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Streptococcus pneumoniae revisited: Laboratory identification and epidemiology

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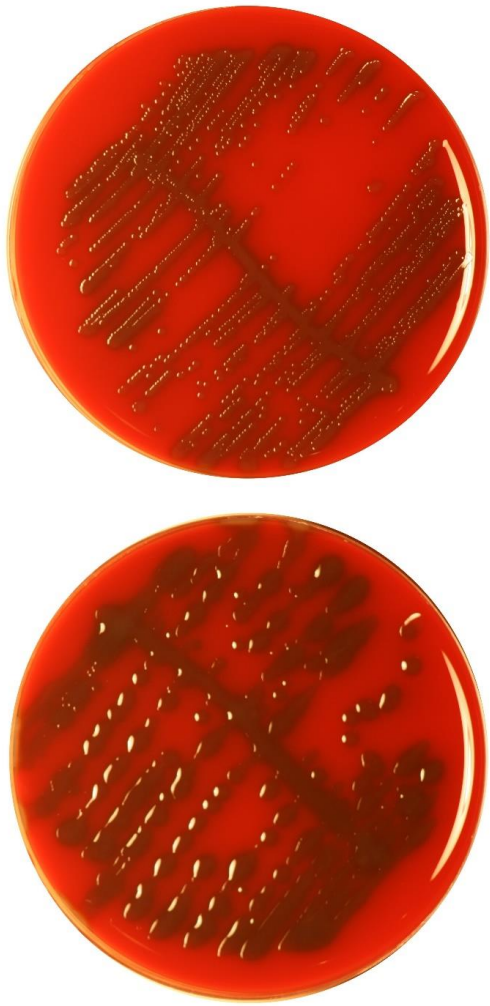
Statens Serum Institut



Front page picture - Appearance of *Streptococcus pneumoniae* colonies on a 10% blood agar plate: Upper picture: “Normal-sized” colonies, which include the majority of known serotypes. Lower picture: Mucoid colonies. Serotype 3, in particular, has this characteristic.
The pictures are printed with kind permission of Kirsten Burmeister.

The Faculty of Health and Medical Sciences at the University of Copenhagen has accepted this dissertation for public defence for the doctoral degree in medicine.

Copenhagen, 28. April 2022, Bente Merete Stallknecht, Head of Faculty.

The defence will take place Tuesday the 14st of June 2022, at 14:00 p.m. in auditorium “Foredragssalen”, room 043-219, Statens Serum Institut, Artillerivej 5, 2300 Kbh S.

Leader of ceremony
Professor Claus Ernst Moser, University of Copenhagen
Chairperson
Professor Jørgen Anders Lindholm Kurtzhals, University of Copenhagen.
Opponents
Professor Sven Hammerschmidt, University of Greifswald, 1. Opponent.
Professor emeritus Mogens Kilian, Aarhus University, 2. Opponent.

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Contents

Main references.

This thesis is based on the following papers

A. Slotved HC, Facklam RR, Fuursted K. Assessment of a novel bile solubility test and MALDI-TOF for the differentiation of *Streptococcus pneumoniae* from other mitis group streptococci. Sci Rep. 2017 Aug 2;7(1):7167. doi:10.1038/s41598-017-07772-x (1).
B. Hansen CB, Fuursted K, Valentiner-Branth P, Dalby T, Jørgensen CS, Slotved HC. Molecular characterization and epidemiology of *Streptococcus pneumoniae* serotype 8 in Denmark. BMC Infect Dis. 2021 May 5;21(1):421. doi: 10.1186/s12879-021-06103-w (2).
C. Kavalari ID, Fuursted K, Krogfelt KA, Slotved HC. Molecular characterization and epidemiology of *Streptococcus pneumoniae* serotype 24F in Denmark. Sci Rep. 2019 Apr 2;9(1):5481. doi: 10.1038/s41598-019-41983-8 (3).
D. Slotved HC, Kaltoft M, Skovsted IC, Kerrn MB, Espersen F. Simple, rapid latex agglutination test for serotyping of pneumococci (Pneumotest-Latex). J Clin Microbiol. 2004 Jun;42(6):2518-22 (4).
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Preface

Purpose of the dissertation.

The purpose of this dissertation is to describe the modern pneumococcal laboratory procedure for identification of *S. pneumoniae* isolates and to describe the epidemiology of pneumococci with respect to carriage and invasive pneumococcal disease (IPD) in Denmark.

Acknowledgements

I am very grateful that my section manager and mentor Dr. Kurt Fuursted, MD, DMCc, encouraged me to write this thesis. Within the hour of him planting the idea with me, I began with the dissertation. Dr. Fuursted has provided me with invaluable advice and support, and our Friday meetings have been of great inspiration and had a profound impact on the dissertation.

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This Thesis is made within the Department of Bacteria, Parasites and Fungi under the management of director, Lars Villiam Pallesen. It is an inspiring workspace where it is possible for researchers like me to evolve and conduct resulting in this thesis. I am very thankful for being part of this department.

Finally, I am very grateful for the patience and understanding that my family, Theis, Mark, Jon and my wife Lillian Sztuk Slotved have shown me whilst working long hours on this thesis.

Abbreviations

AOM: Acute Otitis Media

CDC: Centers for Disease Control and Prevention

CPS: Capsular PolySaccharide

CSF: CerebroSpinal Fluid

DCM: Departments of Clinical Microbiology

FDA: Food and Drug Administration

IPD: Invasive Pneumococcal Disease

IVAC: International Vaccine Access Center, **https://www.jhsph.edu/ivac/**

MiBa: The Danish Microbiology Database

MLSA: MultiLocus Sequence Analysis

MLST: MultiLocus Sequence Typing

MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization–Time Of Flight Mass Spectrometry

NSRlab: National Streptococcal Reference laboratory

OD value: Optical Density value

PCR: Polymerase Chain Reaction

PCV: Pneumococcal Conjugate Vaccine

PPV: Pneumococcal Polysaccharide Vaccine

PCV-7: PCV covering serotype 4, 6B, 9V, 14, 18C, 19F and 23F

PCV-10: PCV covering PCV-7 + serotypes 1, 5 and 7F

PCV-13: PCV covering PCV-7 + serotypes 1, 3, 5, 6A, 7F and 19A

PPV-23: Pneumococcal Polysaccharide Vaccine covering 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

qPCR: Quantitative Polymerase Chain Reaction

rtPCR: realtime Polymerase Chain Reaction

SNPs: Single Nucleotide Polymorphisms

SSI: Statens Serum Institut

TH broth: Todd-Hewitt broth

VGS: Viridans Group Streptococci
WGS: Whole Genome Sequencing
WHO: World Health Organization

1. English summary

Streptococcus pneumoniae (pneumococcus) is considered a worldwide human pathogen, which continues to cause considerable morbidity and mortality even after more than 100 years of investigation. This thesis adds to our knowledge on detection, prevention and treatment of *S. pneumoniae* by presenting a refined pneumococcal identification, and understanding of pneumococcal epidemiology in Denmark. The use of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) combined with peak-specific analysis with a newly developed objective bile solubility test (Paper A) provides a more precise and accurate differentiation of *S. pneumoniae* from other species within the *Streptococcus* Mitis group. The use of whole genome sequencing (WGS) is shown to provide a wide range of novel opportunities by providing a more precise species identification based on multilocus sequence analysis (MLSA) and by resolving the specific nucleotide position 203 in the 16s rRNA sequence. However, it also reveals the challenges associated with the use of WGS data for surveillance due to the phenotype/genotype complexity (Paper B and C).

The phenotypic Pneumotest Latex kit (Papers D and E) has proven to be a fast and simple serotype identification method for *S. pneumoniae* that serves as an alternative to the more labor-demanding Neufeld test; and the combination of WGS-based genotyping and the phenotypic capsular typing system is expected to become the new phenotyping standard.

The various identification and typing procedures (Papers A, B, C, D, E) were used to analyze the epidemiology of pneumococcal carriage and disease in the Danish population. The overall pneumococcal carriage in children aged 8-19 months was shown not to be influenced by introduction of pneumococcal conjugate vaccines (PCV-7 and PCV-13), although the serotype distribution was altered, and the PCV-related serotypes almost disappeared in these children (Paper F).

Invasive pneumococcal diseases (IPD) are greatly reduced in PCV-vaccinated children < 5 years, and a reduction is observed in other age groups owing to flock protection. However, while IPD cases have disappeared from vaccinated children due to PCV-included serotypes, these serotypes

(especially serotype 3) still cause IPD in non-vaccinated age groups (Papers G, H, I, J). An increase in non-PCV serotype IPD cases has also been observed in all age groups (Paper G).
The observed epidemiology suggests that vaccinated children < 2 years are not the main carriers and transmitters, and that some serotypes are carried and transmitted by other age groups (Paper K). This implies that we need to consider alternative vaccination strategies including vaccination of other age groups, and further reduction in the overall IPD burden requires higher-valent conjugate or common antigen vaccines.

2. Danish summary

Streptococcus pneumoniae (pneumococci) betragtes som et verdensomspændende humant patogen, som efter mere end 100 års studier fortsat medfører stor sygdom og dødelighed. Denne afhandling tilføjer ny viden om forebyggelse og behandlingsstrategi ved at præsentere en forbedret identifikation af pneumokokker og viden om pneumokokepidemiologi i Danmark. Kombineret anvendelse af MALDI-TOF MS peak-specifik analyse med en nyudviklet objektiv galdeløselighedstest (Artikel A) tilvejebringer en mere præcis og nøjagtig differentiering af *S. pneumoniae* fra andre arter inden for *Streptococcus* Mitis-gruppen. Anvendelsen af helgenomsekventering (WGS) har resulteret i en lang række nye muligheder, såsom en mere præcis identifikation af arter baseret på multilokus-sekvensanalyse (MLSA) og identifikation af den specifikke nukleotidposition 203 i 16s rRNA-sekvensen. Imidlertid afdækker det også de udfordringer, der er ved at bruge WGS-data til overvågning grundet fænotype/genotype-kompleksiteten (Artikel B and C).
Den fænotypebaserede Pneumotest Latex kit (Artiklerne D og E) har vist sig at være en hurtig og enkel metode til serotypeidentifikation af *S. pneumoniae* som et alternativ til den mere arbejdskrævende Neufeld-test. Kombinationen af WGS-baseret genotyping og den fænotypebaserede identifikation af den serotypespecifikke kapsel forventes at være den fremtidige nye standard for fænotypning.
De udviklede identifikations- og serotypemetoder (Artiklerne A, B, C, D, E) er blevet brugt til at analysere epidemiologien for pneumokokbærerrate og -sygdom i den danske befolkning. Data har vist, at den samlede pneumokokbærerrate hos børn i alderen 8-19 måneder ikke er ændret efter introduktionen af pneumokokkonjugerede vacciner (PCV-7 og PCV-13) til trods for, at serotypedistributionen er ændret, og at de PCV-relaterede serotyper næsten er forsvundet hos børn (Artikel F).

PCV-vaccination af børn <5 år har reduceret raten af invasive pneumokoksygdomme (IPD) kraftigt i denne gruppe, og en reduktion er ligeledes observeret i andre aldersgrupper grundet en potentiel flokbeskyttelse. Selv om IPD-tilfælde grundet PCV-inkluderede serotyper er forsvundet fra vaccinerede børn, forårsager de PCV-inkluderede serotyper stadig IPD i de ikke-vaccinerede aldersgrupper, især serotype 3 (Artiklerne G, H, I, J). En stigning af IPD-tilfælde med serotyper, der ikke er inkluderet i PCV-vaccinen, er også observeret i alle aldersgrupper (Artikel G).
Den observerede ændring af pneumokokepidemiologien antyder, at vaccinerede børn <2 år ikke er de vigtigste bærere og smittespredere, men at nogle serotyper bæres og spredes af andre aldersgrupper (Artikel K). Dette indebærer, at vi er nødt til at overveje alternative vaccinationsstrategier, herunder vaccination af andre aldersgrupper, og at der er behov for nye vacciner, som dækker et højere antal serotyper, hvis der skal opnås en yderligere reduktion af den samlede IPD-byrde.

3. Introduction

Streptococcus pneumoniae is a Gram positive bacterium described first time in 1881 by Pasteur from France and Sternberg from the US (12,13). The bacteria are diplococci often observed either as single cells or in some occasions short chains (14).

The bacterium is known for its characteristic alpha-hemolytic appearance on blood-agar plates, bile solubility and optochin-sensitivity (13). A polysaccharide based capsule surrounds the cell and the capsule´s composition defines the type, also known as the serotype to which the bacterium belongs (15).

3.1. Pneumococcal diseases

S. pneumoniae is known to cause different diseases classified into two main groups, non-invasive and invasive diseases (15). Figure 1 presents a diagram with the most common known pneumococcal diseases. While the invasive pneumococcal diseases (IPD) are considered the most severe forms, the non-invasive pneumococcal infections are the most commonly observed forms (16). Pneumonia is often classified as non-invasive or invasive, and it is estimated that pneumococcal pneumonia is the foremost reason for pneumococcal death both among children and adults around the world (16–19). This is followed by the severe invasive bacteremia and meningitis which is the main segment of IPD (18,19). The less severe, however painful acute otitis media (AOM) among children is often caused by pneumococcus. It is estimated that more than half of the Danish children experience AOM, which is frequently treated with antibiotics with questionable effect and overuse (20,21). Besides these well-known pneumococcal related diseases, pneumococci are also associated with other common infections such as non-invasive sinusitis, and bronchitis, and invasive diseases such as infection of the heart valves (endocarditis), peritonitis, and septic arthritis (22).

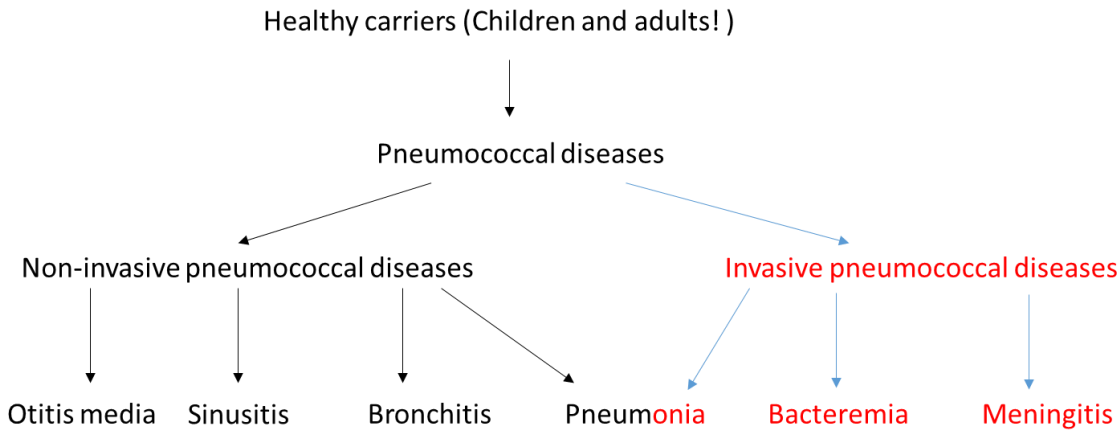


Figure 1. Pneumococcal diseases divided between non-invasive and invasive diseases (in red).

3.2. Pneumococcal history

Since 1875, when *S. pneumoniae* (pneumococci) was first observed by Klebs (13), interest in this species has escalated dramatically. A PubMed search (performed 17 December 2020) on "*Streptococcus pneumoniae*" reveals more than 35,378 published papers, and globally the World Health Organization (WHO) (23) in 2000 estimated that pneumococci were the cause of 14.5 million episodes of invasive disease, while a study estimated that 294,000 children aged 1-59 months died from pneumococci worldwide in 2015 (19).

Defined as one species (15), *S. pneumoniae* consists of at least 100 different serotypes that can be classified by the structure of their polysaccharide capsule (15,24,25). In 1933, the Quellung reaction by Neufeld was introduced as a method to detect the various pneumococcal serotypes (26), and today both phenotypical and molecular methods have been developed (27,28)(Paper A).

Pneumococcal epidemiology is often divided into two stages; a carriage stage preceding the disease stage (29) and the disease stage pre-empting the pneumococcal disease (29,30). The introduction of pneumococcal vaccines has had a considerable impact on the epidemiology of pneumococci (31) (Paper F, Paper G). The first vaccine was developed in 1911 when Wright tested a crude whole-cell vaccine on South African gold miners (32). However, due to the efficacy of antimicrobial agents, interest in pneumococcal vaccines dwindled (32). In 1974, a vaccine was reintroduced as a 14-valent pneumococcal polysaccharide vaccine (PPV), which was replaced in 1983 by the PPV-23 (32). The introduction of a pneumococcal conjugate vaccine (PCV) in 2000, PCV-7, provided infants with effective protection against invasive pneumococcal diseases (IPD) (33,34)(Paper G,

Paper H). New PCV vaccines have since been introduced, the PCV-10 in 2010 followed by the PCV-13 later in 2010 (35). Today, several pneumococcal vaccines covering different proportions of the known pneumococcal serotypes are under development (36). Even so, no pneumococcal vaccine covers all known pneumococcal serotypes (37,38).

Table 1 provides an overview of historical findings related to pneumococci.

Table 1. A selected historical timeline of *S. pneumoniae* from the first descriptions to the introduction of PCV.

Year	Discovery	Reference
1881	Pasteur from France and Sternberg from the US published their findings on a bacterium, which would later be described as *S. pneumoniae* isolates. Both Pasteur and Sternberg isolated the bacterium from carriers.	(12) (22) (13)
1882	Günther and Leyden recovered pneumococci directly from lungs.	(13)
1883	Talamon isolated pneumococci directly from blood.	(13)
1886	Weichselbaum described the role of pneumococcus in pneumonia.	(13)
1900	Neufeld described that pneumococcus was soluble in bile, which was later found to be a species-specific feature that could be used in the process of identifying *S. pneumoniae* isolates.	(39) (13) (22) (Paper A)
1902	Neufeld described the Quellung reaction, which was presented as a method for serotyping pneumococcal isolates in 1931.	(26) (13) (40) (41)
1910	Neufeld and Händel described two different serotypes of pneumococci.	(26) (42)
1910-1913	Serum therapy was described as a treatment of invasive pneumococcal infections.	(43)
1911	The first crude whole-cell pneumococcal vaccine was developed.	(32)

1915	Rochs recommended the use of optochin for differentiating *S. pneumoniae* from other streptococcal species.	(39)
1931	The Quellung reaction was developed as a technique for serotyping of pneumococcal isolates.	(26) (42) (40)
1939	Prince Valdemar died of a pneumococcal infection due to a new serotype, which later was named serotype 9V.	(44) (15)
1940-1945	Sulfonamides and penicillin revolutionized the treatment of pneumococcal infections.	(12) (32)
1950's	Pneumococcal vaccines were removed from the market due to the efficacy of the antimicrobial agents.	(32)
1954 - 1980	The Danish type system versus the American type system. According to Henrichsen it was decided to use the Danish system worldwide at a Food and Drug Administration (FDA) meeting in 1980.	(15) (12) (45)
1977	The 14-valent pneumococcal polysaccharide vaccine (PPV) was introduced.	(32)
1978	Eighty-three different pneumococcal serotypes were listed by Lund.	(46)
1983	The 23-valent PPV replaced the 14-valent PPV.	(32) (15)
2000	The PCV-7 was licensed and introduced in the US.	(15)
2007	Introduction of PCV-7 in Denmark.	(34)
2008	The PCV-10 was licensed.	(15)
2010	Introduction of PCV-13 in Denmark.	Paper G
2020	Reduction in pneumococcal IPD in Denmark due to the Covid-19 caused lockdown on March 13th in Denmark	(47)
2020	Around 100 pneumococcal serotypes have been described	(24)
2020	The PPV-23 is included in the Danish vaccination program for individuals aged 65 year and older and for risk groups	(48)

4. Identification of *Streptococcus pneumoniae*

S. pneumoniae belongs to the Mitis group of the genus *Streptococcus* consisting of up to 20 species (49,50), the majority of which are considered commensal inhabitants, with *S. pneumoniae* being the major exception causing severe infections in humans (49–51).

S. pneumoniae identification is commonly based on the optochin susceptibility test, the bile solubility test, the Omni serum (SSIDiagnostica.dk, Denmark), and the latex agglutination test (SSI Diagnostica, Denmark) as confirming tests (51–53)(Paper A). New identification procedures include phenotypical methods based on spectra analyzing procedures using the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) or various molecule-based methods (49,51)(Paper A).

4.1. Phenotypic identification of *Streptococcus pneumoniae* using the optochin test, bile solubility test and MALDI-TOF

The optochin susceptibility test

Species identification of *S. pneumoniae* by applying a simple optochin disk on the blood agar plate for isolate culturing and observing for optochin susceptibility is easy to perform and therefore the common choice for screening for *S. pneumoniae* at clinical microbiology laboratories (39,54,55). Optochin also known as ethyl hydrocupreine hydrochloride is an antimicrobial chemical, too toxic to be used for human treatment (56). By using the differences in optochin sensitivity within the Mitis group, with *S. pneumoniae* showing the greatest sensitivity, it has been possible to differentiate pneumococci from the other Mitis species based on the isolates optochin sensitivity (39,54,56). With the finding of *S. pseudopneumoniae* in 2004 (54), optochin identification of pneumococci became more complicated, since this species appeared to be optochin sensitive; however, it was observed that *S. pseudopneumoniae* was optochin-sensitive only in ambient atmosphere but resistant in a 5% CO_2 atmosphere (54,57). Other factors have been shown to reduce the specificity of the optochin test: optochin-resistant pneumococci, *S. pseudopneumoniae* not showing clear optochin resistance, and other species within the Mitis group showing inconclusive optochin susceptibility; moreover, it has been observed that culture media and incubation time affect the optochin results (54,55,57). Recently, it was recommended not to use the optochin test as the only diagnostic modality due to a high percentage of optochin-resistant pneumococcal isolates (58) (Paper A).

Bile solubility test

The bile solubility test is a simple and reliable method for identification of *S. pneumoniae* (59,60), although visual interpretation holds a risk of bias (60).

Recently, a new bile solubility test was introduced, replacing manual interpretation with a densitometer measuring the optical density value (OD value) (Paper A). Figure 2 presents OD values from different Mitis group species showing how the species are clustered specifically within different OD value ranges (Paper A). Thus, data with an OD value of 2.1 or above correlated with the identification of *S. pneumoniae*, whereas an OD value in the range from 0.9 to 2.0 correlated with *S. pseudopneumoniae*. An OD value below 0.9 correlated with isolates belonging to either *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus sanguinis*, or *Streptococcus australis*. Further investigation revealed that the bile solubility test is robust with only minor time and person variations of the OD value (Paper A). On some occasions, other species were found to be bile soluble showing OD values normally not characteristic for the specific species (Paper A). Three *S. mitis* isolates showed a higher OD value (range from 1.3 to 2.7) than the OD threshold calculated for that species. The explanation may be the possession of the *lytA* gene (encoding autolysin), indicating bile solubility previously reported in other Mitis group streptococci including *S. pseudopneumoniae* and *S. mitis* (54,61). All three *S. mitis* isolates in Figure 2 contained the *lytA* gene (Paper A). Although data are currently very limited, an unusually high OD value observed for an *S. mitis* strain suggests the presence of the *lytA* gene.

The bile solubility test described in paper A, is - to the author´s belief - the first described bile solubility test, which can be performed without human subjective interpretation. The method is used as part of the NSRlab procedure for correct identification of the Mitis group, in which the obtained OD-value is a part of the species identification (Figure 2).

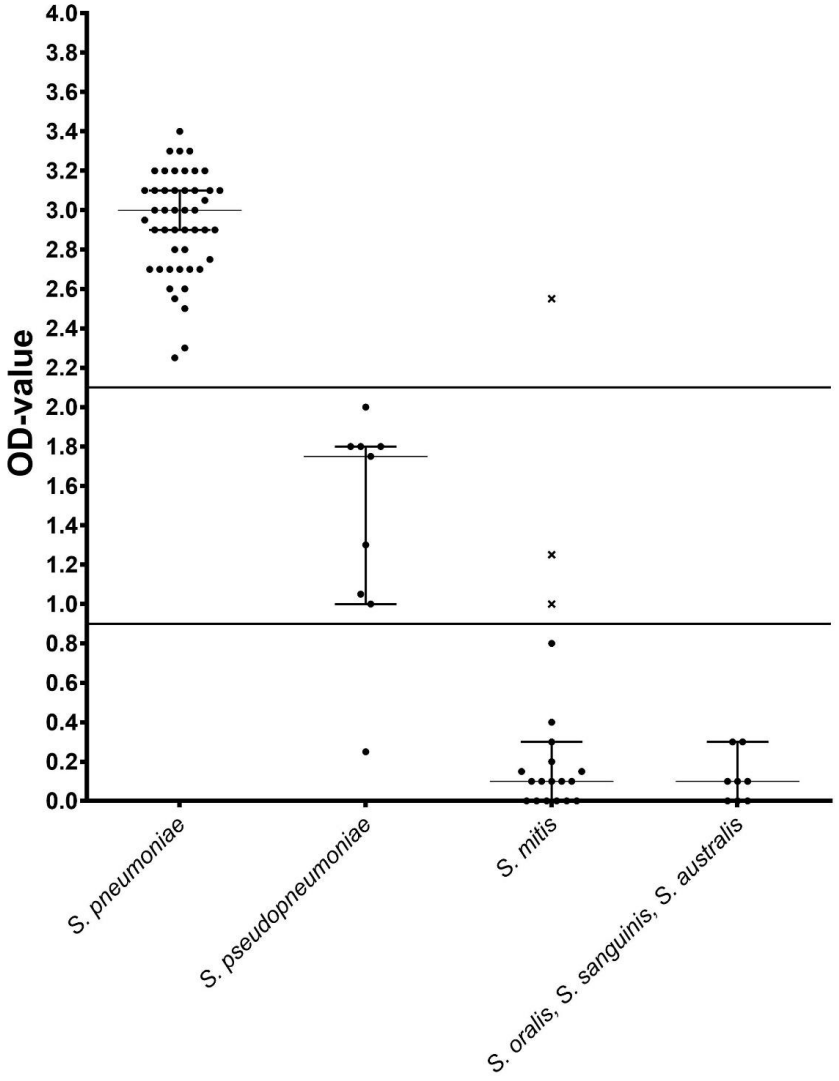


Figure 2. The OD values (median and 95% CI) for six *Streptococcus* species. Lines indicate the calculated cut-off OD values at 2.1 and 0.9. The three *S. mitis* strains containing the autolysin (*lytA*) gene are indicated by an 'x' (copy of Figure 1 from Paper A. For permission see https://creativecommons.org/licenses/by/4.0/).

Matrix-Assisted Laser Desorption Ionization–Time Of Flight Mass Spectrometry (MALDI-TOF MS)

Since 2010, the MALDI-TOF MS has been used extensively in clinical microbiology laboratories for bacterial species identification (62). However, the use of MALDI-TOF for correct species identification of Viridans group streptococci (VGS) has proven to be a challenge (63,64) although addition of VGS reference data to the MALDI-TOF database will reduce misidentification (63,65,66).

Paper A showed that species identification using MALDI-TOF score value data could only be reliable if all the top ten species with a score value above one were of the same species. Misidentification was observed if different species appeared within the top ten scored species (Paper A). By including visual evaluation of the MALDI-TOF spectra, it was possible to correctly identify both *S. pneumoniae* and *S. pseudopneumoniae* based on evaluation of the seven peaks described by Werno et al (2012) (Table 2). False positive identifications were observed as three isolates of *S. mitis* showed peaks characteristic of *S. pneumoniae*; however, all three isolates were bile soluble. No isolates showed false positive identifications as *S. pseudopneumoniae*, and we observed no false negative identifications when using peak evaluation, neither for *S. pneumoniae* nor for *S. pseudopneumoniae* (Paper A).

Table 2. MALDI-TOF profiles based on visual evaluation of the seven peaks described by Werno et al (67).

Defined species	**m/z value**						
	2,625	2,911	2,937.5	5,253	5,824	5,877	6,955
S. pneumoniae	0	0	+	0	0	+	0
S. pseudopneumoniae	+	0	+	+	0	+	0
S. mitis	(+)	(+)	(+)	0	(+)	(+)	+
S. oralis	0	(+)	0	0	(+)	0	+

+: only strains with peaks were detected; (+): strains both with and without peaks were detected; 0: no strains with peaks were detected.

Combining the bile solubility test and MALDI-TOF

A combination of data from the bile solubility test and the MALDI-TOF test improved species identification. On this basis an optimized flowchart was introduced to distinguishi *S. pneumoniae*

from *S. pseudopneumoniae* (Figure 3) with the limitation, however, that identification of other Mitis group species is not possible (Paper A).

This unique use of the bile solubility test combined with MALDI-TOF species ID based on peak-evaluation, has provided the National Streptococcal Reference laboratory (NSRlab) with a quick, simple, and highly reliable procedure for correct species ID within the Mitis group. This is a procedure not described in any other settings.

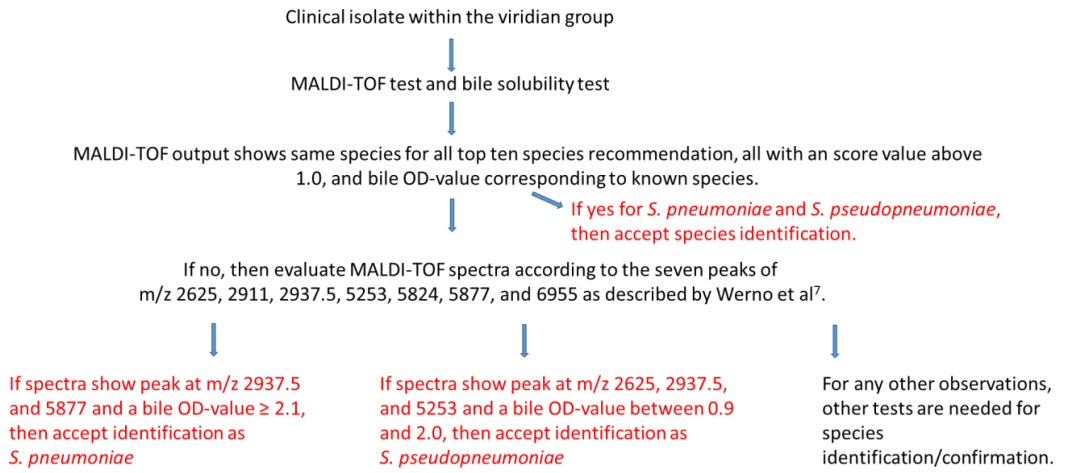


Figure 3. Flowchart identification of *S. pneumoniae* and *S. pseudopneumoniae* using a combination of the MALDI-TOF and the bile solubility test (copy of Figure 2 from Paper A, for permission see https://creativecommons.org/licenses/by/4.0/).

4.2. Molecular identification of *Streptococcus pneumoniae* from the Mitis group

In Paper B and C we tested the presence of several genes used in various molecular identification procedures, including the presence of the genes *lytA* and *ply*, identification of a specific nucleotide position in the 16s rRNA sequence, and the multilocus sequence analysis (MLSA) housekeeping genes (49,68,69). The presence/absence of a gene was based on a cut-off of 80% coverage and 95% identity (25,70).

<u>Identification based on the lytA and ply genes</u>

A real-time polymerase chain reaction (PCR) assay targeting the virulence gene *lytA* (71)(**www.cdc.gov/streplab/protocols.html**, accessed 07 October 2020) is considered the main

procedure for species identification of *S. pneumoniae* in culture-independent samples (59,61,69). The *ply* gene has also been used for rapid species identification of *S. pneumoniae* (71,72). In paper B and C, the presence of *lytA* and *ply* sequences were observed in all tested isolates, thus supporting *S. pneumoniae* identification by the *lytA* and *ply* gene. However, as shown in Paper A, both the *lytA* and the *ply* gene can be detected in various Mitis group species; thus, species identification based on these genes should be assessed critically (58,61,68,73) (Paper A). The recommendation to use multiple tests for species identification also applies for single gene tests (61,73,74).

16s rRNA and identification of cytosine at position 203

16S rRNA gene sequencing has been widely used for species identification of bacteria (75). However, it has also been observed that species within the Mitis group are closely related, with 16s rRNA homology being close to 100% for several species, which should make it impossible to use 16s rRNA for species identification (68,75). Even so, in the study by Scholz et al (68), the authors were able to differentiate *S. pneumoniae* from the other species within the Mitis group, as cytosine was observed only in pneumococcal isolates at position 203 in the 16S rRNA gene. Adapting the procedure for the location of cytosine at position 203, 48 isolates were tested (Paper C) using the 16S rRNA sequence (GenBank: AY485600) identified by Arbique et al (54). In this 16S rRNA sequence, position 189 corresponds with nucleotide position 203 described by Scholz et al (68). Figure 4 shows examples of test results from different Mitis group species, illustrating the presence or absence of cytosine at position 203. Both in paper B and C, the identification of cytosine at position 203 proved to be a simple identification procedure for *S. pneumoniae*, particularly because the identification is based on evaluation of one sequence only. Thus, the data in paper B and C provided a 100% correct identification; yet with the limitation that it is only possible to identify *S. pneumoniae*. The identification of cytosine at position 203 in the 16S rRNA sequence has proven effective in the NSRlab, as a tool to rapidly screen correct species identification of a large number of pneumococcal WGS data, thereby providing a reliable pneumococcal species confirmation (personal experience).

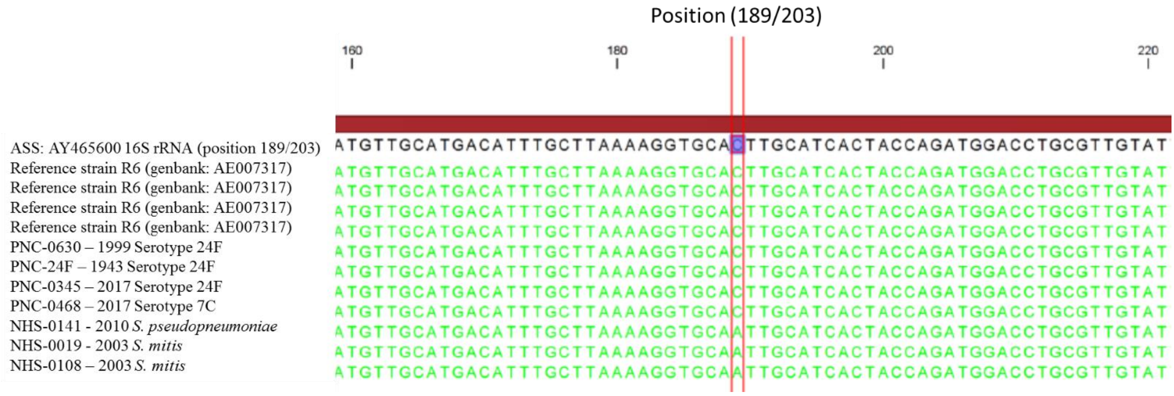


Figure 4. 16S rRNA identification of cytosine at position 203 using the 16S rRNA gene (AY465600). Position 189 in the 16S rRNA sequence for AY465600 corresponded with position 203 described by Scholz et al (68). The isolates were selected as examples for species differentiation.

MultiLocus Sequence Analysis (MLSA) identification based on housekeeping genes

MLSA is based on identification of housekeeping genes (76), with today's standard MLSA test using seven genes, *map*, *pfl*, *ppaC*, *pyk*, *rpoB*, *sodA*, and *tuf*, as described by Bishop et al. (76) and Kilian et al. (77). These genes have been compared with other housekeeping genes such as the seven MLST genes; and although comparison of clustering trees revealed considerable similarity, the MLSA genes demonstrated superior species differentiation (76). The addition of further housekeeping genes did not improve the tree clustering for species identification within the Viridans group streptococci (76) (Figure 5). Seven housekeeping genes, *map, pfl, ppaC, pyk, rpoB, sodA*, and *tuf* were blasted against the pneumococcal isolates in paper C, showing identification above 99% for all seven genes. Investigation of another two housekeeping genes (*ddl*, *gdh*) (77) showed 97% identification for the *gdh* gene for all isolates, whereas the *ddl* gene showed >99% identification for the majority of isolates and identified just above 94% for four of the isolates. Table 3 shows an example of MLSA test results from seven different isolates and the R6 reference strain (GenBank: AE007317). The MLSA test using seven genes (76) was found to provide a good species identification of pneumococcal isolates (Paper C). Ribosomal Multilocus Sequence Typing (rMLST) based on 53 genes encoding the bacterial ribosome protein subunits (*rps* genes) (78), and single nucleotide polymorphisms (SNP's) analysis of the core genome (79) are also used for species

identification. However, as presented in figure 5, no major differences were observed in the tree clustering from using seven MLSA genes versus additional genes/whole gene sequence.

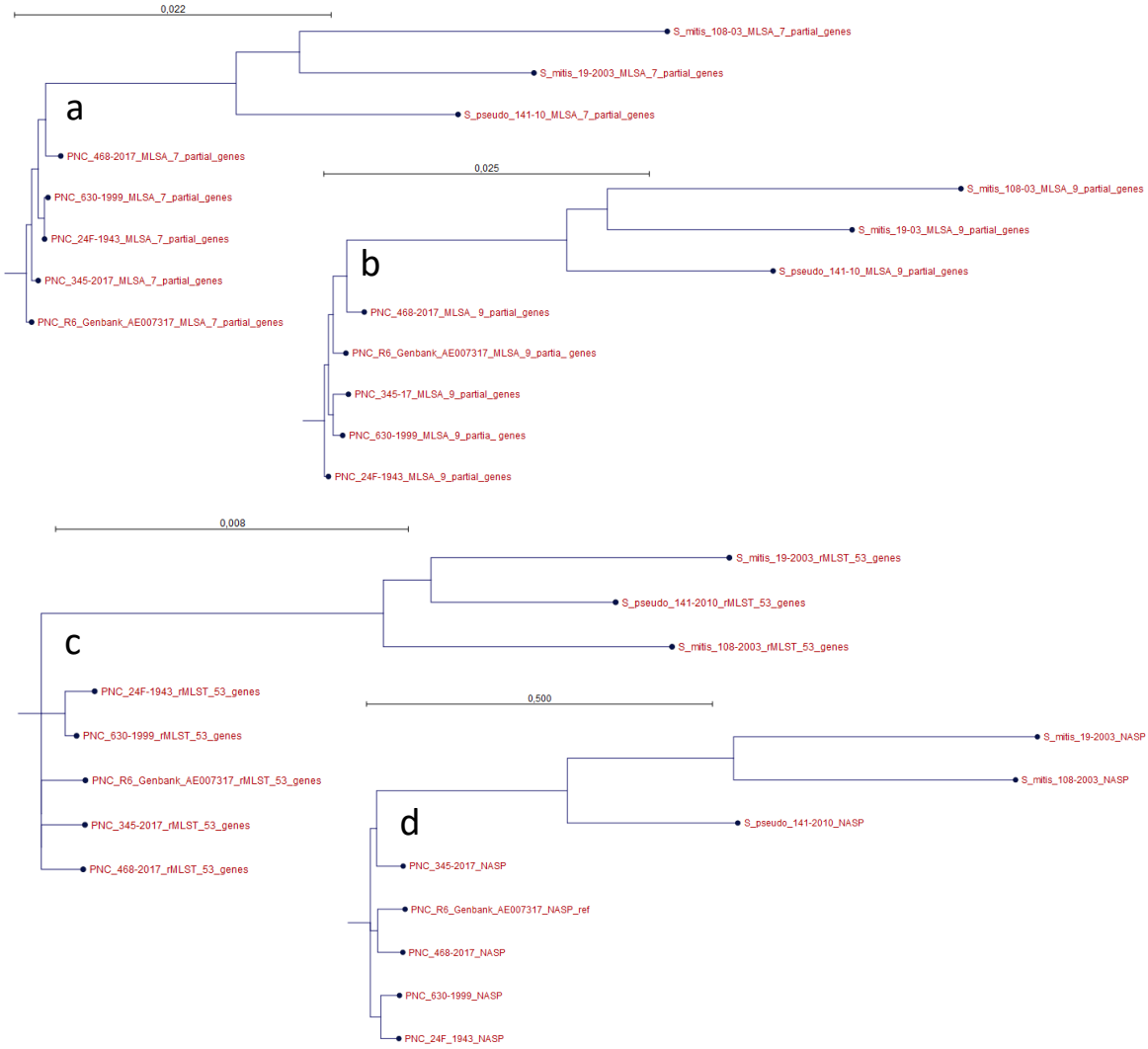


Figure 5. Comparing phylogenetic trees based on seven different isolates and the R6 reference strain (GenBank: AE007317). Four different phylogenetic trees were created using seven housekeeping genes (partial sequences from *S. parasanguinis* strain SK264) (a) (76), two additional housekeeping genes (partial MLST sequences from TIGR4 (GenBank: AE005672)) (b) (77),

rMLST based on 53 genes encoding the *rps* genes (c) (78), and SNP's analysis of the core genome (d) (79).

Table 3. MLSA blasting results of nine housekeeping genes (76,77). Each of the nine used housekeeping genes (complete sequence) are from *S. pneumoniae* TIGR4 (GenBank: AE005672). The grey area represents genes with an overlap or identity lower than 95%. The isolates were selected as examples of using MLSA for species differentiation.

Species origin	**Strain number**	**HOUSEKEEPING GENES (Overlap % - Identity %)**								
		Two additional housekeeping genes (77)		Seven housekeeping genes listed by (76).						
		Ddl	***gdhA***	***map***	***pfl***	***ppaC***	***pyk*F**	***rpoB***	***sodA***	***tuf***
S. pneumoniae	R6 (GenBank: AE007317)	99.8% - 99.4%	100% - 99.9%	100% - 100%	100% - 99.4%	99.8% - 99.4%	100% - 99.8%	99.9% - 99.42%	100% - 99.8%	99.8% - 100%
S. pneumoniae	0468-2017	100% - 99.4%	100% - 100%	100% - 99.8%	100% - 99.6%	100% - 99.6	100% - 99.7%	99.9% - 99.8%	99.7% - 99.8%	100% - 100%
S. pneumoniae	345-2017	100% - 99.3%	100% - 99.0%	100% - 100%	100% - 99.8%	99.8% - 99.8%	100% - 99.8%	100% - 99.5%	100% - 99.8%	100% - 99.9%
S. pneumoniae	24F-1943	99.8% - 99.0%	100% - 99.3%	99.8% - 99.8%	100% - 99.9%	99.8% - 99.9%	99.9% - 99.8%	99.9% - 99.5%	100% - 100%	99.8% - 99.9%
S. pneumoniae	1999-0630	100% - 99.0%	99.9% - 99.0%	99.8% - 99.8%	99.9% - 99.9%	99.8% - 100%	99.9% - 99.9%	100% - 99.4%	99.7% - 100%	99.8% - 99.9%
S. pseudo-pneumoniae	141-2010	100% - 93%	99.9% - 95.5%	99.8% - 94.2%	99.9% - 96.8%	99.8% - 95.6%	100% - 97.0%	99.9% - 96.1%	99.7% - 99.8%	99.8% - 99.3%
S. mitis	0019-2003	100% - 91%	99.9% - 94.6%	100% - 94.2%	100% - 96.5%	100% - 95.9%	100% - 94.1%	100% - 96.2%	100% - 95.9%	100% - 99.1%
S. mitis	0108-2003	100% - 92.5%	100% - 93.9%	99.8% - 93.7%	99.9% - 96.2%	100% - 95.4%	100% - 92.7%	99.9% - 96.2%	100% - 97.19%	100% - 97.3%

4.3. Typing of *Streptococcus pneumoniae*

Around 100 different pneumococcal serotypes have been described based on their capsular structure (24,80). The Neufeld method, also known as the Quellung or capsular reaction test, was the first method to be used for identification of serotypes and is considered a main reference method even today (28,40,59)(Paper D). However, because the Neufeld method is considered labor-intensive, alternative tests have been introduced (27)(Paper D).

Phenotypical methods, including agglutination-based procedures, multiplex immunoassays, and MALDI-TOF spectra have been tested as alternatives to the Neufeld method with various degrees of success (27,28,81–84)(Paper D).

Also, various molecular methods such as PCR and WGS for identification of capsular genes have been developed (25,27,85,86), and today, standard procedures for detection of genotypes can be

found on the homepage of the Centers for Disease Control (CDC) (**https://www.cdc.gov/streplab/pcr.html**, accessed 26 December 2020).

4.4. Phenotypical identification of *Streptococcus pneumoniae* serotypes

In 2004, a new latex agglutination test was introduced (Paper D) based on in-house latex agglutination tests (87) and the established chessboard pool system (Pneumotest) for Neufeld serotyping of pneumococci (88)(Paper D, **www.ssidiagnostica.dk/produkt/latex-kits/?cid=5**, accessed 18-01-2021). The Pneumotest-Latex kit consists of 14 different pooled pneumococcal antisera coated on latex particles (pools A to I and pools P to T) (Figure 6a), and a positive reaction (Figure 6b) was defined as an agglutination within 5 to 10 seconds. The latex agglutination test is used in combination with broth culture; and therefore four different products were tested with the commonly used Todd-Hewitt broth (TH broth) for pneumococcal culturing (89,90)(Paper D and E), and the enriched serum broth (SSIDiagnostica.dk, Denmark), which is a standard broth for pneumococcal culturing at the Statens Serum Institut (SSI) (91)(Paper D and E). All tested broth media were found operable (Paper E).

When testing the Pneumotest-Latex kit on 352 isolates representing 90 different serotypes (known serotypes in 2004), 336 isolates (95.5%, confidence interval 95% (CI) 93-97%) were typed/grouped correctly (Paper D). As expected, test results for species like *S. oralis* and *S. mitis* isolates showed cross-reactions (68,70)(Paper A, Paper D).

Since then, the Pneumotest-Latex test has been expanded to include latex tests for serotyping and serogrouping (SSIDiagnostica.dk, Denmark), and the detection of multiple serotypes from carriage samples has been improved (91,92)(Paper K). Although the latex test was developed only for confirmed pneumococcal isolates (Paper D), it has proven to be valuable for detection of pneumococci directly in clinical samples (93,94).

The development of the Latex agglutination test (Paper D), has proven to be a successful test kit for serotype identification and is sold worldwide (**www.ssidiagnostica.dk/produkt/latex-kits/?cid=5**, accessed 18-01-2021).

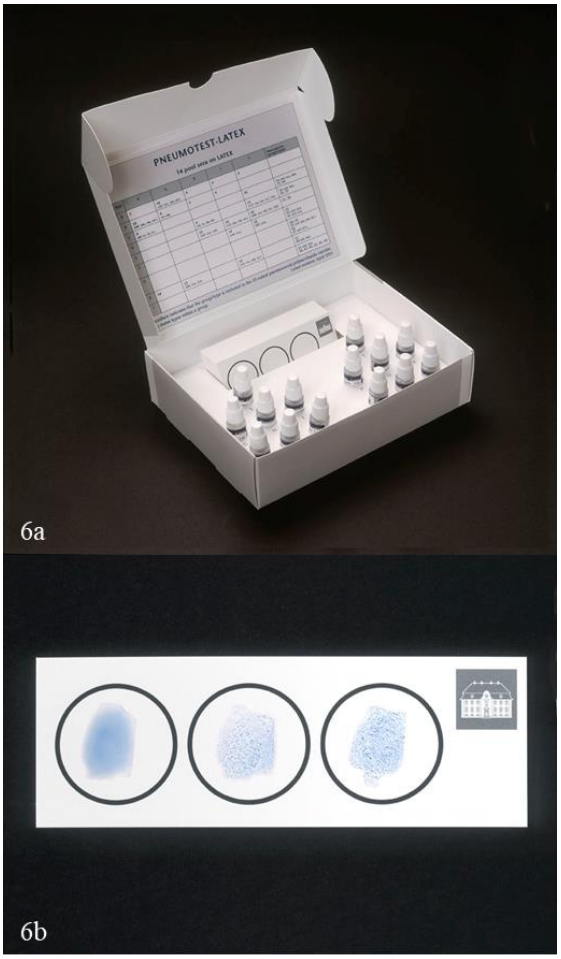


Figure 6. 6a. The commercial version of the Pneumotest-Latex kit consisting of 14 different pooled pneumococcal antisera. 6b. One negative reaction and two positive agglutination reactions.

4.5. Capsular gene identification in *Streptococcus pneumoniae*

Identification of capsular polysaccharide genes (CPS genes) is performed with either PCR or WGS (25,86,95–99). For PCR, protocols from the CDC streptococcal laboratory (25,82)(**https://www.cdc.gov/streplab/pcr.html**, accessed 26 December 2020) are generally used. Two types of PCR protocols are described, a real-time PCR (detects 21 pneumococcal serotypes or serogroups) and a conventional PCR detecting 40 pneumococcal serotypes or serogroups (96,97). The disadvantage of the PCR methods is their limitations as to the number of different serotypes that can be detected (86,96,97).

An estimated 100 CPS sequences have been described, thereby making WGS applicable for genotyping (24,25,85,95,98,100). In paper B 96 phenotypical confirmed serotype 8 isolates were genotyped using the PnemoCaT platform (25) and in all cases the genotype corresponded with the phenotype. However, for some serotypes such as serogroup 24 the identification of the genotype is difficult (25). In paper C sequences from 48 phenotypically confirmed isolates were blasted against 92 CPS genes and this identified 28 isolates as genotype 24F, while 20 isolates were identified as belonging to genogroup 24 (Paper C). Identification of the correct genotype by WGS is a well-known problem (25,101), as the method occasionally picks up new genotypes, which may only later be described phenotypically or may correspond to existing phenotypic serotypes despite being genetically different (24,25,28,102).

4.6. The Danish (local and centralized clinical laboratories) procedure for identification, typing and monitoring of *Streptococcus pneumoniae* isolates

All Departments of Clinical Microbiology (DCMs) and nearly all hospitals in Denmark are public, and all microbiological analyses of human primary specimens are conducted at DCMs (103).
Prior to 2007 it was voluntary to submit invasive pneumococcal isolates to the NSRlab, Statens Serum Institut (SSI). Still, it was estimated that 90-95% of IPD cases were registered in the national laboratory system before 2007, however the older the data, the less documented the quality of the data (34).
With the introduction of PCV-7 in 2007 followed an executive order (BEK nr 1102 af 20/09/2007), rendering it mandatory to submit all invasive pneumococcal isolates to the NSRlab.
The procedure for a DCM after detection of an invasive pneumococcal isolate is to send the isolate to the NSRlab, and the laboratory report of the pneumococcal case is simultaneously recorded in the Danish Microbiology Database (MiBa), a national database registrating and storing all laboratory reports from Danish DCMs (104). The NSRlab checks whether the number of received isolates corresponds to the recorded pneumococcal cases in MiBa. If discrepancies are observed, the respective DCMs are contacted. Based on this system it is estimated that the NSRlab receives all viable invasive pneumococcal isolates in Denmark, and if there is no viable isolate the NSRlab obtains the information from MiBa.

4.7. The National Neisseria and Streptococcus Reference laboratory (NSRlab) capacity

The NSRlab is a laboratory which has the capacity to perform several different techniques for identification of pneumococcal isolates. The routine procedure for pneumococcal isolates is to confirm the species identification with opthocin test and by the use of different antisera tests (ImmuLex™ *S. pneumoniae* Omni or Omni antiserum, SSIDiagnostica.dk, Denmark). Serotype identification is considered as an extra species confirmation. If there is the slightest uncertainties of the species ID, additional tests are performed using the bile solubility test and MALDI_TOF (28)(Paper A). Serotype identification is based on the Pneumotest Latex kit and Neufeldt test (SSIDiagnostica.dk, Denmark)(28)(Paper B, C, D).

For samples with non-viable pneumococcal isolates identification and serotyping is performed by PCR as described by CDC (**www.cdc.gov/streplab/protocols.html**, accessed 07 October 2020) (71,72,105). Recently, whole-genome sequencing (WGS) has been introduced in the laboratory to confirm the pneumococcal species identification, to perform genotyping and identify clonality (Paper B and C). WGS is expected to be the primary method for pneumococcal isolates in 2021, using phenotypical based methods only as additional test for confirmation of WGS results when needed.

5. The epidemiology of pneumococci

Invasive pneumococcal infections data collected since 1938 have been presented in studies describing the Danish epidemiology over many decades (31,34)(Paper G, H, I), and worldwide pneumococcal epidemiology can be followed at the homepage (**http://www.view-hub.org/**, accessed on 06 October 2020), where studies present continually updated data on vaccination, carriage, and IPD from the numerous countries.

After several decades of limited interest in pneumococcal diseases rooted in the belief that pneumococcal diseases were completely curable, a study by Robert Austrian and Jerome Gold on mortality related to pneumococci in 1964 detected a mortality rate between 17% and 25% of the adult population despite penicillin treatment, changing the notion that antimicrobials constituted the sole mode of treatment of pneumococcal diseases (32,106). This study combined with increasing antimicrobial resistance triggered renewed interest in pneumococcal vaccine and epidemiology (32,106–108).

The pneumococcal epidemiology is divided into two phases (29)(Paper K): Phase 1, the carriage stage in which pneumococci are part of the human commensal nasopharyngeal microbiota and

cause no harm; and phase 2, the disease-causing stage instigating both non-invasive and invasive diseases. The phase 1 stage, "carriage", is regarded a prerequisite for a pneumococcal infection, and children have generally been considered the main carriers and transmitters of pneumococcal disease to other age groups (29,30,109,110).

Vaccine	Year introduced in Denmark	Serotypes contained in vaccines
Pneumococcal conjugated vaccine (PCV)		
PCV-7 (Prevnar7, Pfizer)	2007	4, 6B, 9V, 14, 18C, 19F, and 23F
PCV-13 (Prevnar13, Pfizer)	2010	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F
Pneumococcal polysaccharide-based vaccine (PPV)		
PPV-23 (Pneumovax23, Merck)	1983	1, 2, 3, 4, 5, 6B, 7F, 8, 9V, 9N, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F

Table 4. Pneumococcal vaccines used in Denmark. PCV-13 replaced PCV-7 in April 2010 (31). PCV-13 is licensed for all age groups (111). PPV-23 can be used in 2+ age groups (**https://www.ssi.dk/vaccinationer/vaccineleksikon/p/pneumokokvaccine-23-valent**, accessed 06 October 2020).

In Denmark, PCV-7 (Prevnar 7, PCV-7, Pfizer Vaccines) (Table 4) was introduced in October 2007 into the childhood immunization program. The vaccine is administered at the ages of 3, 5, and 12 months (also known as the 2 + 1 schedule), and a catch-up program for children born after April 2006 has been introduced. In 2010, PCV-7 was replaced by PCV-13 (Pfizer Vaccines) (Table 4) (31). Besides the conjugate vaccines, a pneumococcal polysaccharide-based vaccine (PPV-23) containing 23 serotypes (Table 4) is available, although it does not work well in children younger than 2 years of age.

Systematic PCV vaccination data have been available for the PCV-vaccinated children < 2 years of age since the introduction of the PCV in the child immunization program in Denmark (31)(Paper G), whereas pneumococcal vaccination data in adults (> 2 years of age) became systematically available as from November 2015 (112).

The systematic registration of children PCV vaccination together with the high uptake of PCV within this age group (**https://statistik.ssi.dk/**, accessed 29 October 2020), have made it possible to examine and describe the PCV efficacy in the Danish population in details (31)(Paper F, G, H, I, J). Because registration of PPV-23 vaccination first started in 2015 and consequent lack of PPV-23

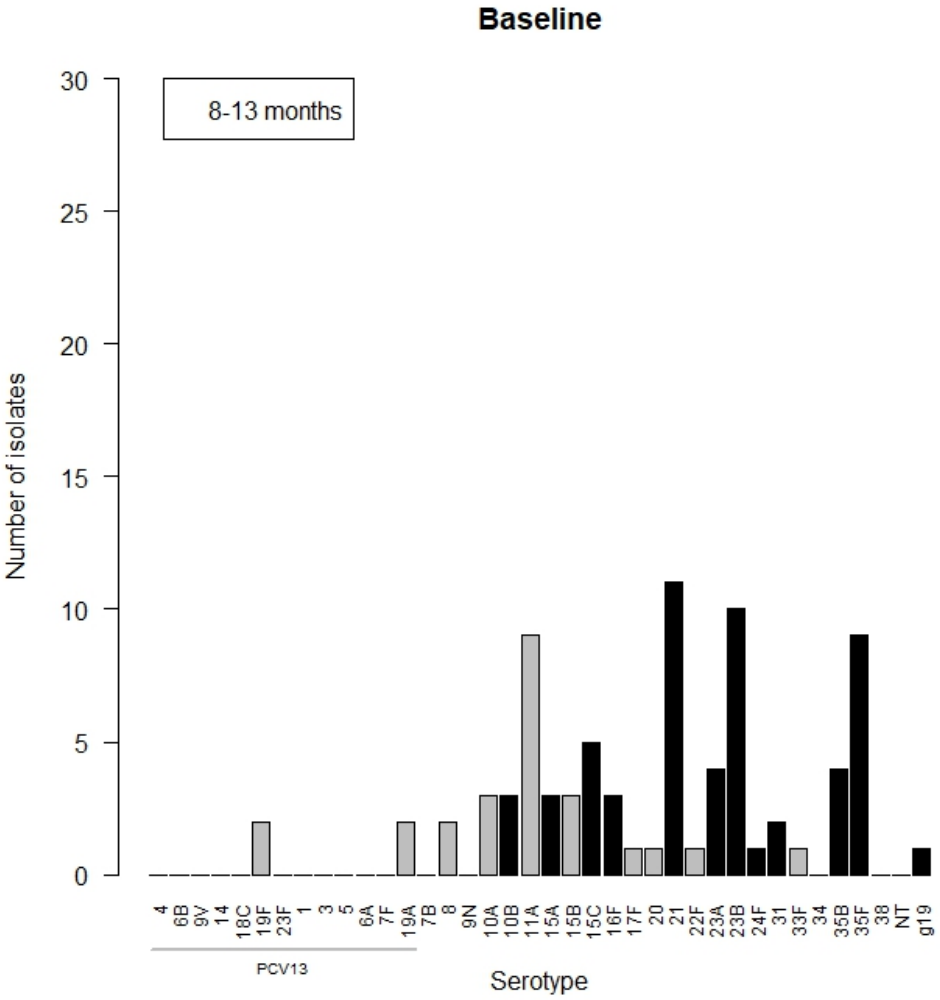
uptake, it is not possible to measure the effect from PPV-23 in the Danish population and thereby perform studies evaluating the PPV-23 effect in Denmark (Paper G).
The PPV-23 vaccination efficacy in adults against IPD has shown to be significant, although the protection is of limited duration (113); several older studies have shown an effectiveness against IPD around 60% (114), whereas a study from 2018 on PPV-23 vaccination of the 64+ age group showed that protection against pneumococcal IPD was only 43% after two years and 23% after five years of PPV-23 vaccination (113). However, general reviews analyzing the vaccine efficacy of both PPV-23 and PCV-13 on IPD, showed that both vaccines protect against IPD, although there are no head-to-head studies comparing vaccine effectiveness of PPV23 and PCV13 (16–18).

5.1. The epidemiology of carriage in Denmark

In 2007, a pneumococcal carriage study was performed before the introduction of PCV-7 in Denmark, showing a carriage rate of 69% in children aged 12 to 23 months attending daycare services, declining with the age of the children. 57% of the carried isolates consisted of serotypes included in PCV-13 (91,115)(Paper K).
In 2014/2015, the first post-PCV carriage study was performed in Denmark on children aged 8-19 months (Figure 7) (Paper F). Nasopharyngeal swab samples were collected from children before (8-13 months) and after (14-19 months) they started in daycare, as attending daycare is considered an important risk factor for increasing the carriage rate in children (115–117). The study showed a carriage rate of 26.0% before attending daycare, and an increase to 67.4% six months after starting to attend daycare. Comparison of the carriage rate from Paper F with the Danish pre-PCV carriage study (91,115) showed no change in the overall carriage rate in children, despite PCV vaccination, as also observed in other countries (118,119). However, whereas the pre-PCV carriage study included 57% PCV-13 included serotypes (115), the post PCV carriage study included only 3% PCV-13 serotypes (Paper F), clearly demonstrating a shift in the serotype distribution (Figure 7). This also explains why the carriage in children < 2 years of age has not been reduced following the introduction of PCV, since a serotype replacement has appeared with a huge increase in non-PCV included serotypes (Paper B, C, F, G).
The serotypes carried in children aged 8-19 months (Figure 7) were observed to cause IPD in children aged 0-4 years in the 2014-2016 period (Figures 8), whereas the main serotypes causing IPD in the elderly (serotypes 8, 3, 7F, 22F, 9N, and 12F) (Figure 8) were not carried or carried only with a low prevalence in the children (Figure 7) (Paper F). Serotypes 3 and 8, which are dominant

causes of IPD in the elderly both in Denmark and other European countries (120,121)(Paper B, G), were only detected in two children (serotype 8) (Figure 7). Similar findings were observed in a UK study, where the authors observed minor carriage of serotypes 3 and 8 in children < 5 years of age (122). In the study by Adler et al (123), healthy adults were found to be carriers with serotype 3 as the dominant serotype, but other serotypes were also found (123). Thus, our carriage study revealed that children aged 8-19 months were not the main transmitters of pneumococci causing IPD in the elderly in Denmark, which is similar to the observation from Israel, where they found that serotype patterns in carriage in older children (aged 24 – 59 months of age) better correlated with IPD in adults compared to the infants (aged less than 24 months) (124).

Since the presentation of the hypothesis (Paper K) stating that other age groups than children below 5 years of age, are carriers of pneumococci and can transmit, several studies have been published suggesting transmission from other age groups as an explanation for the limitation of herd protection from PCV vaccinations of infants (123,125,126)


Baseline
8-13 months
Number of isolates
30
25
20
15
10
5
0
4
6B
9V
14
18C
19F
23F
1
3
5
6A
7F
19A
7B
8
9N
10A
10B
11A
15A
15B
15C
16F
17F
20
21
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23B
24F
31
33F
34
35B
35F
38
NT
g19
PCV13
Serotype

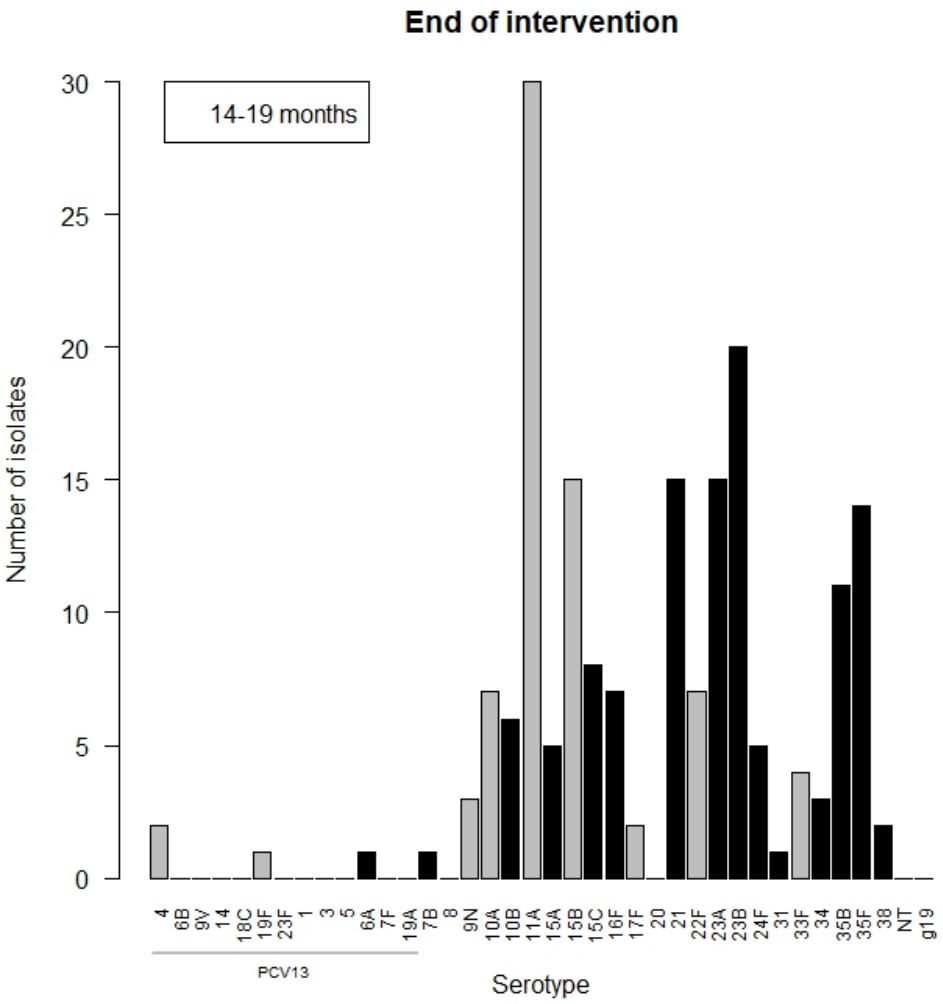
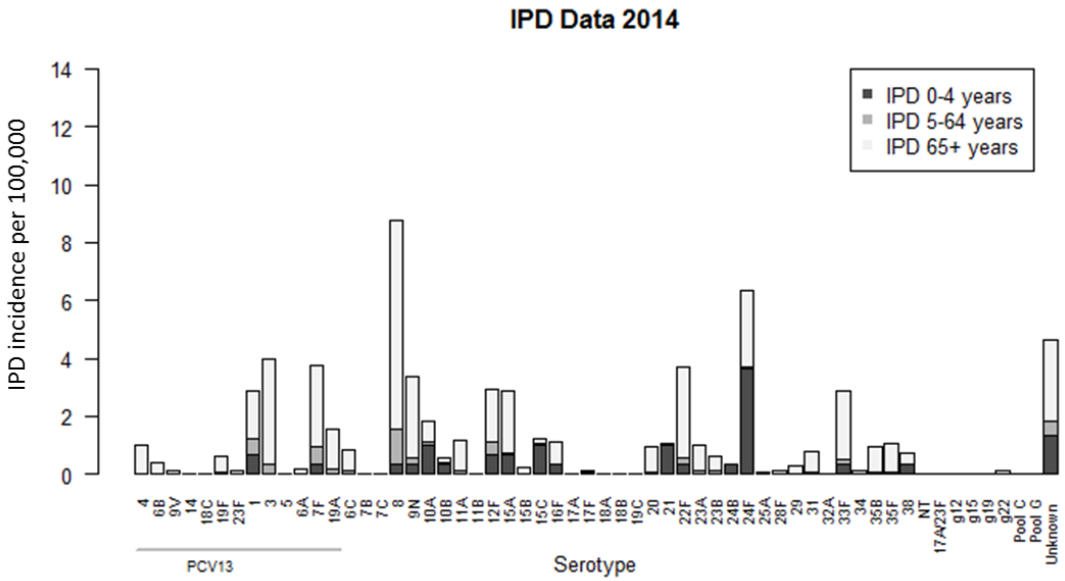
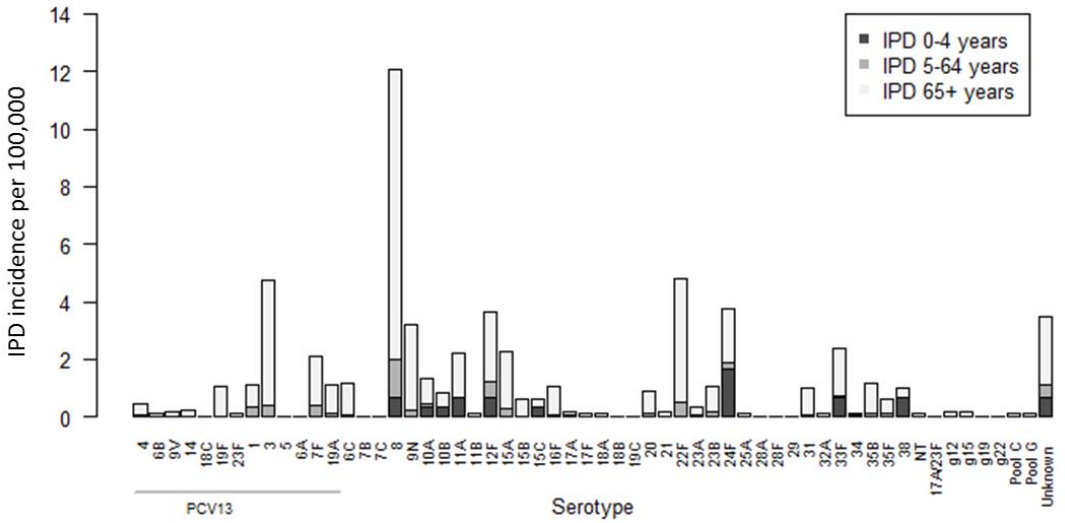


Figure 7. Carriage study and serotype distribution in Danish children at baseline and at the end of the intervention. Grey color indicates PPV23 serotypes, black indicates non-PPV23 serotypes. Serotypes are listed with PCV7-serotypes first, then PCV13 serotypes followed by non-PCV13 serotypes (copy of Figure 1 from Paper F, also see https://creativecommons.org/licenses/by/4.0/).


IPD Data 2014
IPD incidence per 100,000
0
2
4
6
8
10
12
14
IPD 0-4 years
IPD 5-64 years
IPD 65+ years
4 6B 9V 14 18C 19F 23F 1 3 5 6A 7F 19A 6C 7B 7C 8 9N 10A 10B 11A 11B 12F 15A 15B 15C 16F 17A 17F 18A 18B 19C 20 21 22F 23A 23B 24B 24F 25A 28F 29 31 32A 33F 34 35B 35F 38 NT 17A/23F g12 g15 g19 g22 Pool C Pool G Unknown
PCV13
Serotype


IPD Data 2015
IPD incidence per 100,000
0
2
4
6
8
10
12
14
IPD 0-4 years
IPD 5-64 years
IPD 65+ years
4 6B 9V 14 18C 19F 23F 1 3 5 6A 7F 19A 6C 7B 7C 8 9N 10A 10B 11A 11B 12F 15A 15B 15C 16F 17A 17F 18A 18B 19C 20 21 22F 23A 23B 24F 25A 28A 28F 29 31 32A 33F 34 35B 35F 38 NT 17A/23F g12 g15 g19 g22 Pool C Pool G Unknown
PCV13
Serotype

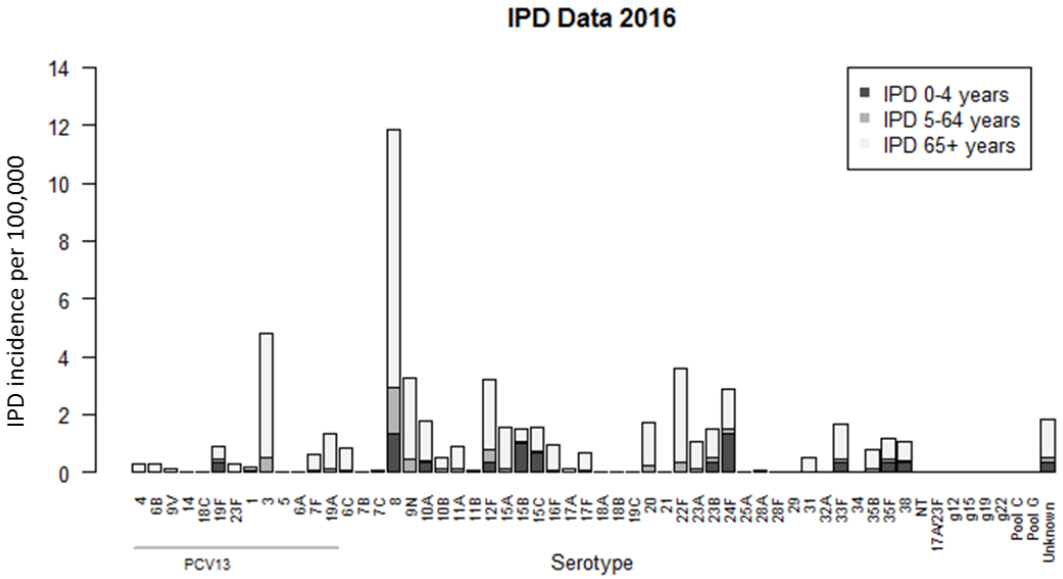


Figure 8. Serotypes found in patients with IPD aged 0-4 years, 5-64 years, and 65+ years. The figures show IPD incidences per 100,000. Serotypes are mentioned with PCV7 serotypes first, then PCV13 serotypes followed by non-PCV13 serotypes (copy of Figure 1 from Paper F, also see https://creativecommons.org/licenses/by/4.0/).

5.2. The epidemiology of invasive pneumococcal disease in Denmark

The definition of a patient with IPD is detection of a culture-positive sample of *S. pneumoniae* in cerebrospinal fluid (CSF), blood, or other normally sterile sites (34).

Before the PCV-7 introduction, the Danish IPD incidence was approximately 16 cases per 100,000 for all age groups, with the majority of cases observed in children and the elderly (Figure 9). With the introduction of PCV-7 and PCV-13 into the childhood immunization program, a sustained reduction in IPD cases was observed for vaccinated children (Figure 9). Additionally, a reduction in the elderly was observed due to herd protection (31,127)(Paper G) (Figure 9).

While the effect of PCV introduction in Denmark on IPD in both vaccinated children < 2 years and children/adults > 2 years has been described in detail, showing changes in the individual PCV-13 serotype-related IPD cases (31,34), the included studies (Papers B, C, G, H, I, K) show the effect of PCV introduction on IPD cases due to non-PCV-13 serotypes, the effect from serotype replacement on IPD in all age groups, and IPD cases in non-vaccinated age groups.

Danish infants (0-90 days) rely on herd protection from vaccinated children > 90 days (128,129)(Paper J). In the study on unvaccinated Danish infants (Paper J), the serotypes causing IPD are common serotypes causing IPD in other age groups in European countries (130)(Paper I, J). It is not possible to ascertain an effect of herd protection in the overall IPD incidence in Danish infants (0-90 days) from 2000 and onwards because of the low number of IPD cases per year (Figure 8) (Paper J). In a large study from the USA, a 30% reduction of IPD cases in unvaccinated infants (0-60 days) was observed, which was most likely due to herd protection from PCV-13 vaccination (129). The serotypes causing IPD in Danish infants were mainly those included in PCV-13, while the serotypes in PCV-7 disappeared from 2010 and onwards (Paper J). In the American study, they still observed IPD cases due to PCV-7 serotypes (129). As observed in the US study, we also found non-PCV serotypes causing IPD in this age group (Figure 10) (129).

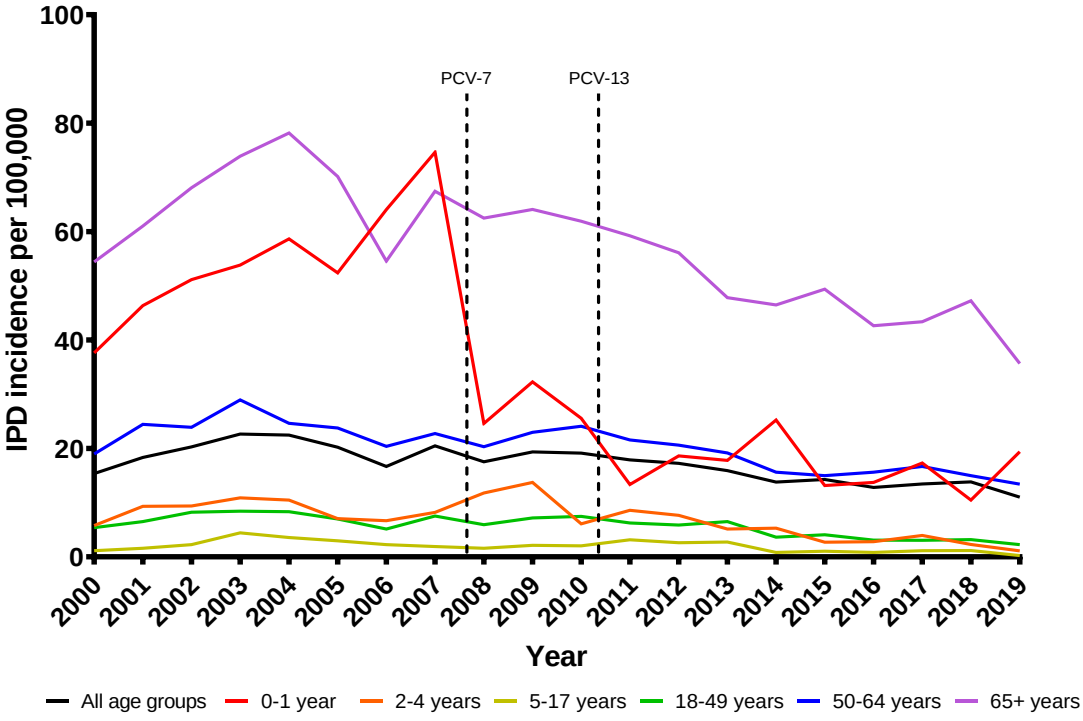


Figure 9. Incidence of invasive pneumococcal disease (IPD) cases per 100,000 in Denmark, 2000-2019, stratified by age group. PCV-7 was introduced in October 2007 and replaced by PCV13 in April 2010 (data from the National Streptococcal Reference laboratory (NSRlab), Statens Serum Institut).

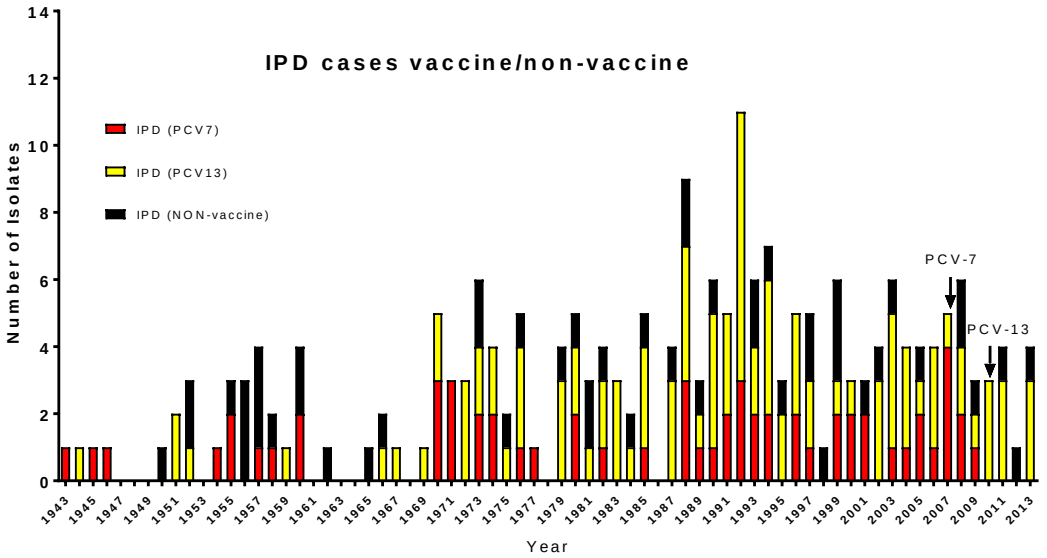


Figure 10. Number of IPD cases in infants below 3 months of age. Red bars represent IPD cases due to serotypes included in the PCV-7, yellow bars represent IPD cases due to serotypes included in the PCV-13, and black bars represent IPD cases due to non-PCV serotypes. Paper J presents a similar figure showing the incidence of IDP cases.

IPD data from 1999 and onwards show the effect of PCV introduction on serotype replacement and the epidemiology of the most common serotypes in Denmark, i.e. serotypes 8, 9N, 11A, 12F, 15A, 22F, and 24F (Figure 9) (Paper B, F, G). For the 0-4-year age group, IPD cases continue to be low, although of the remaining IPD cases more than 91% were due to non-PCV serotypes (Paper G). IPD cases in elderly are now dominated by non-PCV serotypes (Figure 7, Paper B, G), as also reported for other countries (120,131,132). Particularly, the non-PCV serotype 8 is at present the dominant cause of IPD both in Denmark and other countries (Figure 8) (120,133,134)(Paper B, F, G). In Denmark the serotype 8 IPD incidence increased consistently after PCV13 introduction, predominantly affecting the age groups above 65 years. A significant increase was observed from 2012 and the levels have since then continued to be high compared to pre-PCV7 levels (Paper B), and in 2019 the most common IPD causing serotypes in Denmark were still the serotype 8 with 26% of total 622 IPD cases, serotype 3 (11%), serotype 22F (8%), serotype 12F (7%), and serotype 9N (5%) (**https://www.ssi.dk/sygdomme-beredskab-og-**

forskning/sygdomsovervaagning/n/neisseria-og-streptokok-referencelaboratoriet---nsr, accessed 29 October 2020).

British studies observed continued problems with serotypes 3 and 19A included in the PCV-13 (120,122), whereas a Canadian study observed no changes in the overall incidence of IPD in the elderly, although serotype replacements were observed: serotype 3 continued to be a problem in the elderly, whereas serotype 19A decreased (131). IPD cases due to PCV-13 serotypes in Danish children are very rarely observed, whereas serotype 3 continues to be a dominant cause of IPD in the elderly (Paper F, G, H), and overall show no reduction following the PCV-13 introduction (Paper G, H). In 2019 in Denmark there were two cases of serotype 3 IPD in children less than 3 month (pre PCV-13 vaccination), while the remaining IPD cases started to appear from the age group 30+, and there were not reported any PCV-13 vaccine failures involving serotype 3 (data from the National Streptococcal Reference laboratory (NSRlab), Statens Serum Institut). Recently, it was described that serotype 3 might be predominantly carried in adults, an age group normally not vaccinated with PCV-13, providing a possible reason for the lack of effect from PCV-13 in Denmark (123)(Paper K). Because serotype 3 probably not is carried by children, it is not expected that herd protection from PCV vaccination of infants has an effect on the elderly (125). Other studies explain the serotype 3 problem with a low response of PCV-13 against serotype 3 carriage, which cannot, however, be supported in Denmark as the vaccinated group (children < 2 years) has been found not to carry serotype 3 (120,122,131,135)(Paper F).

Other non-PCV serotypes have increased in IPD incidence since the PCV introduction (122,131,135,136)(Paper B, C), which is illustrated by the significant increase in serotype 24F IPD cases in Denmark (Paper C), constituting nearly 3% of the total number of IPD cases in 2019 (data from the National Streptococcal Reference laboratory (NSRlab), Statens Serum Institut). Serotype 24F is not part of any existing pneumococcal vaccines, or scheduled to be included in any planned pneumococcal vaccines (16,36,137). It is therefore important to continue monitoring the epidemiology and susceptibility of serotype 24F and other non-vaccine serotypes to determine if they need to be included in future pneumococcal vaccines (132,138–140).

6. Discussion, concluding remarks and future research

6.1. The identification of *Streptococcus pneumoniae*

National reference laboratories around the world are in a transition from phenotypically based methods to molecularly based methods (25,28,98,99). They are ceasing to use the old phenotypically based methods and replacing them with molecular methods (25,28,98,141,142). A major problem in this transition is international lack of agreement on which minimum requirement is needed for a correct species identification. Many procedures have been suggested, but a clear international agreement on pneumococcal identification has not been established (28,141,142).

A similar situation is present with genotyping versus phenotyping, in which laboratories are basing their pneumococcal serotyping solely on genotyping, with the concern that they do not know if the capsular genes are expressed (24,25,28,98,101).

Because "old" phenotypically based methods often are considered expensive and labor intensive, there is a major threat that these methods will disappear, before understanding how genes express their phenotypical characteristic (28,141).

An additional problem in use of phenotypical methods for serotyping requiring antigen specific rabbit serum is the limitation of commercially available antiserum. At present around 100 serotypes have been described (24), however only 92 serotypes can at present be identified by commercially available antiserum from SSIDiagnostica.dk (https://www.ssidiagnostica.com/, accessed 14-11-2020). This means that at least eight characterized pneumococcal serotypes cannot be phenotypical described by available commercial antiserum. If new commercial antisera are not developed in due time to keep this gap at a minimum between identified serotypes and available corresponding commercial antisera, this may add to the transition towards using only molecular based methods, as the gap increases with missing phenotypical confirmation of new serotypes.

A major advantage for the phenotyping versus genotyping issue is that the pneumococcal vaccine development mainly depends on expression of serotype specific capsular polysaccharides, which rely on the epidemiology of phenotypically verified pneumococcal serotypes. It is the expression and not the presence of capsular genes, which decides the effect of pneumococcal polysaccharide vaccines, and it is therefore crucial to find a procedure that incorporates the benefit from both capsular gene-based typing and capsular phenotyping (28,143).

A solution to preserve the knowledge of phenotypical based methods is to maintain a limited number of international pneumococcal reference laboratories, to which local laboratories can send

isolates for possible phenotypical confirmation of their molecular identification as is already practised in Denmark. This would allow international reference laboratories to receive enough isolates to maintain the knowledge and ability to perform old phenotypically based methods, such as the capsular reaction test.

Until an international agreement has been established for pneumococcal identification and serotyping, is it recommended to follow the below described procedures to obtain a high level of quality data for identification and serotyping.

Basically, it cannot be recommended to perform identification of *S. pneumoniae* based on a single phenotypic method, such as optochin screening, or a single gene identification, as this can cause bias of incorrect identification (60,61,74,141,144)(Paper A).

Based on the conclusions from the studies (28)(Paper A, B, C), a list of recommendations for Mitis group species identification, depending on costs, workload, and laboratory capacity is presented:

1. It is recommended to use the MALDI-TOF as the primary method with the optochin test as a secondary test.
2. In case of discrepancy between the methods in (1), evaluation of the species-specific peaks of the MALDI-TOF spectra is recommended.
3. Additional use of the bile solubility test provides further specificity. Alternatively, the latex agglutination test (ImmuLex™ S. pneumoniae Omni, SSIDiagnostica.dk, Denmark) can be used to increase the species-specific pneumococcal identification.
4. Species identification using PCR for *lytA* and/or *ply* gene detection is recommended mainly for culture-negative isolates. In epidemiological studies testing large numbers of isolates, it is recommended to perform gene detection in combination with phenotypical methods, such as the latex agglutination test (ImmuLex™ *S. pneumoniae* Omni, SSIDiagnostica.dk, Denmark). As both genes can be found in several Mitis group species, it is not recommendable to use PCR on isolates with conflicting results, as this will not provide further conclusive information.
5. If WGS is available, it can provide extensive genetic information, e.g., on the presence of virulence genes, antibiotic resistance genes, and a fully correct species determination. Recently, new homepages presenting easy to use platforms for species identification and other genes has emerged. Suggested site are PubMLST (**https://pubmlst.org/**, accessed 15-11-2020)(145) and Pathogenwatch (**https://pathogen.watch/**, accessed 15-11-2020)(100).

Which method should be used for identification of pneumococcal serotypes depends on the information needed. Phenotypical methods provide information on the expressed capsule, whereas molecular methods detect the presence of a capsule gene, but not whether it is expressed.
Based on the studies (Paper B, C, D, E), it is recommended to follow these protocols for pneumococcal serotype identification:

1. The Pneumotest-Latex kit is a simple phenotypical test recommended for initial serotyping, and its use reduces the need for using the labor-intensive Neufeld method to cases with inconclusive latex results.
2. Real-time PCR or conventional PCR for genotyping is recommended only for non-culture samples, as the complexity of the PCR protocols is similar to that of the WGS.
3. It remains difficult to perform genotyping, particularly within a serogroup, when using WGS. However, the development and presentation of recent freeware using different algorithms for genotype identification such as PneumoCaT (25), SeroBA (95), and the CDC genotyping program (98) makes genotyping easier to perform. Additionally, the development of platforms such as Pathogenwatch (https://pathogen.watch/, accessed 15-11-2020), which have the SeroBA program included, makes it usable for scientists with limit knowledge on bioinformatics.
4. Combining WGS genotyping data with latex agglutination confirmation will offer information on both genotype and phenotype.

6.2. The pneumococcal epidemiology in Denmark

PCV vaccination of children < 2 years of age has changed the pneumococcal epidemiology in Denmark. While ten years of PCV vaccination has shown no impact on the total carriage rate in children < 2 years of age, a major change in the carrier serotype distribution within this age group has been observed with the disappearance of PCV-13 included serotypes and the emergence of non-PCV-13 included serotypes (Paper F). The disappearance of PCV-13 serotypes from children < 2 years can also be linked to the large reduction of IPD in this age group. Furthermore, with the serotype replacement, new non-PCV serotypes seem less virulent and therefore do not fully replace the reduced IPD incidence in children < 2 years of age (Figure 9). The observations of a major reduction in IPD cases in children has been observed in many countries due to PCV introduction, although as previously mentioned some serotypes such as serotype 3 continue to be a problem in children in some countries excluding Denmark (120,146,147), with only two IPD cases of serotype

3 observed in the infant age group, of which both where infants who had not received their first PCV vaccination (data from the National Streptococcal Reference laboratory (NSRlab), Statens Serum Institut).

With the disappearance of PCV-13 serotypes in Danish children (Paper F, G) continued PCV-13 vaccination seems pointless. Alternative strategies for pneumococcal vaccination of children is therefore of interest. Abandoning PCV-13 vaccination of children could be one strategy for Denmark. This has however indirectly been tested in Belgium, where it was decided to change their child vaccination program from PCV-13 to PCV-10, with the effect that serotype 19A IPD cases in children increased dramatically (148). A similar situation will most likely appear in Denmark, since PCV-13 included serotypes still appear in the general Danish population. In 2019 IPD cases were detected with serotype 1, 3, 4, 6A, 7F, 14, 18C, 19F, 19A, 23F (Paper G, data from the National Streptococcal Reference laboratory (NSRlab, Statens Serum Institut, **https://www.ssi.dk/-/media/arkiv/dk/sygdomme-beredskab-og-forskning/sygdomsovervaagning/referencelaboratorier/aarsrapport-2019-luftvejsinfektioner-og-meningitis.pdf?la=da**, accessed 23-September-2020), showing that the majority of PCV-13 serotypes still are present and causing pneumococcal diseases.

In England an alternative vaccination strategy has been introduced with a change from 2 +1 vaccination strategy to a 1 + 1 strategy by January 2020 (**https://www.nhs.uk/conditions/vaccinations/pneumococcal-vaccination/**, accessed 19-September-2020). A study on comparison of 2 +1 and 1 + 1 PCV-13 vaccination was performed in UK, and it was modelled that removing one primary PCV-13 dose would have limited impact on overall IPD of all age groups (149,150). The benefit of 1 + 1 PCV-13 schedule is relevant for Denmark, since Denmark can be considered to be in a similar situation as England (149).

In an Australian study the PCV efficacy was measured using both the 3 + 0 (No booster vaccination) and the 2 + 1 schedule (151). The study showed that both vaccination schedules presented similar vaccine effectiveness, however after 24 month from vaccination start the booster vaccination schedule showed higher efficacy than the no booster vaccination schedule. Based on this study the Australian child vaccination schedule was changed to 2 + 1 for children (151). The inclusion of a PCV booster vaccination is therefore considered to be important for the long term vaccine efficacy in children (151,152).

Although children have greatly benefited from PCV vaccination, with a significant reduction in IPD cases, and the elderly through herd protection have benefited from a reduction in IPD cases (figure

9) (31) there is still a circulation of PCV serotypes in adults and serotype replacements both in Denmark (Figure 9) (Paper B, C, G) and in many other countries (122,126,146).
In recent years new studies have shown that other age groups than children are carriers and transmitters, and the PCV vaccination of children will have a limited effect of herd protection for some serotypes, such as serotype 3 (123,124,126,153,154). The carriage rate observed in adult age groups varies between 0% to 20% depending on age group (123,153,155–158). The studies also show discrepancies between the serotypes causing IPD in elderly and those carried by children (154), and the adults are found to be carriers of both non-PCV-13 serotypes (serotype 8) and some serotypes included in the PCV-13, such as serotype 3, 19A (123,153,156) (Paper F). Also in Denmark the serotype distribution shows differences between the PCV-vaccinated children and the unvaccinated age groups, especially as depicted by the carriage/IPD serotypes in children aged 8 to 19 months and the IPD serotypes in the 65+ age group. The major causes of IPD in the 65+ age group, serotypes 3 and 8, are rarely observed as causes of IPD in children 0-4 years or observed as carrier isolates in children aged 8 to 19 months (Paper B, F). This observation is still valid for Denmark in 2019, where as previously mentioned two cases of serotype 3 in infants were observed and one case of serotype 8 in a nearly one year old child, the remaining cases were observed in adults and particular the elderly (data from the National Streptococcal Reference laboratory (NSRlab, Statens Serum Institut). The failure of PCV-13 on reducing serotype 3 IPD might therefore be due to the fact that protection of the elderly is based on herd protection and not direct protection by PCV vaccination (123,125,126)(Paper H, K).
The profound increase of serotype 8 in Denmark after the PCV13 introduction was not predicted in any Danish published IPD and pneumococcal carriage data up to December 2013 (31,34,115). In 2014, there was indication of the dominance of serotype 8, although at that time point, it was not clear that serotype 8 would continue to be the leading cause of IPD (Paper G). The two Danish carriage studies in children below 5 years of age pre- and post- PCV (115) (Paper F) did not show any indication of high carriage of serotype 8, explaining the transmission to the elderly. The epidemiological data do not provide an explanation for the dominance of serotype 8 IPD cases observed in Denmark (31,34,115) (Paper F, G). The pneumococcal polysaccharide vaccine (PPV) - 23 vaccine includes the serotype 8, and has shown a significant vaccine efficacy against this serotype , although the protection is of limited duration (113). The duration of protection can explain the PPV23´s limited effect in England against serotype 8 IPD despite a national PPV23 immunization program for 65+ age group since 2003 (113,120). Based on the English data, it can

therefore not be expected to observe a greater decline in the serotype 8 IPD incidence, even with the introduction of PPV-23 into a vaccination program for risk groups and the elderly 65+ (159). Future studies are therefore needed to identify possible markers for improving the prediction of the next non-PCV serotype which could increase the incidence of IPD in Denmark and other countries.

It has been shown that carrier-induced hypo-responsiveness in children due to PCV vaccination is common and could be an explanation for the lack of reduction in serotype 3 IPD cases and the continued appearance of other PCV-13 included serotypes (160,161). However, in Denmark this seems not to be the case, as PCV-13 serotypes are rarely carried by children and causing IPD in this age group (Paper F, G, H). The explanation for the continued appearance of PCV-13 serotypes is more likely due to carriage and transmission from unvaccinated age groups (124,154), than carrier-induced hypo-responsiveness.

With these reservoirs of pneumococci in other age groups than the vaccinated children, there will be a limitation for adults in the herd protection effect from PCV vaccination of children (123,125,126) and alternative strategies need to be considered to reduce the incidence of IPD cases among the risk groups such as the 65+ age group (123,126).

Adult vaccination with either PCV-13 or PPV-23 prevents both pneumococcal pneumonia and IPD in adults (16,18). However, the general decrease in PCV-13 included serotypes and increase in non-PCV-13 serotypes, of which several are included in the PPV-23, favors the use of PPV-23 in adults (16,132). The uptake of PCV vaccination of children has been recorded to 87% in 2008 increasing to around 95% in recent years (**https://statistik.ssi.dk/**, accessed 13-10-2020). The PPV-23 uptake in Denmark has not been systematically recorded before 2015 (**https://www.ssi.dk/vaccinationer/boernevaccination/vaccinationsdaekning-og-aarsraporter/det-danske-vaccinationsregister-ddv**, accessed 13-10-2020), and in mid-2020 the PPV-23 was introduced as a part of the vaccination program for adults, also due to the covid-19 situation. (**https://www.ssi.dk/vaccinationer/risikogrupper/invasiv-pneumokoksygdom**, accessed 13-10-2020). There is therefore at present no data on the uptake of PPV-23 in adults, although it is believed to be low (Paper G).

In several countries with USA as the best described example, serotype 19A increased after the PCV-7 introduction and decreased following introduction of the PCV-13, this included a significant reduction of serotype 19A antibiotic non- susceptible strains (162). Also the previously mentioned Belgian study on replacing the PCV-13 with PCV-10, showed the importance of specific vaccination against serotype 19A (148). In addition, some developing countries observed a

reduction in the prevalence of antibiotic non-susceptible pneumococcal isolates, e.g. a decline in penicillin non-susceptible pneumococcal isolates was observed in Ghana (163) and in Angola, and continued use of PCV is expected to result in a decreasing rate of penicillin-non susceptible pneumococcal serotypes (PNSP) (164). However, the overall effect of PCV vaccination on the prevalence of non-susceptible pneumococcal isolates seems to be limited due to the emergence of non-PCV non-susceptible serotypes (164,165).

The pneumococcal vaccination effect on pneumococcal antibiotic susceptibility in Denmark is difficult to evaluate, since the level of non-susceptible isolates over the years has been low (166,167). Furthermore, a general effect of PCV and PPV-23 on the antibiotic resistance level of pneumococci can only be based on weak data, due to the previously mentioned lack of vaccination data and general changes in antibiotic treatment regimens over the period. National Danish Monitoring of pneumococcal antimicrobial susceptibility has been presented in DANMAP since 1997 (**www.danmap.org/reports**, accessed 12-01-2021). From 1990 to 1999 an increase in pneumococcal resistance against penicillin and erythromycin was observed (166), and since 1999 the prevalence of non-susceptibility fluctuated between 2 – 7% (166,167). Based on erythromycin and penicillin resistance data from 1993 to 2019 there was no obvious effect on the number of non-susceptible isolates, which might be related to the introduction of PCV-7 or PCV-13 in Denmark (167). Yet, it can be speculated that the introduction of PCV-13 in 2010 may have had an effect on the reduction in the number of serotype 19A isolates including non-susceptible 19A isolates observed in Denmark since 2010 (31,167)(Paper G).

The epidemiological effect of PCV introduction in Denmark can be summarized as follows:

1. Children < 2 years of age still benefit from PCV vaccination, although no further reduction in the total number of IPD cases within all age groups seems to appear. However, importantly, a reduced level of IPD cases has been reached. PCV serotypes have disappeared in children and have been replaced by non-PCV serotypes both as carried isolates and as causes of IPD.
2. It is recommended not to stop PCV-13 vaccination of children, as several studies clearly show that PCV included serotypes will reemerge after stop of PCV vaccination. A change in the PVC-13 vaccination schedule in the child vaccination program from 2 + 1 to 1 + 1 can however be recommended, based on the experience from England.

3. No net effect from herd protection has been observed on the total number of IPD cases in Danish infants younger than 90 days, although IPD cases due to PCV-7 serotypes have decreased and have not been observed since 2009.
4. Overall, the serotype 3 IPD incidence has not changed with the introduction of PCV-13; on the other hand, serotype 3 has never been a problem in children.
5. An increase in non-PCV serotype IPD cases post-PCV introduction has been observed due to serotype 8, serotype 24F, and other non-PCV serotypes.
6. With the vaccines presently available, a further reduction of IPD in Denmark will require systematic pneumococcal vaccination, in particular of the 65+ years age group as seen with the child vaccination program.
7. By performing a continued PCV-13 vaccination of children and the start of a general PPV-23 vaccination of adult age groups in Denmark, a further reduction in the total number of IPD cases can be expected, although this will not include the counter effect of serotype replacement.
8. Due to limited data on non-susceptible pneumococcal isolates, is it not possible to evaluate the PCV effect on non-susceptible pneumococcal isolates pre- and post PCV introduction. It is therefore of crucial importance to continue monitoring the epidemiology of pneumococcal serotypes in Denmark.

6.3. Concluding remarks and future research

The use of WGS in national reference laboratories has presented new approaches to analyze pneumococcal epidemiology (98,100,145). Future identification of pneumococcal isolates will automatically include correct species identification, reliable genotype, clonal relationship and genetic antibiotic resistance profiles (Pathogenwatch (version 3.7.5, https://pathogen.watch/, accessed 17-11-2020) (100), PubMLST (**https://pubmlst.org/**, accessed 15-11-2020)) (145). Examples of how this information is useful, can be illustrated by the recent years´ outbreak cases of pneumococcal infections among shipyards in several countries, for which knowledge of serotype/clone was important for controlling the outbreak (168,169). Also the observed serotype replacement of the highly non-susceptible serotype 19A in USA after the PCV-7 introduction (162), will be easier to detect and follow (99).

Studies are trying to link resistance related genes with antibiotic susceptibility, thereby providing quick and valuable information on isolate antibiotic susceptibility (98,100,170). However, the

complexity of genes related to the phenotypical susceptibility remains, and detection of genes alone can still not be used without the phenotypically based methods to describe the antibiotic susceptibility of a pneumococcal isolate. Further investigation to improve the genotypic methods is obvious.

The epidemiology of pneumococci in the Danish population is changing due to natural serotype variation and the effect of vaccine introductions (Paper B, C, F, G, J). As exemplified with serotype 3 (Paper H, K) we do not fully understand the carriage and transmission of pneumococci, and epidemiological information is lacking for many of the emerging serotypes causing infections in humans. Recent studies show that older age groups carry pneumococci, and potentially are able to transmit pneumococci (124,154), a finding mainly observed due to introduction of new molecular based identification methods (171), and further, nasopharyngeal swabs also include other types of swab samples such as oropharyngeal samples (144,156,172). This relatively new identification of other age groups as carriers due to improvement in sampling methodology, shows how connected the pneumococcal method development is for epidemiological data, which again can explain the different observations of pneumococcal serotype transmission patterns in a population.

Improvement of pneumococcal identification and typing methods are therefore vital for monitoring pneumococcal epidemiology in order to provide information for the pharma-vaccine industry and for health authorities to make the correct decisions on recommendation for pneumococcal vaccination.

It is recommended to continue the PCV-13 vaccination of the children in Denmark, to prevent the reappearance of PCV-13 IPD cases in children, as observed with 19A in Belgium (148). It can, however, be advised to change the child vaccination program from 2+1 schedule to 1+1. It is also recommended to vaccinate the Danish adult risk group with the PPV-23 every 6 years, since the PPV-23 has shown significant vaccination efficacy in adults (113), in order to prevent pneumococcal pneumonia and IPD (16,132,173), and it seems that the herd protection effect from vaccinated children has reached its limitation (126). With the Covid-19 pandemic and the wish of reducing the restraints on the Danish health system, the recommendation of introducing PPV-23 into a vaccination program for risk groups and the elderly 65+ has been lifted to be an part of the vaccination program for elderly (159).

New pneumococcal vaccines to be expected soon, are based on inclusion of additional limited number of serotype specific polysaccharides, while non serotype based vaccines still are in the

experimental stage (36). The appearance of non-PCV serotypes such as serotype 24F (Paper C), and other serotypes, will therefore still be a health issue (36,165).

The restrictions enacted on Denmark and many other countries since spring 2020 due to the Covid-19 pandemic (174) has shown a dramatic decline in IPD cases in Denmark and parts of the world (47,175,176). In 2020 Denmark will probably observe the lowest number of IPD cases ever seen (47) (Personal observation). The dramatic change in the daily lifestyle worldwide will provide new opportunities to investigate the epidemiology of pneumococci in Denmark and the rest of the world, in particular the transmission from person to person. Effects such as keeping distance and prevent socializing between different age groups already show its effect on reduction of pneumococcal diseases. Soon new data will be presented, analyzing IPD and pneumonia cases in different age groups before and after social lockdown due to Covid-19, and will thus reveal more information on the dynamics of pneumococci. The Covid-19 pandemic is a terrifying burden on human beings, however the pandemic provides the scientific community with massive new information on many diseases´ epidemiology, which in the long term hopefully will compensate for the losses and pain many people at present are suffering.

With the ongoing transformation, new laboratory procedures, and additional studies leading to more epidemiological data are required within these topics:

1. Identification of *S. pneumoniae*, including separation of other species within the Mitis group, is based on various methods with variable specificity. These methods require improvement, refinement, and standardization of procedures for correct species identification. Species identification ought to be based on housekeeping genes as with different MLSA protocols, both of which are based on identification of seven or more housekeeping genes. It should be feasible to improve the MLSA protocols, making it as simple as screening for single genes such as *LytA* or a single nucleotide in 16s rRNA (cytosine versus adenine at position 203). This will, at the same time, provide a far better species identification and make us able to identify not just pneumococci but also other species such as *S. pseudopneumoniae* and *S. mitis*, thus providing a high specificity for species identification and at the same time a very accurate description of clonal association.
2. Guidelines for PCR procedures on non-culture samples are needed with regard to the numbers and types of required genes for an acceptable species identification within the Mitis group. The number of selected genes should not exceed two in order not to make the PCR excessively complex.

3. With the possibility of serotype identification based exclusively on the presence of capsular genes, the need for information will increase on how to spot either unknown capsular genes or factors that inhibit the expression of known capsular genes. This will require comprehensive studies and enhanced understanding of the association between the gene expression and the expressed phenotype, thereby allowing us to predict the capsular polysaccharide profile and identify novel phenotypically important genes.
4. To determine if phenotype-based serotyping methods can be discarded, more studies are needed to show how representative genotyping compares with the expressed phenotype, and how representative genotyping is for proving epidemiology data for informing future vaccine decisions. Furthermore, these studies may help to decide whether only international reference centers need to include phenotype-based methods in their portfolio of typing methods for quality programs, research, and handling of epidemiological data, while allowing clinical laboratories to focus on molecularly based methods for epidemiological monitoring.
5. Besides in nasopharyngeal samples, pneumococci have also been detected in oropharyngeal and sputum samples. Further studies are therefore needed to elucidate how representative nasopharyngeal swap samples are for carriage studies compared with swap samples from oropharyngeal and sputum samples.
6. Studies have already shown that the dynamics of different pneumococcal serotypes are very different. An increasing number of data shows that it is not always the same serotypes that cause IPD in children as in other age groups. More pneumococcal studies are needed to better understand the dynamics between the carriage stage and the disease-causing stages for all known serotypes.
7. Being able to predict the next dominant non-PCV serotype will be of help to foresee which measurement needs to be in place. It is important for the health system to know, if it is a susceptible serotype such as serotype 8 or a non-susceptible serotype such as serotype 19A which will dominate in the future.
8. The lockdown of many countries and the following change in the human behavior, will provide opportunities to see if pneumococcal carriage will change with regard to serotype distribution, and what will happen when the restriction is lifted. Also the lockdowns major effect on reduction of IPD will provide new information on the effect of human behavioral changes.

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