicrob Chemother*, 70(8),2203-2208.

Declaration of contributions

Martha Á. Hjálmarsdóttir (MÁH) started her PhD studies in 2005, which were performed along with her duties as associate professor and Head of the Department of Biomedical Sciences, Faculty of Medicine, University of Iceland and as a biomedical scientist at the Department of Clinical Microbiology, Landspitali University Hospital. Parts of the laboratory work were performed by biomedical scientists at the Department of Clinical Microbiology or by students at BS, MS and PhD levels and supervised by MÁH, Prof. Karl G. Kristinsson (KGK) and Dr. Gunnsteinn Haraldsson, PhD (GH).

Paper I: The study of the epidemiology of penicillin non-susceptible pneumococci (PNSP) in Iceland, 1995-2010, was initiated by KGK and MÁH as a retrospective surveillance study of antimicrobial susceptibility for the years 1995-2004, in order to close a gap since the last publication on PNSP in patients and then adding prospective data for 2005-2010. MÁH collected data from the records of the Department of Clinical Microbiology, verified results, repeated and/or performed analyses when needed and updated and maintained the PNSP database for 1995-2015. MÁH wrote the manuscript on the 1995-2010 study, which was completed under the supervision of KGK.

The 2011-2015 results are from an on-going unpublished study and collected and analysed by MÁH.

Paper II: The study for comparing serotype prevalences of pneumococci isolated from the middle ear, lower respiratory tract and from invasive disease prior to vaccination in Iceland was initiated by MÁH. Molecular serotyping methods were mainly performed by Sigríður Júlía Quirk (SJQ), a MS and PhD student. Serotyping using conventional methods was performed by the biomedical scientists at the Department of Clinical Microbiology for a part of the PNSP isolates and isolates from invasive diseases; the remainder was performed by MÁH and Arna Ágústsdóttir, BS student. MÁH initiated strain collection, collected data, set up a database, analysed results and wrote most parts of the manuscript, completed in collaboration with all co-authors. MÁH and SJQ share first authorship.

Paper III: The study on co-colonization of pneumococcal serotypes in healthy children, – molecular compared to conventional methods, was

initiated by MÁH and GH. Samples from an existing carriage study were used. In a separate carriage study, Árni Sæmundsson, medical student, collected nasopharyngeal swabs from healthy children attending day care centres, supervised by professors Ásgeir Haraldsson, KGK and clinical professor Helga Erlendsdóttir, who was also responsible for the identification, susceptibility testing and serotyping using conventional methods. The swabs and resulting isolates were kindly made available for our studies on cocolonization and detection of pili genes. Pálína F. Guðmundsdóttir, MS student, performed serotyping using molecular methods. MÁH analysed the results and wrote the manuscript, completed in collaboration with all co-authors.

Paper IV: The study on the prevalence of pilus genes in pneumococci isolated from healthy preschool children in Iceland: association with vaccine serotypes and antibiotic resistance was initiated by GH, MÁH and KGK. The detection of genes encoding for pili was performed by Brynhildur Pétursdóttir, MS student. MÁH analysed the data and wrote the manuscript, which was completed in collaboration with all co-authors.

1 Introduction

Streptococcus pneumoniae, the pneumococci, have probably always been a worldwide and major cause of morbidity and mortality among humans. They cause infections that range in severity from otitis media and pneumonia, to life-threatening septicaemia and meningitis. Thus it is not surprising that pneumococci were among the first bacteria to be identified, cultured and studied. Those early studies heralded advances in bacteriology and genetics (Austrian, 1981a; Watson & Musher, 1999). The natural habitat of pneumococci is the nasopharynx, especially in children. They are commonly colonized by pneumococci and sometimes by more than one serotype. This provides a source for pneumococcal disease and is an optimal environment for intra- and interspecies genetic recombination (Bogaert et al., 2004; Leino et al., 2008; Mitchell et al., 2015).

The individuals most at risk for pneumococcal infections are young children, the elderly and patients with impaired immune status (Austrian, 1999; Cartwright, 2002). Distribution of pneumococcal serotypes and clones, both in disease and carriage, is a dynamic process harbouring many variables, like time, geographical area, infection site, virulence factors, and host-related factors (Brueggemann et al., 2004; ECDC, 2012; Henriques-Normark et al., 2008). Distribution is further influenced by antimicrobial usage, selecting for resistance and vaccination, selecting for non-vaccine serotypes (Alanee et al., 2007; Cartwright, 2002; Dagan, 2009; Kristinsson, 1997).

Widely spread international clones are the main force behind increased resistance. They are likely to harbour properties that enhance their success in distribution. Besides resistance to antimicrobials they may harbour virulence factors, like pili, that aid their attachment to the respiratory epithelial cells (Barocchi et al., 2006).

1.1 Pneumococci

1.1.1 General characteristics

Pneumococci are Gram positive α-haemolytic cocci with a typical lancet-like morphology and are usually seen in pairs and chains. They are facultative anaerobe and grow well on blood-agar in a 5% CO_2 enriched atmosphere where they produce their typical concave colonies, due to their production of

autolytic enzymes. Like many other streptococci they grow even better under anaerobic conditions where their colonies tend to be larger, slimier and less concave compared to those grown in CO_2 (Bergey & Holt, 1994). Furthermore, anaerobic conditions and media containing gentamicin are helpful in inhibiting growth of other species commonly found in respiratory samples.

Pneumococci are catalase negative and sensitive to optochin that serves as the key-test in their identification when using conventional methods. They are soluble in bile (2% sodium deoxcholate) and the bile solubility test is used when results for optochin are indefinite. Identification systems, like API strep, and MALDI-TOF are not useful in the identification of pneumococci (Garcia, 2010; Jorgensen & Pfaller, 2015).

Pneumococci harbour several diverse virulence factors, of which most aid adherence of the pneumococci to the host cells, while others play important roles in invasion and spread in the host tissues. The major virulence factor of pneumococci is the capsule. In all the studies related to this thesis the serotype, representing the type of capsule, is part of the analyses. Pili are another virulence factor of special interest in this thesis.

1.1.2 Capsule

The capsule is recognized as the major virulence factor of pneumococci and the target of pneumococcal vaccines. When pneumococci enter the nasopharynx the negatively charged capsule counteracts their entrapment in the mucus (Nelson et al., 2007b). However, when the pneumococci have reached the epithelial cells a thick capsule is a hindrance to adherence. To overcome this they express a thinner capsule at that stage to permit attachment (Hammerschmidt et al., 2005).

The capsule inhibits phagocytosis of the pneumococcus. The negative charge provides a resistance against attachment of phagocytes to the pneumococci. In addition, the capsule protects them from opsonisation by preventing the attachment of IgG and C3b complement components to the pneumococcal surface (Abeyta et al., 2003).

Clinical isolates from invasive disease and most other infections are capsulated (Austrian, 1981a). However, uncapsulated strains are known to cause superficial infections, especially conjunctivitis (Hilty et al., 2014; Valentino et al., 2014). The virulence of different strains or serotypes is related to the thickness of their capsule. Commonly carried serotypes are more likely to have thick capsules and are therefore better protected against

phagocytosis (Weinberger et al., 2009). Capacity for causing disease is also related to a difference in composition of the pneumococcal capsular polysaccharides, or to their specific serotype (Austrian, 1981b; Brueggemann et al., 2004; Hanquet et al., 2010). Furthermore, the capsule provides a protection against lysis induced by treatment with penicillin and vancomycin and has thus a role in antibiotic tolerance (Fernebro et al., 2004).

1.1.2.1 Capsular polysaccharides

The capsule consists of capsular polysaccharides (CPS) that form a thick layer anchored in the peptidoglycan and surrounds the bacterial cell. The capsular polysaccharide is synthesised by the wzx/wzy-dependent pathway except for serotypes 3 and 37 that are synthesised by the synthase pathway (Cartee et al., 2001; Llull et al., 1999).

The genes for the wzx/wzy-dependent pathway are located on the same capsular biosynthetic locus, *cps*. The genes *dexB* and *aliA* flank the locus and seem to be common to all serotypes (Bentley et al., 2006; Garcia et al., 2000). The regulatory and processing genes *wzg, wzh, wzd* and *wze* (*cpsABCD)* are common to all serotypes. In most serotypes the next gene in the locus is *wchA* (*cpsE)* that encodes the initial glucose phosphate transferase that links activated glucose phosphate to a lipid carrier. The *wzy* gene encoding for the polysaccharide polymerase, and *wzx* encoding for the flippase, follow along with a set of genes for glycosyl transferases, acetyl transferases, nucleotide diphosphate sugar biosynthesis and modifying enzymes that like *wzy* and *wzx* can vary according to the serotype (Bentley et al., 2006).

The synthesis of the pneumococcal capsule thus involves several steps. First, the assembly of a repeat unit on a lipid carrier that is located in the interior of the cytoplasmic membrane. Subsequently, this compound is transferred to the other side of the membrane. The third step involves the polymerization of repeat units into long-chain polymer. Finally, the polymer is transferred through the peptidoglycan and anchored on its surface (Yother J., 2008).

1.1.2.2 Serotypes

Pneumococci are classified into serogroups and serotypes according to their reactions of the diverse polysaccharides to factor sera (antisera) (Henrichsen, 1995). The classification represents immunological similarity, where serogroups are labelled with numbers, and serotypes within a serogroup are labelled with letters. The first serotype within a serogroup is

labelled F and the following in alphabetic order (Henrichsen, 1999). Currently, approximately 100 serotypes are known.

In 2006 Bentley *et al.* sequenced the genes of the capsular biosynthetic locus from all the 90 serotypes known at the time, and related their genetics to their reactions to factor sera (Bentley et al., 2006). Many diverse mechanisms are responsible for the antigenic diversity in capsular polysaccharides that do not necessarily reflect the genetic variety. Thus, a serotype within a serogroup may be genetically more related to other serogroups than to other serotypes in its own serogroup (Bentley et al., 2006).

With increased use of sequencing of the *cps* loci still more new serotypes have been revealed. Thus since 2006, five new serotypes of serogroup 6 have been described (Baek et al., 2014; Jin et al., 2009a; Ko et al., 2013; Oliver et al., 2013; Park et al., 2015; Park et al., 2007).

1.1.2.3 Serotyping methods

Immunological methods are the conventional methods used for serotyping of pneumococci, and the foundation of their classification into serogroups or serotypes. They are performed either using agglutination or the Quellung method where the isolate is mixed with factor antisera. In the agglutination method the antibodies are most commonly attached to latex particles to enhance the visibility of the reaction. Using the Quellung method the capsule becomes visible under the microscope when the reaction is positive (Henrichsen, 1999; Sørensen, 1993). Those methods are performed on pneumococcal cultures and are considered highly specific and sensitive.

Increasingly, molecular methods, using single and/or multiplex polymerase chain reactions (PCR) with serotype specific primers, either alone or in combination with conventional immunological methods, are performed in serotype detection. It is most common to use subculture of a single colony for PCR, or a sweep of an agar plate when looking for co-colonization of multiple serotypes in the same sample (Dobay et al., 2009; Jin et al., 2009b; Pai et al., 2006). Co-colonization is a phenomenon not easily detected by conventional methods that rely on the phenotypes of the pneumococcal colonies. This may be very similar between serotypes (Huebner et al., 2000; Saha et al., 2015). PCR methods are only reliant on detecting the DNA sequences that their primers recognize and are therefore more sensitive for detection of co-colonization or mixed infections.

Whole genome sequencing (WGS) is becoming increasingly available and less expensive. Serotype specific sequences as well as other characteristics can be extracted from the genetic information obtained using the WGS and can therefore be used for serotyping (Bentley et al., 2006).

1.1.3 Pili

The pilus genomic islet 1 (PI-1) was first described in pneumococci in 2002 (Hava & Camilli, 2002). It was predicted to be involved in attachment to mucosal surfaces (Hava et al., 2003). Pilus genomic islet 2 (PI-2) was described in 2008 (Bagnoli et al., 2008). The pilus islets encode for pili that are flexible hair-like filaments that stick out from the pneumococcal surface. They mediate adherence to the host cells and generate immune response and are thus surface-extended virulence factors that some clones of pneumococci harbour (Bagnoli et al., 2008; Barocchi et al., 2006).

Murine models suggest that pili also promote invasiveness of pneumococci. Pilus adhesion RrgA of PI-1 pili interacts with complement receptor 3 enhancing phagocytosis and prolonging the survival of pneumococci after the phagocytosis (Orrskog et al., 2012). Along with other components produced by pneumococci, like pneumolysin and hydrogen peroxide that are toxic to macrophages, this might result in the death of the macrophage while the pneumococci are still viable and could thus aid the transfer of the pneumococci from the nasopharyngeal mucosa or alveoli into the bloodstream (Orrskog et al., 2012; Rubins & Janoff, 1998).

1.1.3.1 PI-1 pili

Electron microscopy has shown PI-1 pili to be elongated, flexible and rather sticky filaments of 1 µm in length. They appear both singly and in bundles all over the surface of piliated pneumococci (Barocchi et al., 2006; Hilleringmann et al., 2008).

The pilus genomic islet 1 is 12 kb and consists of 7 genes encoding for PI-1 pili. The transcription regulating gene of PI-1 is named *rlrA*, or *rofA*-like regulator, because of its similarity to the *S. pyogenes rofA* gene that regulates for the adherence protein F. Three genes, *rrgA (rlrA*-regulated gene), *rrgB,* and *rrgC,* encode for the subunits of pilin, the protein of which the pili consist. Additionally there are three genes that encode for three sortases (Barocchi et al., 2006; Hava & Camilli, 2002; Hava et al., 2003). The sortase genes were originally named *srtB, srtC, srtD,* but are classified as class C sortases, which are responsible for the co-valent pilin association. Hence, they are also referred to as srtC-1, srtC-2 and srtC-3, encoded for by

the *srtC-1*, *srtC-2* and *srtC-3* genes (El Mortaji et al., 2012). The pilus islet is flanked by *IS*1167 that contains inverted repeats that are typical for mobile genetic elements (Barocchi et al., 2006; Hava et al., 2003).

The backbone of the pilus consists of the major pilin, RrgB. Its assembly is catalysed by SrtB (srtC-1). The RrgA is a minor pilin that is located on the external end of the pili, and plays a main role in the adhesion to host cells. SrtC (srtC-2) catalyses the assembly of the RrgA pilin and its association to the RrgB. Together RrgA and RrgB make up the shaft of the pilus. All the sortases are involved in connecting the shaft of the pilus to the RrgC. The RrgC is a still smaller pilin and is the foundation of the pilus and anchors it in the peptidoglycan, catalysed by the housekeeping sortase SrtA that finally signals that the pilus is prepared (El Mortaji et al., 2012; Falker et al., 2008; Hilleringmann et al., 2008; Manzano et al., 2008; Nelson et al., 2007a; Shaik et al., 2014).

Due to variations within the *rrgB* gene three different variants of the PI-1 pili have been described, which divide it into the subsets clade I - III (Moschioni et al., 2008). The genetic similarity of the genomic islet within each clade is over 98%, but between clades it is 88-92%. The *rrgC* is conserved, but the *rrgA* is found in two variants (Moschioni et al., 2008). The variation in the *rrgA* gene is restricted to the “head” domain of the RrgA protein where the adhesion motives are located, - however, both variants show similar binding abilities (Moschioni et al., 2010b).

1.1.3.2 PI-2 pili

The pilus genomic islet 2 consists of 5 genes. Two genes encode for sortases, *srtG1* and *srtG2*, *sipA* encodes for a signal peptidase-related product and finally the *pitA* and *pitB* genes that encode for the pilus proteins (Bagnoli et al., 2008).

The backbone of the PI-2 pilus is the PitB protein and its polymerization depends on the activities of SrtG1 and SipA. PitA and SrtG2 seem to be unnecessary in the formation of PI-2 pili and could be considered pseudogenes that have lost their abilities. The properties for adherence seem therefore to rely on the pilus backbone protein alone (Bagnoli et al., 2008).

1.1.3.3 Prevalence of pili

The existence of pilus-encoding genomic islets is a clonal property (Aguiar et al., 2008). Pili are more commonly found in clones of penicillin non-susceptible pneumococci (PNSP) than in penicillin susceptible pneumococci

(PSP) and widely distributed international PNSP clones are known to harbour pili. PI-1 is most commonly detected in clones of commonly carried serotypes like 6B, 9V and 19F (Henriques-Normark et al., 2008; Sjöström et al., 2007), but PI-2 in clones of serotypes like 1, 7F, 19F and 19A (Vainio et al., 2011; Zahner et al., 2010).

The prevalence rate of PI-1 in pneumococci is 15-35% in nasopharyngeal samples from healthy children (Basset et al., 2007; Regev-Yochay et al., 2010; Turner et al., 2012), 20-30% in acute otitis media (Moschioni et al., 2010a; Vainio et al., 2011), and 10-33% in invasive disease (Basset et al., 2007; Moschioni et al., 2008; Selva et al., 2012; Zahner et al., 2010). The rates are usually lower for PI-2 than PI-1, and have been reported to range from 0-21% (Bagnoli et al., 2008; Moschioni et al., 2010a; Vainio et al., 2011; Zahner et al., 2010).

So far piliated pneumococci have most commonly been of the vaccine serotypes, but piliated PNSP of non-vaccine serotypes have emerged and spread, causing increasing concern. This was for example seen with clones of serotype 19A in the era of 7-valent vaccine (Siira et al., 2014; Zahner et al., 2010). Pili have been considered to be candidates for vaccine targetting (Gianfaldoni et al., 2007; Moschioni et al., 2012) (see section 1.3).

1.1.4 Other virulence factors

Autolysins are choline binding proteins (CBP) and like other CBPs they are bound to the teichoic or lipoteichoic acid of the pneumococci. They are hydrolytic enzymes released by bacterial lysis. The main role of the major autolysin, LytA, is to degrade the peptidoglycan of the pneumococcus if newly started peptidoglycan synthesis is halted, either because of lack of nutrients, or by β-lactam antibiotics. It is used in fratricide to kill non-competent siblings, meditates antibiotic induced-lysis, releases the intracellular toxin pneumolysin and facilitates distribution of factors involved in defences against immune responses (Jedrzejas, 2001; Mellroth et al., 2012).

Choline binding protein A enhances adhesion and binds to immunoglobulin receptors on the respiratory mucosa (Hammerschmidt et al., 1997). Furthermore it acts against the complement system (Hammerschmidt, 2006).

Hyaluronate lyase is a major surface protein that is anchored onto the peptidoglycan. It is an enzyme that degrades hyaluran, an important component of connective tissue. Hyaluran degradion aids the spread of

pneumococci in the host tissues and is important in the process of disease propagation (Rigden & Jedrzejas, 2003).

Neuraminidases (NanA, B and C) are sialidases, enzymes that remove sialic acid from glycoconjugate receptors on cell surfaces exposing the receptors in similar way as the influenza A virus. They show synergy to the neuraminidases of the virus, increasing the severity in co-infection (Walther et al., 2016). NanA is the most effective and found in all pneumococci. It is anchored to the peptidoglycan and is also secreted, NanB is mainly found within biofilms and NanC is less commonly expressed. Neuraminidases aid to upper respiratory tract colonization and play a role in biofilm production (Brittan et al., 2012; Walther et al., 2016).

Pneumolysin is a cytoplasmic pore-forming enzyme that is released by autolysin when the cell wall is degraded. It is toxic to ciliated bronchial epithelial cells, disrupts alveolar epithelium and plays thus an important role in pneumonia and facilitates invasive pneumococcal disease (Jedrzejas, 2001). It can inhibit phagocytosis and the functions of other immune cells (Rubins & Janoff, 1998).

Surface protein A, is a CBP and is protective against the complement system of the host and inhibits phagocytosis (Briles et al., 1988).

Other factors have not been defined as virulence factors as such but are, however, important for the genetic development of pneumococci, or for their protection. The ability of pneumococci and other respiratory pathogens and species of the normal flora to form biofilms is well known. Within the biofilms the pneumococci are protected against the immune system and antimicrobials. There they can co-exist with other pneumococcal strains or even other species, surrounded by a pool of DNA, in an optimal environment for intra- and interspecies genetic recombination (Hall-Stoodley et al., 2004; Marks et al., 2012a; Marks et al., 2012b; Moscoso et al., 2006). Pili of type IV are recently discovered surface extended filaments that pneumococci harbour. They mediate DNA binding and uptake (Balaban et al., 2014; Laurenceau et al., 2013).

1.2 Pneumococci in carriage and disease

Pneumococci most commonly colonize the nasopharynx without causing disease, but nasopharyngeal colonization is a precursor to pneumococcal disease. Distribution of serotypes in carriage and disease is a dynamic process encompassing many variables like time, area, infection site, and host-related factors (Alanee et al., 2007; Cartwright, 2002; Henriques-

Normark et al., 2008). It is further influenced by antimicrobial usage that selects for resistance (Kristinsson, 1997) and vaccinations that select for non-vaccine serotypes (Browall et al., 2014).

Pneumococci are a major cause of pneumonia and invasive disease with high morbidity and mortality. The World Health Organisation estimates the mortality to be 1.6 million persons each year and of those 0.7-1 million are children ≤5 year old (WHO, 2007). It was estimated that in the year 2000 14.5 million children ≤ 5 year old had severe pneumococcal disease and that of those 826.000 died. Pneumococci cause 11% of the deaths of all causes in this age group (O'Brien et al., 2009). Relatively milder infections, like otitis media, are the most common cause for antimicrobial treatment of children.

1.2.1 Carriage

Pneumococci are most commonly found as colonizers of the nasopharynx of healthy children, which is their natural habitat. Most children become colonized for the first time during their first year and become colonized by more than one serotype before they become two years old (Gray et al., 1980; Leach et al., 1994; Meats et al., 2003; Syrjanen et al., 2001). The carriage rate is usually at the highest when they are 2-3 years old and common carriage rates are 55-75% (Bogaert et al., 2004). When children attend day care where many children are together in close surroundings, or live in families of many siblings, the environment is optimal for an exchange of bacterial flora (Gudnason et al., 2014; Kristinsson, 1997; Leach et al., 1994).

In most instances carriage does not lead to pneumococcal disease and pneumococci can thus be considered to be a part of the normal flora, capable of causing opportunistic infections (Simell et al., 2012; Sjöström et al., 2006). However, nasopharyngeal colonization is considered to be a precursor to pneumococcal disease (Dhoubhadel et al., 2014; Mastro et al., 1993; Syrjänen et al., 2005) and the causative pneumococci are usually found in the nasopharynx of the patient, which is especially evident in otitis media (Leach et al., 1994; Syrjänen et al., 2005; Syrjänen et al., 2006). Furthermore, newly acquired serotypes are more likely to be the cause of disease than serotypes carried for a long time (Gray et al., 1980; Syrjänen et al., 2005).

The serotypes typically carried by healthy children, like 6A, 6B, 14, 19F and 23F, are considered to have a low invasive potential and those of a high invasive potential are rarely seen in carriage (Hjaltested et al., 2003; Sandgren et al., 2004; Tomasson et al., 2005). It is relatively common that

children carry PNSP and multi-resistant international clones are a well known cause of high rates of PNSP in carriage (Dagan et al., 1996; Lo et al., 2003; Sa-Leao et al., 2000a; Tomasson et al., 2005).

Vaccination has not caused a decrease of the pneumococcal carriage in the population but has decreased carriage of the VTs. Thus, it selects for NVTs that replace the VTs (Cohen et al., 2011; Devine et al., 2015; Rodrigues et al., 2009; Wyllie et al., 2016). It is therefore important to monitor serotype distribution in carriage, especially among healthy children, in order to be able to predict the development of the distribution in pneumococcal disease and to evaluate the effect of vaccination. Preferably the monitoring should include sensitive methods to detect co-colonization (Wyllie et al., 2016).

1.2.2 Co-colonization

Co-colonization refers to the situation when more than one serotype of pneumococci habit the nasopharynx at the same time. They coexist there along with other species of the normal flora and possible respiratory pathogens (Bosch et al., 2016; Garcia-Rodriguez & Fresnadillo Martinez, 2002; Marks et al., 2012b).

Colonization with one pneumococcal strain aids the colonization with additional strains, making the likelihood of co-colonization higher than previously thought (Marks et al., 2012b). Furthermore, it creates possibilities for formation of biofilms that consist of more than one pneumococcal strain. Within those is the optimal environment for intraspecies horizontal genetic transfer (Hall-Stoodley et al., 2008).

It is difficult to detect co-colonization using conventional methods as there may be little difference of the colony morphology or other phenotype-related characteristics of different serotypes (Huebner et al., 2000). The detection can be improved by using molecular methods and those studies show co-colonization rates in the nasopharynx of healthy children up to 40% (Ercibengoa et al., 2012; Kamng'ona et al., 2015; Wyllie et al., 2016).

Co-colonization is important in the light of antimicrobial resistance as intraspecies competition is more likely to be favourable for non-susceptible strains than susceptible (Mitchell et al., 2015). In addition, co-colonization with strains of different antibiograms provides a natural environment for intraspecies transfer of genes encoding for low affinity PBPs and other resistance genes (Coffey et al., 1991). Co-colonization is also important with

regard to vaccination, as transfer of capsular genes might result in serotype switching (Croucher et al., 2014a).

1.2.3 Disease

Most commonly seen pneumococci cause relatively mild mucosal infections like acute otitis media (AOM), conjunctivitis and sinusitis, usually following viral upper respiratory tract infections. These infections are among the most common infections in children and most have had one or more episodes of AOM by the age of 5 years (Arason et al., 2002b; Eskola et al., 2001; Syrjänen et al., 2005). Accordingly, it is the most common reason why parents seek health care services for their young children and the most common background factor for antibiotic treatment of children in high-resource countries (Arason et al., 2005; Klein, 2000; Kristinsson, 1999).

Pneumococci, *Haemophilus influenza* and *Moraxella catarrhalis* are the most common cause of AOM and cause similar symptoms. However, pneumococci are more likely to cause more severe infection, with fever, bulging tympanic membranes and spontaneous perforation, than the others (Palmu et al., 2004). Serotypes have differently high invasive potentials and this influences their distribution according to infection site and the immunological status of the host (Sandgren et al., 2004). In non-invasive disease, like otitis media, serotypes that are often carried by healthy children are common (Alonso et al., 2013; Horácio et al., 2014). However, differences in clinical presentation in otitis media are not related to serotypes (Palmu et al., 2005).

Lower respiratory tract infections, like community-acquired pneumonia, are most often caused by pneumococci and are a common cause for hospitalization and antimicrobial therapy. It is especially a burden for the elderly and the associated mortality is high (Cartwright, 2002; Palmu et al., 2014b; Polverino et al., 2013). Pneumococci are also the most common cause of nosocomial pneumonia (Paradisi et al., 2001). The severity and mortality of pneumonia is increased if sepsis follows. Other unfavourable prognostic factors are being very young or old, having underlying chronic diseases, like cardiac, lung and renal diseases and having an impaired immune status, either because of diseases related to the immune system or therapy that weakens it (Christensen et al., 2012; Dwyer et al., 2014; Loeb, 2015; O'Brien et al., 2009; Örtqvist et al., 2005).

Invasive pneumococcal diseases (IPD), like sepsis and meningitis, are the most severe pneumococcal infections, always calling for antimicrobial

treatment and are the reason for pneumococcal vaccination (Austrian, 1999; CDC, 2005; WHO, 2007). Sepsis most commonly occurs following pneumonia, but meningitis is most commonly caused by haematogenous dissemination from the nasopharynx. Serotypes that are considered to have a high invasive potential, like serotypes 1, 4, 7F and 9V, are common causes of IPD (Brueggemann et al., 2003; Brueggemann et al., 2004; Sandgren et al., 2004). These serotypes are rarely carried by children and are also rarely seen in milder infections (Alonso et al., 2013). In spite of that, the serotypes causing IPD can in most instances be found in the nasopharynx of the patient, supporting that colonization is also a precursor for IPD caused by highly invasive serotypes, although the carriage state may be short (Lloyd-Evans et al., 1996; Mastro et al., 1993).

For individuals with an impaired immune status, such as children and the elderly and immunocompromised patients, serotypes of low invasive potential which are common in their surroundings can be relatively common in IPD (Hanage et al., 2005; Sandgren et al., 2004). Furthermore, patients with IPD caused by NVTs are more likely to have underlying diseases, to have an impaired immune status and have generally worse health prognosis than those infected with VTs (Browall et al., 2014).

Infant vaccinations have reduced the burden of pneumococcal disease, but select for NVTs (Brueggemann et al., 2007; Wagenvoort et al., 2016; Whitney et al., 2003). This is most clearly seen in children, but also through herd immunity in the older population (Hammitt et al., 2006; Regev-Yochay et al., 2015; van Deursen et al., 2012). Replacement of NVTs for VTs is increasingly seen in all kinds of pneumococcal disease (Alonso et al., 2013; Beall et al., 2011; De Schutter et al., 2014; Horácio et al., 2014; Imohl et al., 2015; Regev-Yochay et al., 2015).

1.3 Antimicrobial susceptibility

Pneumococcal infections are often severe and require antimicrobial treatment, therefore the development of resistance is important and a cause for concern (Austrian, 1981a; Kristinsson, 1995; Kyaw et al., 2006).

Penicillin is the antimicrobial of choice when the isolates are sensitive or even intermediately susceptible, while in the case of penicillin allergy macrolides have been the alternative choice (Appelbaum, 2002; Austrian, 1981a; Cremers et al., 2014). In spite of increased resistance the treatment of pneumococcal disease is still dependent on penicillins, aminopenicillins and third generation cephalosporins (Appelbaum, 2002; Rivera & Boucher,

2011). Macrolides and trimethoprim and trimethoprim-sulfamethoxazole have been commonly used, especially in treatment of children and resistance to those antibiotics is common (Appelbaum, 2002; Kristinsson, 1997).

Penicillin non-susceptible pneumococci were first reported 1967 from patients with invasive pneumococcal diseases in Durban, South Africa, and those isolates were also resistant to chloramphenicol (Hansman, 1967). The development was rapid and in 1978 the first multi-resistant strains were reported. Among those were isolates resistant to penicillin, erythromycin, clindamycin, tetracycline and chloramphenicol (Jacobs et al., 1978). Multi-resistant clones then spread rapidly and new emerged, resulting in a major challenge in the management of pneumococcal disease. However, their rates differ between areas and countries (Appelbaum, 1987; Bean et al., 2004; Butler et al., 1996; ECDC, 2012; Shi et al., 1998) and mostly reflect the spread of highly successful international PNSP clones associated with several serotypes (Henriques-Normark et al., 2008; Klugman, 2002). Their propagational success on the other hand is related to selective antibiotic pressure and a favourable environment related to risk factors (Mitchell et al., 2015).

Several factors influence the risk for carriage and disease caused by PNSP. Among those are being of young age, attending day-care centres, having young siblings and having previously received antibiotic treatment (De Lencastre & Tomasz, 2002; Kristinsson, 1997; Regev-Yochay et al., 2003). This risk is reduced by vaccinations as the serotypes best known for resistance are included in the vaccines (Daana et al., 2015; Kyaw et al., 2006).

1.3.1 Resistance to β-lactams

Penicillin susceptibility is defined by the penicillin minimal inhibitory concentration (MIC) of the isolate and standards for susceptibility testing define breakpoints that determine whether an isolate is considered to be sensitive, intermediate sensitive, or resistant. The criterion for sensitive isolates is a penicillin MIC $\leq$0.06 mg/L according to the most commonly used standards, i.e. those of the Clinical and Laboratory Standard Institute, CLSI (CLSI, 2012) and the European Committee on Antimicrobial Susceptibility Testing, EUCAST (EUCAST, 2012). However, the breakpoints for penicillin resistance differ slightly between the standards. While in infections other than meningitis, the CLSI criterion for resistant isolates is a penicillin MIC of ≥2.0 mg/L, it is >2 mg/L according to EUCAST. Thus, the CLSI criteria for intermediate sensitive isolates is a penicillin MIC of 0.12-1 mg/L, while it is

0.12-2 mg/L according to EUCAST. For meningitis the criterion for sensitive is the same as for non-meningitis, but for resistant isolates it is >0.06 mg/L.

The cause of non-susceptibility to β-lactam antimicrobials in pneumococci is alterations of their target, the penicillin binding proteins (PBP), resulting in reduced affinity for the β-lactams. In pneumococci non-susceptibility is not caused by production of β-lactamases that split up the β-lactam ring in the molecule of the antimicrobial and inactivate it, as is for example well known for *Staphylococcus aureus*. Therefore, penicillin can be used for treatment purposes in spite of reduced affinity if the MIC of the strain remains within the definition for intermediate sensitivity. For this purpose the EUCAST standard includes guidelines for dosing of penicillin in these instances, and there is a recommendation for higher and more frequent doses according to the MIC of the pneumococci (EUCAST, 2015).

PBPs are transpeptidases that are responsible for the cross-linking of the peptidoglycan in the cell wall (Zighelboim & Tomasz, 1980). They transfer the peptides needed in the pentapeptide bridges that link the N-acetyl muramic acid molecules in the peptidoglycan layer and thus enhance its strength (Garcia-Bustos et al., 1988). Among the peptides is D-alanyl-D-alanine that has a similar structure as the β-lactam ring, which is the key component of β-lactams. The β-lactams covalently link to the PBPs instead, and block the completion of the pentapeptide bridges (Garcia-Bustos et al., 1988). This results in osmotic instability, triggers autolysis of the cell wall and the bacterium is killed (Novak et al., 1998) .

The pneumococci have 6 different PBPs: PBP1a, PBP1b, PBP2a, PBP2x, PBP2b and PBP3. Changes in the genes that encode for the PBPs result in altered PBPs that have lower affinity for β-lactams. (Engel et al., 2014; Hakenbeck et al., 1986; Hakenbeck et al., 1999; Zighelboim & Tomasz, 1980). The main targets of β-lactams are the PBP2b and PBP2x, - thus alterations in the genes encoding for those PBPs are the main causes for low affinity (Krauss et al., 1996). The resistance increases considerably if a pneumococcal strain harbours a low affinity variant of PBP1a along with variants of either PBP2b or PBP2x (Reichmann et al., 1996). The scale of the changes is reflected in the MIC values. Sometimes they are subtle enough for the isolate to remain within the breakpoints for sensitive, but in other instances the isolate will be defined as PNSP, either of intermediate sensitivity or resistant to penicillin (Hakenbeck et al., 1986). It is possible to predict the MIC of pneumococci without performing susceptibility testing, by

sequencing the PBP genes and assigning them to PBP sequence types that have been matched to defined MICs (Li et al., 2016).

Low affinity PBPs in pneumococci are encoded for by so-called mosaic genes, where all, or parts of the genes involved, are replaced (Laible et al., 1991; Sibold et al., 1994). Thus, several events may have been needed, and in more than one PBP, to make changes that are sufficient to reduce the affinity to be considered non-susceptible. That mosaic PBP genes are not found in penicillin susceptible isolates, and transfer of altered genes has been shown to be possible between strains *"in vitro",* supports this theory (Dowson et al., 1989). The origin of genetic changes in PNSP is thought to be point mutations in related streptococci in the normal upper respiratory tract flora. Interspecies horizontal transfer follows through transformation of resistance determinants and recombination into the genes that encode for PBP in pneumococci (Coffey et al., 1991; Dowson et al., 1989). Intraspecies horizontal transfer, also via transformation, is then responsible for rapid development of PNSP (Mitchell et al., 2015).

Although the PBPs of the PNSP are changed, they are capable of cross-linking the subunits of the peptidoglycan and creating a solid cell-wall, but it´s structure contains more branched peptides (Garcia-Bustos & Tomasz, 1990).

Besides altered PBPs, penicillin tolerance in pneumococci can also be caused by changes that lead to loss or downregulation of autolysin (Novak et al., 1998).

1.3.2 Resistance to other antimicrobials

Increased prevalence of resistance of pneumococci against macrolides causes a risk for treatment failure. Macrolide resistance is widespread, although the rates differ between areas as for PNSP (Dagan et al., 1998; Farrell & Jenkins, 2004; Kristinsson, 1997; Kyaw et al., 2006).

The target of macrolides is the 23S ribosomal RNA in the 50S ribosomal subunit resulting in inhibition of protein synthesis, a bacteriostatic activity. Macrolide resistance in pneumococci is mediated through target modification and/or efflux pumps (Sutcliffe et al., 1996). Target modification is caused by methylation encoded for by the *erm*(B) gene causing alteration of the target that prevents the binding of the macrolide (Leclercq, 2002). This causes resistance to macrolides, lincosamides and streptogramin B and can be either constant or induced (Leclercq & Courvalin, 1991). In pneumococci the efflux pumps are encoded for by *mef*(A) genes and cause resistance at low or moderate levels to macrolides (Leclercq, 2002). The *erm*(B) are the most

common cause for resistance in pneumococci (Angot et al., 2000; Sutcliffe et al., 1996; Weisblum, 1995) but the *mef*(A) genes are also a common cause (Bley et al., 2011). Dual resistance encoded for by both these genes has been described in serotypes 19A and 19F of CC 320 along with high resistance rates to other antimicrobials, like penicillin, tetracycline and trimethoprim (Farrell & Jenkins, 2004). Use of macrolides, especially azithromycin, is related to increased resistance to this antibiotic class (Bergman et al., 2006).

Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal subunit preventing the access of aminoacyl tRNA to its site on the ribosome, resulting in inhibition of protein synthesis, a bacteriostatic activity (Goldman et al., 1983; Speer et al., 1992). Resistance to tetracycline is due to the *tet*(M) or *tet*(O) genes that encode for ribosome protection proteins, which are similar to elongation factors and allow the binding of aminoacyl tRNA in spite of high tetracycline concentrations (Izdebski et al., 2007; Speer et al., 1992; Widdowson & Klugman, 1998). It is very common that isolates are dually resistant to tetracyclines and macrolides due to insertion of the *erm*(B) gene into transposons that carry the *tet*(M) gene (Cochetti et al., 2008; Croucher et al., 2014a).

Resistance to chloramphenicol is mediated through production of chloramphenicol acetyltransferase, encoded for by the *cat* gene that is carried on transposons. The chloramphenicol acetyltransferase alters the chloramphenicol to a non-functional derivative (Korona-Glowniak et al., 2015; Widdowson & Klugman, 1999).

Resistance to trimethoprim is very common in pneumococci. In contrast to other routinely tested antibiotics, resistance to trimethoprim is not due to transformation, but is mediated through mutations in the gene encoding for dihydrofolate reductase. Strong correlations are between resistance to trimethoprim and sulfamethoxazole and the mutations are the main cause for resistance to both trimethoprim alone and trimethoprim-sulphamethoxazole (Adrian & Klugman, 1997).

1.3.3 PNSP in Iceland

In Iceland the first PNSP isolate was found in December 1988 in a specimen from the sinus maxillaris of a woman with sinusitis. It was of serotype 18 and had a penicillin MIC of 2 mg/L. The second isolate was identified in April 1989 in a middle ear specimen from a child. It was of serotype 6B and had a penicillin MIC of 2 mg/ml. Both were found through screening for penicillin

non-susceptibility that was regularly done to find possible PNSP in the era prior to these first identifications in the country (Kristinsson et al., 1992). The screening was done by testing oxacillin susceptibility of at least 100 respiratory isolates a year and performing MIC testings on all oxacillin resistant isolates. After these two occasions, susceptibility testing of all clinical pneumococcal isolates was implemented.

PNSP rapidly gained high prevalence, with prevalence rates of 2.3% of all clinical pneumococcal isolates in 1989, 2.7% in 1990, 8.4% in 1991, 16.3% in 1992 and peaked at 19.8% in 1993. Then a decline to 16.9% was seen in 1994 (Kristinsson, 1995). The increase of PNSP up to 1993 was mainly due to the expansion of a multi-resistant clone that was at the time identified as 6B, the Spain^{6B}-2, ST90, CC90 (PMEN2) and included 75% of the PNSP isolates during these years (Soares et al., 1993).The clone emerged in the 1960s and has been found throughout Western Europe from the 1980s and as well in the other continents. It seems to have entered Iceland from Western Europe, but not necessarily from Spain. The initial transmission into the country was very successful and the clone rapidly expanded in Reykjavik and surrounding communities, followed by distribution throughout the country (Arason et al., 2002a; Croucher et al., 2014b). Recent studies of van Tonder *et al* have revealed that the CC90 actually belongs to the newly described serotype 6E (van Tonder et al., 2015), but it is still debated if the serotype should be named 6E or if it is a variant of serotype 6B, 6Bii (Burton et al., 2016). In this thesis it is referred to as 6Bii/E.

Isolates of CC90 were intermediately sensitive to penicillin, with a MIC of ~1.0 mg/L and resistant to erythromycin, tetracycline, chloramphenicol and trimethoprim/sulfamethoxazole (Kristinsson, 1995). The high PNSP rates in Iceland were unique in the Nordic countries (Croucher et al., 2014b; Henning et al., 1997; Magnus & Andersen, 1995; Nissinen et al., 1995; Renneberg et al., 1997).

Other PNSP serogroups identified in patients in Iceland during 1988-1995 were serogroups 19 and 23, of which most of the isolates had slightly reduced susceptibility to penicillin with a MIC just above the breakpoint (0.12 mg/L). A few strains of serogroup 23 were multi-resistant, belonging to the Spain^{23F}-1 clone (Sa-Leao et al., 2002b; Vilhelmsson et al., 2000).

In a carriage study among healthy children, performed in five daycare centres in the Reykjavik capital area in 1992-1999, the PNSP rates were 13.9%, and most of serotypes 6B, 6A and 23F (Tomasson et al., 2005). Another study performed in five communities in the countryside in 1992-1993

showed PNSP rates of 9.7%, with most of serogroups being 6, 23 and 19 (Arason et al., 1996).

In 1989-1995 antimicrobial consumption in Iceland was high and higher than in the other Nordic countries, especially the consumption of trimethoprim-sulphamethoxazole (Kristinsson, 1997). This created a favourable environment for distribution of successful multi-resistant clones in the country (Kristinsson, 1997). Furthermore, there was no direct selective pressure because of vaccination. Although protein-conjugated vaccines had been on the market since 2000 they were not introduced to the childhood vaccination scheme in Iceland until April 2011 (Sóttvarnalæknir, 2011). Prior to that protein-conjugated vaccines were only recommended for children in high risk groups and their use was limited (Sóttvarnarlæknir, 2007).

1.4 Pneumococcal immunization

Pneumococcal vaccines had been implemented as part of the infant vaccination programs of 103 countries by the end of 2013 according to reports of the World Health Organization. The global coverage was 25% (WHO, 2015).

Pneumococcal vaccination using (PHiD-CV10) vaccine was implemented in the infant vaccination program in Iceland in April 2011, thus scheduled for all children born in 2011 and onwards. The vaccine is given in a 2 + 1 schedule (3, 5 and 12 months) and was initiated without a catch-up (Sóttvarnalæknir, 2011).

1.4.1 Polysaccharide vaccines

Capsular polysaccharides can be protective against specific serotypes and purified capsular polysaccharides are the antigens used in pneumococcal vaccines. However, the number of serotypes included in the vaccines is limited (Felton & Prescott, 1939).

The first polysaccharide vaccines were marketed in 1977 and contained polysaccharides from 14 serotypes, or 14-valent pneumococcal vaccines (Fedson, 1998; Hilleman et al., 1981). Their selection was based on epidemiological information, mostly from USA, parts of Europe and South Africa (Robbins et al., 1983). Soon the manufacturers developed 23-valent vaccines, with polysaccharides from 13 of the 14 serotypes included the first vaccines and from 10 other serotypes in addition. The 23-valent vaccine Pneumovax® contains polysaccharides of the serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and

33F (VT-23). The polysaccharide vaccines have been on the market since 1983 and been recommended for use in the elderly and other risk groups in this country as in many other (Fedson, 1998; Sóttvarnarlæknir, 2007). Unfortunately, polysaccharide vaccines are not effective in young children (Douglas et al., 1983).

1.4.2 Pneumococcal conjugated vaccines

In infancy the immune system is still insufficiently developed in order to react to polysaccharide stimuli (Timens et al., 1989). Therefore purified polysaccharide vaccines are poorly immunogenic in this age group (Harding et al., 1991; Hilleman et al., 1981). However, immunogenic proteins are capable of initiating immune response in infants (Black et al., 2000). If polysaccharides are conjugated with these proteins (Avery & Goebel, 1931) the immune response provoked is also aimed at the polysaccharides and immunological memory is generated (Black et al., 2000). Protein-conjugated vaccines (PCV) were therefore developed for use in infant pneumococcal vaccination and are effective (Chevallier et al., 2009; Palmu et al., 2013; Schneerson et al., 1980; Silfverdal et al., 2016). PCVs include polysaccharides of fewer serotypes than pure polysaccharide vaccines. The serotypes originally chosen were mainly serotypes seen in paediatric invasive diseases in North America and Europe (Robbins et al., 1983) and were considered to have good potential for clinical impact in all regions (Hausdorff et al., 2000). At the same time those serotypes are common in other childhood diseases such as AOM. Importantly the serotypes in the PCVs include the most common multi-resistant pneumococci (Black et al., 2000; Klugman et al., 2003). Serotype replacement in invasive pneumococcal disease, starting soon after initiation of the first PCV, resulted in further development of the conjugated vaccines to include more serotypes.

Prevenar/Prevnar® (Pfizer, Philadelphia, USA), the first conjugated pneumococcal vaccine, was licensed in 2000. It included purified polysaccharides of 7 serotypes, the serotypes 4, 6B, 9V, 14, 18C, 19F and 23F (VT-7), conjugated to diphtheria toxoid (PCV-7).

Synflorix® (GlaxoSmithKline Vaccines, Rixensart, Belgium), was licensed in Europe in 2009. It is ten-valent and includes polysaccharides of the same serotypes as the seven-valent vaccine and additionally of serotypes 1, 5, 7F (VT-10). The serotype-specific polysaccharides are conjugated to protein D from non-typable *Haemophilus influenza,* diphtheria toxoid and tetanus toxoid (PHiD-CV10).

In 2010 Prevenar 13® (Pfizer, Philadelphia, USA), was licensed. It includes the polysaccharides of the same serotypes as the ten-valent vaccine and in addition those of serotypes 3, 6A and 19A (VT-13), all conjugated to diphtheria toxoid (PCV-13).

After the initial launching of the PCV-7, it was noted that the incidence of invasive pneumococcal disease decreased in vaccinated children and with the implementation of PCV-10 or PCV-13 an added protection was seen (Black et al., 2000; De Wals et al., 2012; Jokinen et al., 2015; Metcalf et al., 2015; Palmu et al., 2013; Steens et al., 2013; Whitney et al., 2006). Decreased incidence of pneumococcal disease was also noted, although at lower rates in non-vaccinated individuals because of herd immunity (Hammitt et al., 2006; Regev-Yochay et al., 2015). At the same time non-vaccine serotypes (NVT) emerged as main cause for invasive pneumococcal disease (Brueggemann et al., 2007; CDC, 2005; Croucher et al., 2014a; Metcalf et al., 2015). The replacement of the NVTs has since been well described, including expansion of the serotypes 19A, 7F and 6C following vaccination with PCV-7 and then their decline following implementation of PCV-13 (Cohen et al., 2011; Kaplan et al., 2010; Metcalf et al., 2015). This was especially described for 19A, which when the rates of the vaccine serotypes decreased, emerged as the most common cause of IPD and antimicrobial resistance in the USA (Moore et al., 2008; Pai et al., 2005).

To give an example of a comprehensive study of vaccine effects, the Finnish randomised double-blind cohort study, the Finnish otitis media vaccine trial, is chosen. The researchers investigated, prior to implementation of pneumococcal vaccine, the effect of the seven-valent conjugated vaccine on otitis media and compared the results with a control group receiving hepatitis vaccine. Their results showed lower overall prevalence of otitis media in the vaccinated group, but especially a reduction in prevalence of vaccine serotypes (Eskola et al., 2001), reduction in tympanostomy tube placement (Sarasoja et al., 2013) and persistence of antibodies for a long time (Ekstrom et al., 2013). Furthermore, the replacement with NVTs or non-typable *Haemophilus influenzae* was noticeable soon after the first vaccination (Jokinen et al., 2012). Similar results were noted in another large study in California (Black et al., 2000).

Another Finnish study, the Finnish invasive pneumococcal disease vaccine trial, is also a good example for studies on the vaccines that followed. There the effectiveness of PHiD-CV10 against invasive disease in young children was addressed prior to implementation of the vaccine into the

national infant vaccination schedule. The study was also a randomised, double-blind trial, using either two or three doses for initial vaccination, followed by one boost. Hepatitis vaccines were used for the control groups. It showed high effectiveness in both the groups of vaccinated children (Palmu et al., 2013), a reduction in antimicrobial purchases for vaccinated children (Palmu et al., 2014a), a reduction in carriage of vaccine types (Vesikari et al., 2016) and like in the former study there was a reduction in tympanostomy tube placements (Palmu et al., 2015a), However, there was only a trend towards reduction of acute otitis media (Vesikari et al., 2016). Evaluation of the impact of the vaccine after its initiation in two plus one doses confirmed a high level of reduction of invasive disease in vaccinated children and showed also reduction, although later and at lower levels, in unvaccinated children (Jokinen et al., 2015).

Cross-protection between VTs to related NVTs has been described. This is especially evident in the case of the recently described 6E that has identical serological properties as the VT 6B and can thus actually be considered as VT (Burton et al., 2016; van Tonder et al., 2015). Cross-protection from vaccine polysaccharides of serotype 6B has also been described towards 6A and 6C (Grant et al., 2013). However, previously serotype 6C was identified as 6A, and in a study where 6A isolates were re-identified, only cross-protection to 6A was seen, but not to serotype 6C (Palmu et al., 2015b). Cross-protection from vaccines including 19F to 19A has also been reported (De Wals et al., 2012; Grant et al., 2013; Lee et al., 2011) Furthermore, the VTs may differ in power to generate sufficiently high titres for protection (Poolman et al., 2009).

1.4.3 Vaccine candidates

Diverse proteins related to virulence factors of pneumococci have been studied as possible targets in the search for new vaccine candidates and as well the whole pneumococcal cell. Several proteins are under investigation and may possibly become available in the future. Among those are vaccines that have pili as their target.

Piliated pneumococci generate immune response (Barocchi et al., 2006) and proteins of the PI-1 pili have been investigated as possible vaccine targets (Gianfaldoni et al., 2007; Moschioni et al., 2012). All the pili subunits, RrgA, RrgB and RrgC, have been shown to evoke immune response in mice, and both passive and active immunization with sera raised against each of the proteins and the combined pili proteins are known to be protective in mice (Gianfaldoni et al., 2007).

In a mouse model, a fusion protein of the three variants, clades I-III, of the RrgB was tried and found to induce an immune response (Moschioni et al., 2012). The fusion protein was protective, while proteins of each clade were not cross-protective between the RrgB clades (Harfouche et al., 2012). The two variants of the RrgA promoting adhesion, were protective in mice and also cross-protective between the RrgA clades (Moschioni et al., 2010b). In a chinchilla model, variation of pilus expression was shown to protect pneumococci against the effects of a vaccine including RrgB and that they had developed mechanisms for regulation of non-essential surface proteins in order to overcome the host defences (Figueira et al., 2014). Thus possible vaccine targets need to be studied in detail and their function understood and monitored "*in vivo*".

Antibody titers against RrgA and RrgB are increased in humans following colonization (Ahmed et al., 2014). The antibody titers, especially against RrgA, were higher in children not carrying pneumococci than in the ones that did. This indicates that the proteins might be protective against pneumococcal carriage (Ahmed et al., 2014).

1.5 Genetic diversity and major PNSP clones

Pneumococci are highly competent and their genetic diversity is thought to be due to a high rate of recombination rather than mutations (Feil et al., 2000). Selective pressure and good accessibility to intraspecies and even interspecies DNA increase the possibilities for recombination (Marks et al., 2012b). Their ability to form biofilms is very useful in this regard as the accessibility of foreign DNA is high within their closed environment (Hall-Stoodley et al., 2008; Marks et al., 2012a). Processes within the biofilm stimulate DNA transfer and competent pneumococci even directly increase the DNA pool by fratricide and thus kill non-competent pneumococci (Claverys et al., 2007).

1.5.1 Sequence typing

Multi-locus sequence typing (MLST) where seven housekeeping genes, encoding for basic metabolic functions are sequenced is used to analyse the relatedness of pneumococcal strains. These genes are chosen as they are not expected to be affected by selective forces and evolve slowly. That makes them better candidates for showing the relatedness between strains of the same species than other genes that are forced to change because of selective factors like vaccination, antimicrobial use and changes in their environment (Enright & Spratt, 1998).

MLST is done by sequencing the seven housekeeping genes after they have been amplified in PCR reactions. They are increasingly obtained using whole genome sequencing, WGS, where the sequences of the housekeeping genes are extracted from the WGS data (Everett et al., 2012).

When the sequences of the housekeeping genes have been analysed, the results are compared to data in an internationally available databank, pubmlst.org, and either defined as known sequence types (ST), their relatedness with known clones and clonal complexes (CCs) being defined, or they are defined as novel STs/CCs and in that case information is added to the database (Jolley & Maiden, 2010).

STs of a specific CC detected by MLST are most commonly of the same serotype, but not necessarily so, as capsular genes can spread through horizontal DNA transfer and those changes evolve faster than changes in the housekeeping genes (Enright & Spratt, 1998). Specific properties are related to CCs. Thus widely distributed successful clones often carry genes encoding for resistance against antimicrobials and virulence factors like pili that are not harboured by all pneumococci (Brueggemann et al., 2003; Croucher et al., 2014b).

1.5.2 Major global PNSP clones

Major global antimicrobial-resistant clones of pneumococci are named according to the Pneumococcal Molecular Epidemiology Network (www.pneumogen.net/pmen/) using the name of the country of the origin of the first described strain of the clone, its first serotype detected and a sequential number, and when serotype variants are detected those are added to the name, e.g. Spain^{23F}-1-19F (McGee et al., 2001). That clone caused a rise in PNSP rates in many countries (Klugman, 2002), but was detected at low rates in Iceland in the nineties (Sa-Leao et al., 2002b).

Clones are increasingly referred to just using the number of their clonal complex. Sometimes their sequence type and their relation to the named global clone is used, such as CC90, ST90, Spain^{6B}-2 (Ardanuy et al., 2009; Beall et al., 2006; Siira et al., 2012b). CC90 was the main cause for high rates of PNSP in Iceland in the nineties (Croucher et al., 2014b; Sa-Leao et al., 2002a; Vilhelmsson et al., 2000).

Major multi-resistant clones are the main driving force behind high rates of resistance to routinely tested antimicrobials, as experienced with the above mentioned clones. Therefore, the rates of PNSP serotypes are mainly dependent on the distribution of diverse clones in different areas. This has

been described for CC320 causing increased rates of PNSP of serotypes 19A and 19F in Finland prior to vaccination (Siira et al., 2012a) and for an increase of PNSP of serotype 19A in invasive disease in USA post PCV-7 (Beall et al., 2011). In Sweden CC156 drove the increase of PNSP of serotype 9V (Sjöström et al., 2007) and the same was noticed in the Czech Republic (Zemlickova et al., 2006).

1.6 Background and research questions for the study

The main research aims of this study were to describe the epidemiology of non-susceptible pneumococci and possible effects of pneumococcal vaccination. In that context the target was to describe the prevalence of PNSP in patient samples. Previously, this has been described for patients in 1988 to 1994. Accordingly, there was a gap in the history of PNSP that needed to be closed. The research question pertaining to this was therefore: *How did PNSP develop after 1994 and how was the development during the study period?*

All the other aims of the study result from the desire to gain a better understanding of PNSP. Previously, Icelandic studies had reported serotype distributions of PNSP, in invasive pneumococcal diseases and in carriage. Here the central research question was: *How is the distribution of all serotypes in patient isolates, regardless of susceptibility? Do serotypes with high proportions of PNSP progress differently from those that do not have the same attribute? How is the distribution of serotypes in different diseases, or age groups?*

Carriage of more than one pneumococcal serotype might lead to increased possibilities for intraspecies genetic exchange, or treatment failure. For this the research question was: *How commonly do children who harbour pneumococci carry more than one serotype?*

Pili are virulence factors of pneumococci that were discovered in the beginning of the study period. Accordingly, very little was known about their distribution in pneumococci and we have no information of this in strains circulating in Iceland. The research question on this was: *Could pili play a role in the development of PNSP?*

Vaccination with conjugated pneumococcal vaccine had not started when the study started but the vaccines were available and would obviously be used in the future. Here the research question was: *What possible effect could vaccination have on all those issues?*

2 Aims

The specific aims of the studies described in the thesis were to:

I. Establish the prevalence of non-susceptibility, especially to penicillin, in pneumococcal isolates from patients in 1995-2015 and their distribution by time, sampling site, age groups and vaccine serotypes (paper I 1995-2010, unpublished data 2011-2015)

II. Find the prevalence of serotypes in patient samples and compare serotype distribution of pneumococci isolated from the middle ear, lower respiratory tract and in invasive pneumococcal disease prior to vaccination (2007-2011), analyzed by time, sampling site, age groups, antimicrobial susceptibility and serotypes (paper II).

III. Determine co-colonization of pneumococcal serotypes in samples from the nasopharynx of healthy, unvaccinated children attending daycare centres in Reykjavik and the surrounding metropolitan area in 2009, and compare the results in terms of age, daycare centre, antimicrobial susceptibility and serotypes. Furthermore, for this purpose to use molecular methods for serotype detection on DNA extracted directly from the specimens and compare this with conventional methods (paper III).

IV. Investigate the prevalence of pilus genes in pneumococci isolated from the nasopharynx of healthy, unvaccinated children attending daycare centres in Reykjavik and the surrounding area in 2009, by antimicrobial susceptibility and serotypes (paper IV).

3 Materials and methods

3.1 Study population and samples

The study population included inhabitants of the greater capital area of Reykjavik, where the Department of Clinical Microbiology, Landspitali, Reykjavik, serves as the primary microbiology laboratory. In 1995 this area was inhabited by 160.000 individuals, or 60%, of the 268.000 populating Iceland. Children younger than 6 years represented about 10% of the population. In 2010 the study population in the capital area numbered 201.000 (of 318.000; 63%) (http://www.statice.is/). Inhabitants from other areas of the country often seek both general and specialist health services in the capital and were thus included. The laboratory also serves as reference laboratory for the whole country. It is estimated that for practical purposes the laboratory serves 85% of the population.

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

The thesis is based on studies done on pneumococcal isolatesfrom two groups, i.e. a) patients of all age-groups and b) healthy children attending daycare centres.

Infant vaccination against pneumococci did not start in Iceland until April 2011, when vaccination with PHiD-CV10 was introduced. Catch-up vaccinations for older children were not done. Therefore, the study population can be considered unvaccinated until 2011, but children born that year and later have been vaccinated.

3.1.1 Patients (papers I and II)

The patient samples were clinical samples of any kind collected by health personnel at health institutions/health centres and sent for analysis to the Department of Clinical Microbiology, Landspitali. The laboratory provided guidelines for sampling, specimen containers and/or swabs with transport medium. The samples were analysed by the professional microbiology staff at the laboratory. Isolates and resulting data from the department were used in the study.

Culture collections already existed for PNSP and IPD. All databases, i.e. already existing or new, were based on the laboratory data.

The PNSP collection included PNSP from all kinds of specimens from all patients. The collection database was started in 1988 and covers the era

since PNSP emerged in the country. The database used for the PNSP had thus been set up prior to the commencement of the data collection used for this study, but it was further developed and maintained. It includes information on all samples from patients where pneumococci were identified, regardless of their susceptibility. Isolates of the same phenotype, identical serotype and antibiogram from the same individual within a 30 day interval were excluded. Where possible the isolate representing the most severe infection was used, in the order 1) isolates from invasive disease (IPD), 2) lower respiratory tract (LRT), 3) middle ear (ME), and 4) other upper respiratory tract isolates (URT). No other exclusions were made. The study on the epidemiology of PNSP isolates from patients was done in two parts. In the first part of the study isolates from the years 1995 -2010 were used (paper I). In the second one isolates from 2011-2015 were used. It was an extension, using all the same criteria as for the first cohort and this is unpublished.

The IPD collection consists of all viable isolates from invasive disease, the oldest from 1987.

An additional collection of all non-invasive susceptible isolates was started in 2005, but the data were already included in the PNSP database. Isolates of the same phenotype from the same individual within the 30 day interval already mentioned were excluded. For comparison of serotype prevalence of pneumococci isolated from the middle ear, lower respiratory tract and from invasive disease prior to vaccination all isolates from those same sampling sites during 2007-2011 and met the stuy criteria were used (paper II).

3.1.2 Healthy children (papers III and IV)

Nasopharyngeal swabs (NPH) were collected from healthy children aged 2-6 years attending 15 representative daycare centres (DCC) in the Reykjavik metropolitan area during March and April 2009. They were collected by the same person and according to WHO recommendations (Satzke et al., 2013) using Coban® swabs with a flexible shaft and including Stuart agar gel transport medium (Brescia, Italy).

The DCCs had participated in our previous carriage studies and were selected to optimally represent children from all parts of the study area. All the children attending the selected DCCs were invited to participate. Informed consent was obtained from the parents/guardians of all participating children. The study was approved by the National Bioethics Committee (license no: 08-185-S1).

The parents were not allowed to bring the children to the DCCs if they were ill. Thus the children attending on the day of sampling were considered healthy.

Over 95% of 2-5 year old children in this area attend DCCs (http://www.statice.is/). Normally the children are enrolled in the DCC in early autumn in the year they become 2 years of age, explaining lower attendance of that age group, or 46%. The children leave the daycare centres the year when they become 6 years old, therefore the study only comprised children who reached the age of 6 during the first months of 2009.

3.2 Isolation and identification

3.2.1 Primary culture and identification

3.2.1.1 Patient samples (papers I and II)

All patient specimens were cultured on two 5% horse blood agar plates (Oxoid, Hamshare, UK), one incubated in a 5% CO_2 enriched atmosphere and the other under anaerobic conditions. Identification was done by morphology and susceptibility to optochin. All isolates were stored at -80^{o}C in glycerol broths.

3.2.1.2 Samples from healthy children (papers III and IV)

The nasopharyngeal swabs were plated for selective culture on blood-agar containing 5 mg/L of gentamicin within 5 hours and incubated anaerobically overnight. Pneumococci were identified by morphology and susceptibility to optochin. Pneumococcal isolates were kept frozen in glycerol broths at -80°C for molecular serotyping (paper III) and for the identification of pili (paper IV). After plating, the swabs were kept frozen at -20°C for DNA extraction for molecular serotyping (paper III).

3.3 Antimicrobial analysis

3.3.1 Antimicrobial susceptibility testing (papers I-IV)

Disk susceptibility testing was performed on all the pneumococcal isolates using the CLSI/NCCLS Performance Standard for Antimicrobial Disk Susceptibility Tests, appropriate at any given time (CLSI, 2009). The isolates were screened for penicillin non-susceptibility with 1 μg oxacillin discs and their susceptibility to chloramphenicol, erythromycin, tetracycline and trimethoprim/sulfamethoxazole was tested. Oxacillin sensitive (≥20 mm zone) isolates were defined as sensitive to penicillin and other β-lactams.

The minimal inhibitory concentration (MIC) for penicillin and ceftriaxone was measured for all oxacillin resistant isolates using the E-test® (Solna, Sweden) (AB-Biodisk, 1998). Interpretation of results was according to CLSI/NCCLS Performance Standards for Antimicrobial Susceptibility Testing valid at any given time (CLSI, 2009). For comparison the penicillin MICs were also interpreted according to the EUCAST criteria for clinical breakpoints (EUCAST, 2012).

3.3.2 Antimicrobial and vaccine usage (paper I)

Information on antimicrobial and vaccine usage was obtained from the Icelandic Medicines Agency (http://www.ima.is/) and the Directorate of Health´s Chief Epidemiologist Office (http://www.landlaeknir.is/english/).

Sale statistics were based on total sales through wholesalers and represent the total antimicrobial use for humans in the country. The data were presented as defined daily doses (DDD) per 1000 inhabitants per day using the ATC/DDD system (http://WHO.int).

3.4 DNA isolation

3.4.1 From bacterial culture (papers I-IV)

The frozen isolates were subcultured on blood-agar (Oxoid, Hamshare, UK) at 37°C in 5% CO_2 overnight. Suspension of 2-4 colonies was made in 500 µl of 5% Celex 100® (Bio-Rad laboratories, Hercules, CA, USA), heated at 100°C for 10 minutes and centrifuged at 14.000 rounds per minute. The supernatant including extracted DNA was kept at -20°C.

3.4.2 From nasopharyngeal samples (paper III)

DNA was extracted directly from the NPH samples that had previously been used for plating, using QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany). The content of the swab was suspended in 300 µl of DNAase and RNAase free water (Sigma-Aldrich Company, Irvine, UK) and the DNA extracted according to the manufacturers guidelines. Extracted DNA was kept at -20°C.

3.5 Serotyping

Serotyping using conventional methods was routinely done on all IPD and PNSP isolates at the Department of Clinical Microbiology during the study period and from 2010 in combination with PCR reactions when needed. From 2007 and onwards all sensitive isolates that were included in this study were

also serotyped using conventional methods in the first year, after which PCR reactions were mainly used.

In many instances serotypes were classified with regard to whether they belonged to vaccine serotypes (VTs) or non-vaccine serotypes (NVTs). VTs refer to the serotypes of either 10-valent (VT-10) or 13-valent (VT-13) protein-conjugated vaccines (PCV). Non-vaccine serotypes (NVTs) are serotypes that are not included in VT-13 unless further specified as NVT-10 and NVT-13.

3.5.1 Conventional serotyping (papers I-IV)

Conventional methods for serotyping were agglutination using antisera against capsular polysaccharides (Statens Serum Institut, Copenhagen, Denmark). The co-agglutination method (Kronvall, 1973), was used until 2005 when it was replaced by the Pneumotest-Latex and/or latex tests for specified serotypes (Slotved et al., 2004).

3.5.2 Serotyping using PCR (papers I-IV)

Molecular serotyping with PCR was done either using DNA extracted from pneumococcal culture (papers I-IV) or directly from nasopharyngeal swabs (paper III).

Monoplex PCR was used in combination with conventional methods, or to confirm each serotype if more than one was detected in a sample.

For the study on co-colonization with pneumococcal serotypes (paper III) a multiplex PCR panel was designed to detect the selected serotypes or serogroups 3, 4, 5, 6, 7F, 9V, 11A, 14, 15B/C, 18A/B/C/F, 19A, 19F, 22F/A, 23A, and 23F (Dias et al., 2007; Dobay et al., 2009; Pai et al., 2006). The selected serogroups/types were all the VTs (except serotype 1 was not included), as well as the most commonly detected NVTs in our previous studies. The panel consisted of five reactions where each included primers targeting three different serotypes/serogroups. In all reactions, primers targeting the pneumococcal *cpsA* gene were used as a positive control for capsulated pneumococci (Appendix, Table 9). In addition a series of PCR reactions was used for serogroup 6, i.e. for the serotypes 6A, 6B and 6C (Jin et al., 2009b) (Appendix, Table 10).

For the study on comparison of the serotype prevalence in patient samples from the middle ear, lower respiratory tract and invasive pneumococcal disease in 2007-2011 (paper II) the panel used for detection of co-colonization was extended to include the additional serotypes 1, 19B/C,

20A/B, 23B, 29, 33F/A, 33B/D, 34, 35F/47F, 35B, 42(35A7C) (Dobay et al., 2009; Pai et al., 2006; Zhou et al., 2007). Furthermore, primers for *lytA* were included as a control (Sourav et al., 2010). The series of PCR reaction to separate the serotypes of serogroup 6 was extended to include serotypes, 6D and 6E (Jin et al., 2009a; Kawaguchiya et al., 2015). Thus the extended panel and serogroup 6 reactions included 33 primer-pairs for serotypes/groups and 2 primer-pairs for controls (Appendix, Table 9 and 10).

The single and multiplex PCRs were performed in 25 µl volumes using QuickLoad *Taq 2X* Master mix (New England Biolabs Inc., Ipswich, MA, USA), except for the co-colonization analysis (paper III) where Multiplex PCR 5x Master mix (New England Biolabs Inc., Ipswich, MA, USA) was used. Each reaction contained 0.375 µM of each *cpsA* primer, 0.75 µM of each serotype specific primer and 5 µl of DNA extract. Thermal cycling was performed under the following conditions: 94°C for 4 min followed by 30 amplification cycles at 94°C for 45 s, 54°C for 45 s, and at 65°C for 2 min 30 s (Dobay et al., 2009; Pai et al., 2006)

Samples containing pneumococci of serogroup 6 were serotyped using a set of three PCR reactions. The PCR series for separation of the serotypes of serogroup 6 were performed in 25 µl volumes using Amplitaq Gold® PCR Master Mix (Applied Biosystems, Foster City, California, USA) (Jin et al., 2009a; Jin et al., 2009b).

PCR products were analysed by gel electrophoresis in 1.5% agarose gels in 0.5x TBE buffer, stained with ethidium bromide and photographed in UV light.

3.6 Molecular typing (papers I and II)

3.6.1 PFGE (paper I)

At the beginning of the study period Pulsed Field Gel Electrophoresis (PFGE) was the general method used to identify pneumococcal clones (Sa-Leao et al., 2000b).

All multi-resistant isolates of serogroup 19 in 1998-2006, and 2007-2010 a random collection of isolates from the lower respiratory tract and middle ear and all isolates from invasive disease, regardless of susceptibility, were subjected to Pulsed Field Gel Electrophoresis, PFGE, (paper I and II).

3.6.2 MLST (paper I and III)

Multi Locus Sequence Typing (MLST) (Aanensen & Spratt, 2005; Enright & McKenzie, 1997) was performed on a selection of PNSP isolates of defined PFGE clones. Information on clonal distribution in IPD isolates was available in the IPD database. STs were organized into CC and their relatedness to other ST/CC compared using automated query of the PubMLST database (http://pubmlst.org).

Representative PFGE clones of PNSP were subjected to MLST, including multi-resistant isolates of serotypes 6B/6E and 19F (paper I and II).

3.7 Detection of pilus islets, PI-1 and PI-2 (paper IV)

The presence or absence of PI-1 and PI-2 was determined using a modification of previously published PCR methods with primers specific for genes of the pilus islets PI-1 (Moschioni et al., 2008; Paterson & Mitchell, 2006) and PI-2 (Bagnoli et al., 2008). The absence of the pilus islets was confirmed using primers flanking the islets that can only give positive reaction when the islets are absent (Aguiar et al., 2008; Bagnoli et al., 2008).

PCR was performed in a 25 µl total volume with Quick-Load Taq 2X Master Mix (New England BioLabs Inc., Ipswich, MA, USA), 1 µl of each primer (0,4 µM) and 5 µl of DNA suspension. All primers came from TAG Copenhagen A/S (Copenhagen, Denmark).

In short, the presence of PI-1 was detected using the primers srtD for and srtD rev and the absence was confirmed using the primers PFL for and PFL rev. The presence of PI-2 was detected using the primers P06 for and P06 rev and the absence was confirmed using the primers 1008 for and 1009 rev. All PI-1 were further classified to clades using specific primers for rrgB clade I-III (Moschioni et al., 2008) (Appendix, Table 11).

Pilus islets PCRs were performed on all the pneumococcal isolates from healthy children isolated in 2009.

3.8 Statistical analysis

Pearson's chi-squared and Fisher´s exact test were used for statistical analysis. P-values of ≤ 0.05 were considered significant.

4 Results and Discussion

4.1 The epidemiology of PNSP (paper I)

The results for the years 1995-2010 are presented and discussed in chapters 4.1.1 - 4.1.7 (paper I) and 2011-2015 in chapter 4.1.8 (unpublished).

4.1.1 Demographics

Pneumococcal isolates fulfilling the criteria of the study for the years 1995-2010 were 13.937 from 10.915 patients.

This study covered ~85% of population of the country and was therefore not likely to be biased or unrepresentative. The isolates were all from patients, the study period was long, the catchment area of our laboratory remained the same during the study period and it complemented previous studies from Iceland (Arason et al., 2006; Kristinsson, 1995; Kristinsson et al., 1992; Sa-Leao et al., 2002a; Vilhelmsson et al., 2000). Furthermore, the same methods were used for data capture and susceptibility testing during the whole period. This is considered to be main strength of this study.

The annual number of pneumococcal isolates was highest in 1995, 1296 isolates, then gradually decreased to the lowest number of 579 isolates in 2003 (Table 1). Of the 13.937 pneumococcal isolates 629 (4.6%) originated from invasive pneumococcal diease, 2266 (16.3%) from the lower respiratory tract, 2562 (18.4%) from the middle ear, 6034 (43.3%) from nasopharynx and 1038 isolates (7.4%) from other sites, mostly from conjunctiva and sinuses. Exact information on the sampling site was missing for 1408 (9.7%) of the isolates, mainly from the City Hospital (Borgarspitali, now part of the Landspitali University Hospital) during the first years of the study (Table 1).

Relatively large proportions of the isolates originated from the nasopharynx of children, but those were children considered to have respiratory tract infections or otitis media. Isolates from carriage studies were not included, but our data can be used to compare the PNSP rates in these groups. Furthermore, our previous studies included nasopharyngeal specimens as well and that also influenced the decision to include the nasopharyngeal samples in this study.

Most of the isolates, 5622 (40.3%), were from 0-1 year old children, 3045 (21.8%) from 2-6, 600 (4.3%) from 7-17, 2687 (19.3%) from 18-64 and 1983 isolates (14.2%) from ≥65 years old patients (Table 2).

Table 1. Annual number of all clinical isolates (n) of pneumococci and PNSP, 1995-2010, by sampling site and with their proportions (%).

	IPD		ME		LRT		NPH		Other		Unknown		Total	
Year	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)
1995	45	6 (13.3)	183	50 (27.3)	198	55 (27.8)	527	149 (28.3)	70	16 (22.9)	273	37 (21.1)	1296	313 (24.1)
1996	37	6 (16.2)	175	67 (38.3)	153	44 (28.8)	559	164 (29.3)	88	13 (14.8)	259	33 (20.4)	1271	327 (25.7)
1997	36	8 (22.2)	140	54 (38.6)	132	30 (22.7)	459	114 (24.8)	60	12 (20.0)	220	8 (21.0)	1047	226 (21.6)
1998	36	5 (13.9)	162	52 (32.1)	158	40 (25.3)	460	88 (19.1)	67	12 (17.9)	171	9 (16.2)	1054	206 (19.5)
1999	45	2 (4.4)	159	39 (24.5)	137	27 (19.7)	357	56 (15.7)	68	13 (19.1)	163	7 (17.5)	929	144 (15.5)
2000	35	3 (8.6)	162	24 (14.8)	114	28 (24.6)	361	50 (13.8)	78	12 (15.4)	135	10 (15.2)	885	127 (14.3)
2001	41	3 (7.3)	117	26 (22.2)	111	17 (15.3)	299	35 (11.7)	63	7 (11.1)	146	17 (18.8)	777	105 (13.5)
2002	36	2 (5.6)	207	45 (21.7)	156	29 (18.6)	288	47 (16.3)	58	8 (13.8)	5	2 (0.7)	750	133 (17.7)
2003	36	3 (8.3)	124	32 (25.8)	138	19 (13.8)	231	42 (18.2)	46	6 13.0)	4	1 (0.7)	579	103 (17.8)
2004	46	8 (17.4)	99	24 (24.2)	141	38 (26.9)	305	68 (22.3)	60	10 (16.7)	3	2 (0.5)	654	150 (22.9)
2005	41	5 (12.2)	148	53 (35.8)	157	29 (18.5)	281	103 (36.7)	57	6 (10.5)	6	3 (0.9)	690	199 (28.8)
2006	47	4 (8.5)	152	60 (39.5)	137	25 (18.2)	316	106 (33.5)	72	15 (20.8)	15	4 (2.0)	739	214 (29.0)
2007	40	4 (10.0)	179	82 (45.8)	150	45 (30.0)	340	157 (46.1)	66	12 (18.2)	1	0 (0.1)	776	300 (38.7)
2008	40	5 (12.5)	168	76 (45.2)	133	48 (36.1)	404	149 (36.9)	66	7 (10.6)	7	4 (0.9)	818	289 (35.3)
2009	31	1 (3.2)	209	97 (46.4)	128	40 (31.2)	426	147 (35.5)	60	9 (15.0)	0	0 (0.0)	854	294 (34.4)
2010	37	5 (13.5)	178	81 (45.5)	123	51 (41.5)	421	165 (39.2)	59	13 (22.0)	0	0 (0.0)	818	315 (38.5)
Total	**629**	**70 (11.1)**	**2562**	**862 (33.6)**	**2266**	**565 (24.9)**	**6034**	**1640 (27.2)**	**1038**	**171 (16.5)**	**1408**	**137 (9.7)**	**13937**	**3445 (24.7)**

4.1.1 Susceptibility to penicillin

Information on penicillin susceptibility was available for 13.884 isolates (99.6%). Oxacillin resistant were 3809 isolates (27.4%). These were further tested for penicillin MIC. In total 3445 (24.8%) isolates from 2818 patients were defined as PNSP, or 90.4% of the oxacillin resistant isolates. In 1995 there were 313 isolates (24.2%) defined as PNSP, increasing to 327 (25.9%) in 1996, then gradually decreasing to 105 isolates (13.6%) in 2001. After 2003, there was a rapid increase in PNSP to 315 isolates (38.6%) in 2010 (Table 1).

The Department of Clinical Microbiology switched from using the CLSI standard to EUCAST in June 2012. Both standards have the same

breakpoints for the definition of penicillin sensitivity for non-parenteral treatment of pneumococci (PSP), or penicillin MIC ≤0.06 mg/L. Isolates with higher MIC were accordingly defined as PNSP. The breakpoints between intermediate and resistant differed slightly between the standards as the CLSI breakpoint for resistant were ≥2.0 mg/L and the EUCAST >2.0 mg/L. Using CLSI interpretation 2589 isolates (18.6%) were defined as intermediate and 856 (6.2%) were resistant. Using EUCAST interpretation 3288 isolates (23.7%) were defined intermediate and 157 (1.1%) as resistant (Figure 1).

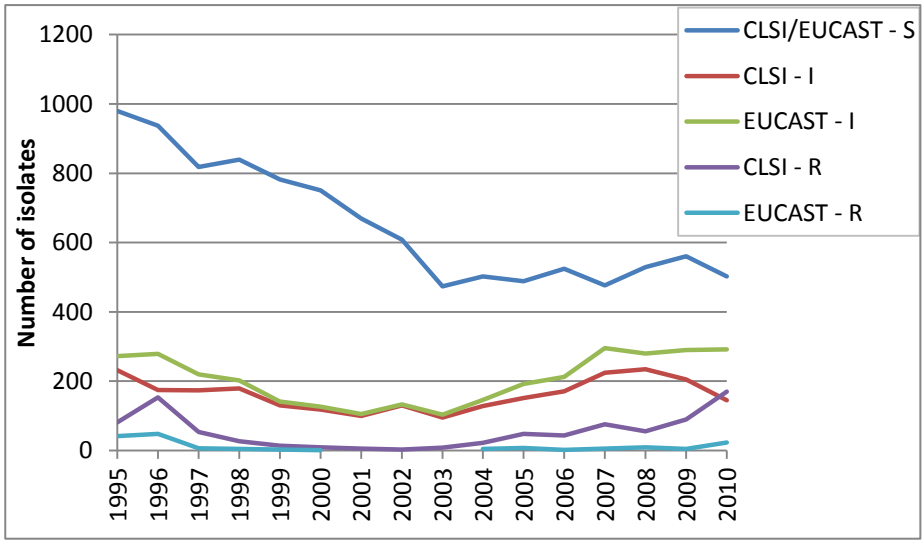

Figure 1. Effects of the different breakpoints of the CLSI and EUCAST standards for interpretation of results of susceptibility tesing with penicillin, by year.

Although the breakpoint for penicillin resistance only differed by one dilution between the standards, it had a marked influence on the resistance rates as the prevailing clones had their MIC close to the breakpoint. This needs to be taken into consideration when resistance rates are being compared in the future. This was especially noted in the beginning of the study period and again after 2004. Thus, in 1996 there were 174 isolates (13.8%) defined as intermediate and 153 (12.1%) as resistant according to the CLSI standard, as opposed to 279 (22.1%) intermediate and 48 (3.8%) resistant by the EUCAST standard. In 2010 there were 145 isolates (1.7%) defined as intermediate and 170 (20.8%) as resistant according to the CLSI standard, as opposed to 292 (35.7%) intermediate and 23 (2.8%) resistant by EUCAST (Figure 1).

4.1.2 Susceptibility to other routinely tested antimicrobials

Ceftriaxone

The ceftriaxone MIC was measured for all oxacillin resistant isolates. All the isolates were sensitive except 13 isolates of serotype 19F that were intermediate to ceftriaxone with a MIC of 2 mg/L. The penicillin MICs for those isolates was 1-8 mg/L, all isolated after 2005.

Erythromycin

Information on erythromycin susceptibility was available for 12.429 (89.2%) isolates. Non-susceptible isolates were 2925 (23.5%), thereof 2882 resistant and 43 intermediate. Of the intermediate isolates, 17 were from 1995 and in the other years between none and three. In 1995 non-susceptible isolates were 174 (17.0%), a gradual decline was seen to 95 (13.0%) by 2000, but then a rapid increase occurred after 2004 to 326 isolates (39.9%) in 2010 (Figure 2).

In 1995-1997 <1% of PSP were non-susceptible to erythromycin. Their rate increased rapidly to 9.1% in 2003, and then a decline was seen after 2006 to decline to 7.0% in 2010.

Tetracycline

Information about tetracycline susceptibility was available for 12.376 isolates (88.8%). Non-susceptible isolates were 2647 (21.4%), thereof 2592 resistant and 55 intermediate, i.e. none to eight within individual years. Temporal trends were similar as for penicillin and erythromycin (Figure. 2).

Tetracycline non-susceptibility in PSP was not as common as erythromycin resistance.

Trimethoprim/sulfamethoxazole

Information about trimethoprim/sulfamethoxazole susceptibility was available for 11.657 isolates (83.6%). Its use in susceptibility testings started in the autumn of 1995; information was available for 90.3%. Non-susceptible isolates to trimethoprim/sulfamethoxazole were 4372 (41.0%), thereof 4372 resistant and 406 intermediate. Temporal trends were similar as for penicillin, erythromycin and tetracycline, - however, at higher rates (Figure. 2).

Trimethoprim/sulfamethoxazole non-susceptibility in penicillin susceptible isolates was more common than for other routinely tested antimicrobials.

Chloramphenicol

Information about chloramphenicol susceptibility was available for 12.328 isolates (88.4%). Non-susceptible isolates were 981 (8.0%), thereof 927 resistant and 46 intermediate. The rate of chloramphenicol non-susceptibility did not follow same temporal trend as seen with other antimicrobials. It was highest at 165 (16.2%) in 1995, then gradually declined to nine isolates (1.1%) in 2010 (Figure. 2).

The steadily declining rates of chloramphenicol non-susceptible isolates during the whole period reflect the continuous decline of the multi-resistant CC90.

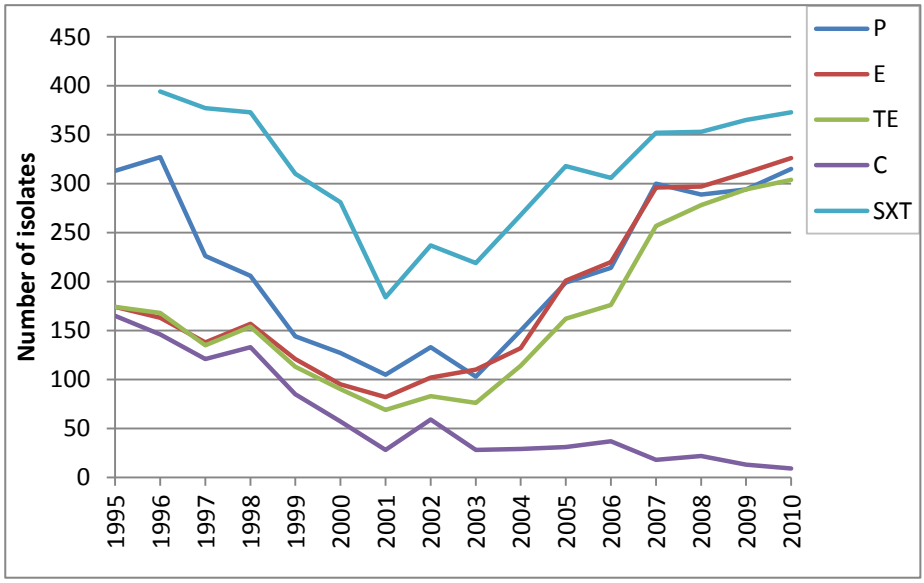

Figure 2. Annual number of non-susceptible isolates against the routinely tested antimicrobials 1995-2010. P=penicillin, E=erythromycin, TE=tetracycline, C=chloramphenicol, SXT= trimethoprim-sulfamethoxazole (SXT started 1996).

Dual or multiple resistance

The isolates were often non-susceptible to more antimicrobials than penicillin. In some instances dual resistance was seen, most commonly to penicillin and trimethoprim/sulfamethoxazole. The majority of the PNSP were multi-resistant. Multi-resistance was mainly caused by two widely spread known international clones. The first one, CC90, was widely distributed in the

country prior to this study while the second one, Taiwan^{19F}-14, CC236/271/320 (CC320), emerged and expanded during the study period. These clones will be further discussed in section 4.1.5.

4.1.3 Prevalence of PNSP by age and sampling site

Prevalence of the 3445 PNSP isolates, 1995-2010, according to patient age group showed that 1849 (53.7%) isolates were from 0-1 year old children and 617 (17.9%) from 2-6 year old children. Thus, 71.6% of PNSP isolates originated from children under 7 years of age. According to sampling site 1640 (47.6%) originated from the NPH and 862 (25.0%) were from ME. The prevalence of PNSP was highest in ME isolates from 0-1 year old children, i.e. 709 isolates (39.4%), while it was 19 isolates (19%) in IPD, where this age group also had highest rates of the PNSP. In isolates from ≥65 year old patients the highest rates of PNSP were from LRT, 325 (27.2%) (Table 2).

Table 2. Number of all clinical isolates of pneumococci (n) and PNSP, 1995-2010, by sampling site and age-groups with the proportions of PNSP (%)

	0-1		2-6		7-17		18-64		≥65		Total	
Site	n	**PNSP (%)**	n	**PNSP (%)**	n	**PNSP (%)**	n	**PNSP (%)**	n	**PNSP (%)**	n	**PNSP (%)**
IPD	100	19 (19.0)	50	5 (10.0)	15	1 (6.7)	223	20 (9.0)	241	25 (10.4)	629	70 (11.1)
ME	1799	709 (39.4)	658	142 (21.6)	53	5 (9.4)	38	3 (7.9)	14	3 (21.4)	2562	862 (33.6)
LTR	23	7 (30.4)	57	19 (33.3)	59	17 (28.8)	932	197 (21.1)	1195	325 (27.2)	2266	565 (24.9)
NPH	3071	1025 (33.4)	1851	397 (21.4)	355	47 (13.2)	650	144 (22.1)	107	27 (25.1)	6034	1640 (27.2)
Other	329	41 (12.5)	193	29 (15.0)	55	11 (20.0)	365	77 (21.1)	96	13 (13.5)	1038	171 (16.5)
Unkn.	300	48 (16.0)	232	25 (10.8)	62	4 (6.4)	479	35 (7.3)	335	25 (7.5)	1408	137 (9.7)
Total	**5622**	**1849 (32.9)**	**3041**	**617 (20.3)**	**599**	**85 (14.2)**	**2687**	**476 (17.7)**	**1988**	**418 (21.0)**	**13937**	**3445 (24.7)**

Most published population-based studies on PNSP only include IPD, samples from otitis media, or samples from children and are therefore not fully comparable to studies encompassing isolates from all sites (Bruinsma et al., 2004; Castañeda et al., 2009; Kim et al., 2012). Furthermore, most surveillance systems only report susceptibility in IPD. Important changes in the prevalence of PNSP may thus not be detected or reported. Our data support this theory. Iceland has in recent years had one of the lowest reported rates of PNSP in IPD among European countries according to the EARS-Net survey in 2008-2011 (ECDC, 2012), while overall PNSP rates are considerably high. Some European countries have reported high rates of PNSP in IPD while also reporting high rates for non-invasive disease (Fenoll

et al., 2000; Gaudelus et al., 2000; Kempf et al., 2011). However, countries reporting a low prevalence in IPD, such as Iceland, have not reported as high a prevalence of PNSP in non-invasive disease as seen in Iceland (Eriksson et al., 2000; Principi & Marchisio, 2000).

4.1.4 Prevalence of PNSP by serotypes and genotypes

Of the 3445 PNSP isolates 2987 (86.7%) were serogrouped and 2266 isolates (65.8%) were fully serotyped. Of the serogrouped PNSP, 1505 isolates (50.4%) belonged to serogroup 19, followed by 1128 (37.8%) of serogroup 6, while 128 isolates (4.3%) were of serogroup 9. There were considerable temporal changes in the serogroup prevalences (Figure 3).

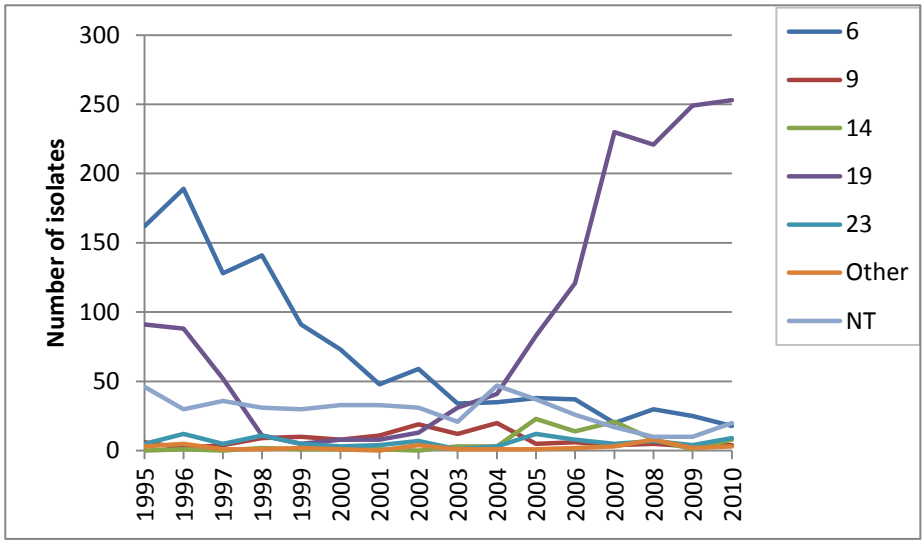

Figure 3. Annual number of isolates of PNSP, 1995-2010, by serogroups. NT=not-typed and non-typable.

The most common serogroup in the beginning of the study period was serogroup 6. In 1995, 60.7% of PNSP isolates were of serogroup 6, or 13.5% of all pneumococcal isolates of the year. After 1996, their rate declined steadily to become 5.7% of PNSP in 2010. Most of these isolates were identified as serotype 6B, belonging to the multi-resistant Spain^{6B}-2 clone, CC90, which appeared in Iceland in 1989. Recently it was confirmed that these isolates belong to the newly described serotype 6E (van Tonder et al., 2015). Their susceptibility was in concordance with our previous studies, intermediate to penicillin, most commonly with a MIC of 1.0 mg/L, resistant to

erythromycin, tetracycline, trimethoprim/sulfamethoxazole and chloramphenicol.

Isolates resembling CC90 represented 80% of PNSP of serogroup 6, highest at 90% during the few first years declining to 55% in 2010. It should be kept in mind that the proportions do not tell the whole story as the number of PNSP of serotype 6 declined extensively during the study period. Thus CC90 peaked in 1996 and then there was a decline which occurred over a longer period and was relatively equal in all age groups. This most likely reflects the natural dynamics in a decline of a successful pneumococcal clone due to herd immunity to that particular clone (Austin et al., 1999). Reduced antimicrobial use may also have played a role in the speed of the decline (Arason et al., 2002a). The decline of CC90 was reflected in the rates of chloramphenicol resistance.

The temporal distribution of serogroup 19 showed two events. In the beginning of the study period PNSP of serotype 19 were relatively common, then again and especially after 2004, when a rapid increase of PNSP of serotype 19F occurred.

First in 1995 91 isolates (34.1%) of serogrouped PNSP belonged to serogroup 19. There was a gradual decline to 27.4% in 1997, but in 1998 the rate was 6.3%. All the isolates from that time, with one exception, showed intermediate susceptibility to penicillin, most commonly with MICs of 0.12-0.25 mg/L. They were all susceptible to erythromycin, tetracycline and chloramphenicol.

The later event involving serogroup 19 started in 1998 when a multi-resistant isolate of serogroup 19 was identified. Then another was identified in 2000, and four in 2001 followed by a constant increase to become 85.6% of serogrouped PNSP by 2010, or 30.9% of all pneumococcal isolates in that year. The majority of the isolates of serogroup 19 were multi-resistant isolates of serotype 19F most commonly intermediate to penicillin, with MICs of 1-2 mg/L, such as 94.9% in 2010, resistant to erythromycin, tetracycline and trimethoprim/sulfamethoxazole, but chloramphenicol sensitive.

PFGE analyses of multi-resistant 19F isolates showed that most belonged to a single PFGE clone not previously recognized in Iceland. MLST of the first representative isolates demonstrated sequence types 271 and 1968, respectively, or single and double locus variants of the international clone Taiwan^{19F}-14, CC236/271/320, named CC320 in this thesis.

Genotyping of all PNSP was not feasible because of cost. Previously, isolates of multi-resistant serotype 6B isolates had been submitted to PFGE and MLST and identified as CC90. The vast majority of the isolates shared the identical antibiogram, easily detected as it also includes resistance to chloramphenicol. It can thus be fairly securely estimated that isolates of serogroup 6 sharing that unique antibiogram belong to CC90 and thus it can be considered to be a useful tool as it´s phenotype marker. In the instance of CC320 this was not as clear-cut, but very rarely isolates of 19F having the same antibiogram as the genotyped isolates were of other clonal complexes. No other serotypes of serogroup 19 were identified with this antibiogram. Thus, it can be used as a phenotype marker for the clone, although not as specific as the antibiogram of CC90. Furthermore, our unpublished data on MLST of all the isolates from the first years, followed by selection of every fourth or every second isolate (by year) in the remaining period supports this. Other STs were detected, but the vast majority belonged to CC320 and were either single or double locus variants of the Taiwan^{19F}-14 clone.

The PNSP rates were lowest during 2000-2003 when the rates of isolates of CC90 had declined considerably and the rates of isolates of CC320 were still relatively low. An increasing prevalence of PNSP that followed was due to the rapid expansion of CC320. This was especially seen in isolates from the middle ear and respiratory tract, but not in invasive isolates. There the mean PNSP prevalence rate for the period was 11.1%, and remained virtually the same for the first and second half of the study period. Thus the mean prevalence of PNSP from the middle ear was 33.6%, increasing from 27.5% to 38.5% from the first to the second half of the period. This indicates that this clone, like the former dominant CC90, had a predilection for the middle ear and was not common in IPD (Arason et al., 2002a; Arason et al., 2005; Kristinsson, 1995).

PNSP of other serotypes were seen at low and relatively constant rates during the period. Most commonly they were of serogroups 9, 14 and 23 and usually intermediate to penicillin and susceptible to other tested antimicrobials, except trimethoprim/sulfamethoxazole where their susceptibility varied. Multi-resistant isolates of serotype 14, 23F and 9V were occasionally identified. Only 6 isolates of multi-resistant serotype 19A were isolated, all from the respiratory tract. This is in concordance with previous studies (Kristinsson, 1995; Sa-Leao et al., 2002b).

High prevalence of only one PNSP clone at the same time possibly indicates that the Icelandic population is not large enough to sustain more

clones at any given time. The reason remains speculative, for example competition between clones in the nasopharynx and/or herd immunity to surface proteins, such as pili.

It is of interest to look at pili in the context of the decline of CC90 and the rise of CC320, its follower as the dominant clone in the country (Bagnoli et al., 2008; Barocchi et al., 2006; Hava & Camilli, 2002). Pili are known to evoke immune response, including production of specific antibodies against the different clades of pili that are not cross-protective between the clades (Harfouche et al., 2012; Moschioni et al., 2012). Herd immunity to a clone of a given clade could therefore select for unpiliated clones or clones of other clades (more about pili in chapter 4.4).

4.1.5 Antibacterial sales

The sales of antibacterial drugs (ATC J01, system antibacterials for systemic use, except antimycobacterials and classified according to the anatomical therapeutical chemical classification system (ATC)) for treatment of both hospitalized and non-hospitalized patients, was highest close to the beginning and the end of the study period. This amounted to 23.0 defined daily doses/1000 inhabitants/day (DID) in 1996 and 23.4 DID in 2006. It was lowest at 19.8 DID in 2001 (Figure 4).

For penicillins (J01C) the peaks and troughs in sales followed similar timeline as overall sales, and were high, 10.9 DID, in the beginning, lowest with 10.5 DID in 2001, and increased again in to become 12.6 DID by end of the study period. Sales of extended spectrum penicillins followed the general trend, but sales of combinations with β-lactamase inhibitors gradually increased during the period and sales of β-lactamase sensitive penicillins declined.

Sales of macrolides (J01F) followed a similar temporal trend, i.e. they were highest at 2.0 DID in 1996, gradually declining to 1.5 in 2000 and increasing to 1.8 in 2007. During the study period, there was a constant decline in erythromycin sales and an increase in azithromycin and clarithromycin sales to peak at 0.8 DID in 2008 and 0.4 DID in 2007, respectively. According to a report from the Chief Epidemiologist of the Directorate of Health, azithromycin was most commonly used in the age group 0-4 years old for the period 2007-11 (Landlæknisembættið, 2012).

Sales of trimethoprim/sulfamethoxazole (J01E), decreased and sales of tetracyclines (J01A) increased during the whole period. The respiratory quinolones have never been licensed in Iceland.

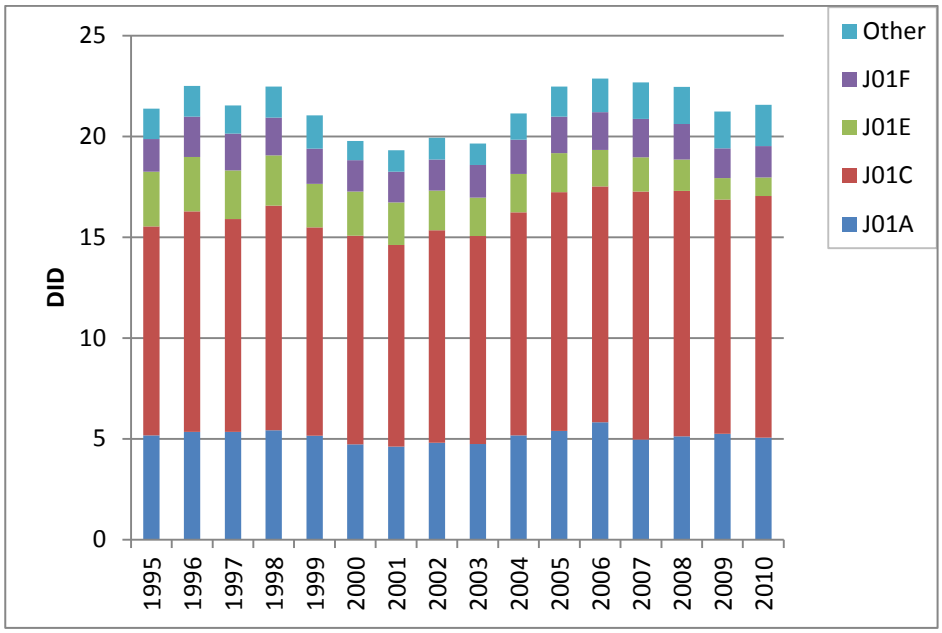

Figure 4. Annual sales of antimicrobials, 1995-2010, presented in defined daily doses/1000 inhabitants/day. J01A = tetracyclines, J01C = β-lactams, penicillins, J01E = sulphonamides and trimethoprim, J0F = macrolides, lincosamides and streptogramins.

After the CC90 started to decline and until the CC320 started to increase the use of antimicrobials remained relatively low, then it started to increase again, or from 20.5 DID to 22.2 DID.

Although an increase in total use of antimicrobials at the same time as CC320 expanded may have contributed to its overall expansion, it is possible that an increased use of macrolides, especially azithromycin in young children, had a greater effect. Azithromycin has been shown to significantly increase the prevalence of macrolide resistant pneumococci (Albrich et al., 2004; Bergman et al., 2006). Increased macrolide resistance during the same time in penicillin susceptible isolates supports this suggestion.

4.1.6 Vaccine sales and prevalence of vaccine serotypes

When protein-conjugated pneumococcal vaccines became available vaccination was recommended for children that were immunocompromised, or had certain heart and lung diseases. Until PCV was implemented in the infant vaccination program in 2011 the sales of PCV were limited, i.e. 118 single doses in 2007, and 230 in 2008, 348 in 2009 and 1623 in 2010.

The recommendations for the use of polysaccharide vaccines in adults were similar as for children, with the addition that it was also recommended for all who were 60 years old or older. Those recommendations remained the unchanged for the whole period.

Of the serotyped PNSP 2036 (89.8%) and 2122 (93.6%) belonged to the serotypes of the 10- and 13-valent vaccines, respectively. Most PNSP isolates of VTs belonged to the dominating multi-resistant PNSP clones of CC90 and CC320. The disappearance of CC90 was prior to vaccination and therefore not vaccine driven.

4.1.7 PNSP post vaccination, 2011-2015 (unpublished results)

Pneumococcal vaccination with PHiD-CV10 was implemented into the infant vaccination program as of April 1st 2011. As there was not a catch-up vaccination only half of the children born in 2011 can be expected to have received the vaccine twice by the end of that year.

The number of all pneumococcal isolates from all patients in 2011-2015 were 2455, of those 784 (32.0%) were PNSP. In 2011, 336 isolates (42.7%) belonged to PNSP, a higher proportion of pneumococcal isolates than ever seen in the country. In 2015 the number of PNSP had declined to 53 isolates (22.0%) (p<0.0001). The number of all pneumococcal isolates in 2011 was 786 and it was comparable to previous years, but then there was a constant decline in their number to become 241 (23.8%) in 2015 (p<0.0001) (Table 3). Thus between 2011 and 2015, the number of PNSP decreased six-fold, while the number of total pneumococcal isolates, decreased three-fold.

A decline in the number of PNSP and samples from otitis media was noted already between the years 2011 and 2012 (p=0.006). This was also seen in nasopharyngeal samples (p=0.01), but those numbers may be biased as recommendations were made to only sample patients who had been positive for PNSP and/or those having recurrent infections. It is likely that, as in the case of middle ear samples, some of the decline was actually caused by the PNSP decrease, and culture positive recurrent infections declined.

There was a decline in the number of pneumococcal isolates and of PNSP in IPD samples until 2013, but then an increase was seen. All these PNSP isolates were from adults, except one in 2011 that stemmed from a child born in 2010 and therefore most likely unvaccinated.

Table 3. Annual number of all clinical isolates of pneumococci (n) and PNSP, 2011-2015, by sampling site and with their proportions (%).

	IPD		ME		LRT		NPH		Other		Total	
Year	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)
2011	33	6 (18.2)	191	96 (50.3)	89	30 (33.7)	415	189 (45.5)	58	15 (25.9)	786	336 (42.7)
2012	25	1 (4.0)	133	46 (34.6)	108	44 (40.7)	360	132 (36.7)	65	17 (26.2)	691	240 (34.7)
2013	17	3 (17.6)	147	35 (23.8)	89	35 (39.3)	145	24 (16.6)	57	3 (5.2)	455	100 (22.0)
2014	24	2 (8.3)	68	14 (20.6)	75	25 (33.3)	73	13 (17.8)	42	1 (2.4)	282	55 (19.5)
2015	26	6 (23.1)	46	10 (21.7)	89	25 (28.1)	44	6 (13.6)	36	6 (16.7)	241	53 (22.0)
Total	**125**	**18 (14.4)**	**585**	**201 (34.4)**	**450**	**159 (35.3)**	**1037**	**364 (35.1)**	**258**	**42 (16.3)**	**2455**	**784 (31.9)**

Following vaccination, a PNSP decrease was first seen in isolates from 0-1 year old children. PNSP were 234 (56.5%) in 2011 and declined to 121 (28.1%) in 2012 (p<0.0001) and then the decline gradually continued to become 9 isolates in 2015. A year later significant decrease in PNSP was seen for 2-6 years old children (p=0.01). Evidence of herd effect were seen in 2014 for 18-64 years (p=0.04), but cannot be seen for the group ≥65 years (Table 4).

Table 4. Annual number of all clinical isolates of pneumococci (n), 2011-2015, the number of PNSP and their proportions (%) within age groups.

	0-1		2-6		7-17		18-64		≥65		Total	
Year	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)	n	PNSP (%)		PNSP (%)
2011	431	234 (56.5)	164	46 (34.6)	26	4 (36.4)	101	29 (23.2)	65	23 (22.8)	787	336 (42.7)
2012	315	121 (29.2)	161	48 (36.1)	17	5 (45.5)	129	45 (36.0)	69	21 (20.8)	691	240 (34.7)
2013	174	36 (8.7)	126	21 (15.8)	15	1 (9.1)	79	25 (20.0)	61	17 (16.8)	455	100 (22.0)
2014	94	14 (3.4)	53	8 (6.0)	8	1 (9.1)	63	9 (7.2)	63	23 (22.8)	281	55 (19.5)
2015	55	9 (2.2)	35	10 (7.5)	13	0	55	17 (13.6)	83	17 (16.8)	241	53 (22.0)
Total	**1069**	**414 (52.8)**	**539**	**133 (17.0)**	**79**	**11 (1.4)**	**427**	**125 (15.9)**	**341**	**101 (12.9)**	**2455**	**784 (31.9)**

A similar decrease was seen in the number of non-susceptible isolates to erythromycin, tetracycline and trimethoprim/sulphamethoxazole as for penicillin (Figure 5).

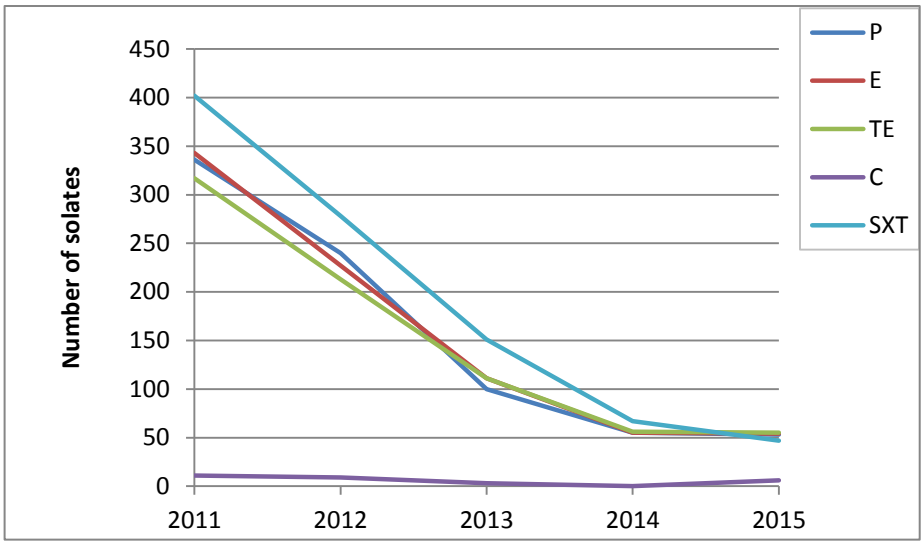

Figure 5. Annual number of non-susceptible isolates against the routinely tested antimicrobials 2011-2015. P=penicillin, E=erythromycin, TE=tetracycline, C=chloramphenicol, SXT= trimethoprim-sulfamethoxazole.

PNSP isolates available for serogrouping were 743 (94.8%). Serogroup 19 was the most common with 587 (79.0%) isolates, of which 551 (93.9%) belonged to serotype 19F. In 2011, 269 isolates, 83% of the PNSP, were of serogroup 19F. This declined to 19 isolates, 41.3% of the PNSP, by 2015 (p=0.0001) (Figure 6). PNSP of VT-10 were 280 (86.4%) in 2011 and declined to only 23 (50%) in 2015.

The decline of isolates of serotype 19F that mainly belonged to the CC320 differed from the decline in serotype 6B, or the CC90. The rate of decline was faster, started in the youngest age group and followed virtually the age of the vaccinated children.

The effect of infant vaccination with PHiD-CV10 on PNSP so far has mainly been seen in a reduction of multi-resistant 19F in children, reflecting a reduction in otitis media and respiratory tract infections. The PNSP rates in IPD in children were low and disappeared in vaccinated children, in concordance with observations of others (De Wals et al., 2012; Jokinen et al., 2015; Kaplan et al., 2004; Kyaw et al., 2006; Singleton et al., 2013; Tomczyk et al., 2016). Other studies on vaccine effects on PNSP in otitis media report different results. Studies from Finland (Eskola et al., 2001) and Spain (Alonso et al., 2013) report decreases of PNSP as shown here. However, a study

from USA reports unchanged PNSP rates relating to vaccine serotypes in otitis media (McEllistrem et al., 2005) and a study from Israel shows vaccine escapes of piliated VTs, including 19F, in carriage (Regev-Yochay et al., 2016).

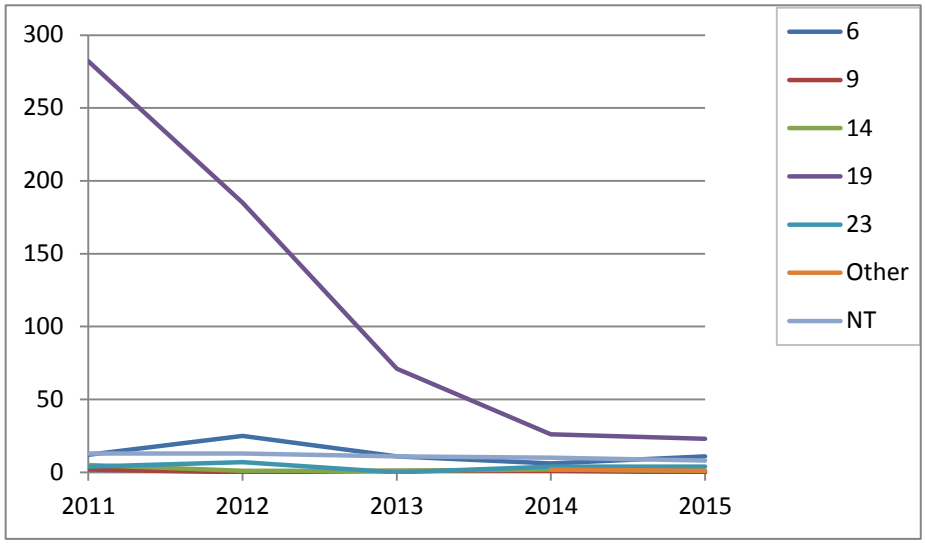

Figure 6. Annual number of isolates of PNSP, 2011-2015, by serogroups. NT=non-typable.

Studying visits of children to the Children´s Hospital, Landspitali, Sigurdsson *et. al.* reported a decrease in the incidence of acute otitis media in 1-2 year old children and of pneumonia in <2 year old children. This development is in concordance with the decline of 19F (Sigurdsson et al., 2015).

Vaccination has thus already shown a high impact on the prevalence of PNSP in Iceland and has reduced the burden of pneumococcal disease in young children. Serotype replacement of PNSP in patient samples is still subtle and the need to for continuing surveillance of PNSP is obvious.

4.2 Comparison of serotype prevalence (paper II)

4.2.1 Demographics

In total 1711 pneumococcal isolates originating from ME, LRT and IPD samples from 2007-2011 fulfilled the study criteria. Of those 1616 (94.4%) were available for serotyping. Distribution according to specimen sites and age groups did not differ between the available and the 95 (5.6%) non-available isolates (not stored or non-viable) from ME and LRT samples, but all except one isolate from IPD cases were available. Temporal changes in number of pneumococcal isolates were noted: The highest number of isolates was 357 (22.1%) in 2007 and gradually declined to 283 in 2011 (17.5%).

The ME isolates were 879 (54.4%) and the vast majority originated from children under seven years of age. Isolates from 0-1 year old children were more often from ME than other sampling sites (p<0.0001). The ME isolates were also more often from 0-1 than from 2-6 year old children (p<0.0001) (Table 5), mostly from patients with perforated tympanic membranes or implanted tympanic tubes, therefore representing the common childhood condition of otitis media.

The LRT isolates were 564 (34.9%), mainly from sputum samples and bronchoalveolar lavage fluids. Isolates from ≥65 year old patients were more commonly of LRT origin than from other sampling sites (p<0.0001) (Table 5). The age distribution reflects the risk groups for pneumococcal infection other than young children. It is difficult to obtain LRT specimens from young children and that explains the low number of isolates, but the rates do not reflect actual rates of LRT pneumococcal infection in children.

The IPD isolates were 173 (10.7%), and included blood, cerebrospinal fluid and joint fluid. About half of the isolates were from the youngest and oldest age groups, respectively (Table 5). The relatively equal age distribution in IPD specimens reflects all risk groups in addition to the influence provided by serotypes of high invasive potential.

Because of the different age distribution of the isolates according it must be kept in mind when comparing isolates from different sampling sites that they partly reflect differences between disease conditions. Thus ME samples mainly represent otitis media in children, LRT samples pneumonia in older adults, while IPD samples represent invasive disease in all risk groups.

Table 5. Number of all clinical isolates from middle ear, lower respiratory tract and invasive disease (n), 2007-2010 and their proportions (%), by sampling site and age groups, number of PNSP and their proportions (%).

	ME		LRT		IPD		Total	
Age	n (%)	PNSP n (%)	n (%)	PNSP n (%)	n (%)	PNSP n (%)	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 (31.6)
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	114 (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)**

4.2.2 Prevalence of serotype

The isolates were from a total of 52 serotypes. The most common was serotype 19F with 583 (36.1%) isolates, followed by 23F with 157 (9.7%) isolates (Table 6). The vast majority of 19F isolates were multi-resistant and had an identical antibiogram as CC320 (paper I). There were 24 multi-resistant isolates (1.5%) of the previously dominating serotype 6Bii/E with an identical antibiogram as CC90. Of the serotypes detected 19 were seen in fewer than five isolates each.

Of the isolates, 1052 (56.1%) belonged to VT-10 and 1359 to VT-13. Isolates of VTs were more common than NVTs (p<0.0001). The proportion of isolates belonging to VTs was highest in ME and lowest in LRT (Table 6). Most other studies mainly report prevalence of VTs in IPD, or only in isolates from children, while in this study we reported results based on all pneumococcal isolates from selected sampling sites and from a nationwide sampling process. There are opportunities for epidemiological studies in a country with relatively few inhabitants and good health and reporting systems that do not present elsewhere.

The ME isolates were of 34 serotypes. The most common was serotype 19F with 400 (45.5%) isolates, more so in ME than in LRT and IPD (p<0.0001). Of all 19F isolates 322 (60.2%) originated from 0-1 year old children and 62 (11.6%) from 2-6 year old children. Serotype 19F was thus a dominant serotype with a predilection for the middle ear cavity in young children. Other serotypes were mainly those who are also common in otitis media and are commonly carried by children. Isolates of serotypes with a

high invasive potential were rare, as expected (Brueggemann et al., 2004; Sjöström et al., 2006). The prevalence of the eight most common serotypes ranged from 45.5-1.6% (Table 6).

Table 6. Ranking of the eight most common serotypes in middle ear, lower respiratory tract and invasive disease, 2007-2011, their number (n) and proportions (%) by sampling site. Vaccine and non-vaccine serotypes and their proportions by 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)	14	28 (5.0)	7F	12 (6.9)	19A	99 (6.1)
6B	47 (5.3)	NT*	23 (4.1)	19F	11 (6.4)	3	86 (5.3)
3	28 (3.2)	6B	22 (3.9)	3	9 (5.2)	6B	77 (4.8)
9V	14 (1.6)	19A	21 (3.7)	6B	8 (4.6)	9V	47 (2.9)
Other	84 (9.4)	Other	167(29.6)	Other	61 (35.3)	Other	328 (20.3)
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 (15.9)

* NT (non-typable) = uncapsulated isolates (positive in PCR reactions for *lytA* and *cpsA*) negative in serotype specific reactions or negative in Pneumotest Latex.

Apart from the exceptionally high rates of isolates of serotype 19F in the ME isolates our findings were in concordance with other studies (Alonso et al., 2013; Hanage et al., 2004; Mavroidi et al., 2007; Mayanskiy et al., 2015; McEllistrem et al., 2005; Reijtman et al., 2013). The ME isolates showed the highest rate of VTs. This can mostly be explained with the high rates of 19F and in addition relatively high rates of other commonly carried VTs. Those serotypes were selected to be included in the PCV intended for children, since they are known for high rates of PNSP (Black et al., 2000; Klugman et al., 2003). They are known to be commonly carried serotypes, especially by

children (Ercibengoa et al., 2012; Gudnason et al., 2014; Sa-Leao et al., 2000b).

The LRT isolates were of 45 serotypes. The most common was 19F with 172 (30.5%) isolates. The prevalence of the eight most common serotypes ranged from 30.5%-3.7% and isolates of high invasive potential were infrequently seen. The lowest VTs rate was among LRT isolates and is explained by the rate of 19F which was lower than in ME. The serotype diversity was higher than in ME and IPD. Furthermore, the rates of commonly carried VTs were lower in LRT isolates, mainly originating from adults and the elderly, compared the ME isolates that were mostly from children.

The IPD isolates were of 29 serotypes and the most common was serotype 14 with 28 (16.2%) isolates. Half of the isolates were from the youngest and oldest patients. The prevalence of the 8 most common serotypes ranged from 16.2% down to 4.6%. The lower rate of VTs in IPD than in ME was related to the high rate of 19F in ME. Higher rates of VTs in IPD than in LRT, was expected since the main purpose of PCV is to protect against IPD.

Interestingly, the two most common serotypes in IPD were serotype 14 and 19A that are generally not considered to be of high invasive potential (Brueggemann et al., 2004; Sjöström et al., 2006). This may be related to clonal properties, as different clones of serotype 14 have been reported to be more associated to IPD than to carriage and vice versa (Sjöström et al., 2006). Another possible explanation for the relatively high rate of serotypes of moderate or low invasive potential is the patients' age. Young children and the elderly have a less competent immune system and in addition older patients are more likely to be immunocompromised due to chronic diseases or immunosuppressive therapy. Therefore, in addition to infections caused by highly invasive serotypes those patients are more likely to develop opportunistic infections caused by serotypes of lower invasive capacity (Brueggemann et al., 2003; Henriques-Normark & Tuomanen, 2013).

The next three serotypes in rank were serotypes 4, 7F and 9V that are considered to have high invasive potentials (Alanee et al., 2007; Brueggemann et al., 2004; Hanage et al., 2005; Henriques-Normark & Tuomanen, 2013). The age distribution of the patients diagnosed with the highly invasive serotypes tended to be more evenly distributed. This is in concordance with other studies (Cartwright, 2002; Sandgren et al., 2004).

Serotypes that are commonly carried by children and known as a cause of relatively mild infections were thus a relatively common cause for IPD (Alonso et al., 2013; Hjalmarsdottir et al., 2016; Horácio et al., 2014).

Overall, the most common NVT was 11A, with 41 isolates (2.5%). It will be of interest to see how these proportions will develop in the vaccination era.

4.2.3 Antimicrobial susceptibility

Penicillin non-susceptible pneumococcal isolates were 651 (40.3%). The number of PNSP isolates remained stable during the study period, i.e. 128-138 isolates each year, while the number of all isolates gradually decreased from 357 to 283. Accordingly, the proportions of PNSP gradually increased from 36.7% in 2007 to 44.9% in 2011.

Of the PNSP isolates 428 (48.7%) were from ME, 205 (36.3%) from LRT and 18 (10.4%) from IPD (Table 7). This was in concordance with other studies showing that antimicrobial resistance is more likely detected in milder infections than in invasive disease, except for individuals at high risk (Hanage et al., 2005; Sjöström et al., 2006).

The PNSP were of 18 serotypes with serotype 19F being the most common, i.e. 535 (82.2%) isolates. Other common serotypes were also serotypes that are frequent colonizers or the cause of milder infections. This is concordant with other studies (Alonso et al., 2013; Hermans et al., 1997; van der Linden & Reinert, 2010). Serotype 19F was the most common PNSP serotype in all specimen groups (p<0.0001), it was largely found in ME with 378 (88.3%) isolates, while in LRT it was present in 150 (73.2%) and in IPD in 7 (38.9%) PNSP isolates (Table 7). The majority of the serotype 19F isolates, or 501 (93.6%) isolates, had an identical antibiogram to CC320 and was confirmed as such by MLST typing of selected isolates (Quirk S. J., 2016) (and unpublished results). The remaining 34 (6.4%) isolates of PNSP serotype 19F had diverse antibiograms.

PNSP isolates of VT-10 were 611 (93.4%) and 626 (96.2%) isolates were of VT-13. The temporal changes were similar as for all pneumococcal isolates, but tended to fluctuate. PNSP of VTs were more common in ME than in LRT (p<0.0001) and also more common than in IPD (p=0.0025). The VTs were more common than the NVTs among PNSP in all instances (Table 7).

Table 7. Ranking of the four most common PNSP serotypes, by 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)	6E	21 (3.2)
6E	11 (2.6)	9V	10 (4.9)	14	2 (11.1)	14	20 (3.1)
6B	8 (1.9)	6E	9 (4.4)	23F	2 (11.1)	9V	14 (2.2)
Other	19 (4.4)	Other	23 (11.2)	Other	5 (27.8)	Other	61 (9.4)
Total	**428 (65.7)**	**Total**	**205 (31.5)**	**Total**	**18 (2.8)**	**Total**	**651**
VT-10	416 (97.2)	VT	181 (88.3)	VT	14 (77.8)	VT	611 (93.9)
NVT-10	10 (2.8)	NVT	24 (11.7)	NVT	4 (22.2)	NVT	40 (6.1)
VT-13	426 (99.5)	VT-13	185 (90.2)	VT-13	15 (83.3)	VT-13	626 (96.2)
NVT-13	2 (0.5)	NVT-13	20 (9.8)	NVT-13	3 (16.7)	NVT-13	25 (3.8)

* NT (non-typable) = uncapsulated isolates (positive in PCR reactions for *lytA* and *cpsA*), negative in serotype specific reactions or negative in Pneumotest Latex®.

The second most common serotype in all isolates was serotype 23F with 157 isolates (9.7%) and of these isolates 147 (93.6%) were fully susceptible. The third in rank 6A with 122 (7.5%) isolates of which 116 (95.1%) were susceptible. These serotypes next were by far outnumbered by 19F. It is likely that a dominating serotype must include clones that have properties which make them successful. The prescription of antimicrobials in the country is relatively high and brings selective pressure for resistant clones that along with other properties of successful clones, like pili, can influence the rates of serotypes (papers I and IV)

4.3 Co-colonization of serotypes (paper III)

4.3.1 Demographics

For detection of pneumococci and serotyping with conventional and molecular methods, nasopharyngeal swabs were collected from 516 children of the 1227 that were invited to participate in the study. Samples from two of the children were only available for conventional method, thus samples from 514 children (41.9%) met the study criteria. The children attended 15 daycare centres in Reykjavik and neighbouring communities in 2009. Their age was from 1.2 year to 6.3 years and the median age was 4.2 years (Table 8).

Table 8. Number of children attending selected day care centres 2009, by age, median age, number of children participating with proportions (%) of the attending children.

DCC	1 year	2 year	3 year	4 year	5 year	Total	Median age	Participants n (%)
DCC1	6	24	19	21	19	89	3.7	53 (59,6)
DCC2	4	22	14	15	33	88	4.6	51 (58,0)
DCC3	3	8	12	8	4	35	3.5	18 (51,4)
DCC4	12	35	21	21	29	118	4.7	34 (28,8)
DCC5	6	17	17	19	19	78	4.1	31 (39,7)
DCC6	0	25	15	16	22	78	4.6	40 (51,3)
DCC7	13	23	36	29	21	122	4.6	45 (36,9)
DCC8	0	7	9	10	11	37	4.9	29 (78,4)
DCC9	17	10	14	18	15	74	4.4	31 (41,9)
DCC10	7	17	16	11	11	62	3.0	37 (59,7)
DCC11	23	14	15	14	11	77	3.7	27 (35,1)
DCC12	6	11	18	13	16	64	4.6	28 (43,6)
DCC13	3	12	31	27	34	107	4.6	32 (29,9)
DCC14	12	30	26	23	27	118	4.5	26 (22,0)
DCC15	16	26	17	14	7	80	3.5	32 (40,0)
Total	**128**	**281**	**280**	**259**	**279**	**1227**	**4.2**	**514 (41,9)**

4.3.2 Carriage and co-colonization

By using conventional methods for isolation, identification and serotyping, pneumococci were found in NPH swabs from 371 children (72.2%). With molecular methods for pneumococcal detection on DNA extracted directly from the same swabs, pneumococci were detected in 391 children (76.1%). There was not any difference in detection rates between the methods (p=0.2). Pneumococci were only found when using conventional methods in samples from three children, but from 27 children when using the molecular method. The carriage rate detected by the combined methods was 76.6%. This high rate has not previously been reported in carriage studies within the country (Gudnason et al., 2014; Tomasson et al., 2005). Compared to other countries this is among the highest reported (Bogaert et al., 2004; Ercibengoa et al., 2012).

Co-colonization was detected in 30 (8.1%) of the children carrying pneumococci when using conventional methods. In all instances the children were colonized by two serotypes, thus the conventional methods yielded 401 isolates from 371 carriers. Using molecular methods, co-colonization was detected in 92 (23.5%) of the children carrying pneumococci. Most commonly, or in 79 children, they were colonized by two serotypes, but 12

children were colonized by three and one child by four serotypes. Thus molecular methods yielded 500 strains from the 391 children carrying pneumococci. The detection rate of co-colonization was higher using molecular than conventional methods (p<0.0001).

The high rate of co-colonization using molecular methods is in concordance with other studies, but in those the DNA was extracted from a sweep of pneumococcal culture (Ercibengoa et al., 2012; Rivera-Olivero et al., 2009). It is also in concordance with observations of others that conventional methods, where only a few colonies from each sample are serotyped, are not a good indicator of co-colonization as colony morphology and other phenotypic properties are in most instances similar between serotypes (Huebner et al., 2000; Saha et al., 2015).

When this study was performed we had not previously seen studies describing serotyping from DNA extracted directly from nasopharyngeal swabs for the purpose of detecting co-colonization. The main reason for using directly extracted DNA was to avoid potential problems like different growth rates of different serotypes, avoid possible variations in performing the sweepings and save time and resources spent on freezing and replating the swabs prior to extraction. Furthermore, molecular methods are not as dependent on experience with reading the results compared to what is required with the conventional methods. The molecular methods worked well and have been further developed since this study was performed and are mainly used for serotyping of pneumococcal cultures.

The prevalence of co-colonization in the children that were carriers of pneumococci was from 12.0% in six year old children to 26.9% in three year children (Figure 7).

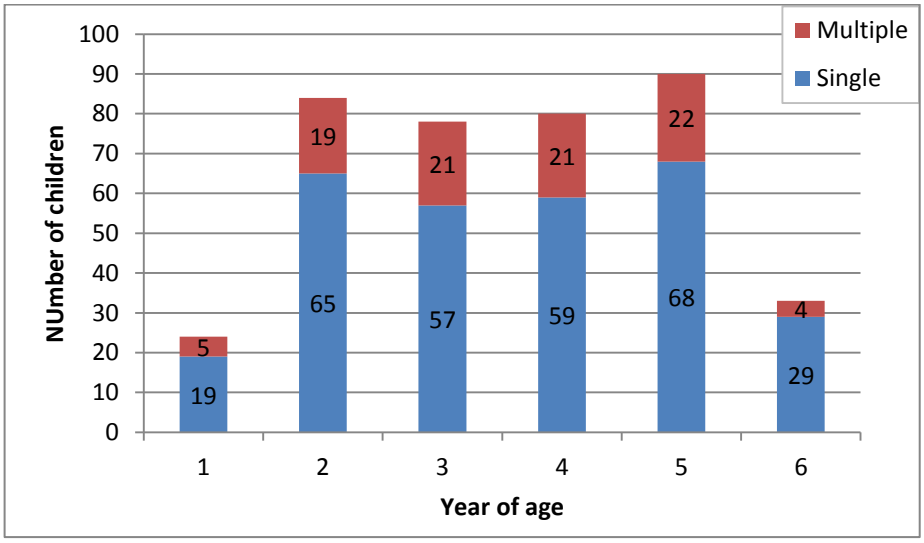

Figure 7. Number of children colonized by one or more serotypes, according to age.

Prevalence of co-colonization according to DCCs was highest at 40% in DCC2 and lowest at 8.3% in DCC15 (Figure 8). In DCC2 the median age of the sampled children was 4.6 years but in DDC15 it was 3.5 years. In DDC10 where the children were youngest (median age 3.0), 10.8% were co-colonized. In DCC8 with the oldest children (median age 4.9), it was 10.3%. Thus the rates of co-colonization seemed to vary more from one DCC to another than by the age of the children.

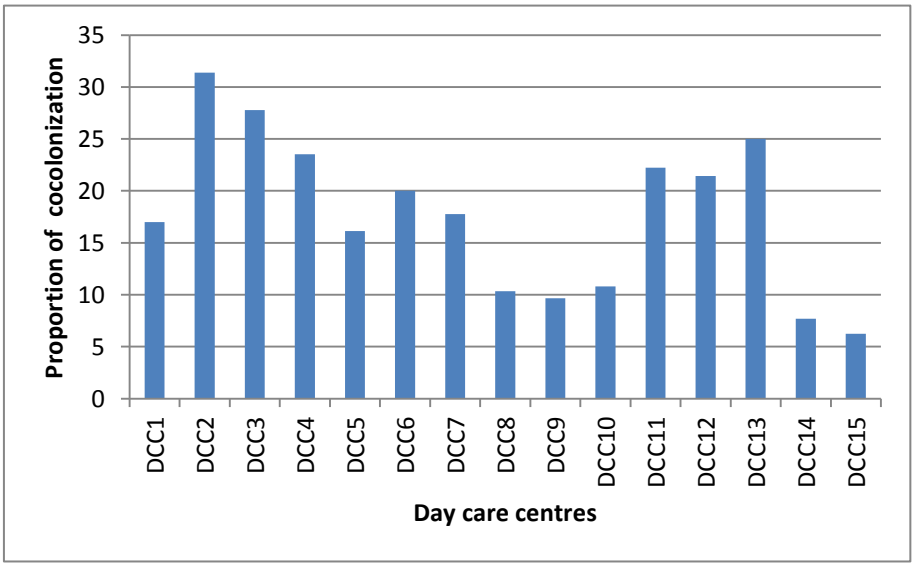

Figure 8. Proportions of co-co-colonized children, by day care centres.

4.3.3 Prevalence of serotypes

When using conventional methods the most common serotypes were 6B with 53 (13.2%), 23F with 51 (12.7%) and 19A with 45 (11.2%) isolates. While using the multiplex PCR panel most common serotypes were 23F with 65 (13.0%), 19A with 61 (12.2%) and 6B with 55 (11.0%) strains. The differences in rates of serotypes between the methods were in no instance significant, but their ranking order differed slightly (Figure 9).

The rates of serotypes indicate that no serotype was dominant in contrast to what was seen in patient samples from children during the same period (papers I and II). Mostly the children were colonized by serotypes that are common colonizers in childhood and the results were in concordance with other studies (Brueggemann et al., 2003; Ercibengoa et al., 2012). It is of interest that serotype 19F, which was at that time the dominant serotype in pneumococcal disease and especially in children, was seen in less than a tenth of the isolates (papers I and II).

Particular serotype combinations were not detected in co-colonized children and the serotypes found reflected the overall serotype rates.

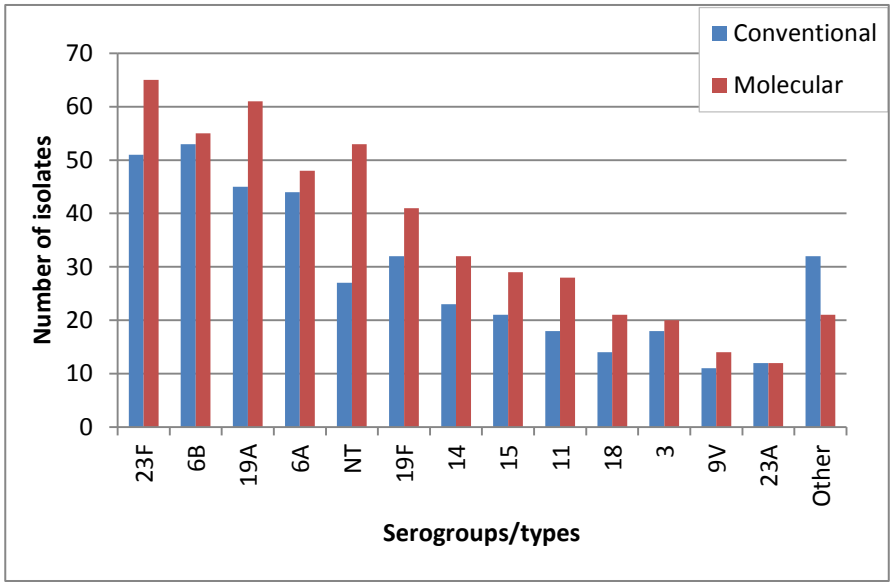

Figure 9. Number of isolates by serogroups/types detected in 514 healthy children attending day care centres, 401 isolates by conventional methods and 500 by molecular methods.

VT-10 were identified in 186 (46.4%) and VT-13 in 293 isolates (73.1%) when using conventional methods, and using the PCR panel in 234 (58.4%) and 363 strains (90.5%), respectively. Of the co-colonized children 74 (80.4%) and 91 (98.9%) carried one or more of the serotypes of VT-10 and VT-13, respectively. The sampling was done in 2009, or two years prior to implementation of the PHiD-CV10 into the infant vaccination program and thus the results represent an unvaccinated population.

The rates of VTs were high and the main differences between the rates of VT-10 and VT-13 were due to high rates of 19A and 6A that are only included in the 13-valent vaccine. It can be debated that this does not reflect actual protection as there is cross-protection of the 6B antigens for 6A and of the 19F antigens for 19A (Grant et al., 2013; Lee et al., 2011). Furthermore, we now know that a small part of the isolates identified as 6B were actually of serotype 6Bii/6E, but these serotypes have identical serological properties (Burton et al., 2016). The isolates serotyped as 6B will be further analysed, but four isolates had the antibiogram of CC90.

4.3.4 Differences in detection by methods

Besides the different abilities of the methods to detect co-colonization, more specific differences were also seen. Using conventional methods, serotype 14 was identified in six (20%) of co-colonized children compared to 24 (6%) of all isolates (p<0.01). Using molecular methods a serotype-specific difference was not seen. Serotype 14 has different morphology and it is in concordance with observations of others that serotypes of differing morphology are more likely to be detected in co-colonization (Huebner et al., 2000; Saha et al., 2015).

When using conventional methods 27 isolates (6.5%) were non-typable. Those were all negative in agglutination against all Pneumotest® pools. *CpsA* and *lytA* genes were detected using molecular methods in all the isolates. Serotypes that were not included in the PCR panel were found in 25 samples (6.2%) using conventional methods. No isolate of serotype 1 was identified, - the only VT not included in the PCR panel. When using molecular methods 18 samples were only *cpsA* and *lytA* positive, but negative in all serotype-specific PCR reactions. Serotypes were detected in the remaining 7 samples indicating the presence of more than one serotype in those samples. Furthermore, it can be assumed that although isolates of the serotypes that were not included in the panel were rare, they could be more often present in co-colonization than detected by either of the methods. Other studies have reported rare serotypes to be more commonly found in co-colonization than

in carriage of a single serotype (Ercibengoa et al., 2012). It can thus be assumed that the actual rate of co-colonization was higher than detected.

In 27 of the samples where there was no growth of pneumococci using conventional methods, the molecular method still detected pneumococci. In one of those samples it detected three serotypes and in another there were two serotypes. Of the samples where pneumococci were not detected using molecular methods, pneumococci were found in three samples when using conventional methods. Two were of serotypes included in the PCR panel and one was not.

When using molecular methods, 53 strains (9.6%) were non-typable. Those were all *cpsA* positive, but negative in all serotype-specific PCR reactions. Among those, conventional methods yielded 19 non-typable isolates, 18 isolates of other serotypes than included in the PCR panel, one isolate of serotype that was included in the panel and no growth of pneumococci in the remaining 15 samples.

The limited number of serotypes in the PCR panel was a limitation. However, it may be regarded as strength to have succeeded in designing a PCR panel with only five reactions that detected the serotype of 447 strains and *cpsA* genes in additional 53 strains, while agglutination of pneumococcal culture yielded 374 serotyped and 27 non-typable isolates. Furthermore, the PCR panel covered 94% of the serotypes detected by conventional methods.

4.4 Prevalence of pilus genes (paper IV)

4.4.1 Demographics

Pneumococcal carriage and serotype distribution of this study was already described in section 4.3, as the participants were the same, healthy children attending day care centres in 2009. The isolates obtained by the conventional method described in the section were used in this study on prevalence of pilus genes. Of these three isolates were not available. Thus, 398 isolates from healthy children attending 15 DDCs were available for PI detection.

4.4.2 Pilus islet 1

Of the 398 isolates in the study 134 (33.7%) carried the PI-1 genes, while 261 (65.6%) did not. The prevalence rate is in concordance with previously reported rates of 15%-35% in children who are healthy carriers (Basset et al., 2007; Regev-Yochay et al., 2010; Turner et al., 2012). It is also comparable to reported rates of 20%-30% in acute otitis media (Moschioni et al., 2010a;

Vainio et al., 2011) and 10%-33% in invasive disease (Aguiar et al., 2008; Moschioni et al., 2008; Selva et al., 2012; Zahner et al., 2010). Confirmed result could not be obtained for three isolates (0.8%). These showed negative results in PCR with the primers specific for the PI-1 genes. However, the absence of PI-1 could not be confirmed by a positive reaction using the PFL primers. They bind to the sequences flanking the PI-1 and can only show positive reaction when it is absent.

Prevalence of genes encoding for PI-1 varied according to serotypes and within a serotype. Of the five serotypes that were most commonly carried by the children the prevalence of PI-1 genes was highest in serotype 19F where the vast majority, 96.8% (30/31) was positive. However, the highest number of PI-1 positive isolates was seen within serotype 6B, though at lower but still high rates, 82.8% (48/58). Pili genes were seen in other serotypes in lower numbers although in some serotypes at high rates (Figure 10). Isolates of serotype 19F were mostly multi-resistant and had an identical antibiogram to CC320 (paper I). The only isolate of serotype 19F that did not carry the PI-1 genes was susceptible, and in accordance with carriage of pilus genes being a clonal property. Of the isolates that were identified as serotype 6B four had the antibiogram of CC90. All carried PI-1 genes of clade II.

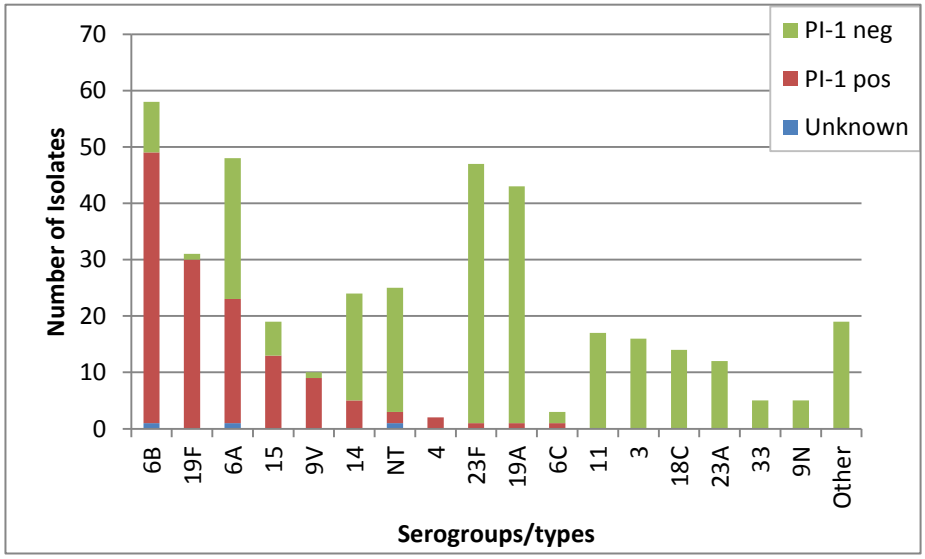

Figure 10. Prevalence of PI-1 genes, by serogroups/types. Unknown were negative in pili specific reactions, but their absence could not be confirmed with positive reaction using primers for the flanking sequences.

Isolates of VT-10 were 95 (70.9%) and of VT-13 there were 128 isolates (88.1%). VTs more commonly carried PI-1 than NVT (P<0.0001). As the majority of isolates carrying PI-1 genes were of VTs, vaccination is likely to reduce the rates of piliated pneumococci. Other studies have reported a decrease in the prevalence of piliated pneumococci in IPD following vaccination (Aguiar et al., 2012; Regev-Yochay et al., 2010) and one study reported vaccine escapes of piliated pneumococci (Regev-Yochay et al., 2016). PI-1 positive isolates of the NVTs were most commonly of serogroup 15, i.e. 13 isolates (9.7%). It will be of interest to see the development in the era after implementation of vaccination, as besides vaccine escape of piliated pneumococci, replacement of piliated NVTs has been reported (Regev-Yochay et al., 2010).

All the isolates carrying PI-1 genes were further analysed for detection of their class of clade, but for three isolates the class could not be determined. Of the remaining 131 isolates 73 (55.7%) were of clade I, 41 (31.3%) of clade II and 17 (13%) of clade III and their distribution varied according to serotypes (Figure 11).

Piliated pneumococci evoke immune response to their clade and therefore possibly provide selective pressure against pneumococci harbouring pili of the same clade (Henriques-Normark et al., 2008). It can therefore be

expected that when a piliated clone has been dominating in an area for some time herd immunity against pili of its clade arises. Thus, the succeeding clone would be likely to be without pili, having pili of another class or pili of another clade within the same class. What supports this theory is that 40 (83.3 %) of the isolates carrying genes for PI-1 of serotype 6B were of clade II and all the isolates of serotype 19F were of clade I. It was interesting to see that most of the 6B isolates carrying PI-1 genes of clade II are susceptible to antimicrobials. This might be caused by exchange of pili genes from CC90. The non-vaccine serogroup 15 had pili of clade III, making its future development interesting.

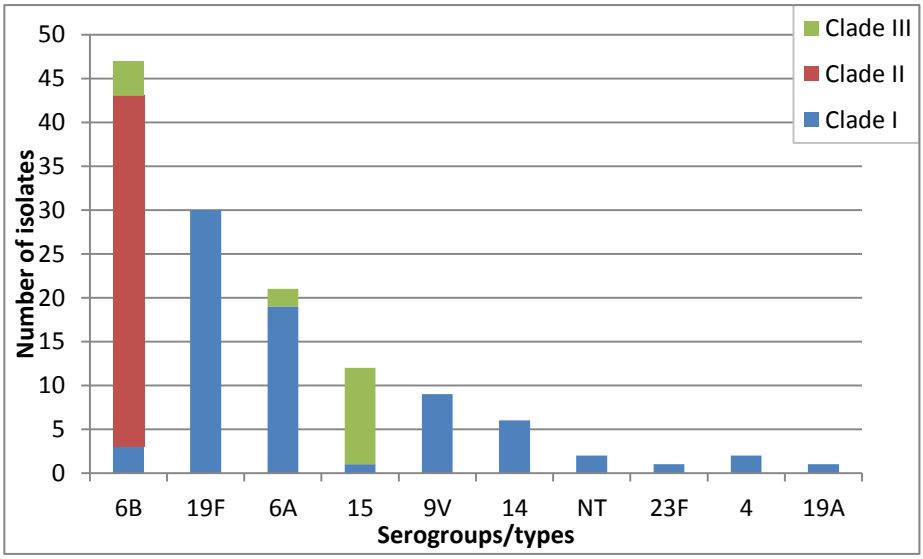

Figure 11. Prevalence of clades of PI-1, by serogroups/types.

4.4.3 Pilus islet 2

Of the 398 isolates tested 38 (9.5%) carried the PI-2 genes and 355 isolates (89.2%) did not. This rate is comparable to other studies on mixed, IPD and ME collections of pneumococcal isolates that have reported rates from 0%-21% (Bagnoli et al., 2008; Moschioni et al., 2010a; Vainio et al., 2011; Zahner et al., 2010). A confirmatory result could not be obtained for five isolates. Those showed negative results with the PI-2 specific primers. However, the absence of PI-2 could not be confirmed by a positive reaction to the 1008for and 1009rev primers for the flanking sequences.

Prevalence of genes encoding for PI-2 varied according to serotypes and within a serotype. The highest number and prevalence were seen within serotype 19F, i.e. 27 isolates (87.1%). All carried also genes for PI-1, clade I. Of the 19F PI-2 negative isolates, three carried PI-1 genes, one was negative in both, but all had different antibiograms from the positive ones. PI-2 genes were found in three other serotypes. Most were in serogroup 11, the only PI-2 positive NVT, 7 isolates (41.2%). VTs more commonly carried PI-2 genes than NTV (p<0.0001). Of the 38 isolates that carried the PI-2 genes 30 (78.9%) were of VT-10 and 31 (81.6%) of VT-13 (Figure 12).

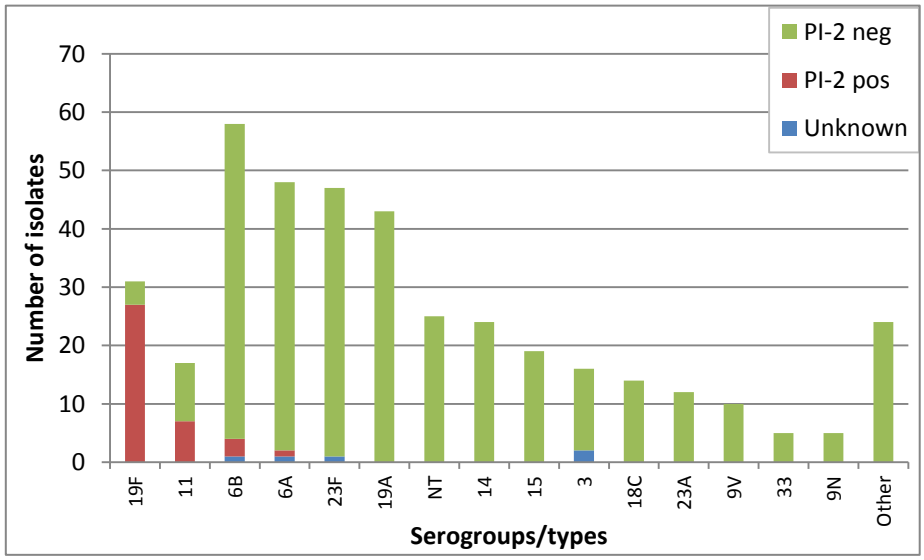

Figure 12. Prevalence of PI-2, by serogroups/types. Unknown were negative in pili specific reactions but their absence could not be confirmed with positive reaction using primers for the flanking sequences.

4.4.4 Association with antimicrobial susceptibility

Of the 398 isolates in the study, 340 (85.4%) were penicillin susceptible (MIC ≤0.06 mg/L) and 58 (14.6%) were PNSP. None were fully resistant to penicillin. This is a lower rate than seen in nasopharyngeal samples from patients, where it was 34.5% in 2009 (p<0.0001) (paper I). The most common PNSP serotype was serotype 19F with 28 isolates (48.3% of PNSP) followed by 6B with 7 isolates (12.1%) (Table 9). These were also the most common PNSP serotypes in patient samples in this year.

Table 9. Serotype distribution, according to susceptibility to penicillin of isolates carrying PI-1 and PI-2 genes.

Type	PNSP total	PNSP positive for PI, n (%)		PSP total	PSP positive for PI, n (%)	
		PI-1	PI-2		PI-1	PI-2
19F	28	28 (100)	26 (92.9)	3	2 (66.7)	1 (33.3)
6B	7	5 (71.4)	1 (14.3)	51	43 (84.3)	2 (3.9)
6A	1	1 (100)	1 (100)	47	21 (44.7)	0
15	0	0	0	19	13 (68.4)	0
9V	0	0	0	10	9 (90.0)	0
11	0	0	0	17	0	7 (14.2)
14	6	5 (83.3)	0	0	0	0
NT	13	2 (15.4)	0	0	0	0
4	0	0	0	2	2 (100)	0
19A	1	0	0	42	1 (2.4)	0
23F	1	0	0	46	1 (2.2)	0
6C	0	0	0	3	1 (33.3)	0
Other	1	0	0	100	0	0
Total	**58**	**41**	**28**	**340**	**93**	**10**

Isolates carrying the PI-1 genes were relatively more common in PNSP than in PSP, i.e. 41 isolates (70.7%) and 93 (27.4%), respectively (p<0.001). The same held true for isolates carrying PI-2 genes, with 28 PNSP isolates (48.3%) and 10 PSP isolates (2.9%) (p<0.001). The PNSP carrying PI-1 genes were most commonly of serotype 19F, i.e. 28 isolates, and the PSP of serotype 6B with 43 isolates. PI-2 positive PNSP were most oftenof serotype 19F, 26 isolates, and of the PSP serogroup 11, or 7 isolates (Table 9).

The dominant PNSP, CC320, in Iceland and its close relatives of serotype 19A, CC320, carry both PI-1 and PI-2 genes (Bagnoli et al., 2008; Zahner et al., 2010). The PI-1 of CC320 was of different clade than its preceding PNSP clone, CC90.

5 Summary and conclusions

The main findings of the study were that one in four pneumococcal isolate identified in samples from Icelandic patients was defined as penicillin non-susceptible during 1995-2015. Temporal changes reflected the rates of the multi-resistant isolates of two international multi-resistant clones. We saw the earlier clone that had caused health problems in Iceland since 1989, reach its peak and then decrease slowly and equally in all age groups, most likely in concordance with the natural dynamics of pneumococci. We saw the second clone reach considerably higher prevalence and then decrease rapidly. This development was exceptional compared to other countries in northern Europe. Importantly, following implementation of vaccination with ten-valent pneumococcal *Haemopilus Influenzae* protein D conjugated vaccine into the infant vaccination program in Iceland in 2011, the number of PNSP decreased dramatically in vaccinated children.

In 1996 the clone Spain^{6B}-2, CC90, that had been dominant in the country for years, reached its peak and then gradually declined in all age groups, most likely reflecting the natural dynamics of a successful clone. This development completed the first wave of PNSP in the country.

The second wave of PNSP started in 1998 when a new clone, Taiwan^{19F}-14, CC320, emerged and expanded rapidly to cause higher rates of PNSP than ever seen before. It reached its peak in the year in which the vaccinations started. Therafter a rapid decline occurred. The number of isolates of this clone declined, along with the number of isolates of other vaccine serotypes, and in all age groups except in the oldest patients. Thus, following vaccination more than a three-fold decrease in the number of pneumococcal isolates and six-fold in the number of PNSP isolates occurred within four years. In accordance with this the burden of pneumococcal disease in the country has decreased directly through vaccination and indirectly through herd effect.

If, or when, the third wave occurs, depends on the development of pneumococcal vaccines as serotype substitution and serotype replacement has already been described in countries that have longer experience of vaccination than we do in Iceland.

Serotype distribution in clinical isolates during 2007-2011, or in the last five years prior to prior to vaccination, was dominated by the multi-resistant isolates of serotype 19F, CC320. It was by far the most common serotype in all age groups, in middle ear samples and in samples from the lower

respiratory tract. Serotype 19F was especially common in isolates from the middle ear. These isolates were almost solely from young children and there the vaccine serotypes were the most common. Serotype diversity was most prevalent in samples from the lower respiratory tract which mainly originated from adults and the oldest patients and there the rates of vaccine types were the lowest. However, serotype 19F was ranked number six in samples from invasive disease where serotype 14 was the most common.

Nasopharyngeal swabs from healthy children collected in 2009 were used to get an overview of pneumococcal carriage and especially to investigate co-colonization of different serotypes. For this purpose we used molecular methods on DNA extracted directly from the swabs. The carriage was among the highest reported and three out of four children were found to be carrying pneumococci. Every forth of the carriers had more than one serotype, most carried two serotypes, but up to four were detected in the same child. Thus, the opportunities for intraspecies genetic exchange are more than can be expected from most carriage studies. It is known that children are the source for pneumococci and that nasopharyngeal colonization is a prerequisite for pneumococcal disease. If a patient carries multiple serotypes, then insufficient treatment or a wrong choice of antibiotic is likely to be conductive towards a favourable environment for PNSP. Interestingly serotype 19F was seen at lower rates in nasopharyngeal isolates from these healthy children than from children seeking health services. The method of extracting DNA directly from the swabs and using molecular methods for detection of serotypes worked well.

Pili were first described in 2006 and 2008 and it was interesting to see if the pneumococci circulating in Iceland were piliated. We used the isolates from the carriage study in 2009 to investigate this. Genes encoding for pili were most commonly found in the most common PNSP serotypes including the multi-resistant clones. Isolates of serotype 6B carried genes for pilus islet 1 of clade II and isolates of serotype 19F carried both genes for pilus islet 1, clade I and pilus islet 2. Carriage of pili is a clonal property and part of the explanation for the success of CC90 and CC320. Pili initiate antibody responses and thus play a role in creating herd immunity to pneumococcal clones. This lends support to the explanation that CC90 declined because of increased herd immunity with time. In the same way it supports that when a dominant clone is replaced it is likely to be by a clone that is either not piliated or to have a different kind of pili, such as is the case with the CC320.

The impact of the two dominant clones on pneumococcal epidemiology and antimicrobial resistance in Iceland was remarkable. When vaccination started resistance was higher than ever because of the 19F clone. Following vaccination it declined markedly, especially in children, where the clone has almost disappeared.

We hope that our study will provide important information on the nature of penicillin non-susceptible pneumococci to enhance patient diagnosis and treatment, resulting in better outcome from patients with pneumococcal diseases. We also hope that our study provides important information on the value of pneumococcal vaccination. We highlight the need for further studies to monitor the effect of the pneumococcal vaccines and the studies may be conducive to further vaccine developments.

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Original publications

Paper I

Hjálmarsdóttir M.A., Kristinsson K.G. (2014). Epidemiology of penicillin-non-susceptible pneumococci in Iceland, 1995-2010. *J Antimicrob Chemother*, 2014,69(4), 940-946.

Paper II

Hjalmarsdóttir M.A.*, Quirk S.J.*, Haraldsson G., Erlendsdóttir H., Haraldsson A., Kristinsson K.G. Comparison of serotype prevalence of pneumococci isolated from middle ear, lower respiratory tract and invasive disease prior to vaccination in Iceland.
(*shared first authorship). Manuscript submitted.

Paper III

Hjalmarsdóttir M.A., Gumundsdóttir P.F., Erlendsdóttir H., Kristinsson K.G., Haraldsson G. (2016). Co-Colonization of Pneumococcal Serotypes in Healthy Children Attending Day Care Centres: Molecular Versus Conventional Methods. *Pediatr Infect Dis J*, 35(5), 477-480.

Paper IV

Hjalmarsdóttir M.A., Pétursdóttir B., Erlendsdóttir H., Haraldsson G., Kristinsson K.G. (2015). Prevalence of pilus genes in pneumococci isolated from healthy preschool children in Iceland: association with vaccine serotypes and antibiotic resistance. *J Antimicrob Chemother*, 70(8),2203-2208.

Appendix I
Primers

Table 10. Oligonucleotide primers used in multiplex PCR and/or single PCR reactions. Primers for the *cpsA* gene were included in each multiplex PCR reaction (paper III) and/or primers for the *lytA*gene (paper II).

Serotype	Size	Dir.	Sequence	References
1	280	f	CTCTATAGAATGGAGTATATAAACTATGGTTA	Pai et al, 2006
		r	CCAAAGAAAATACTAACATTATCACAATATTGGC	
3	371	f	ATGGTGTGATTTCTCCTAGATTGGAAAGTAG	Pai et al, 2006
		r	CTTCTCCAATTGCTTACCAAGTGCAATAACG	
4	430	f	CTGTTACTTGTTCTGGACTCTCGATAATTGG	Pai et al, 2006
		r	GCCCACTCCTGTTAAAATCCTACCCGCATTG	
5	362	f	ATACCTACACAACTTCTGATTATGCCTTTGTG	Pai et al, 2006
		r	GCTCGATAAACATAATCAATATTTGAAAAAGTATG	
6	250	f	AATTTGTATTTTATTCATGCCTATATCTGG	Pai et al, 2006
		r	TTAGCGGAGATAATTTAAAATGATGACTA	
7F/A	826	f	CCTACGGGAGGATATAAAATTATTTTTGAG	Pai et al, 2006
		r	CAAATACACCACTATAGGCTGTTGAGACTAAC	
9V/A	753	f	CTTCGTTAGTTAAAATTCTAAATTTTTCTAAG	Pai et al, 2006
		r	GTCCCAATACCAGTCCTTGCAACACAAG	
11A/D	463	f	GGACATGTTCAGGTGATTTCCCAATATAGTG	Pai et al, 2006
		r	GATTATGAGTGTAATTTATTCCAACTTCTCCC	
14	189	f	GAAATGTTACTTGGCGCAGGTGTCAGAATT	Dias et al, 2007
		r	GCCAATACTTCTTAGTCTCTCAGATGAAT	
15B/C	496	f	TTGGAATTTTTTAATTAGTGGCTTACCTA	Pai et al, 2006
		r	CATCCGCTTATTAATTGAAGTAATCTGAACC	
18	285	f	GCC GTG GGA AGC TTA TTT TT	Dobay etal, 2009
		r	CCT GCC TAA AGG CAA CAA TG	

Serotype	Size	Dir.	Sequence	References
19F	304	f	GTTAAGATTGCTGATCGATTAATTGATATCC	Pai et al, 2006
		r	GTAATATGTCTTTAGGGCGTTTATGGCGATAG	
19A	478	f	GTTAGTCCTGTTTTAGATTTATTTGGTGATGT	Pai et al, 2006
		r	GAGCAGTCAATAAGATGAGACGATAGTTAG	
19B/C	354	f	AGAATTCGGAGATTTGTGGTA	Zhou etal, 2007
		r	AATCCCAGATCAAATGTTCC	
20A/B	453	f	TTCACCTGACAGCGAGAAG	Based on Pai etal, 2006
		r	TCTGAAAATGCAAACGTCCT	This study
22F/A	643	f	GAGTATAGCCAGATTATGGCAGTTTTATTGTC	Pai et al, 2006
		r	CTCCAGCACTTGCGCTGGAAACAACAGACAAC	
23F	384	f	GTAACAGTTGCTGTAGAGGGAATTGGCTTTTC	Pai et al, 2006
		r	CACAACACCTAACACTCGATGGCTATATGATTC	
23A	853	f	GATTTGGAGCGGATCGATTA	Dobay etal, 2009
		r	AATGGGTAATGGAGGGGAGT	
23B	268	f	GTGGGTTGACGCATAAGAAT	This study
		r	GATAATAAAGAAATTACTAACCATGTCGT	Zhou etal, 2007
29	654	f	CCGAAAATTGTTCACAGGATAC	Zhou etal, 2007
		r	AAAAGAATTGTTTGATCCGAGA	This study
33F/A	338	f	GAAGGCAATCAATGTGATTGTGTCGCG	Pai et al, 2006
		r	CTTCAAAATGAAGATTATAGTACCCTTCTAC	
33B/D	264	f	TCG TTG GAT GAC AAA ACT CTT AC	Zhou etal, 2007
		r	CCT CCC TGA GCC AAA ATA AC	
34	409	f	CTTTTGTAAGAGGAGATTATTTTCAC	Based on Pai etal, 2006
		r	CCCAATCCGACTAAGTCTTC	
35F/47F	521	f	CCCATCTATCTTGATGATGAAC	Based on Pai etal, 2006
		r	TTCCTAGAGCGAGTAAACCAA	
35B	688	f	TTGGATAAGTCTGTTGTGGAGA	Based on Pai etal, 2006
		r	CGCAGCTTCTTTCCAGATAA	
38(25F)	568	f	CGTTCTTTTATCTCACTGTATAGTATCT	Based on Pai etal, 2006
		r	GAATTAAAGCTAACGTAACAATCC	
42(35A/C)	492	f	TCCCTTTTTCAGACGTAGC	Based on Dobay etal, 2009
		r	CAAGAAATTGATCCGCTTG	

Serotype	Size	Dir.	Sequence	References
47A	789	f	TGCCATAACGGACTCTAGAAC	Based on Zhou etal, 2007
		r	CTGTCCCTTAGCTCTGTCCA	This study
cpsA	160	f	GCAGTACAGCAGTTTGTTGGACTGACC	Pai et al, 2006
		r	GAATATTTTCATTATCAGTCCCAGTC	
lytA	318	f	CAACCGTACAGAATGAAGCGG	Based on Sourav et. al, 2010
		r	TTATTCGTGCAATACTCGTGCG	

Table 11. Oligonucleotide primers used for detection of serotypes within serogroup 6. (paper II and unpublished). 6A: 6A/C positive and 6C/D negative. 6B: 6B/D positive, 6C/D negative. 6C: 6A/C positive and 6C/D positive. 6D: 6B/D positive, 6C/D positive. 6E: 6AB, 6E and 6E specific positive.

Serotyope	Size	Dir.	Sequence	References
6A/C	149	f	ATTTATATATAGAAAAACTGGCTCATGATAG	
		r	GCGGAGATAATTTAAAATGATGACTAGTTG	(Jin et al., 2009a)
6B/D	155	f	AAGATTATTTATATATAGAAAAACTGTCTCATGATAA	
		r	GCGGAGATAATTTAAAATGATGACTAGTTG	(Jin et al., 2009a)
6C (6D)	359	f	ATCTCTAAATCTGAATATGAAGCGGCTCAATC	
		r	GAACTGAGCTAAATAATCCTCTGGATTATCCACC	(Jin et al., 2009a)
6E reactions				
6AB	215	f	CACAGGCAAAATTGGATTC	Based on Kawaguchiya et al. 2014
		r	AACAGAATTGCGAATATCTC	Kawaguchiya et al. 2014
6E	578	f	TGATATTCATTCGCATTGTC	Kawaguchiya et al. 2014
		r	TATGAACCAAATCACGCTCCAAG	Kawaguchiya et al. 2014
6E specific	573	f	AGGTGAAATTAGAACTTGCG	Kawaguchiya et al. 2014
		r	GCTTCCCAGTTTGTTCTATC	Based on Kawaguchiya et al. 2014

Table 12. Oligonucleotide primers used for pili detection and classification.

Primer	Sequence	Purpose	Reference
srtD for	CATGTCTTTTTCCGCCATTT	Presence of PI-1	This study
srtD rev	CGTAGTAAACGTGCTAGCTTCC		
PFL for	CTCATTGACTACACAAGTATCACCTC	Absence of PI-1	Modified from Aguiar
PFL rev	AGCATACTCCAACTCATAAATATGTG		
P06 for	CGTGGGTATCAGGTGTCCTATGATAA	Presence of PI-2	(Bagnoli et al., 2008)
P06 rev	GCCTCGTCTTCTAATKACTGTTAC		
1008 for	GCTGGATCGAGTTTGAAACCAGAA	Absence of PI-2	(Bagnoli et al., 2008)
1009 rev	TAAGGATCACCAAAGTCCAAGGCA		
Clade I for	AACAGATGGGGATATGGATAAAATTG	Clade I	(Moschioni et al., 2008)
Clade I rev	AATGGTAATTCAATTTCAATTGGA		
Clade II for	AATCCATAAGTTACTGCTCTCAGA	Clade II	(Moschioni et al., 2008)
Clade II rev	ATCCATAGCTACATTATTCAAAGT		
Clade III for	GACAGATCAAGAGCTTGACGCTTG	Clade III	(Moschioni et al., 2008)
Clade III rev	CTGGATCTACGAAACCTGCTGCAG		