View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Repositório Aberto da Universidade do Porto

FERNANDA MARIA PEREIRA RODRIGUES

PAEDIATRIC NASOPHARYNGEAL ECOLOGY IN THE ERA OF CONJUGATE VACCINES

Tese de Candidatura ao grau de Doutor em Ciências Médicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Professor Doutor Guilherme Gonçalves
Categoria – Professor Associado
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Co-orientador – Professor Doutor Jorge Saraiva
Categoria – Professor Catedrático Convidado
Afiliação – Faculdade de Medicina da Universidade de Coimbra.

The studies presented in this thesis were performed at the Hospital Pediátrico de Coimbra, Centro Hospitalar e Universitário de Coimbra, Portugal, at Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal, under the supervision of Prof. Doutor Guilherme Gonçalves, at Nuffield Department of Medicine, University of Oxford, UK, in collaboration with Dona Foster, at Division of Clinical Sciences, St. George's, University of London, UK, in collaboration with Jason Hinds and at Bristol Children's Vaccine Centre, Schools of Clinical Sciences and Cellular and Molecular Medicine, University of Bristol, UK, in collaboration with Prof. Adam Finn.

Tese elaborada de acordo com o Regulamento Geral do 3º Ciclo de Estudos Conducente ao Grau de Doutor em Ciências Médicas do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Aos meus queridos pais

Resumo

O pneumococo faz parte de um conjunto complexo de microrganismos que colonizam transitoriamente a nasofaringe, podendo por vezes causar doença. A colonização é considerada um pré-requisito para a doença e é a fonte de transmissão entre os indivíduos.

As vacinas conjugadas pneumocócicas (VCPs) foram licenciadas em 2000 e demonstraram ter impacto sobre a doença em grande parte através da redução da colonização e transmissão entre indivíduos de serotipos vacinais, os quais são substituídos por outros não incluídos nas mesmas. Portugal não introduziu estas vacinas no seu Programa Nacional de Vacinação, estando disponíveis no mercado privado. Muitas famílias têm optado por imunizar as suas crianças. Assim, formulámos a hipótese de que o impacto da imunização com a VCP7-valente sobre a colonização nasofaríngea por *S. pneumoniae*, em comunidades com coberturas vacinais mais baixas, a aumentar lentamente e com distribuição geográfica heterogénea, será diferente daquele observado em países onde a vacina foi implementada de forma consistente ao longo do tempo, com distribuição geográfica uniforme e taxas de cobertura elevadas.

Entre 2007 e 2010, numa série de estudos transversais anuais em crianças a frequentar infantários em Coimbra, Portugal, observámos, uma tendência de redução ou desaparecimento de serotipos vacinais em colonização nasofaríngea, exceto o 19F, que não seguiu este padrão e, mais recentemente aumentou, tornando-se o mais prevalente. Estes resultados demonstram que os efeitos indiretos da VCP podem ser observados com as taxas moderadamente elevadas de utilização da mesma em Portugal, mas que, pelo menos para o serotipo 19F, os atuais padrões de uso da vacina não parecem ser suficientes para reduzir ou eliminar este serotipo. Observámos também que, após vários anos de uso da VCP, não houve substituição completa de serotipos e a sua diversidade tem-se mantido consistentemente perto do valor mais alto observado noutros estudos e sem tendências progressivas ao longo do tempo. A utilização de uma nova técnica molecular de *microarray* para serotipagem de pneumococo, que permite deteção e quantificação do ADN que codifica para o *locus* capsular de mais do que um serotipo ou estirpe na mesma amostra, mostrou co-colonização por mais do que uma estirpe capsulada em cerca de 10% das crianças em infantários. Permitiu identificação de estirpes presentes em baixa densidade e reconhecimento de oportunidades para transferência génica entre bactérias.

O sucesso da colonização nasofaríngea pneumocócica poderá depender da coexistência com outras bactérias que ocupam o mesmo nicho e os vírus poderão

também desempenhar um papel nesta dinâmica. A nasofaringe é uma importante fonte de secreções que poderão facilitar a transmissão de bactérias entre os indivíduos. Formulámos a hipótese de que poderá haver relações entre as diferentes espécies bacterianas, vírus respiratórios e rinite, que poderão contribuir para o sucesso da colonização nasofaríngea e da transmissão na comunidade.

Através da recolha prospetiva de informação sobre a presença de rinite, associada a análise microbiológica de várias espécies bacterianas e de um painel de vírus respiratórios nas secreções nasofaríngeas, explorámos as relações entre colonização bacteriana, deteção de vírus e existência de rinite. Demonstrámos associações positivas entre a deteção de rinovírus e a presença e densidade de colonização nasofaríngea por pneumococo, sugerindo que a presença do vírus pode facilitar a aquisição e/ou proliferação bacteriana. Demonstrámos também associação entre a presença de *H. influenzae* e rinite, a qual poderá promover a transmissão, com vantagens para a bactéria.

A otite média aguda (OMA), presumivelmente precedida por colonização nasofaríngea, é frequentemente causada por pneumococo. Formulámos as hipóteses de que a co-colonização por múltiplos serotipos na nasofaringe poderá também ocorrer no ouvido e que a presença e densidade de colonização bacteriana na nasofaringe poderão ser diferentes em crianças saudáveis e crianças com OMA, contribuindo para a patogenia desta infeção.

Investigámos a microbiologia do ouvido médio em crianças com otite média aguda supurada (OMAS) através de cultura e utilizámos a técnica molecular de *microarray* para serotipagem de pneumococo nas secreções nasofaríngeas e do ouvido. Detetámos mais do que uma estirpe capsulada na otorreia em cerca de 20% das crianças com identificação de pneumococo. A grande maioria das crianças com identificação de pneumococo na otorreia tinha também pneumococo nas secreções nasofaríngeas e, nestes casos, em todas, pelo menos um serotipo foi encontrado simultaneamente nos dois locais. A comparação das taxas e densidades de colonização nasofaríngea por *S. pneumoniae*, *H. influenzae* e *M. catarrhalis* foram semelhantes no grupo de crianças em infantários e crianças com OMAS mas, em análise multivariada, a densidade de colonização por *H. influenzae* e *M. catarrhalis* foi menor no grupo com otite, sugerindo que uma disrupção no equilíbrio entre estas bactérias poderá estar associada com a doença.

Em conclusão, estes estudos fornecem informação adicional sobre os efeitos da utilização da VCP na colonização nasofaríngea a nível populacional, e sobre a biologia da colonização em crianças saudáveis e com doença. Poderão ser úteis na definição de futuras estratégias de prevenção da doença e para melhor compreensão da patogenia da OMA.

Palavras- chave: pneumococo, colonização nasofaríngea, vacinas conjugadas pneumocócicas, interações microbianas, otite média aguda

Abstract

S. pneumoniae is a transient coloniser among a complex nasopharyngeal microbiota and can sometimes cause disease. Presence in the nasopharynx is considered a pre-requisite for disease and is the source of transmission between individuals.

Pneumococcal conjugate vaccines (PCVs) were licensed in 2000 and appear to be impacting on disease to a great extent through reduction in colonisation and transmission of vaccine serotypes, which are duly replaced by non-vaccine serotypes. Portugal has not included PCVs in the national immunisation schedule offered to all children. Many families have chosen to have their children immunised through private provision. We hypothesised that the impact of immunisation with the seven-valent PCV on nasopharyngeal colonisation by *S. pneumoniae*, in a setting with lower, slowly rising and heterogeneous vaccination coverage would be different from that seen in countries where PCVs have been implemented at high rates, consistently over time and evenly geographically.

Between 2007 and 2010, in a series of annual cross sectional surveys in children attending daycare nurseries in Coimbra, Portugal, we have charted downward trends in nasopharyngeal colonisation with PCV serotypes, except for 19F which failed to disappear and latterly has risen to become the most prevalent serotype. These findings illustrate that indirect effects of PCVs can be seen even at the only moderately high vaccine uptake rates seen in Portugal but that, for 19F at least, the present patterns of vaccine usage may not be sufficient to reduce or eliminate this serotype. We have also shown incomplete serotype replacement after several years of PCV use and that serotype diversity has been consistently close to the highest seen in other settings and without progressive trends over time. The use of a novel pneumococcal molecular serotyping microarray that allows detection and quantification of DNA coding for the capsular locus of more than one serotype or strain in each sample, showed co-colonisation with more than one encapsulated pneumococcus in approximately 10% of the children in nurseries. We detected serotypes in low abundance and thus identified an ecology that offers opportunities for horizontal gene transfer.

Successful colonisation may also depend on successful coexistence with other bacterial species occupying the same niche. Viruses may also play a role in these dynamics. The nasopharynx is a major source of secretions that may facilitate transmission of bacteria between individuals. We hypothesised that there may be relationships between bacterial colonisers, respiratory viruses and

rhinitis that may contribute to successful colonisation and transmission in the community.

By prospectively collecting data on rhinitis and analysing nasopharyngeal samples both for several bacterial species and for a panel of respiratory viruses, we have explored the relationships between bacterial colonisers, viral detection and rhinitis. We have shown positive associations between detection of rhinovirus and both the presence and the density of pneumococcus in the nose suggesting that the virus may enhance bacterial acquisition and/or proliferation. We have also shown an association between presence of *H. influenzae* and rhinitis which could promote transmission and thus be of advantage to this species.

Acute otitis media (AOM), presumed to be preceded by nasopharyngeal colonisation, is frequently caused by pneumococcus. We hypothesised that just as nasal co-colonisation with multiple strains of *S. pneumoniae* occurs, this may also be the case in the ear and that the rates and densities of nasopharyngeal bacterial colonisation may differ between healthy children and children with AOM, in ways which may contribute to the pathogenesis of the disease.

We investigated the middle ear microbiology in children with AOM with spontaneous otorrhoea (AOMSO) by culture and applied the same pneumococcal molecular serotyping microarray to nasopharyngeal and aural discharge samples. More than one pneumococcal serotype was found in approximately 20% of the otorrhoea samples of the children that cultured pneumococcus. The great majority of children with pneumococcus in the ear also had it in the nose and in all of those, at least one serotype was found simultaneously in both places. Nasopharyngeal colonisation rates and densities of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* did not differ between healthy children and children with AOMSO although multivariate analysis showed that the densities of *H. influenzae* and *M. catarrhalis* were lower in otitis, suggesting that a disruption of the equilibrium between these species may be associated with the disease.

In conclusion, these studies further clarify the effects of pneumococcal vaccination on nasopharyngeal carriage at the population level and the biology of colonisation during health and disease. Our findings may prove useful in the development of future disease prevention strategies and to better understanding of AOM pathogenesis.

Keywords: pneumococcus, nasopharyngeal colonisation, pneumococcal conjugate vaccines, microbial interactions, acute otitis media

Acknowledgements

I would like to express my deep gratitude to Prof. Doutor Guilherme Gonçalves the supervisor of this work. I thank him for his help and monitoring over the years. I am also very grateful to Prof. Doutor Jorge Saraiva for his critical comments.

Part of the research studies leading to this Ph.D. thesis were conducted at Bristol Children's Vaccine Centre, Schools of Clinical Sciences and Cellular and Molecular Medicine, University of Bristol, UK, in collaboration with Prof. Adam Finn. I am very thankful for all his critical comments and suggestions that contributed immensely to the studies included in this thesis.

I am especially grateful to Prof. Carmona da Mota, my professor of Paediatrics and my mentor, for teaching me scientific rigour and for constant encouragement over the years.

A special thanks to all those working in the laboratories in Coimbra, Oxford and Bristol who contributed to generating the data presented here.

I am also very grateful to children and their parents for their participation and nurses and staff of day care centres for their dedicated work in the studies.

Finally my deep gratitude to my family, especially my parents, for their constant support and simply for making everything possible.

Abbreviations

AOM	Acute otitis media
AOMSO	Acute otitis media with spontaneous otorrhoea
cps	Capsule polysaccharide synthesis
D index	Simpson's index of diversity
DCCs	Day-care centres
DNA	Deoxyribonucleic acid
H. influenzae	*Haemophilus influenzae*
Hib	*Haemophilus influenzae* type b
M. catarrhalis	*Moraxella catarrhalis*
MenC	*Neisseria meningitidis* serogroup C
NT	Non-typeable
PCR	Polimerase chain reaction
PCV	Pneumococcal conjugate vaccine
PCV7	Seven-valent pneumococcal conjugate vaccine
PCV10	Ten-valent pneumococcal conjugate vaccine
PCV13	Thirteen-valent pneumococcal conjugate vaccine
plyNCR	Pneumolysin non-coding region
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
S. aureus	*Staphylococcus aureus*
S. pneumoniae	*Streptococcus pneumoniae*
STGG	Skim milk-tryptone-glucose-glycerin
WHO	World Health Organization

Publications

The original papers included in this thesis are:

Rodrigues F, Foster D, Caramelo F, Serranho P, Gonçalves G, Januário L, Finn A. Progressive changes in pneumococcal carriage in children attending daycare in Portugal after 6 years of gradual conjugate vaccine introduction show falls in most residual vaccine serotypes but no net replacement or trends in diversity. *Vaccine* 2012;30(26):3951-6.

Rodrigues F, Morales-Aza B, Holland R, Gould K, Hinds J, Gonçalves G, Januário L, Finn A. Resurgence of serotype 19F carriage in pre-school children in Portugal in the context of continuing moderate conjugate pneumococcal vaccine uptake. *Clinical Infectious Diseases* 2013;57(3):473-4.

Rodrigues F, Foster D, Nicoli E, Trotter C, Vipond B, Muir P, Gonçalves G, Januário L, Finn A. Relationships between rhinitis symptoms, respiratory viral infections and nasopharyngeal colonization with *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* in children attending daycare. *The Pediatric Infectious Diseases Journal* 2013;32(3):227-232.

Rodrigues F, Morales-Aza B, Turner K, Sikora P, Gould K, Hinds J, Gonçalves G, Januário L, Finn A. Multiple *Streptococcus pneumoniae* serotypes in aural discharge from children with acute otitis media with spontaneous otorrhea. *Journal of Clinical Microbiology* 2013;51(10):3409-11.

Rodrigues F, Morales-Aza B, Sikora P, Gonçalves G, Januário L, Finn A. Nasopharyngeal pneumococcus is neither commoner nor more abundant in children with acute otitis media with spontaneous othorrhoea than healthy children; but other otopathogens may have lower density. 2013 *(in preparation)*

The original data presented have been published in international peer-reviewed journals with the exception of those in the Appendix to Chapter 2 and in Chapter 6 which are in preparation for publication. The text of the other results chapters are exactly as published except that the page format was adapted to conform with the layout of the thesis and the reference numbers adjusted to a single bibliography of uniform style. As a consequence there is some overlap amongst these chapters and between them and the introductory chapter which reviews the literature comprehensively and defines the objectives of the overall research plan. The dissertation is completed by a general conclusion and suggestions for future work.

Table of Contents

Resumo v
Abstract xi
Acknowledgements xv
Abbreviations xvii
Publications xix
1. General Introduction 1
1.1 Introduction to the microflora of the nasopharynx 3
1.1.1 *Streptococcus pneumoniae* 5
1.1.2 *Haemophilus influenzae* 11
1.1.3 *Moraxella catarrhalis* 12
1.1.4 *Staphylococcus aureus* 13
1.1.5 Respiratory viruses 14
1.1.6 Methods for detecting nasopharyngeal colonisation 16
1.2 The determinants and dynamics of nasopharyngeal colonisation 19
1.3 The relationship between nasopharyngeal colonisation and disease 24
1.4 Nasopharyngeal colonisation and transmission to the community 27
1.5 Pneumococcal conjugate vaccines 27
1.6 The impact of pneumococcal conjugate vaccine on pneumococcal nasopharyngeal colonisation 31
1.6.1 Direct effects of pneumococcal conjugate vaccines on nasopharyngeal colonisation 33
1.6.2 Indirect effects of pneumococcal conjugate vaccines on nasopharyngeal colonisation 33
1.6.3 Replacement 34
1.6.4 Effects of pneumococcal conjugate vaccine on strain diversity 34
1.6.5 Modelling the effects of vaccination on *S. pneumoniae* nasopharyngeal colonisation 35
1.6.6 Observational nasopharyngeal colonisation studies in Portugal 36

1.7 Interactions between bacterial pathogens 37
1.7.1 Interactions between *S. pneumoniae* and *S. aureus* 39
1.7.2 Interactions between *S. pneumoniae, H. influenzae* and *M. catarrhalis* 39
1.7.3 Interactions between *S. aureus* and *M. catarrhalis* 39
1.7.4 Other associations between bacterial species 39
1.8 The impact of pneumococcal conjugate vaccines on other potential pathogens colonising the nasopharynx 41
1.9 Interactions between bacterial pathogens and respiratory viruses 43
1.10 Nasopharyngeal colonisation and acute otitis media 44
Objectives 47
2. Progressive changes in pneumococcal carriage in children attending daycare in Portugal after 6 years of gradual conjugate vaccine introduction show falls in most residual vaccine serotypes but no net replacement or trends in diversity 49
2.1 Abstract 51
2.2 Introduction 52
2.3 Methods 53
2.3.1 Recruitment, data collection, sampling and storage 54
2.3.2 Laboratory methods 54
2.3.3 Statistical analysis 55
2.4 Results 55
2.5 Discussion 59
2.6 Appendix to Chapter 2 63
3. Resurgence of serotype 19F carriage in pre-school children in Portugal in the context of continuing moderate conjugate pneumococcal vaccine uptake 67
4. Relationships between rhinitis symptoms, respiratory viral infections and nasopharyngeal colonization with *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* in children attending daycare 71
4.1 Abstract 73
4.2 Introduction 74
4.3 Methods 75
4.3.1 Study population 75
4.3.2 Study approval and consent 75
4.3.3 Clinical data 75
4.3.4 Nasopharyngeal swab collection 76

4.3.5 Bacterial culture 76
4.3.6 Viral PCR 77
4.3.7 Statistical analysis 78
4.4 Results 79
4.4.1 Characteristics of the study group 79
4.4.2 Bacterial carriage 80
4.4.3 Association between rhinitis symptoms and bacterial carriage 82
4.4.4 Viral nucleic acid detection 83
4.5 Discussion 83
5. Multiple *Streptococcus pneumoniae* serotypes in aural discharge from children with acute otitis media with spontaneous otorrhea 87
6. Nasopharyngeal pneumococcus is neither commoner nor more abundant in children with acute otitis media with spontaneous otorrhoea than healthy children; but other otopathogens may have lower density 97
6.1 Abstract 99
6.2 Introduction 100
6.3 Methods 102
6.4 Results 103
6.5 Discussion 106
7. General conclusions 109
References 119

Figures

Figure 1.1. *Streptococcus pneumoniae*.. 6
Figure 1.2. *Streptococcus pneumoniae* serotype 3. 6
Figure 1.3. *Haemophilus influenzae*... 12
Figure 1.4. *Moraxella catarrhalis*.. 13
Figure 1.5. *Staphylococcus aureus*... 14
Figure 1.6. Quellung reaction. 18
Figure 1.7. Anatomy of the nasopharynx. 24
Figure 1.8. Nasopharyngeal epithelial cell and *S. pneumoniae* attachment to the cell.. 25
Figure 2.1. Proportions of all children studied carrying pneumococcus in 2008 and 2009 compared to 2007 data. 57
Figure 2.2. Proportions of all children studied carrying PCV7 serotypes in 2008 and 2009 compared to 2007 and proportions carrying the non-PCV7 serotypes detected in 2008 and 2009. 58
Figure 2.3. Ranked frequency distribution curves for the commonest 20 serotypes in each year. 59
Figure 2.4. Proportions of all children studied carrying pneumococcus from 2007 to 2010.. 64
Figure 2.5. Rank frequency distribution of the only or predominant serotype detected in the 2010 samples. 64
Figure 2.6. Individual serotype and non-typeable distribution of single and co-colonised samples.. 65
Figure 2.7. Percentage of DNA of the predominant strain (grey line) and the other strain(s) (black line) in co-colonised children. 66
Figure 3.1. Percentage of serotype 19F DNA in 18 children co-colonized with 19F and at least 1 other serotype... 70
Figure 4.1. Age distribution of detected bacterial carriage in the study population. 79
Figure 4.2. Proportions of children with each SNOT score who were nasally colonized with Sp, Hi and Sa. 80
Figure 4.3. Relationships between SNOT score and density of colonization for (A) Sp, (B) Hi and (C) Sa.. 81
Figure 5.1. Microbiological characteristics of 28 children with acute otitis media with spontaneous otorrhea who were culture positive for pneumococcus. 93

Tables

Table 1.1. Selected studies of rates of *S. pneumoniae* nasopharyngeal carriage in children. 20
Table 1.2. Selected studies of rates of *H. influenzae* nasopharyngeal carriage in children. 21
Table 1.3. Selected studies of rates of *M. catarrhalis* nasopharyngeal carriage in children. 21
Table 1.4. Characteristics of licensed pneumococcal vaccines. 29
Table 1.5. Vaccine coverage with ≥ 3 doses at 24 months of age in the Central Region of Portugal and Coimbra's District by birth cohort, 2008-2011. 31
Table 1.6. Studies of associations between co-colonising bacteria in the nasopharynx in children. 40
Table 1.7. Studies of the impact of pneumococcal conjugate vaccines on nasopharyngeal colonisation of *H. influenzae*, *M. catarrhalis* and *S. aureus*. 42
Table 2.1. Characteristics of the 2008 and 2009 subjects (compared with the 2007 sample). 56
Table 4.1. Primers and probes for RSV and PIF real time PCR 77
Table 6.1. Characteristics of children with acute otitis media with spontaneous otorrhoea and children in day care centres. 103
Table 6.2. Colonisation rates and mean density scores of culture-positive children for *S. pneumoniae, H. influenzae, M. catarrhalis S. aureus* and *S. pyogenes* and mean number of bacterial species detected in univariate analysis. 104
Table 6.3. Colonisation rates for *S. pneumoniae, H. influenzae, M. catarrhalis*, *S. aureus* and *S. pyogenes* in multivariate analysis. 105
Table 6.4. Colonisation density for *S. pneumoniae, H. influenzae, M. catarrhalis*, *S. aureus* and *S. pyogenes* in multivariate analysis. 106

1. General Introduction

1.1 Introduction to the microflora of the nasopharynx

The five most common bacterial families in the human nasopharynx are *Moraxellaceae*, *Streptococcaceae*, *Corynebacteriaceae*, *Pasteurellaceae* (including the genus *Haemophilus*), and *Staphylococcaceae* (1-3). *Streptococcus pneumoniae* (*S. pneumoniae*), *Haemophilus influenzae* (*H. influenzae*) (mostly non-typeable strains), *Moraxella catarrhalis* (*M. catarrhalis*) and *Staphylococcus aureus* (*S. aureus*) are transient colonisers of this complex microbiota that can also cause disease (2, 3).

Although there is no universally accepted terminology, the following definitions are used in this work:

– Exposure: contact of the upper respiratory tract mucosal surface with the microbe, which may or may not persist and proliferate;

– Acquisition: the process of establishment of a strain within the host on the mucosal surface;

– Carriage or colonisation: overarching terms used interchangeably to include both the acquisition and the stable, detectable presence of the microbe in the nasopharynx of an individual over a period of time (a condition usually persisting for weeks or months).

Bacteria rapidly colonise the human nasopharynx soon after birth and can be acquired and eliminated many times over the life of a person (4-7). This is a dynamic process, during which microbes interact with each other and with the host and its maturing innate and specific immune responses (8). This human bacterial ecosystem is thought to be beneficial to the host, by stimulating the immune system, promoting the development of mucosal structure and function and providing a protective barrier against pathogen invasion (9). Viruses can also frequently be detected in the nasopharynx of children (10-16).

Nasopharyngeal colonisation is affected by the complex interplay of host and environmental factors, immune responses induced by organisms and vaccines, bacterial and viral characteristics and their interactions (17).

Several host and environmental factors have been shown to influence or be associated with colonisation: age, immunity, attendance at day care centres (DCCs), exposure to tobacco smoke and recent antibiotic use being examples (18-20). Infants and young children are more commonly colonised with *S. pneumoniae* and other bacteria, with rates peaking at around the age of 1-2 years (18). They have longer carriage episodes (21), more frequent rhinitis symptoms

and behaviours that make them more promiscuous with their respiratory and oral secretions, facilitating transmission both to other young children and to their family contacts. Day-care centre attendance by preschool children has consistently been associated with increased frequency of colonisation with *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (18) and DCCs are considered to be a unique setting for the transmission of nasopharyngeal bacteria (22).

Successful colonisation also depends on successful coexistence with other bacteria (23). The acquisition, presence and clearance of bacterial species may not occur entirely independently of one another and interactions seem to influence the species which persist in the nasopharynx (24, 25). Bacteria may be positively or negatively associated with each other (19, 23, 25-27) and associations may shift from negative to positive when additional bacterial species are present (17). It is also increasingly acknowledged that human diseases including respiratory tract infections can be polymicrobial resulting from synergistic and competing interactions between pathogens (28). More recently there is a growing interest in possible interactions between potentially pathogenic bacteria and commensals. The latter might prevent disease by inhibition of colonisation and expansion of pathogens, and by inducing immune modulation and stimulation of both mucosal maturation and barrier function (9). A better understanding of the dynamic relationships which exist between commensals and potential pathogens in the nasopharynx may provide insight into the pathogenesis of respiratory infectious diseases. The evolution of gene sequencing technologies has enabled detailed analyses of the nasopharyngeal microbiota of children by amplification and sequencing of the V5 and V6 hypervariable regions of the 16S ribosomal RNA (ribonucleic acid) gene (3).

In 2000, a seven-valent pneumococcal conjugate vaccine (PCV) was licensed in the USA and one year later in Europe. This vaccine has both direct and indirect effects on nasopharyngeal colonisation (29-31), resulting in reduced carriage rates of the serotypes contained in the vaccine, but with little or no long-term impact on the overall prevalence of pneumococcal carriage as other non-vaccine serotypes subsequently become more common (32). The approach to and timing of adoption of PCV immunisation of children has varied between countries and this may have resulted in distinct epidemiological effects on nasopharyngeal ecology.

The biological success of pneumococcus can be attributed to cellular components that are useful for colonisation and transmission (33). While the host

generally exists in harmony with the commensal microflora, changing conditions in the nasopharynx may affect the equilibrium of these components, and may also determine if and when the pneumococcus becomes a pathogen (34). For example, episodes of acquisition of new microbes, bacterial or viral, may potentially disrupt the equilibrium of this ecosystem, creating the conditions for invasion and causation of local or systemic disease (28, 35).

S. pneumoniae remains an important cause of serious bacterial disease among children worldwide. Presence in the nasopharynx is thought to be a prerequisite for disease, which only occurs in a small percentage of people who are colonised, and is also considered to be the source of pneumococcal transmission between individuals. Since children have the highest carriage rates, the highest transmission rates and are the recipients of PCVs, to examine bacterial colonisation in this age group is key to understanding transmission, interactions between colonising species and the impact of pneumococcal vaccination on nasopharyngeal ecology. Since the dynamics of microbial interactions may influence disease development, examining bacterial carriage during respiratory tract infections is a useful strategy for studying changes in nasopharyngeal ecology that may have a role in the pathogenesis of these infections.

1.1.1 *Streptococcus pneumoniae*

S. pneumoniae is a Gram-positive coccus, occurring as pairs (diplococcus), often arranged in chains (Figure 1.1.). Pneumococci grow on blood agar. Samples are commonly inoculated onto streptococcal selective colistin-blood agar (COBA) or gentamicin containing media and incubated at 37ºC in 5% CO_2. Identification of *S. pneumoniae* from culture is achieved by accurate observation of both its morphologic appearance (elongated cocci) and four main phenotypic characteristics: alpha-haemolysis of blood agar, catalase negativity, optochin susceptibility and bile solubility. Identification of suspected alpha-haemolytic colonies is confirmed by growth inhibition around optochin discs on subculture and solubility in bile salts (36). Culture-based methods have a number of advantages, including the ability to provide antibiotic susceptibility information.

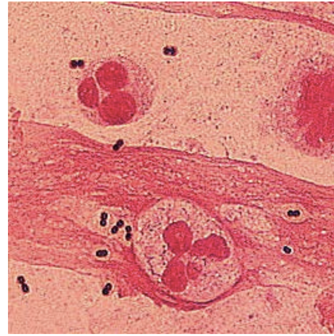


Figure 1.1. *Streptococcus pneumoniae***.**

Gram-stain showing Gram-positive elongated cocci seen as pairs (reproduced with permission from: MicrobeLibrary, American Society for Microbiology, distributed under a Creative Commons Attribution-Noncommercial-Share Alike 3.0 License)

Almost all pathogenic strains of pneumococci express a polysaccharide capsule. Currently, more than 90 immunologically distinct pneumococcal serotypes are recognised (37) on the basis of their polysaccharide capsules (Figure 1.2.) that can also provoke type-specific protective immune responses. Serotypes 6C, 6D and 11E are the most recently discovered (38-40).

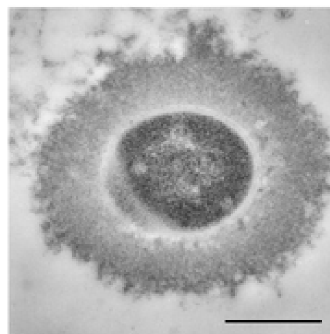


Figure 1.2. *Streptococcus pneumoniae* serotype 3**.**

Transmission electron microscopy image. Scale bar: 0.5 μm. Adapted from Poolman et al. (41) (reproduced with permission from: Elsevier)

Serotypes with chemically similar polysaccharide capsules are classified together into serogroups (there are 46 serogroups) - for example serogroup 6 consists of serotypes 6A to 6D. The genes involved in capsule synthesis are located in a region labelled the capsular polysaccharide synthesis (*cps*) locus (42). There are pneumococcal isolates that cannot be typed due to lack of capsule and are designated non-typeable pneumococci. Lack of capsule production may happen because of down regulation of gene expression of existing functional *cps*

genes or by the acquisition of genetic lesions that render capsule polysaccharide biosynthesis non-functional (43).

S. pneumoniae frequently colonises the nasopharynges of healthy people, particularly young children. Transmission occurs from person-to-person by inhalation of respiratory tract droplets or by direct contact with respiratory tract secretions. Although transmission is very common, clinical illness occurs relatively infrequently among contacts. None-the-less, because it is so ubiquitous, *S. pneumoniae* is a leading cause of disease in young children worldwide (44-46), imposing a significant burden of morbidity and healthcare costs in developed as well as developing countries where it is also an important cause of mortality. Pneumococcus can cause invasive infection including meningitis, sepsis and bacteraemia and is also a common cause of acute otitis media (AOM), pneumonia, sinusitis and conjunctivitis. Occasionally it causes mastoiditis, periorbital cellulitis, endocarditis, osteomyelitis, pericarditis, peritonitis, arthritis and soft tissue infection. Haemolytic-uraemic syndrome can also complicate invasive disease (47). The risk of pneumococcal disease is generally highest among young children, the elderly, immunocompromised patients and people who have chronic medical conditions, such as heart or lung disease, diabetes and asplenia (48, 49). Day care centre attendance among children aged <2 years is a risk factor for invasive pneumococcal disease (50, 51). Most cases of disease are sporadic and outbreaks are uncommon (see section 1.1.1.2) but may occur in closed populations such as nursing homes or DCCs (52-54).

1.1.1.1 *S. pneumoniae* serotypes in nasopharyngeal colonisation

In the pre-PCV era, the rank order of serotypes found in colonisation was reasonably stable across different populations (8, 55). Certain serotypes such as 6A, 6B, 9V, 10, 11, 13, 14, 15, 18C, 19A, 19F, 23F, 33 and 35, accounted for the majority of nasopharyngeal isolates from children; others, like serotypes 1 and 5 were rarely detected (8, 56-58). This tendency of certain serotypes to be undetectable in the nasopharynx may be associated with their low density and duration of carriage (59). Weinberger et al. (60) showed an association between increased carriage prevalence and resistance to non-opsonic neutrophil-mediated killing: the more prevalent serotypes, such as 19F and 23F, were most resistant to killing, while types rarely isolated from carriage, such as types 4 and 5, were more efficiently killed. Serotypes that were resistant to killing, tended to be more heavily encapsulated. An association between polysaccharide structure and

carriage prevalence was also identified - commoner serotypes had fewer carbons per repeat unit and thus lower energy expended on capsule generation. A significant association between metabolic cost and degree of encapsulation and a trend between metabolic cost and resistance to non-opsonic killing was demonstrated (60). Other microbial factors, such as adhesins, toxins and proteins that avoid host immune effectors, are also likely to influence the carriage prevalence of a serotype (61). A model to estimate rates of clearance and competitive ability of different pneumococcal serotypes was used in a longitudinal study in Kenya (62). Children aged 3–59 months were swabbed several times until two swabs were negative for the original serotype. Time to clearance ranged from 28 to 123 days. Among the 27 commonest serotypes, the lowest susceptibility to competition, defined as the rate at which individuals carrying that serotype switch to carry another one, relative to the rate at which that other serotype colonises an uncolonised person, was found in 19F and for this serotype, acquisition of other types was 52% less likely compared to a non-colonised individual. Highly prevalent serotypes had the longest time to clearance and the lowest susceptibility to competition. Duration of carriage declined with age for most serotypes (by 41 months of age, the mean time to clearance was less than half of that for children younger than 22 months) and differences between clearance rates were attenuated as children became older and capable of more rapid clearance of the longest-lived serotypes, postulated to be due to development of immune responses targeted at antigens other than the capsule (62).

It is interesting that serotypes that are less fit, with lower prevalence, higher clearance rates and less competitive ability, can persist at all. While strong immune responses have been shown for some serotypes (individuals who have carried type 14 are ~90% less likely to carry it again), prior carriage appears to be much less protective for others (63-65). There is also evidence for serotype-independent protective acquired immunity that reduces the rate of new colonisation and which is stimulated in young children by previous pneumococcal carriage (66). Cobey and Lipstich (67) used a model to explain the coexistence of pneumococcal serotypes based on serotype-specific and non-specific acquired immunity. Serotypes to which few hosts are immune have a relative advantage that partially compensates for lack of fitness. The authors propose that weak serotype-specific immune responses allow repeated colonisations by the same type and anticapsular immunity alone appeared to be insufficient to overcome

differences in serotype fitness in their model. As described above (62) and demonstrated by Högber et al. (68), as individuals acquire non-specific immunity to pneumococcal non-capsular antigens, the duration of carriage declines and becomes more similar for all serotypes. As consequence of this, the relative advantage of the serotypes with lower clearance rates is reduced, decreasing fitness variation between serotypes and contributing to the higher carriage serotype diversity that is observed in older children and adults (67, 69).

An age-related serotype distribution, with some serotypes (19F, 6B, 6A, 9V, and 23F) peaking at the age of 1–2 years, and others (especially serotypes 3, 8, 10, 11, and 15) peaking at an older age, was described by Bogaert et al. (70).

Antimicrobial resistance also tends to be commoner in certain serotypes. Due to the prolonged nature of carriage compared to invasive disease, selection for antibiotic-resistant strains is likely to occur in the nasopharynx, where bacteria are likely to be exposed to antibiotic selection pressure at lower drug concentrations and also exposed to other species and strains with the ability to pass on antibiotic-resistance genes (71). The frequency of detection of certain serotypes correlates with their likelihood of becoming resistant (59, 72). For example, serotype 1, rarely detected in carriage, remains highly susceptible to antibiotics.

Non-typeable pneumococci have been reported in nasopharyngeal colonisation studies in the USA, ranging from 5% of pneumococcal isolates in Navajo and White Mountain Apache children (73) to 1.8% in children in Massachusetts (74). We previously reported rates of colonisation with non-typeable pneumococci of 5.2% among all pneumococcal carriage isolates in DCCs in Coimbra (75). As these strains do not, by definition, express capsular polysaccharide antigens, they are not subject to anti-capsular immunity either induced by carriage or vaccination. They do express other species antigens and colonisation rates likewise fall with increasing age in childhood (75).

1.1.1.2 *S. pneumoniae* serotypes in invasive and mucosal disease

Although all common serotypes can cause any form of pneumococcal disease, some are more frequently associated with certain presentations than others.

Most paediatric invasive disease around the world was caused by ten serogroups, with 1, 6, 14, 19, and 23 among the most prominent, but with some variations in the proportions in different populations (55). A meta-analysis

showed that the invasive disease potential of a specific serotype, defined as the proportion of carriage episodes that results in a case of invasive disease, was stable over time and geographically (57). However different serotypes had different invasive disease potential - serotypes 1, 5, and 7 were more invasive than 3, 6A, and 15 (57). In an analysis that took into account the prevalence of invasive pneumococcal disease caused by different serotypes in a community to the extent of exposure of that population to these serotypes, it was found a significant inverse correlation between invasive disease and carriage prevalence, with the most invasive serotypes being the least commonly detected in carriage and the most frequently carried the least invasive (57).

A review of pneumococcal serotype distribution showed that the most common serotypes causing AOM globally in children aged <18 years were 3, 6A, 6B, 9V, 14, 19A, 19F and 23F (76). Unlike for invasive disease, there are only small differences between the propensities of different serotypes to cause AOM, most causing it at a frequency that is proportional to their prevalence in nasopharyngeal carriage (77).

Due to the difficulty in obtaining adequate samples for culture in pneumonia, the identification of aetiological agents has remained a challenge. A study to estimate pneumococcal serotype-specific disease potential in paediatric community-acquired alveolar pneumonia, compared nasopharyngeal pneumococcal colonisation during disease with colonisation in healthy children, reporting that serotypes 1, 5, 22F, 7F, 14, 9V and 19A had higher odds of being carried during radiologically diagnosed community-acquired alveolar pneumonia compared to healthy controls, with lower odds for serotypes 6A, 6B, 23A, and 35B (78). A review of serotype-specific properties reported a high proportion of complicated pneumonia cases being caused by serotypes 1 and 3 (79).

Serotypes also differ in the age distribution of those affected and their tendency to cause outbreaks. The incidence of disease caused by serotypes 1 and 5 remains constant or even increases slightly after 2 years of age, in contrast to the pattern followed by other common serotypes causing invasive disease. This is observed in settings with a high incidence of serotype 1 (Alaskan natives, South Africa, and Israel) and also in lower incidence settings (USA and Western Europe) (79). Outbreaks are both caused by serotypes that tend to be frequent colonisers of the nasopharynx (i.e., 4, 9V, 14, and 23F) and by the more rarely carried types 1 and 3 (79), suggesting that independent properties drive the phenomenon in the two distinct scenarios.

Long-term secular trends can also result in changes in serotype prevalence (80, 81). Serotype 1, for example, has shown long-term fluctuations (82, 83). A study of historical trends (1928-1998) in serogroup distribution in the USA, showed that the proportion of paediatric pneumococcal infections caused by serogroups 4, 6, 9, 14, 18, 19 and 23 increased significantly from 53% to 87% while infections caused by serogroups 1–3 and 5 decreased significantly from 18% to 2%. These differences could be due to patterns of antibiotic use, socioeconomic conditions, the immunocompromised status of populations and also be confounded by changes in blood-culture practices (71). Another analysis of temporal trends (1938-2007) in pneumococcal invasive disease and serotypes in Denmark, a country with low antibiotic use, showed that some serotypes/serogroups also increased (4 and 9), others decreased (18C), while others remained stable (6, 7F, 14, and 23F) and serotype 2 nearly disappeared. Before the 1960s, serotypes 1, 2, 3, and 5 peaked every 2-3 years. Peaks became less frequent during the 1970s, occurring every 7-10 years (83).

Non-typeable pneumococci have been associated with conjunctivitis in outbreaks or sporadic cases (84, 85). Occasionally, they have been implicated in other types of pneumococcal disease, such as otitis, respiratory infections and invasive disease (43, 84, 86). Between 2001 and 2006, 3% of invasive disease isolates from Navajo children and adults, were reported to be non-typeable (87). Recently, a microarray serotyping method was applied to study non-typeable isolates identified by the Quellung reaction from invasive disease in children and showed that, although invasive disease caused by "true" non-typeables that lack capsule gene loci can occur, it is very rare. Most of the non-typeable invasive isolates studied by the microarray, had complete *cps* genes for known serotypes present, suggesting that capsule production was fully down regulated *in vitro*, resulting in the negative Quellung reactions (43).

1.1.2 *Haemophilus influenzae*

H. influenzae is a Gram-negative coccobacillus, variable in length with marked pleomorphism and with random arrangements (Figure 1.3.). It is a fastidious organism that requires factors that are present in blood, specifically haemin (X factor) and nicotinamide-adenine-dinucleotide (NAD, known as V factor) for growth. Samples are commonly inoculated onto Bacitracin chocolate agar plates and incubated at 35-37ºC in 5% CO_2. Identification of suspect colonies

is confirmed using manufactured discs with X and V factors in the base plate. *H. influenzae* appear as round, colourless-to-grey, opaque colonies (36, 88).

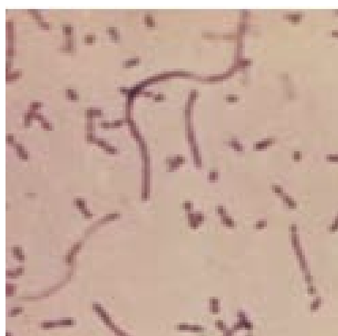


Figure 1.3. *Haemophilus influenzae*.
Gram stain showing Gram-negative coccobacilli, with no specific arrangement (reproduced with permission from: Kenneth Todar, PhD)

There are encapsulated strains that express antigenically distinct capsular polysaccharides (a to f) and non-encapsulated strains designated non-typeable.

The nasopharynx is the natural habitat of *H. influenzae* in humans. Transmission occurs through contact with respiratory tract or oral secretions.

H. influenzae type b (Hib) was a major cause of meningitis, bacteraemia, pneumonia, epiglottitis, septic arthritis, cellulitis, AOM, and other less common infections, such as endocarditis, osteomyelitis and peritonitis. Non-type b encapsulated *H. influenzae* can, more rarely, cause disease similar to type b infections. Non-typeable strains cause infections of the respiratory tract and, less often, bacteraemia and meningitis (89).

The widespread use of conjugate Hib vaccines in infancy has resulted in a dramatic decline in invasive Hib disease in children. There is no evidence of substantial replacement disease with non–b encapsulated *H. influenzae* in young children subsequent to widespread vaccine use (90). Most of the residual disease burden, which is caused by non-b *H. influenzae*, occurs in the youngest and oldest age groups and those with underlying immunocompromising conditions (90, 91).

1.1.3 *Moraxella catarrhalis*

M. catarrhalis is a Gram-negative coccus occurring singly or in pairs (Figure 1.4.). For culture, samples are commonly inoculated onto non-selective blood agar plates and incubated at 35-37ºC in 5% CO_2. Suspect colonies are smooth,

flat, uniform, buff colonies, 1-2 mm in diameter and oxidase-positive. Identification is confirmed by detection of butyrate esterase activity by a positive tributyrin test (92).

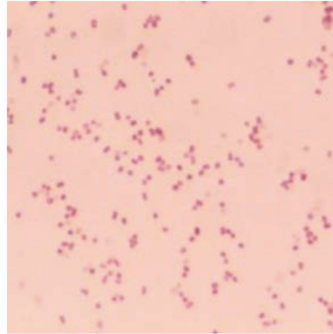


Figure 1.4. *Moraxella catarrhalis.*
Gram-stain showing Gram-negative cocci and diplococci (reproduced with permission from: Public Health Image Library (PHIL), Center for Disease Control and Prevention; ID#15012)

Exclusive to humans, it is part of the normal flora of the upper respiratory tract and is rarely pathogenic. Transmission is presumed to occur through direct contact with respiratory or oral tract secretions. Symptomatic infections are most common in infants and young children and include AOM and sinusitis. In patients with chronic lung disease or immunodeficiencies it can also cause bronchopulmonary infections. Rare manifestations include bacteraemia, endocarditis, pneumonia, preseptal and orbital cellulitis, osteomyelitis, septic arthritis and neonatal meningitis (30). Unlike *S. pneumoniae*, *H. influenzae* and *S. aureus*, *M. catarrhalis* rarely causes bacteraemic illness.

1.1.4 *Staphylococcus aureus*

S. aureus is a Gram-positive coccus, occurring singly, in pairs, tetrads and in irregular clusters (Figure 1.5.). They grow on a variety of non-selective agars when incubated in 5% CO_2 at 35-37ºC and are capable of growing on selective agars containing high concentrations of sodium chloride. Colonies are opaque and may be white or cream and are occasionally yellow or orange. They are catalase positive and oxidase-negative (do not contain cytochrome c oxidase). There are three major characteristics that in combination differentiate *S. aureus* from coagulase negative staphylococci. Firstly, identification can be made by agglutination on Latex tests, which confirms the presence of bound coagulase (although some coagulase negative staphylococci can also give a positive result). Secondly, a positive coagulase test detects the presence of free coagulase. Finally, *S. aureus* produces a DNAase that is detected by incubation of the suspect colony

on a DNAase plate, which then produces a zone of clearance around the organism when the plate is developed with hydrochloric acid (36, 93).

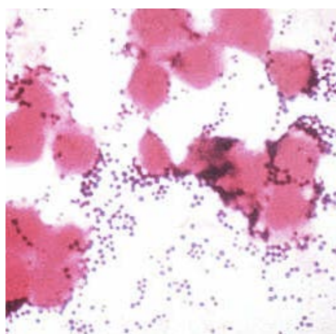


Figure 1.5. *Staphylococcus aureus.*
Gram stain showing positive cocci forming irregular clusters (reproduced with permission from: Kenneth Todar, PhD)

S. aureus colonises epithelial surfaces, of which the anterior nares are the most frequent carriage sites but can also commonly be found on the skin. Colonisation is associated with a higher risk of infection, with more than 80% of health care-associated *S. aureus* infections being endogenous (94-97). S*. aureus* is an important cause of skin and soft tissue infections, pneumonia, bloodstream infections, bone and joint infections, meningitis, endocarditis and urinary tract infections in both children and adults.

1.1.5 Respiratory viruses

Viral respiratory infections are very frequent in young children, peaking in the first two years of life (98). They show seasonality, more clear for some virus (respiratory syncytial virus and influenza virus) than others (rhinovirus), and DCC attendance is a major risk factor for those infections (98). The most common symptoms and signs are nasal congestion, runny nose, cough and sneezing (99).

Human bocavirus, polyomaviruses, respiratory syncytial virus (A and B), human influenza virus A and B, parainfluenza virus 1–4, picornaviruses (human rhinoviruses and enteroviruses), adenovirus, human coronavirus and human metapneumovirus are well known causes of respiratory infections and can frequently be detected in nasopharyngeal samples of children with respiratory symptoms but also in children without clinical manifestations, as shown in several studies (10-16, 100-103).

Despite variations in the relative proportions of different viruses causing respiratory infections, depending on factors such as age, season, sampling and detection methods, rhinoviruses have been consistently found to be the most common in all age groups (98). Results of virological and serological analysis in a cohort of children during the first 2 years of life showed that, by the age of 6 months, more than 20% had had at least one episode of rhinovirus detection. By the age of 2 years, rhinovirus infection had been documented in 79% and 91% of the children had antibodies to it (104).

The use of highly sensitive molecular techniques, such as polymerase chain reaction (PCR), has greatly improved detection of these viruses. With this method, a pair of synthetic oligonucleotides or primers hybridises to a single stranded deoxyribonucleic acid (DNA) target (generated by reverse transcription in the case of RNA viruses), with the pair spanning a region that will be exponentially reproduced. The hybridised primer acts as a substrate for a DNA polymerase that creates a complementary strand via sequential addition of deoxynucleotides. With real-time PCR, in contrast to conventional assays, the detection of the amplicon can be visualised using fluorescently labelled probes which release the label from an adjacent quencher on polymerisation so that as the amplification progresses the emergence of the target can be accurately detected by association with a number of cycles ("viral load"). Multiplex real-time PCR uses multiple primer pairs to allow amplification of multiple templates within a single reaction (with multicolour fluorescent labelling) (105). However, despite all the benefits of these methods, they can also make the interpretation of results more difficult, raising the question whether the presence of small amounts of viral nucleic acid has clinical relevance. One prospective case-control study using PCR in symptomatic and asymptomatic young children, confirmed that asymptomatic carriage of respiratory viruses occurs frequently although viral loads were higher in cases than in controls for all viruses. In contrast to rhinovirus, respiratory syncytial virus was rarely detected in asymptomatic children, suggesting that a positive test is almost always of clinical relevance, independently of the viral load. Accordingly, a causal inference based on the detection of rhinoviruses in symptomatic patients should be made with caution and assessment of viral load could potentially assist the interpretation of positive results. The establishment of cut-off levels for diagnosing significant infections using these molecular tests has been proposed (15). A study of viral respiratory infections in hospitalised and community control children in Alaska also showed that respiratory syncytial virus,

parainfluenza virus, human metapneumovirus and influenza virus nucleic acid detection was significantly more common in hospitalised cases than controls. Rhinovirus and adenovirus were detected in two-thirds of hospitalized children, but their frequent detection in control children made their role in respiratory hospitalisation uncertain. Respiratory syncytial virus and human metapneumovirus virus detection were associated with more severe illness (106).

The presence of viruses in the nasopharynx of asymptomatic children may have several explanations: it may be detected during the incubation period, the presence of minor respiratory symptoms may be missed or ignored by the parents, it may indicate a subclinical infection in which the presence of a low viral load may only trigger a minimal inflammatory response without symptoms or the presence of the virus may be detected at the end of an expiring infection after symptoms have resolved (12, 15, 107).

1.1.6 Methods for detecting nasopharyngeal colonisation

The sensitivity of detection of *S. pneumoniae* colonisation depends on the sampling technique used, the handling of the specimen and the methodologies used to culture and/or identify and serotype the organism, all of which can therefore contribute to variability between observed rates of carriage. The World Health Organization (WHO) has established standardised methods for the study of pneumococcal nasopharyngeal colonisation, intending to reduce or eliminate such artefactual variation (108, 109).

It is recommended that samples should be deep nasopharyngeal swabs. For studies in children, swabs should be of paediatric size with a calcium alginate or a Dacron polyester tip and a flexible shaft. The type and supplier of the swab should be consistent throughout the study. Once obtained, the swab can be immersed in 0.5 or 1mL of skim milk-tryptone-glucose-glycerin (STGG) broth, a transport medium the use of which has been validated against the standard method of direct inoculation of the nasopharyngeal swabs onto culture plates (110, 111). There are several benefits of using STGG broth over direct plating: the possibility of long term storage of the original specimen at -70ºC; the opportunity to inoculate multiple plates from the original sample and to conduct multiple assays on a single specimen; with homogenous dispersion of the nasopharyngeal specimen in the broth sample, the ability to quantitate the growth of organisms; transport of nasopharyngeal specimens from the site of collection to a distant laboratory; and cost-efficient laboratory analysis of large numbers of

nasopharyngeal specimens in batches. STGG is inexpensive and remains stable for at least 6 months after sterilization (110). After collection, samples containing swabs should be maintained and transported on wet ice to the laboratory within 8 hours. Storage should be at -70ºC. Samples can be kept for long periods of time up to 2 and even 6 years after collection (112).

The culture techniques used in this project are described in sections 1.1.1, 1.1.2, 1.1.3 and 1.1.4. The isolates are also stored in glycerin at -70ºC.

Conventional serotyping can be done from purified isolates immediately after culture or recultured after being stored frozen.

Detection of respiratory viruses by PCR assay of nasopharyngeal swabs stored in STGG is an alternative approach to using nasal aspirates stored in viral transport medium, with similar sensitivity and specificity (113). Thus a single swab can permit both detailed bacteriological and virological analysis.

1.1.6.1 Pneumococcal serotyping – new techniques to detect co- colonisation

The Quellung reaction, that involves mixing a loopful of a pure pneumococcal culture with an equal quantity of specific antiserum and then examining microscopically for capsular swelling (Figure 1.6.), provided the basis for the development of widespread serotyping (114).

It has been recognised for a long time that simultaneous colonisation with different pneumococcal strains and serotypes can occur (115, 116). However, most studies have relied on conventional culture techniques and serotyping individual colonies, detecting only the most abundant ones and thus underestimating simultaneous colonisation with multiple serotypes. Detection of co-colonisation is important for understanding vaccine effectiveness, transmission, replacement and opportunities for horizontal gene transfer that may lead to change of the capsular serotype or acquisition of antibiotic resistance. Several methods for detection of multiple serotypes have been proposed, including swab enrichment culture followed by Quellung typing (117) or multiplex PCR (118) from the broth, immunoblot (119) or multiplex PCR (120) from the primary culture plate, multiplex PCR direct from the nasopharyngeal swab transport medium (121) and pneumolysin non-coding region (plyNCR) PCR followed by terminal restriction fragment length polymorphism (RFLP) determination direct from the swab (122). When more sensitive methods are applied, the rate of multiple *S. pneumoniae* serotypes found in the same sample ranges from 10–50%: it has been shown to be relatively common in areas with a

high burden of *S. pneumoniae* colonisation and disease, however many studies report rates at the lower end of that range (118, 120, 122-126).

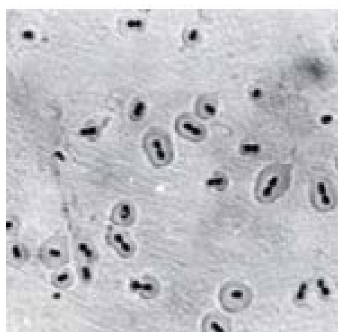


Figure 1.6. Quellung reaction.
Capsular swelling demonstrating the presence of a specific capsular type (reproduced with permission from: Kenneth Todar, PhD)

In 2009, Hinds et al. (127) presented a new microarray detection and serotyping method, based on genomic DNA hybridisation, that is able not only to detect but also quantify DNA from multiple serotypes in the same sample. Molecular serotyping is performed on the DNA extracts using the B_G@S SP-CPS v1.4.0 microarray (Bacterial Microarray Group at St. George's, University of London, London, United Kingdom - B_G@S; http://bugs.sgul.ac.uk). An evaluation of methods to facilitate direct analysis of nasopharyngeal swabs by microarray showed a reduction in sensitivity for detecting additional serotypes at low abundance when directly analysing DNA from the broth, associated with low amounts of DNA recovered, the bacterial load of the pneumococcus and other pathogens and the presence of contaminating host DNA. The method has evolved to culturing STGG prior to DNA extraction, as an initial enrichment and amplification step to increase yields of pneumococcal or bacterial DNA prior to microarray analysis (128). The microarray includes reporters to represent all *cps* genes involved in capsule polysaccharide biosynthesis of the serotypes known to date. The serotype is determined by the combination of *cps* genes found to be present in the isolate. This method detects *S. pneumoniae* with full complement of *cps* genes required to produce the polysaccharide capsule (serotypes) and is also able to detect the presence of the collectively called non-typeables that include non-encapsulated pneumococci (usually *S. pneumoniae* lacking any *cps* genes that are found in carriage such as NT2, NT3, NT4 and also *S. pneumoniae* containing incomplete *cps* gene complement for serotype so also non-encapsulated) and closely related *Streptococcus* species such as *S. mitis*, *S. oralis*,

S. pseudopneumoniae that may contain homologues of *cps* genes. Identifying non-pneumococcal streptococci using microarray is not uncommon in pneumococcal carriage studies (43), which is also important because they may contribute *cps* or antimicrobial resistance genes to the pool available for genetic exchange in the nasopharynx. A recent study using this methodology in children and adults showed that colonisation with multiple strains of *S. pneumoniae* was associated with higher overall density of nasopharyngeal carriage and was more frequent at younger age (125). Another study showed no association of multiple colonisation with age and a pattern that often consisted of a dominant and one or more minor serotype populations (129). A comparison of nasopharyngeal colonisation by use of conventional methods (WHO protocol) (108, 109), latex agglutination and molecular serotyping by microarray, showed that co-colonisation detection rates were lower for the first (11.2%), significantly underestimating multiple-serotype carriage, and similar between microarray (48.8%) and latex agglutination (43.2%). However, latex agglutination failed to detect serotypes at low abundance. The authors concluded that microarray serotyping, although more costly and technology dependent, is more sensitive for such low-density serotypes (123).

1.2 The determinants and dynamics of nasopharyngeal colonisation

Several factors determine whether individuals within a population carry a microorganism in their nasopharynges. First whether, how often and at what dose they are exposed to it. Second, how resistant they are to it as it arrives, which may be affected by epithelial integrity and thus also physical environmental conditions (which may also directly affect the viability of the organism) and both innate and specific existing mucosal immunity. Third, how efficient they are at eliminating it once carriage is established, principally through development of specific immunity, in concert with innate immune mechanisms. The presence or absence of other microorganisms and antimicrobial drugs within the niche may also affect this process. All the factors known to be associated with colonisation operate through one or more of these mechanisms. Additionally, when comparing studies, different sampling techniques and laboratory methodologies need to be taken into account because they can be responsible for differences in reported colonisation rates (8, 18).

Any individual is likely to be colonised with potential pathogens many times during his or her life (8). Children usually acquire several different strains over time. In a 10-month longitudinal household study of *S. pneumoniae* nasopharyngeal carriage, in the 0–2 year olds, 39% were found to carry pneumococcus more than five times during the follow-up period (130). Another 1-year longitudinal study with 11 sampling periods, performed among 47 children attending a single DCC, reported that children were sequentially colonised with up to five *S. pneumoniae* serotypes and nine *H. influenzae* clones, suggesting a high rate of acquisition and turnover of strains (131).

The mean age of first acquisition of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* is 6 months (1–30 months) (5, 18, 132), although in certain populations *S. pneumoniae* can be found as early as the first month (63) and even on the first day of life (133). In developing countries and indigenous populations children universally acquire pneumococcus in the first few months of life (109).

The duration of *S. pneumoniae* carriage varies between serotypes, previous immunological exposure and the host's age and immunocompetence (see section 1.1.1.1). A longitudinal study in Thailand showed a median duration of 31 days in adults and 60.5 days in children (134). However, duration periods of more than 30 weeks have been observed (135) and a recent African paediatric study using intensive sampling, found a mean duration of carriage of 31.3 days, with serotype specific means ranging from 6.7 to 50 days (136). Colonisation with the initial strain of non-typeable *H. influenzae* was reported to persist from 1-5 months with a median duration of 2 months (137).

Nasopharyngeal carriage studies in children have been conducted in various settings and populations around the world, reporting different rates, summarised in Tables 1.1., 1.2. and 1.3. The studies were selected to represent different regions of the world.

Table 1.1. Selected studies of rates of *S. pneumoniae* nasopharyngeal carriage in children.

Country	Study population	Year	Carriage rate
USA (Buffalo) (4)	306 healthy children or with otitis media; 1Y	1997	54%
Sweden (138)	1129 healthy children; 2-7Y 2Y/4Y/7Y	1995	50%/42%/21%
Portugal (139)	586 healthy children; DCC; 6M-6Y	1996	47%
Greece (140)	1269 healthy children; 2-23M	1997-98	33%

The Netherlands (141)	259 healthy children; DCC; 3-36M	1999	58%
Israel (142)	264 healthy children; DCC; 12-23M	1996-97	78%
Asia and Middle East (143)	4963 healthy children; 5Y	1998-99	22.3%
Indonesia (144)	484 healthy children; 12-25M	1997	51%
India (145)	100 healthy children; 6W-18M	2001	81%
Uganda (146)	191 healthy children; <3Y	1995	62%
Gambia (147)	102 healthy children; 46M	2001	87%

DCC: Day Care Centre; **M:** months; **W:** weeks; **Y:** years

Table 1.2. Selected studies of rates of *H. influenzae* nasopharyngeal carriage in children.

Country	Study population	Year	Carriage rate
USA (Buffalo) (4)	306 healthy children or with otitis media; 1Y	1997	33%
Sweden (138)	635 healthy children; <7Y	1995	32%
Portugal (139)	586 healthy children; DCC; 6M-6Y	1996	72%
The Netherlands (141)	259 healthy children; DCC; 3-36M	1999	37.4%

DCC: Day Care Centre; **M:** months; **W:** weeks; **Y:** years

Table 1.3. Selected studies of rates of *M. catarrhalis* nasopharyngeal carriage in children.

Country	Study population	Year	Carriage rate
USA (Buffalo) (4)	306 healthy children or with otitis media; 1Y	1997	72%
Sweden (138)	635 healthy children; <7Y	1995	42%
Portugal (139)	586 healthy children; DCC; 6M-6Y	1996	54%
The Netherlands (141)	259 healthy children; DCC; 3-36M	1999	81.6%

DCC: Day Care Centre; **M:** months; **W:** weeks; **Y:** years

Several factors are associated with colonisation (4-7, 18, 137). It varies widely with age - *S. pneumoniae, H. influenzae* and *M. catarrhalis* are all more prevalent in children than in adults.

For *S. pneumoniae* the rates of nasopharyngeal colonisation rise from birth, peaking at 50% to >70% around 2 years of age (1, 8). Thereafter, an age related decline is observed starting gradually after the age of 3-5 years in developed countries, to a stable colonisation rate of around 5-10 %, which is reached after the age of 10 years (130, 148, 149), but that can be as high as 25–60% in some developing countries (150, 151) and as low as 1% in Finland (152) and Sweden (153). In developing countries and settings with high disease burden, high *S. pneumoniae* carriage rates persist further into childhood (73, 150).

For *H. influenzae* and *M. catarrhalis*, a study in the USA evaluating nasopharyngeal carriage during the first year of life showed that, at 6 months of age, 9% were colonised with *H. influenzae* and 26% with *M. catarrhalis* and at 1 year of age these percentages increased to 33% and 72% respectively (4). The overall isolation rates for pre-school children, school children and adults found in Sweden were respectively: 13%, 6% and 3% for *H. influenzae* and 27%, 4% and 2% for *M. catarrhalis* (153).

Higher carriage rates in younger children could be explained by close contacts with other young children (often attending DCCs), associated with poorly developed immunity to these pathogens. As discussed in section 1.1.1.1, serotype-specific antibodies to the capsular polysaccharide and non-capsule-specific immune responses are generated in response to carriage of *S. pneumoniae* (63-66, 154). The latter provides a potential biological explanation for the fact that serotype-specific duration of carriage shortens with age. Children, in particular those <2 years of age, are much less likely to develop an immune response to carriage (155, 156).

Colonisation rates with *S. pneumoniae* and *S. aureus* seem to follow opposing age-related trends, the causative mechanisms of which remain controversial and uncertain (25, 70, 157). *S. aureus* nasal colonisation is very common among newborns and decreases rapidly during the first year, while pneumococcus is rare at birth and increases significantly during the first year of life (157). Serial nasal swabs collected in 443 children in The Netherlands showed that the prevalence of *S. aureus* carriage decreased from 52.1% at the age of 1.5 months to 12.9% at 14 months (157). In adulthood, *S. aureus* is found in the nares of up to half of the adult population (158). About 20% of the adult population can carry the same strain for extended periods of time (persistent carriers) and 30% host different strains over time (intermittent carriers) (94, 159). Ten percent of children from 0 to 9 years old and 24% from 10 to 19 years old

were also found to be persistent carriers (160). In a paediatric study that defined persistent carriage as continuous carriage of the same *S. aureus* strain at 1.5, 6, and 14 months, this was rarely found in early infancy (157).

Other independent risk factors that also seem to predict colonisation in healthy populations include:

– Ethnicity (161, 162): African American, native American (Apache and Navajo) and Alaskan native populations are at increased risk of pneumococcal colonisation;

– Socio-economic and environmental factors (5, 8, 18, 163-165): these include family size, income, smoking and recent antibiotic use. Colonisation rates with *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* are higher among infants with siblings (132). The number of siblings and overcrowded living conditions facilitate horizontal transfer of bacteria from one person to another. Parental smoking and passive smoke exposure in general may damage the nasopharyngeal mucosa and impair immune responses, increasing the susceptibility to viral infections and bacterial colonisation (18, 166). The number of older siblings, family size and passive smoking, also identified by some authors to be associated with *S. aureus* nasal colonisation rates (167), were not confirmed by others (157). Antibiotics induce several changes in nasopharyngeal flora. Lower isolation rates of potential pathogens have been observed during and soon after (first weeks) antibiotic treatment (148). However, rapid replacement occurs with either overgrowth of more resistant strains or by newly acquired strains. Antibiotics can also affect the balance between pathogenic bacteria and commensals (18).

– Crowding (8, 18, 141, 163, 168, 169): this has been reported as a major risk factor for carriage and spread of pneumococcus. In young children, DCC attendance, because of closer interpersonal contact, facilitates transfer of bacteria from one child to another. A study investigating colonisation among children attending DCCs and children who did not attend DCCs, showed a 1.6 to 3.4-fold increased risk for nasopharyngeal carriage among the former. Genetic analysis of the pneumococcal isolates revealed 75% clustering among pneumococci isolated from DCC attendees versus 50% among those not attending DCCs, indicating a higher risk for horizontal spread of pneumococci in DCCs than in the general population (141). In Finland, DCC attendees and their family members were followed for 9 months, with monthly sampling of the nasopharynx. For children, the majority of acquisitions, with documented

exposure to homologous pneumococci, resulted from exposure in DCC and much less in the family (170). Studies performed in Portugal showed that the likelihood of being colonised with pneumococcus was higher in larger nurseries (75) and that colonised children shared *S. pneumoniae* and *H. influenzae* clones with others in the same DCCs (131).

1.3 The relationship between nasopharyngeal colonisation and disease

Posterior to the nasal cavity, superior to the oropharynx and connected to the middle ear by the Eustachian tube, the nasopharynx has connections with the mouth, nose (and thence the outside world) and the sinus cavities, middle ear and lower respiratory tract, common sites of symptomatic and clinically significant disease. This complex ciliated epithelial structure is the niche for many microbes, in an environment which includes air (and its gaseous, particulate and dissolved constituents including water), tethered and free mucins and the cells and products of the innate and specific immune systems (34) (Figures 1.7. and 1.8.).

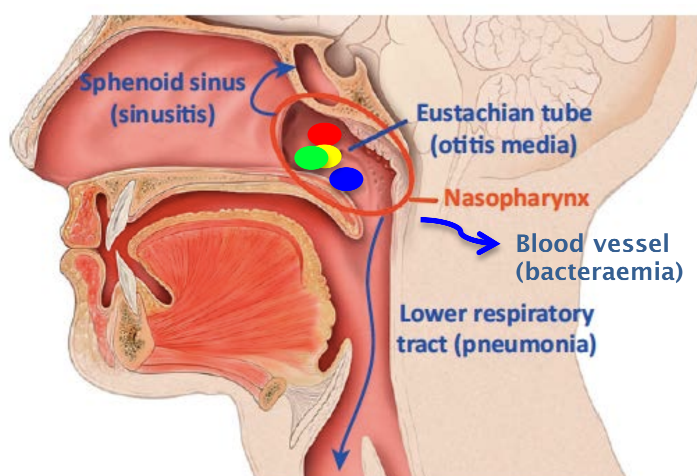


Figure 1.7. Anatomy of the nasopharynx.

Reproduced with permission and slightly modified from: image as adapted by Shak et al. (34) from an illustration by Patrick J. Lynch distributed under Creative Commons Attribution 2.5 License

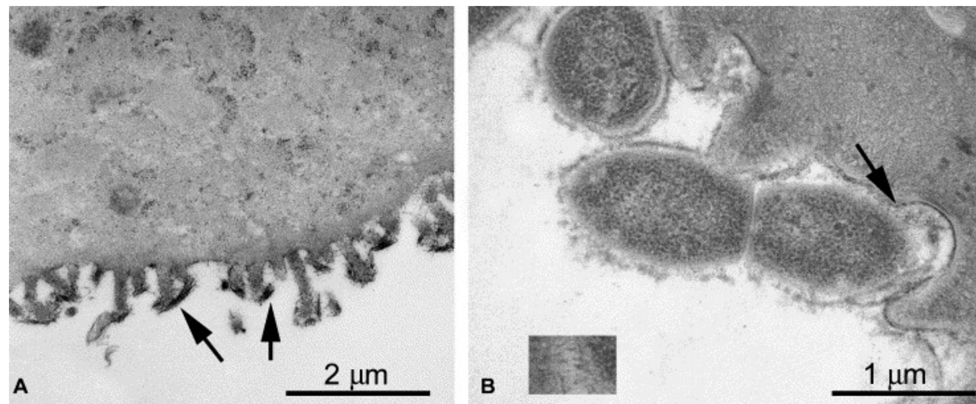


Figure 1.8. Nasopharyngeal epithelial cell and *S. pneumoniae* attachment to the cell.
A) Electron microscopic image of a nasopharyngeal epithelial cell; **B)** Transmission electron micrograph of pneumococcal attachment to nasopharyngeal epithelial cells. Tonnaer et al. (171) (reproduced with permission from: Elsevier)

Although most commonly associated with health, developing (acquisition) or established nasopharyngeal colonisation is a precondition and thus a necessary initial step in the development of disease. For example, pneumococcus can migrate to the ear to cause AOM, to the sinus to cause sinusitis, to the lower respiratory tract to cause pneumonia, and in some cases into the blood vessels through an epithelial surface to cause bacteraemia and thence invasive disease (172-174). However this happens only in a small percentage of children who are colonised.

There are ecological associations between *S. pneumoniae* colonisation and disease. The peak incidence of bacterial AOM happens between 6 and 18 months of age, when the highest rates of *S. pneumoniae* colonisation occur. Studies of children with invasive pneumococcal disease conducted in Pakistan and The Gambia showed that more than 94% of children with invasive pneumococcal disease simultaneously carried *S. pneumoniae*, compared to 52% and 76% respectively in healthy controls (175, 176).

Experimental challenge studies in animal models show that the nasal inoculation of pneumococci can lead to otitis media (177) or invasive disease (178, 179). A temporal relationship between *S. pneumoniae* acquisition and disease manifestation has been shown in prospective studies in children (35, 180-182). Gray et al. (180) followed 82 infants from birth up to 2 years of age, with serial throat swabs, concluding that symptomatic infection usually occurred within 1 month of acquisition of a new serotype and was rarely associated with prolonged carriage. Another study assessing 329 infants, followed from 2 months

up to 2 years of age, with scheduled visits every 3 months up to 18 months of age, also showed that the majority of *S. pneumoniae* AOM events developed in association with newly acquired pneumococcus (35). In a third study, 213 children were swabbed nine times between 2 weeks and 6 months of age showing that pneumococcal acquisition was significantly associated with office visits for non-specific respiratory infections (182). It was also reported that, at the time of *S. pneumoniae* infections such as AOM and pneumonia, there is higher prevalence of carriage among the affected children (148, 183), although others did not find such association (184, 185). Data on the association between colonisation density and disease in children are limited. High-density ($\geq 10^6$ colony-forming units/mL) nasopharyngeal colonisation with any bacterial pathogen in culture was more common in Vietnamese children with pneumonia (49%) than among children with acute bronchitis (29%) or healthy children (17%) (186). Recently Vu et al. showed that the median bacterial load of *S. pneumoniae* in nasopharyngeal samples, measured using a multiplex PCR, was substantially higher in children with radiologically confirmed pneumonia compared with healthy controls or children with other lower respiratory tract infections, although no clinically useful cut-offs could be established for the diagnosis of paediatric pneumonia (184). Furthermore, a recent study in adults showed that patients with community-acquired pneumonia had higher density nasopharyngeal colonisation than asymptomatic controls, based on bacterial cultures and molecular methods, compatible with the hypothesis that higher bacterial loads in the nose may increase the risk of microaspiration thus resulting in lobar pneumonia (187). Although *S. pneumoniae* can be cultured from blood in a minority of children presenting with bacterial pneumonia, nevertheless, it is probable that, in most and perhaps all cases of pneumonia and AOM, the organisms first reaches the lung and the ear by spreading from the nasopharynx, overcoming the mucociliary and innate and specific immune protective mechanisms that are in place to prevent this. The transition from asymptomatic carriage to disease may happen at a critical nasopharyngeal colonisation density. We can speculate that high-density colonisation may be occurring either in an unstable early phase of colonisation not long after acquisition or later, coincident with respiratory viral infection, in an effort to transmit and that, in the latter case, micro-aspiration or migration to normally sterile sites and thus disease may be a by-product of the transmission phase of the organism's life cycle. A mechanistic explanation for this relatively rare occurrence, in the context of high prevalence of nasopharyngeal bacterial

colonisation, might lead to new strategies to prevent pneumonia and other bacterial infections.

1.4 Nasopharyngeal colonisation and transmission to the community

Colonised children serve as reservoirs for person-to-person transmission in the community (8, 96, 180, 188).

Crowding, as occurs in DCCs, hospitals and prisons, increases the risk of horizontal spread of potential pathogens (163, 188-190). The high rates of pneumococcal colonisation and the crowding index found in young children are thought to be the most important factors for transmission of pneumococcal strains within the community (191). A prospective study conducted in Israel to determine the role of DCCs in the spread of *S. pneumoniae* to the community in general, evaluated the association between pneumococcal colonisation among young children cared for at home and among their older siblings who attended DCCs. The similarity between strains from the older and the younger siblings' isolates was noticeable and this was not found when isolates from other DCCs were compared (188). In a Finnish study of DCC attendees and their family members, it was shown that 66% of acquisitions of a new serotype in a family were associated with simultaneous or previous carriage of the same type in the child attending the DCC. Pneumococcal transmission was found to take place, in effect, as "micro-epidemics" driven by the DCCs (170). In a population-based cohort study, a correlation between the colonisation statuses of mothers and children was observed for *S. aureus* and *H. influenzae* (158).

The nasopharynx is a major source of secretions. It is known that viral infections are the principle cause of most wintertime rhinitis in children and adults alike. This biological theory leads us to hypothesise that the efficiency of transmission of nasal bacteria may be enhanced by intercurrent rhinitis, when secretions, coughing and sneezing are increased.

1.5 Pneumococcal conjugate vaccines

S. pneumoniae is an encapsulated bacterium against which vaccine-induced protection can be conferred by antibodies directed against the polysaccharide capsule.

After a trial with a killed whole-cell vaccine in 1911, the first purified

capsular polysaccharide vaccine was tested in the USA in the 1940s (192). Following these results, other preparations were formulated. The currently available pneumococcal polysaccharide vaccine (Pneumovax23®, Merck), contains purified capsular polysaccharides from 23 different serotypes (Table 1.4). Pure polysaccharide vaccines generate a T cell-independent, antibody-mediated response. Antibody responses to most pneumococcal capsular types are poor or inconsistent in children aged <2 years whose immune systems are, for unknown reasons, largely ineffective against these antigens (193). A double-blind, randomized, controlled trial of a 14-valent *S. pneumoniae* polysaccharide vaccine, showed lack of demonstrable benefit in young Australian children (194). Although it can induce mucosal immune responses (195), pneumococcal polysaccharide vaccine is thought to have little or no effect on pneumococcal carriage (196, 197). It also fails to induce immunological memory (198). This vaccine has been widely recommended for adults over 65 years and, despite little or no evidence of clinical efficacy, for children older than 2 years of age with high-risk medical conditions including immunosuppression, asplenia, chronic heart, lung and liver diseases, diabetes mellitus, cerebrospinal fluid leaks and cochlear implants on the basis that it provides broader serotype coverage than conjugate vaccines.

Conjugating the polysaccharide antigens to a carrier protein, recruits T-cell-help for B cell mediated immune responses, stimulating serotype-specific antibody production and immunological memory that can be demonstrated in early infancy, providing protection against disease caused by serotypes included in the vaccine. The first PCV, a seven-valent vaccine (PCV7) (Prevenar®, Wyeth), was licensed in the USA in 2000 and in Europe in 2001. The vaccine included polysaccharide antigens for the seven most common serotypes causing invasive disease in children in the USA (55), conjugated to a protein carrier CRM197, a non-toxic mutant of the diphtheria toxin (Table 1.4). It demonstrated clinical efficacy against vaccine-serotype invasive disease in two randomised, double blind, clinical trials in infants (199, 200). Immunogenicity studies demonstrated that infants receiving three doses 2 months apart (at 2, 4, and 6 months of age), successfully developed antibodies to all seven serotypes; studies including booster doses of polysaccharide at 12-15 months demonstrated that it could also induce immunological memory (201). The estimated vaccine coverage for invasive pneumococcal infections in children ≤5 years of age in Europe was below the estimates for the USA, ranging from 53.8% in a Spanish study to 85% in Denmark

(202, 203). Based on invasive disease isolates from 1999-2002, the potential coverage of PCV7 among infants in Portugal was 63.2% (204).

Since the introduction of PCV7, WHO recommends that approval of new pneumococcal vaccines in terms of protection against invasive pneumococcal disease, should be based on non-inferiority data compared with PCV7, using a serological antibody threshold, demonstration of the functionality of the induced antibodies as measured by opsonophagocytosis (OPA) and demonstration of boostability (205). Based on these criteria, in 2009, a 10-valent pneumococcal conjugate vaccine (PCV10) (Synflorix®, GlaxoSmithKline Biologicals) (Table 1.4) was licensed (206, 207). This vaccine uses a recombinant version of protein D, a non-lipidated form of a highly conserved cell-surface lipoprotein of non-typeable *H. influenzae* as carrier for eight of the ten vaccine serotypes. For the other two serotypes, diphtheria and tetanus toxoids are the carrier proteins. This vaccine also showed efficacy against AOM caused by *S. pneumoniae* and non-typeable *H. influenzae* (208).

In 2010, after studies showing that it was similarly immunogenic against the PCV7-containing serotypes and also against the new serotypes (209), a 13-valent pneumococcal conjugate vaccine (PCV13) (Prevenar13®, Pfizer) (Table 1.4), using the same protein carrier CRM197, was licensed and replaced PCV7.

Table 1.4. Characteristics of licensed pneumococcal vaccines.

Name and producer	Date of availability	Type	Serotypes
PPSV23, Merck	1983	Polysaccharide	1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F
PCV7, Wyeth/Pfizer	2000	Conjugate	4, 6B, 9V, 14, 18C, 19F, 23F
PCV10, GSK	2009	Conjugate	4, 6B, 9V, 14, 18C, 19F, 23F + **1, 5, 7F**
PCV13, Pfizer	2010	Conjugate	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F + **3, 6A, 19A**

PPSV, pneumococcal polysaccharide vaccine; **PCV,** pneumococcal conjugate vaccine; **GSK,** GlaxoSmithKline Biologicals

By 2013, PCVs had been incorporated into routine childhood immunisation programmes in 96 countries (30). However, the chronology and dynamics of their adoption has varied as a result of diverse public health decisions and processes

in different jurisdictions. The original recommended vaccine schedule was three doses in infancy followed by a single booster dose in the second year of life – the so-called 3 + 1 schedule. The UK and subsequently other countries opted instead for a 2 + 1 schedule and both of these regimens are now in use.

The introduction of PCV7 into the routine childhood vaccination schedule in several countries has dramatically reduced the incidence of invasive disease caused by vaccine serotypes (210-214) both in vaccinated and among unvaccinated groups as a result of herd immunity effects (214, 215). A post-licensure surveillance of pneumonia incidence in Israel showed that PCV7 was associated with significant declines in radiologically confirmed alveolar pneumonia among children <6 month (-31%), 6–18 month (-41%) and 18–35 month old children (-34%), respectively (216). A study from Brasil showed an approximately 25% decline in hospitalisations for pneumonia among children aged 2-24 months (217). A 2008-09 study in the UK reported a reduction in the incidence of community-acquired pneumonia of 19% in children aged <5 years, 33.1% in those <2 years and a reduction of 38.1% in hospitalisations, compared to 2001-02 (218). In AOM, PCV7 has reduced the burden of disease and shifted pneumococcal serotypes and the distribution of otopathogens currently reported, with *H. influenzae* being now the predominant organism in some series (219-221). Eight observational post-implementation database studies of PCV have reported an average of 19% reduction in visit rates for all cause AOM (222).

In Portugal, PCVs have been used since 2001 through private sales and have not been included in the nationally funded immunisation programme. The original seven-valent vaccine was replaced in 2010 by PCV13 and, for a period of about one year (2009-2010), PCV10 was given to some children. Both the 2+1 and 3+1 schemes have been commonly followed. All this, combined with the fact that vaccine uptake in Portugal is driven in part by the willingness and ability to pay, so that coverage will probably be much lower in areas of relative poverty, means that the situation in this country with regard to PCV usage is extremely complex and very uneven, placing Portugal in an unusual situation. A survey of immunisation records conducted in the north of Portugal in 2007 showed that the proportion of infants born in 2001 who received three PCV doses by 12 months of age was 23.7%. This rose steadily to 51.2% in 2005. The proportion of the 2001 cohort who received four doses by 24 months was 20.2% and it was 43.1% for the 2004 cohort (223). National PCV sales data corroborate this picture, suggesting that the proportion of fully vaccinated children (based on a notional

3.5 doses per child) rose from around 32% in 2002 to around 65% in 2005 and 79% in 2007, then falling slightly to 75% in 2008 and again to 62% in 2012 (personal communication, Pfizer). Vaccination data from the Central Region of Portugal, obtained from the national vaccination registration system (SINUS – Modulo de vacinação), are presented in Table 1.5.

Table 1.5. Vaccine coverage with ≥ 3 doses at 24 months of age in the Central Region of Portugal and Coimbra's District by birth cohort, 2008-2011.

Birth cohort	Coimbra's District (range)	Central Region of Portugal
2008	67% (26.3-84)	69%
2009	71.6% (26.6-87.5)	71%
2010	73.5% (38.1-100)	73%
2011	71.1% (39.7-87.1)	73%

Courtesy of Viveiros D. Source: Módulo de Vacinação do Programa SINUS, 2013

A study to evaluate the impact of PCV7 use on serotypes causing invasive disease in Portugal, between 1999-2002 and 2003-2005, showed reduction in the proportion of vaccine types 4, 6B, 14 and 23F. Changes in serotype distribution compatible with the introduction of PCV7 were seen for children ≤5 years of age from 2003 onwards. Similarly, significant indirect effects on invasive disease in adults were noted, with reductions in the proportion of invasive disease caused by serotypes 4 and 14. These changes were accompanied by an increase in 19A in all age groups and in 7F in adults (224). A national surveillance programme for paediatric pneumococcal invasive disease reported a continuing decline of PCV7 serotypes between 2006 and 2008, with non-vaccine types 1, 7F and 19A becoming the leading causes of invasive disease after seven years of PCV7 use (225). There are no available data regarding the impact of PCV on community-acquired pneumonia or AOM in Portugal.

1.6 The impact of pneumococcal conjugate vaccine on pneumococcal nasopharyngeal colonisation

Like other bacterial capsular conjugate vaccines, such as those against *H. influenzae* type b and *Neisseria meningitidis* serogroup C (MenC), PCVs were developed and licensed based on their capacity to induce sufficient serum anti-capsular antibodies in young children to protect them against developing invasive

disease in normally sterile sites – principally blood stream infection. However they also induce significant mucosal immune responses (195). At the time when the first widely used capsular polysaccharide-protein conjugated vaccine against *H. influenzae* type b was being introduced in European countries in the 1990s, the observation had already been made by researchers in Finland that these vaccines induced both IgG and IgA capsule-specific antibodies in mucosal secretions (226). Their potential to induce herd immunity by impacting upon colonisation and thus transmission of *H. influenzae* type b, as well as to induce direct protection against invasive disease in the vaccine recipient was anticipated and this expectation was soon borne out by observations that invasive *H. influenzae* type b disease disappeared more rapidly and more completely than could be explained by direct effects alone and also diminished in age groups who had not been immunised (227). The introduction of MenC conjugate vaccines and later PCVs into early childhood immunisation programmes has extended the impact of mass vaccination on paediatric nasopharyngeal ecology. These observations have begun to suggest that this class of vaccines actually owe their success, to a great extent, to mucosal immunogenicity and consequent herd immunity effects (227, 228) and the primary mechanism of effectiveness of many vaccine programmes turns out to be their impact on colonisation and thus transmission. This phenomenon is now thought to be of great importance for the effectiveness of conjugate vaccine programmes at the population level and immunisation schedules are now being modified to take it into account. For example, in Portugal, the MenC programme was recently modified to discontinue vaccine doses in early infancy even though disease incidence peaked in infancy during the pre-vaccine era.

The direct effect of the vaccine is the induced change in the risk of colonisation and disease caused by the vaccine types in the vaccinated individual.

The indirect effect or herd immunity is the protection against vaccine type colonisation and thus disease in the unvaccinated population or in vaccinated individuals for example, after their direct protection has worn off or if it is incomplete. The indirect effect is mediated by a reduction of vaccine-type nasopharyngeal colonisation and thus transmission and disease.

Since vaccinated individuals are also affected by indirect effects, direct effects can be measured only in pre-licensure efficacy trials or soon after implementation of the vaccine. Once vaccines are included in national immunisation programmes and widely used, direct effects can be measured only

combined with indirect effects. Pure indirect effects can still be measured among people who for any reason have never been vaccinated.

A concern that vaccination against targeted serotypes might open ecological niches that would be filled by other serotypes was raised before introduction of PCVs (229, 230).

1.6.1 Direct effects of pneumococcal conjugate vaccines on nasopharyngeal colonisation

Several PCV efficacy trials (231-236) showed reduction of vaccine type nasopharyngeal colonisation, with a magnitude of the direct effect on vaccine type colonisation rates of around 50% (29). This decrease is thought to be achieved primarily by reductions in rates of acquisition and also lower colonisation density among those colonised, rather than by reduction in duration of carriage (234, 237). In a community-randomised controlled trial investigating the effect of PCV7 on nasopharyngeal colonisation among American Indian infants, children who had received PCV7 and were colonised with *S. pneumoniae* vaccine types, had lower density of colonisation than those who received the control vaccine and who were also colonised with vaccine types (234). No effect of PCV7 on the duration of colonisation by any serotype, at any age at sampling was shown, although modest differences in duration could have been missed because of low sampling frequency (234).

Most trials have also shown that the prevalence of non-vaccine type colonisation increases among vaccinated subjects (232, 234, 236), with this colonisation rate consequently being higher in the pneumococcal-vaccine group than in the control group (234, 236). In the American Indian trial, this increase in non-vaccine type colonisation in the PCV7 group was not conclusively evident until 18 months of age (234).

1.6.2 Indirect effects of pneumococcal conjugate vaccines on nasopharyngeal colonisation

Since PCV decreases vaccine type nasopharyngeal colonisation prevalence and density in vaccinated children, this leads to decreased vaccine type transmission to both unvaccinated and vaccinated populations and consequently to generalised decreased colonisation with vaccine types. Reductions in vaccine type colonisation will necessarily also result in reduction of invasive disease caused by these serotypes in both vaccinated and unvaccinated individuals.

Indirect effects usually appear after widespread use of the vaccine for some time, however within communities with close contacts they may emerge promptly (29).

A 2013 review summarising data from 14 countries, showed that introduction of PCV was consistently followed by significant decreases in vaccine type nasopharyngeal colonisation and invasive disease among age groups not targeted for vaccination (30). The same review also showed that although the greatest magnitude of reduction was observed in the first years following vaccine introduction, the decline continued thereafter. This is clearest at high vaccination coverage levels but is also seen to some extent with coverage as low as 40% and is observed across all age groups (30).

Once indirect effects are observed, they affect both unvaccinated and vaccinated children and therefore, as vaccine types decline towards extinction, the differences between the two groups will progressively disappear over time until eventually they become indistinguishable.

1.6.3 Replacement

The first report of serotype replacement came from a randomised trial in The Gambia (229) showing that while vaccine types significantly declined in vaccinated infants, non-vaccine types significantly increased and as a result, the overall effect on pneumococcal nasopharyngeal colonisation rate was minimal. This study was followed by others in Israel (236), South Africa (232), The Netherlands (238) and a subsequent trial in The Gambia (233) that also demonstrated such replacement.

Following widespread introduction of PCV7, several observational colonisation studies from the USA (239-241), Norway (126), France (242), Greece (243) and the UK (244), have also documented that vaccine types have significantly declined or disappeared and non-vaccine serotypes have increased, leaving overall colonisation rates virtually unchanged. However, complete disappearance of vaccine types with large increases in non-vaccine types has only been shown in highly-vaccinated populations to date (239).

1.6.4 Effects of pneumococcal conjugate vaccine on strain diversity

The diversity by serotype of *S. pneumoniae* nasopharyngeal colonisation in any population in the absence of any external pressure is thought to be relatively stable. However, if a population is challenged by vaccination and this leads to a reduction in the dominance of a few highly prevalent vaccine types, it has been

proposed that the serotype diversity will increase - with a larger number of circulating serotypes each, on average, at a lower proportion - and then take some time to return to the previous distribution, now with different serotypes predominating. The Simpson's index of diversity (D) and the concept of a typical distribution for the ranked frequency of the serotypes were proposed by Hanage and colleagues as methods for assessing these changes (245). Two ecological studies in human populations, in the USA (245) and UK (246), have described changes in serotype diversity following introduction of universal vaccination. The group in the USA compared the serotype diversity of pneumococcal nasopharyngeal samples from children in Massachusetts, collected in 2001, 2004 and 2007 with other collections from the pre-vaccine era, in order to describe the population effect of vaccine use. The diversity of each dataset was estimated using the D index. By 2004, coverage with three doses of vaccine among children aged 19-35 months in Massachusetts was close to 90% and has not dropped below 90% since then. The 2004 sample (4 years after vaccine introduction) was significantly more diverse than the pre-vaccine samples (D=0.946 versus 0.9181) while the 2007 sample showed no significant difference in diversity from the pre-vaccine period (D=0.923), although now with different common serotypes. In 2007 the carriage frequency of the non-vaccine type 19A was similar to that of the most common serotype in the pre-vaccine sample (~20%), suggesting that 7-8 years after vaccine introduction, serotype replacement involving 19A may have been complete in Massachusetts, so that further marked increases in common serotypes were unlikely, either in colonisation or disease. In the UK study, where PCV7 was introduced to almost immediate high levels in 2006 with a catch up programme up to the age of 2 years, pneumococcal nasopharyngeal colonisation data in children from 2001-02 were compared with 2008-09 using the same index of diversity: for the 2001-2002 sample, D was 0.891 and increased significantly to 0.960 in 2008-09. The ranked frequency distribution of the serotypes, while similar in the prevaccination era compared to children in Massachusetts, changed to become more diverse after vaccination, suggesting that at the time of this analysis, PCV7-induced changes in pneumococcal population biology were still evolving.

1.6.5 Modelling the effects of vaccination on *S. pneumoniae* nasopharyngeal colonisation

As discussed in section 1.1.1.1, Cobey and Lipstich (67) have created a

model to predict changes in pneumococcal colonisation with age. They also state that this model can be used to predict changes induced by the use of PCV. Unlike naturally occurring immunity following colonisation, the vaccine reliably induces anticapsular immunity that is often protective against future carriage.

Simulating vaccination in the model reproduced rapid and sustained replacement of vaccine types as seen in real life. Serotype diversity briefly increased in the few years after vaccination and the prevalence of total carriage fluctuated before approaching a new equilibrium. Whether the simulated serotype diversity or total carriage prevalence ultimately returned to prevaccine levels depended on the ranks of the targeted serotypes.

Scenarios in which vaccine serotypes might re-emerge because of sustained reductions in anticapsular and/or non-specific immunity after vaccination, include vaccine uptake at a coverage just below what is required to eliminate transmission through herd immunity or re-introduction of those serotypes through immigration.

1.6.6 Observational nasopharyngeal colonisation studies in Portugal

Nasopharyngeal colonisation studies in Portugal started in 1996, in children aged 6 months to 6 years, in DCCs in Lisbon, reporting colonisation rates of 47% for *S. pneumoniae* (139). A repeat study performed in the same area in 1999, showed pneumococcal colonisation rates of 63% (247). In 1998-99, a one-year longitudinal study was performed in a single DCC. Of the samples obtained, 61.4% carried pneumococci. Five PCV7 serotypes were identified, in decreasing order of abundance: 19F (34.2%), 23F (15.2%), 6B (11.3%), 14 (8.6%) and 9V (3.1%). Children were sequentially colonised with up to five serotypes, showing a high rate of acquisition and turnover of strains. Colonised children shared clones with others (131). Between 2001 and 2003 a study to evaluate the impact of PCV7 on carriage of drug resistant *S. pneumoniae* was performed showing that colonisation rates were similar in the intervention (vaccinated with PCV7) and control groups (68%). In the intervention group the frequency of vaccine types decreased significantly (81% to 5%) and the frequency of non-vaccine types increased significantly (19% to 95%); in the control group, the colonisation rate with vaccine types remained high and the rate of non-vaccine types showed no consistent upward or downward trends (248). In 2006, when 57.4% of the children had received at least one dose of PCV7, a point-prevalence study to evaluate PCV7 impact on pneumococcal colonisation was performed in DCCs in

the Lisbon area and results were compared with a similar study carried out in 2001. Colonisation rates of *S. pneumoniae* remained stable (64.9% in 2001, 68.7% in 2006), with the six vaccine types detected decreasing from 53.1% to 11.2% of all pneumococci and non-vaccine types increasing from 46.9% to 88.8%, showing that extensive serotype replacement occurred among both vaccinated and non-vaccinated children, indicating that vaccination had reached a sufficiently high coverage to induce indirect effects in unvaccinated children. Non-vaccine types 1, 6C, 7F, 15A, 16F, 21, 23A, 29 and non-typeable strains increased significantly (249).

In Coimbra, we studied rates of pneumococcal nasopharyngeal colonisation for the first time in 2007 (75). In January-February a cross-sectional study was conducted among children aged 6 months to 6 years attending eight DCCs. Nasopharyngeal swabs were obtained from 507 children of whom 76.7% had received at least one dose of PCV7. The pneumococcal nasopharyngeal colonisation rate was 61.3%. Colonisation rates varied with age: a peak (76.2%) was observed among children 12–24 months and the proportion of colonised children decreased progressively through the older age groups. Colonisation also varied with the number of children attending each DCC: the likelihood of being colonised being higher in larger nurseries. Serotyping results showed that 20.7% were vaccine types, 70.8% non-vaccine types and 8.5% non-typeable. Serotype 19F was the second most frequent serotype, detected in 10.5% of positive samples for pneumococcus. The global carriage rate was not associated with vaccination status, but vaccine types were more frequently found among unvaccinated children.

1.7 Interactions between bacterial pathogens

The microbes colonising the nasopharynx are adapted to be able to survive in that environment and are able to evade clearance mechanisms including mucus and ciliae, to attach to the epithelium, to find nutrients and to avoid elimination by host immune mechanisms at least for long enough to be able to transmit onwards. During these processes, it is likely that they interact with other strains and species occupying the same niche. It is likely that a range of different interactions that can be negative, positive or both have developed and that such relationships might change under different circumstances such as nutrient supply, life cycle phase, presence of other species, state of the host's immune

responses and other variations between individual hosts.

Understanding of the bacterial–bacterial interactions observed in the upper respiratory tract is limited. Several mechanisms have been proposed including production of hydrogen peroxidase (H_2O_2) by some bacteria which is lethal for others (250, 251) and interference with other species' structures that mediate adherence to the epithelium via host receptors. Examples include using neuraminidase to cleave the sialic acid residues that some bacteria require for attachment. Expression of phosphorycholine a cell-surface molecule that mediates attachment to host cell receptors may induce cross reactive specific host immune responses (252, 253). Involvement of the host immune system is also possible in other ways, either triggering it to combat other species or selectively evading it (254). An example of the latter is production of outer membrane vesicles that may be able to inactivate complement (255), a mechanism which might benefit other species as well as those generating the vesicles. However, interspecies interactions are likely to be more complex than the simple interaction between two species (256).

There is growing evidence of the existence of nasopharyngeal biofilms created by several bacterial species, including pneumococcus (257), *S. aureus* (258), *H. influenzae* (259) and *M. catarrhalis* (260). Pneumococcal biofilms start with bacterial attachment to the substratum followed by bacterial aggregation and creation of a matrix composed of extracellular DNA, proteins and polysaccharides (261, 262), linking bacterial cells and attaching them to host cells. The pneumococcus can also be part of multi-species biofilms, for example including *H. influenzae,* with increased biomass (263). *M. catarrhalis* likewise produces more robust biofilms in the presence of *H. influenzae* strains (260). Interspecies biofilms are able to engender passive gene transfer of antibiotic resistance between strains (264) but the role of biofilms in competition or cooperation among microbial species requires further investigation.

Numerous studies trying to establish and quantify the relationships which exist between the different bacterial species that colonise the nasopharynx have reached conflicting conclusions (Table 1.6). This may be due to differences among study samples in the many variables that may have an influence on colonisation including age, season, intercurrent viral infections and other clinical and demographic variables such as levels of exposure to other children, environmental smoke and antibiotics. An overview of published clinical studies is presented in Table 1.6.

1.7.1 Interactions between *S. pneumoniae* and *S. aureus*

A negative association between carriage of *S. aureus* and *S. pneumoniae* (particularly vaccine-type pneumococci) has been demonstrated in cross-sectional studies in different paediatric populations (25, 70). However, other studies have not confirmed this (19, 24, 265).

The mechanisms for this possible negative association between *S. pneumoniae* and *S. aureus* or why vaccine types should be distinct in this regard remain unclear.

1.7.2 Interactions between *S. pneumoniae, H. influenzae* and *M. catarrhalis*

In nasopharyngeal colonisation studies, with few exceptions (17), positive associations between *S. pneumoniae*, *H. influenzae* and *M. catarrhali*s are typically reported in healthy children (24, 266-268). A recent study showed that the densities of *S. pneumoniae* and *H. influenzae* in individual carriers were also positively correlated (269).

1.7.3 Interactions between *S. aureus* and *M. catarrhalis*

There are very few studies analysing interactions between *S. aureus* and *M. catarrhalis*: three showed no association (19, 24, 270), while one (267) showed a negative association.

1.7.4 Other associations between bacterial species

Other significant positive and negative associations between species have not been reported to date.

The presence of a third bacterium may affect the outcome of competition between two species, making the process more complex. Interactions may, for example, shift from negative to positive. Pettigrew et al. described negative associations between *H. influenzae* and *S. pneumoniae* but this competitive interaction changed when *H. influenzae* and *M. catarrhalis* were both present (17).

Different patterns of colonisation and interaction have been observed during symptomatic respiratory infection. Xu et al. found that co-colonisation with different bacteria was more frequent at the onset of AOM than in healthy children and that *H. influenzae* carriage was negatively associated with *S. pneumoniae* and *M. catarrhalis* at the onset of AOM but not in healthy children (270).

Table 1.6. Studies of associations between co-colonising bacteria in the nasopharynx in children.

Study reference	Study population	Sp & Sa	Sp & Hi	Sp & Mc	Hi & Mc	Sa & Hi	Sa & Mc
Bogaert et al.; 2004 (70)	The Netherlands; 3198 children; 1-19Y	Negative (for VT)	ND	ND	ND	ND	ND
Regev-Youchay et al.; 2004 (25)	Israel; 790 children; ≤40M	Negative (for VT)	ND	ND	ND	ND	ND
Zemlickova et al.; 2006 (271)	Czech Republic; 425 children; 3-6Y	Negative	No association	No association	ND	No association	ND
Jacoby et al.; 2007 (24)	Australia; 167 children, 1005 samples; <2Y	No association	Positive (Aboriginal children only)	Positive	Positive	No association	No association
Madhi et al.; 2007 (23)	South Africa; 271 children; mean age 5.7Y	Negative (HIV uninfected children)	Positive	ND	ND	Negative (HIV uninfected children)	ND
Lee et al.; 2009 (265)	USA; 1986 children; 3M-7Y	No association	ND	ND	ND	ND	ND
Abdullahi et al.; 2008 (69)	Kenya; 450 children; <5Y	ND	Positive	ND	ND	ND	ND
Jourdain et al.; 2011 (19)	Belgium; 333 children, 830 samples; 3-6Y	No association	Positive	No association	Positive	Positive	No association
Quintero et al.; 2011 (272)	Venezuela; 250 children; 2-5Y	Negative (for VT)	ND	ND	ND	ND	ND
Verhaegh et al.; 2011 (273)	The Netherlands; 1079 children, 2751 samples; <24M	ND	ND	ND	Positive	ND	ND
van Gils et al.; 2011 (274)	The Netherlands; 1005 children; <24M	Negative	Positive (for VT)	Positive (for VT)	ND	ND	ND
Kwambana et al.; 2011 (268)	Gambia; 30 children, 498 samples; <12M	Negative	Positive	Positive	ND	ND	ND
Dunne et al.; 2012 (266)	Fiji; 161 children; 17M	ND	Positive	Positive	Positive	ND	ND
Bae et al; 2012 (267)	South Korea; 582 children; 3-10Y	Negative	Positive	Positive	Positive	Negative	Negative
Xu et al.; 2012 (270)	USA; 320 children; 6-24M	Negative	No association	Positive	No	No association	No association
van den Bergh et al.; 2012 (103)	The Netherlands 986 children; 6-24M	Negative	Positive	Positive	No association	No association	Negative
Chien et al.; 2013 (269)	Peru; 360 children, 446 samples; ≤35M	Negative	Positive	ND	ND	No association	ND

M, months; **Y**, years; **ND**, not done; **Sp**, *S. pneumoniae*; **Hi**, *H. influenzae*, **Mc**, *M. catarrhalis*; **Sa**, *S. aureus*; **VT,** vaccine types. Adapted from Dunne et al. (275)

1.8 The impact of pneumococcal conjugate vaccines on other potential pathogens colonising the nasopharynx

Given that different bacterial species may interact, vaccines that target specific bacteria and modify colonisation rates and densities may also modify polymicrobial interactions in the nasopharynx and thus affect other species.

The suggested negative association between carriage of *S. pneumoniae* vaccine types and *S. aureus* has raised concerns that widespread use of PCVs and the consequent reduction in vaccine types might be followed by an increase in *S. aureus* colonisation and associated disease (25). In a randomised controlled trial of PCV7 in The Netherlands, an increase in *S. aureus* colonisation was observed at 12 months of age compared with unvaccinated controls but not at the other time points examined (274). A follow-up study comparing colonisation levels from this trial with two additional time periods following PCV7 introduction, showed *S. aureus* colonisation rates were significantly higher in 11 month old infants following widespread vaccine use, but no differences were observed at 24 months of age (276). Observational studies that have evaluated bacterial colonisation before and after PCV introduction report inconsistent results: some found increased prevalence of *S. aureus* (23, 276, 277) and for others it was stable (126, 266, 278). Cross sectional studies performed in Massachusetts between 2003-04 and 2006-07, in the setting of widespread use of PCV and decreasing colonisation with vaccine types, found that *S. aureus* colonisation among children 3 months to <7 years did not change significantly (15.3% to 14.1%) (265).

Regarding other potential pathogens, a randomised controlled trial in Fiji showed that infant pneumococcal vaccination had no effect on overall nasopharyngeal colonisation rates or densities of *H. influenzae* or *M. catarrhalis* at 17 months of age (266). A trial in The Netherlands, reported that PCV7 had no effect on *H. influenzae* colonisation and that *M. catarrhalis* colonisation was lower compared to unvaccinated controls at 12 months of age (277). However these two reports are from randomised controlled trials and effects might be different once more widespread indirect effects have started to become apparent. Spijkerman et al. described an increase in carriage rates of *H. influenzae*, 3 and 4.5 years after PCV7 implementation in The Netherlands, with no consistent changes for *M. catarrhalis* (276). Table 1.7. summarises studies of the impact of pneumococcal conjugate vaccines on nasopharyngeal colonisation of *S. aureus*, *H. influenzae* and *M. catarrhalis.*

Table 1.7. Studies of the impact of pneumococcal conjugate vaccines on nasopharyngeal colonisation of *H. influenzae*, *M. catarrhalis* and *S. aureus*.

Study reference	Country	Study description	Sa	Hi	Mc
Madhi et al.; 2007 (23)	South Africa	Randomised controlled trial of PCV9	No differences between PCV9 and placebo groups	No differences between vaccinated and controls	ND
Prymula et al.; 2009 (235)	Czech Republic and Slovakia	Randomised controlled trial of PCV11	ND	No differences between vaccinated and controls	ND
Lee et al.; 2009 (265)	USA	Prospective observational studies 2-3Y and 5-6Y post-PCV7	Carriage rate: stable (14%)	ND	ND
van Gils et al.; 2011 (277)	The Netherlands	Randomised controlled trial of PCV7	Carriage rates: 10% in the 2+1 dose group and 5% in unvaccinated controls at 12M	No differences between vaccinated and controls	Carriage rates: 61% in the 2+1 dose group and 68% in unvaccinated controls at 12M
Prymula et al.; 2011(279)	Czech Republic	Randomised controlled trial of PCV10	ND	Carriage rates: 10% in the PCV10 group compared to 16% in unvaccinated controls at 24–27M	ND
Dunne et al.; 2012 (266)	Fiji	Randomised controlled trial of PCV7 with or without 23vPPS booster	ND	No differences between vaccinated and controls	No differences between PCV7 and unvaccinated controls
Ho et al.; 2012 (280)	Hong Kong	Cross-sectional study	No difference between PCV7 vaccinated and unvaccinated children	ND	ND
Dukers-Muijrers et al.; 2012 (281)	The Netherlands	Cross-sectional study	No difference between PCV7 vaccinated and unvaccinated children	ND	ND
Spijkerman et al.; 2012 (276)	The Netherlands	Cross-sectional studies, 3Y and 4-5Y post-PCV7, compared to pre-PCV7	Carriage rates: 9% and 14% in post-PCV7 time periods compared to 5% in pre-PCV7 at 11-12M	Carriage rates: 65% and 65% in both post-PCV7 time periods at 11–12M compared to 46% pre-PCV7; 73% and 76% post-PCV7 compared to 52% pre-PCV7 at 24M	Carriage rates: 80% 4–5Y post-PCV7 compared to 59% pre-PCV7 at 24M

M, months; **Y**, years; **PCV7**, 7-valent pneumococcal conjugate vaccine; **PCV9**, 9-valent pneumococcal conjugate vaccine; **PCV11**, 11-valent pneumococcal conjugate vaccine; **23vPPS**, 23-valent polysaccharide vaccine; **Hi**, *H. influenzae*, **Mc**, *M. catarrhalis*; **Sa**, *S. aureus*; **ND**, not done. Adapted from Dunne et al. (275)

1.9 Interactions between bacterial pathogens and respiratory viruses

Viral infection results in vasodilation and increased vascular permeability of the nasal mucosa which cause rhinorrhea and cholinergic stimulation leading to increased mucous secretion and sneezing (98). Some viruses like influenza virus and adenovirus can also cause extensive damage to the respiratory epithelium (98).

The mechanisms by which viruses may influence bacterial colonisation are numerous and diverse: viral presence may render the epithelium more susceptible to bacterial adherence, disruption of the epithelial barrier, up-regulation of adhesion proteins, production of viral factors that increase bacterial adherence and interference with the immune system may all occur (256, 282). By taking advantage of the presence of the virus, the bacteria may proliferate and stimulate or augment rhinitis, mechanisms that may increase bacterial transmission efficiency, but which may also play a role in causation of disease including pneumonia and AOM and promote development of host immunity.

Interaction between viruses and bacteria in the pathogenesis of respiratory tract infections has been extensively reported in the literature in particular the synergism between influenza virus and *S. pneumoniae* (282). Other positive viral-bacterial associations have been described in human studies: for example between human rhinovirus and *S. pneumoniae* (102), human rhinovirus and *M. catarrhalis* (283), human rhinovirus and *H. influenzae* and *M. catarrhalis* (101) and between adenovirus and *H. influenzae* and *M. catarrhalis* (101).

Children are susceptible to secondary bacterial infections during viral upper respiratory tract infections and significant morbidity due to pneumococcal co-infection is associated with viral respiratory infections (284-286). It was shown in animal models that respiratory syncytial virus and influenza virus can alter the immune system and enable *S. pneumoniae* to spread from the nasopharynx into the lungs (282, 287). In addition, it was observed that children with pneumonia carried significantly more *S. pneumoniae* when respiratory syncytial virus, influenza or rhinoviruses were present compared with children with pneumonia without viruses (184). Xu et al. found that among young children, viral upper respiratory tract infections were present at onset of AOM in 93% of the cases (270).

Viral vaccines (e.g. influenza vaccine) might therefore have an impact on incidence of bacterial diseases.

Although most studies point towards viral predisposition to bacterial infection and little information exists regarding bacterial predisposition to viral disease, some report a possible bidirectional synergism with bacterial infection increasing the susceptibility to a consecutive viral infection (288, 289).

In a double-blind, randomized, placebo-controlled trial in South-Africa, it was demonstrated that the 9-valent pneumococcal conjugate vaccine, in addition to reducing pneumococcal disease, substantially prevented viral-associated pneumonia, suggesting that pneumococcus has a major role in the development of pneumonia associated with these viruses and that viruses contribute to the pathogenesis of bacterial pneumonia (290). Epidemiological analysis of pneumonia hospitalisations before and after PCV7 introduction in the USA demonstrated that vaccination was associated with reduced incidence of influenza-associated pneumonia among children in addition to reduction in pneumococcal pneumonia (215).

Further information on the associations between nasopharyngeal bacterial colonisation with different bacterial species and intercurrent respiratory viral infection may contribute to understanding the complex network of relationships that exists connecting these microbial pathogens and infections in the human host.

1.10 Nasopharyngeal colonisation and acute otitis media

Acute otitis media is one the most common bacterial infections among children (291). The incidence is greatest in the first 2 years of life and *S. pneumoniae*, non-typeable *H. influenzae* and *M. catarrhalis* are the major bacterial pathogens (292, 293). Recurrent AOM is common, with as many as 46% of children having three or more episodes before their third birthday (293). Added to this substantial morbidity, the fact that this diagnosis drives a large proportion of antibiotic prescribing to children and thus emergence of antibiotic resistant strains, gives this condition very substantial public health significance.

Nasopharyngeal colonisation by potential middle-ear pathogens is presumed to precede AOM. A strong relationship between the frequency of colonisation and otitis media for each pathogen has been reported (4). Several studies have shown that if *S. pneumoniae* or *H. influenzae* are not found in the nasopharynx during AOM, it is unlikely that they would be found in the middle ear fluid (294-296). Another study to evaluate nasopharyngeal culture in predicting the aetiology of

AOM in children showed that the same pneumococcal serotype grew from both the nasopharynx and the middle ear fluid in all but one of the 158 events in which *pneumococcus* was serotyped in both of the samples (181) and *S. pneumoniae* from the ear showed a close genetic relatedness with their nasopharyngeal counterparts (297). It was also shown that the rate of bacterial nasopharyngeal colonisation is higher during AOM (148, 298) and that co-colonisation with different bacteria is more frequent at the onset of AOM than in healthy children (270). However other studies have reported similar nasopharyngeal rates in healthy children and during AOM (185, 299). An association between nasal bacterial load and the presence of and severity of suppurative otitis media was reported (300).

Obtaining a middle ear fluid (MEF) sample when the tympanic membrane is intact involves tympanocentesis or myringotomy, procedures that cause discomfort. Spontaneous rupture of the tympanic membranes is a complication of AOM that enables a culture of the draining MEF to be obtained. A study to evaluate the reliability of the microbiology of spontaneously draining AOM was performed comparing the bacteria isolated from the external ear canal associated with AOM with spontaneous otorrhoea (AOMSO) with those isolated from the middle ear fluid, in children with uncomplicated AOM (301). The authors concluded that culturing the otorrhoea material is a simple and practical method for obtaining information regarding the potential cause of the AOM. However, this method is 28% less sensitive for positive culture compared to aspiration of the middle ear. It was also reported that, compared to AOM, the relative importance of *S. pneumoniae* and *S. pyogenes* in AOMSO was higher than that of *H. influenzae* and *M. catarrhalis* (302) and that there was no correlation between the bacteria isolated and duration of otorrhoea (301). A study by Ruohola et al. evaluated the dynamics of bacteria in the middle ear during the course of AOM with tympanostomy tube otorrhoea, suggesting evolution over time with different species detected in successive samples (303).

Viral infections may render the middle ear susceptible to infection (284). Virus-induced inflammation of the nasopharynx, with production and release of cytokines and inflammatory mediators, which also extends to the Eustachian tube provoking dysfunction, may alter the equilibrium allowing bacteria and/or viruses to invade the middle ear.

More than one bacterial species can simultaneously infect the middle ear in AOM (220, 302, 304). Recurrent, non-responsive and chronic cases of otitis

media are often polymicrobial with biofilm formation, with frequent involvement of non-typeable *H. influenzae* (305) and pneumococcal serotypes of lower virulence and commonly carried by healthy children (306).

As described previously, conventional pneumococcal culture and serotyping methods underestimate multiple-serotype detection. Microarray-based serotyping has not previously been applied to biological samples from the middle ear.

More detailed studies of the complex microbiology of the middle ear in AOMSO, the relationships that exist between the microbiology of the nasopharynx and middle ear in this condition and evaluation of the differences in nasopharyngeal ecology that exist between healthy children and children with AOM may have the potential to contribute to understanding disease pathogenesis and uncover new strategies for prevention through use of bacterial and viral vaccines.

Objectives

The studies presented in this thesis were driven by the knowledge that nasopharyngeal colonisation is a prerequisite for disease and the source of transmission between individuals, that a complex microbiota shares this ecological niche and that PCVs impact on disease to a great extent through their effects on colonisation and transmission.

The unifying hypothesis is that *S. pneumoniae* nasopharyngeal colonisation is affected by different vaccination strategies and by interaction with other bacterial species and viruses present in the same niche. These dynamics may be important for transmission between individuals and so vaccine effectiveness and in the pathogenesis of common childhood respiratory infectious diseases.

The specific questions addressed in this thesis are:

1. Is the impact of immunisation with PCV7 on nasopharyngeal colonisation by *S. pneumoniae* in settings with lower, slowly rising and heterogeneous vaccination coverage different from those seen in countries where PCVs have been implemented consistently over time, evenly geographically and at high rates?
2. In this context, are there relationships between bacterial colonisers, viruses and rhinitis that may contribute to successful colonisation and transmission within the community?
3. Is the nasopharyngeal ecology in children with acute otitis media with spontaneous otorrhoea distinct from that in healthy children in the same community, in ways that may be associated with the pathogenesis of the disease?

Three main objectives were formulated. First, our goal was to survey changes in *S. pneumoniae* nasopharyngeal colonisation in healthy pre-school children in Coimbra, occurring in the context of moderately high paediatric

uptake rates of PCV, with special reference to serotype replacement and diversity. Second, we sought to evaluate the prevalence of other bacteria and viruses in the nasopharynx of healthy children as well as specific associations between these pathogens and relationships with rhinitis. Finally we aimed to analyse nasopharyngeal ecology during disease and compare it with that in health.

The overarching goal of the research presented in this thesis is to increase our understanding of nasopharyngeal ecology in the era of conjugate vaccines that induce mucosal immune responses to nasal bacteria. The results may advance our understanding of the effect of PCV use on nasopharyngeal colonisation and of the interactions between microorganisms, transmission between individuals and disease pathogenesis, in preschool children.

2. Progressive changes in pneumococcal carriage in children attending daycare in Portugal after 6 years of gradual conjugate vaccine introduction show falls in most residual vaccine serotypes but no net replacement or trends in diversity

Fernanda Rodrigues[1], Dona Foster[2], Francisco Caramelo[3], Pedro Serranho[4], Guilherme Gonçalves[5], Luís Januário[6], Adam Finn[7]

[1]Infectious Diseases Unit and Emergency Service, Center for Clinical Investigation, Coimbra Children's Hospital, Coimbra, Portugal; [2]Nuffield Department of Medicine, University of Oxford, Oxford, UK; [3]Institute of Biophysics/Biomathematics, Laboratory of Biostatistics and Medical Informatics, IBILI, Faculty of Medicine, University of Coimbra, Coimbra, Portugal; [4]ICBAS, Instituto de Ciências Médicas Abel Salazar, University of Porto, Porto, Portugal; [5]Bristol Children's Vaccine Centre, Divisions of Clinical Sciences and Cellular and Molecular Medicine, University of Bristol, Bristol, UK

Vaccine 2012;30(26):3951-3956

Reprinted from *Vaccine*, vol. 30, Issue 26, Fernanda Rodrigues, Dona Foster, Francisco Caramelo, Pedro Serranho, Guilherme Gonçalves, Luís Januário, Adam Finn. Progressive changes in pneumococcal carriage in children attending daycare in Portugal after 6 years of gradual conjugate vaccine introduction show falls in most residual vaccine serotypes but no net replacement or trends in diversity, pages 3951-3956, Copyright 2012, with permission from Elsevier. All rights reserved.

2.1 Abstract

Objectives: To track ongoing trends in pneumococcal (Sp) serotype carriage under the selection pressure of moderate pneumococcal conjugate vaccine (PCV) use, children in a community in Portugal were studied in the same months in 3 consecutive years.

Methods: Nasopharyngeal specimens were collected (children aged 3 months to <7 years) in 8 urban daycare centers in February 2008 (n=561) and 2009 (n=585). Sp isolates were serotyped.

Results: While demographics were similar in 2008-2009 and a previously reported sample in 2007, PCV coverage (at least one dose) in the children studied rose from 76.5% to 84% although national coverage was lower than this. Sp carriage fell from 61% to51% with a concomitant fall in PCV7 serotype carriage from 12.1% to 4.3%. Remaining PCV7 serotypes declined to near (23F) or totally (6B, 14) undetectable levels except 19F which persisted unchanged in around 4% of children. Although carriage of 3 and 6C rose, there was no net increase in non-PCV7 serotypes and no progressive trend in serotype diversity.

Conclusions: Ecological changes induced by PCVs where uptake is moderate appear to be different from high usage settings. We report falling Sp carriage due to PCV7 serotype disappearance with persistence of 19F and no ongoing net replacement after several years of PCV7 use and slowly rising uptake.

Keywords: *Streptococcus pneumoniae*, nasopharyngeal colonisation, replacement, serotype 19F

2.2 Introduction

Pneumococcal (Sp) conjugate vaccines, although not included in the nationally funded immunisation schedule, have been used in Portugal since the seven-valent vaccine containing serotypes 4, 6B, 9V, 14, 18C, 19F and 23F (PCV7) was licensed in Europe in 2001. Evidence from a survey of immunisation records conducted in the north of Portugal shows that the proportion of infants born in 2001 who received three doses by 12 months of age was 23.7%. This rose steadily to 51.2% in 2005. The proportion of the 2001 cohort who received four doses by 24 months was 20.2% and 43.1% for the 2004 cohort (223). No such data are available for the central region of Portugal, where Coimbra is situated, but coverage rates for other vaccines are similar for both regions. National PCV7 sales data corroborate this picture suggesting that the proportion of fully vaccinated children (based on the assumption that immunised children receive, on average, 3.5 doses) rose from around 32% in 2002 to around 65% in 2005 and 79% in 2007, then falling slightly to 75% in 2008 (personal communication, Pfizer). In April 2009, after the completion of the study reported in this paper, a 10-valent vaccine containing serotypes 1, 5 and 7F (PCV10) became available and some infants received it instead of PCV7 that year, while notional PCV7 coverage fell to 62%. From January 2010 a 13-valent vaccine adding serotypes 3, 6A and 19A (PCV13) replaced PCV7 and the large majority of immunised infants have received PCV13 since then (notional coverage in 2010 was 58%) (personal communication, Pfizer).

Researchers in the US have reported disappearance to near extinction of Sp serotypes contained in the vaccine from paediatric carriage over a period of around 5-7 years under selection pressure of mucosal immunity induced by widespread primary schedule immunisation (239, 245). However, the chronology and dynamics of adoption of PCV immunisation has varied as a result of diverse public health decisions and processes, healthcare delivery and funding systems in different countries.

Communities with similar serial cross-sectional surveys to those reported here include Massachusetts in the USA, Southampton in the UK and Nice in France (239, 242, 244). In the USA and UK vaccine use went up rapidly and stayed up. In Massachusetts, the percentage of children ≥12 and <12 months of age, respectively, who received at least one vaccine dose was 38%/83% in 2001, 79%/96% in 2004 and 98%/98% in 2007. Total Sp carriage rates in children aged 3

months to <7 years in primary care practices were 27% in 2001-02, 23% in 2003-04 and 30% 2006-07. Over this period, the proportion of children that carried non-PCV serotypes rose from 15% to 29% (64%-97% of isolates) (239). PCV7 was introduced in the UK in September 2006 as a two dose priming course and a 13 month third dose booster. National coverage at 12 months (2 doses) was 83.7% in 2007-2008 rising to 92.9% in 2009-2010, exceeding 92% in all regions apart from London where it was 86.5%. By 2009-2010 coverage at 24 months (3 doses) had reached 87.6% (307). In the 3 year study (2006-2009) of children aged ≤4 years, in the paediatric outpatients department of a large teaching hospital in Southampton, total Sp carriage rates were 32.2%, 27.1% and 31.1% while the proportion of isolates that were non-PCV7 serotypes rose from 46.2% to 83.3% (244).

In France, PCV7 became available and its cost reimbursed from December 2002. Results of a series of national surveys conducted annually since 2006 show lower coverage which rose more slowly than in the USA and UK, with only 64% having received at least one dose by 12 months in 2006, rising progressively to 86% in 2008 and complete course coverage at 16-24 months peaking in 2008 at only 68% (308). In a detailed study in children aged 3-40 months attending day care centres (DCCs) in Nice, from January to March 1999, 2002, 2004, 2006 and 2008, total Sp carriage rates fell from 54% to 45.2% while the proportion of isolates that were PCV7 serotypes fell from 77% to 4%. Vaccine coverage (≥1 dose) in the study population rose from 37.1% in 2004 to 90.1% in 2008 (242).

The dynamics of the impact of immunisation with this vaccine on the ecology of nasopharyngeal colonisation by Sp in Portugal and other settings may differ from those seen in countries where it has been implemented more consistently over time, more evenly geographically and at higher rates. Previously, we reported carriage rates by age and serotype in a cross-sectional study performed in pre-school DCCs in Coimbra, Portugal in 2007 (75). We subsequently conducted two closely similar follow-up cross sectional studies, at the same time of year (late winter), in similarly-aged samples of children in DCCs, in the same city, in 2008 and 2009, before the newer vaccines became available, in order to track trends in serotype carriage over time.

2.3 Methods

2.3.1 Recruitment, data collection, sampling and storage

Cross-sectional nasopharyngeal specimens were collected from children aged 3 months to <7 years, in 8 urban DCCs (5 sites in all 3 years, 1 in 2007 only, 1 in 2008 and 2009 and 1 in 2009 only) in Coimbra, a city in the central region of Portugal, in February 2008 and 2009. All subjects were healthy enough to attend nursery although some had intercurrent upper respiratory infections. The study was approved by the Ethics Committee of Hospital Pediátrico de Coimbra. Parents or guardians provided written informed consent for their child to participate in all cases.

One week before sample collection, parents or guardians of participating children completed a questionnaire and the following information was obtained: age, sex, number of children in the household who were <6 years of age, presence of smokers at home, use of antibiotics in the preceding month and dates of all PCV7 doses received.

2.3.2 Laboratory methods

Nasopharyngeal swabs were taken by the same trained nurses each year and transported to the local microbiology laboratory. In 2008 (as in 2007), initial isolation of Sp colonies was undertaken locally in Coimbra, thus swabs were transported in Stuart transport medium and plated within 12 hours after storage at 4°C. In 2009, the swabs were transported to the UK for isolation and identification of Sp and other pathogens and were, therefore, inoculated into STGG and stored at -80°C prior to culture. As reported by O'Brien et al. (110), the two methodologies are expected to have closely similar sensitivities. Both laboratories used standard microbiological techniques for the isolation and identification of Sp: briefly, cultures were inoculated onto 5% blood agar plates (Becton-Dickinson, Portugal & E & O Laboratories Ltd, UK) and incubated at 37°C in 5% carbon dioxide for 24 hours. Plates were examined for the presence of Sp with identification determined by morphology, optochin sensitivity, gentamicin resistance (UK only) and bile solubility. When suspected Sp colonies with greater than one morphology were observed, each type was purified for further testing. Isolates were stored at -80°C.

Sp isolates were serotyped using the Quellung reaction (anti-serum provided by the Statens Serum Institute, Copenhagen, Denmark), a multiplex PCR method (adapted from Pai et al.) (309) or a combination of both. PCR was undertaken on crude DNA extracts that were mixed with 3.3μL ImmoMix (Bioline, UK), 1μL

magnesium chloride (Bioline, UK), 0.5μL dNTPs (Invitrogen, UK), various combinations of oligonucleotides (Operon) and water to a final concentration of 25μL. Reactions were run on a DNA engine (PTC-225); 94˚C for 45 seconds, 54˚C for 45 seconds, 65˚C for 2 min 30 seconds for 30 cycles followed by 10 minutes at 65˚C. The reactions were run on a 2% gel containing ethidium bromide for 45 minutes at 120 V and photographed using transilluminator (Biorad, UK). All reactions that were positive for serogroup 6 or 15 were typed by Quellung to give a serotype. All isolates that were identified as serotype 6A underwent PCR using 6C primers and methods previously published (310). The isolates that were not typeable by either Quellung or multiplex PCR were confirmed as Sp using detection of the autolysin gene according to the published method (311).

For some analyses, serotypes were grouped into one of three categories:

- PCV7 serotypes (VT): those that matched serotypes included in the 7 valent vaccine (4, 6B, 9V, 14, 18C, 19F and 23F)
- Non-PCV7 serotypes (NVT): all other serotypes
- Non-typeable (NT).

2.3.3 Statistical analysis

Standard descriptive statistics and Chi-square tests were used to characterise the sample and assess trends in overall carriage and carriage of PCV7 and non-PCV7 serotypes and serotype 19F. Simpson's index for diversity was calculated to analyse the diversity among serotypes in each sample (312). Confidence intervals for this index were computed at 95%.

2.4 Results

561 and 585 children were enrolled respectively in 2008 and 2009. The demographic data of these children are shown in Table 2.1 and compared with those enrolled into the study performed in 2007 (75). These features were similar across the three years, although the proportion of children having one or more siblings <6 years old at home was higher and the proportion that received antibiotics in previous month was lower in 2009. The proportion of subjects who had received at least one dose of the PCV7 vaccine showed a significant upward trend (76.5% to 84%) over the 3 years.

Rates of Sp colonisation varied with age and were highest in the group aged < 12 months in 2008 and in those aged 12–24 months in 2009, falling thereafter with increasing age except that in 2009, carriage was slightly commoner in 5 than 4 year olds.

Table 2.1. Characteristics of the 2008 and 2009 subjects (compared with the 2007 sample).

	2007 (for comparison)	**2008**	**2009**	**P**
Number of subjects recruited	507	561	585	0.055
Male (%)	51.7	55.1	52.5	0.500
Aged <24m, 24- 48m, >48m (%)	24, 37, 39	22, 36, 42	2, 37, 41	0.844
Age (median and range) m	41 (6-81)	43 (3-77)	42 (4-74)	0.800
One or more siblings <6 years old at home (%)	29.2	29.7	38.7	0.001
One or more smokers at home (%)	30.1	27.0	28.6	0.528
Received antibiotics in previous month (%)	24.9	26.8	18.1	0.001
Doses of PCV7 received (%): 0/1/≥2	23.5/4.3/72.2	17.6/1.8/80.6	16.0/1.2/82.8	<0.001

m: months; **PCV7:** 7 valent pneumococcal conjugate vaccine

The overall proportion of children in whose swabs Sp was detected was 55% (311/561) in 2008 and 51% (300/585) in 2009. This compares with 61% (311/507) in 2007 (p=0.004) (Figure 2.1.).

The proportion of PCV7 serotypes also shows a significant downward trend over time such that the fall in the overall carriage rate is accounted for by their disappearance (p<0.0001) (Figure 2.1.), apart from 19F which persists (Figure 2.2.). In contrast to several other published studies (126, 239, 245, 246), no overall compensatory rise in non-PCV7 serotypes is seen (p=0.792) (Figure 2.1.). Among the PCV7 serotypes, only 6B, 14, 19F and 23F (the latter, one isolate only)

had been detected in the 2007 sample (75). Serotypes 4, 9V and 18C were never detected among these children (75). By 2009 both 6B and 23F were no longer found and only one isolate of type 14 (in a child aged 63 months) was identified among 585 children (Figure 2.2.). In contrast, serotype 19F continued to circulate at a frequency of between approximately 3.5% and 6% in this population (p=0.170). Of the 19F carriers, 65-75% had received at least one dose of vaccine and there was no obvious trend in their age over the period studied.

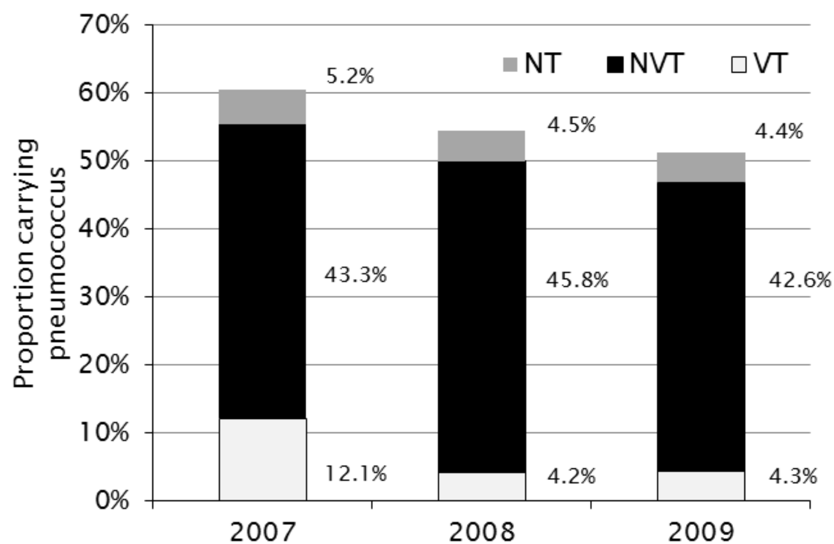


Figure 2.1. Proportions of all children studied carrying pneumococcus in 2008 and 2009 compared to 2007 data.

Bars are subdivided into vaccine types (VT – 4, 6B, 9V, 14, 18C, 19F and 23F), non-vaccine types (NVT - all others) and non-typeable isolates (NT)

Regarding the non-PCV7 serotypes, there was no overall rise, although the numbers of isolates of some of them increased from 2008 to 2009, most notably serotypes 3 (2,22) and 6C (4,16) (Figure 2.2.). Other serotypes which are included in the newer vaccines fluctuated over the three years but did not show obvious trends (1 (5,0,7); 6A (12*,32,8) *includes 6C; 7F (1,0,3); 19A (18,41,31). Other frequently found serotypes were 15B/C, 23A, 23B, 11A, 21, 16F, 35F and 24F. All 3 of the serotypes most commonly associated with invasive disease during this period in Portugal (1, 7F and 19A) were detected (225).

The proportion of children colonised with non-typeable Sp was 5.2%, 4.5%, and 4.4% in 2007, 2008 and 2009, respectively (Figure 2.1.).

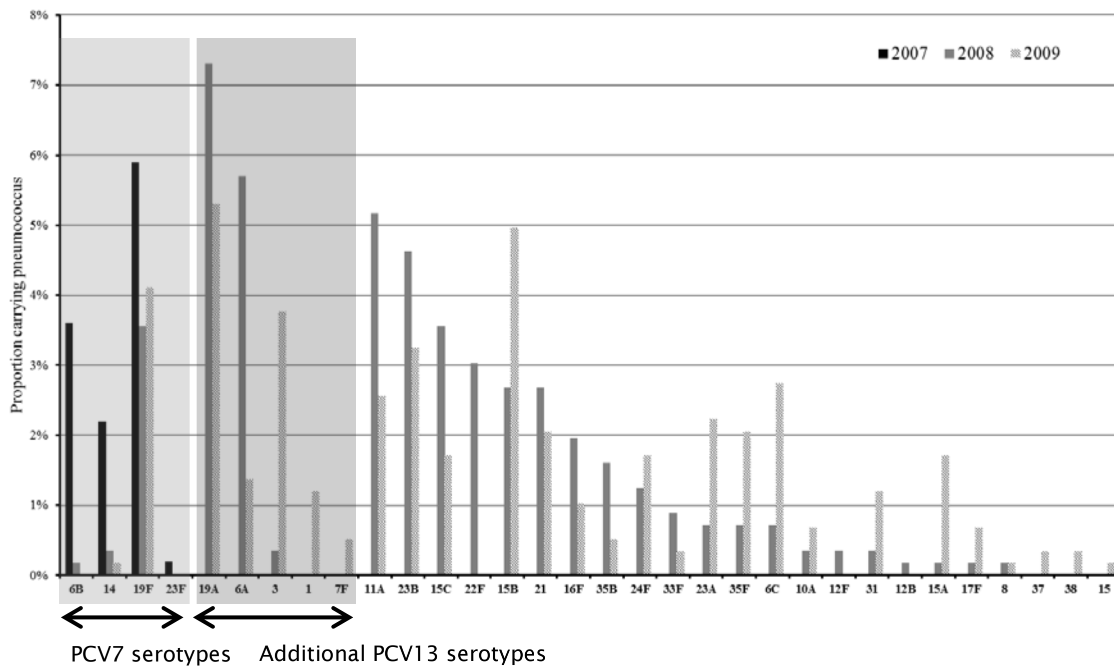


Figure 2.2. Proportions of all children studied carrying PCV7 serotypes in 2008 and 2009 compared to 2007 (data for 4, 9V and 18C are not shown as none were detected) and proportions carrying the non-PCV7 serotypes (additional five PCV13 serotypes and others) detected in 2008 and 2009.

The total number of different serotypes identified was 26 in 2008 and 27 in 2009 having been 27 in 2007. The corresponding Simpson indices (D) of diversity were: 2007 - D = 0.9366, 95% CI = (0.9361, 0.9372); 2008 - D = 0.9220, 95% CI = (0.9213, 0.9226); 2009 - D = 0.9405, 95% CI = (0.9400, 0.9410), confirming, despite an apparent transient fall in diversity in 2008, no progressive trend over this period. This is illustrated in the ranked frequency distribution curves (Figure 2.3.) which are presented so as to permit visual comparison with two recent papers which have shown data in this way (245, 246). Relative to the other curves which almost superimpose, the 2008 curve shows greater relative abundance of the commonest serotypes (left end of curve) and lower frequency of the more uncommon ones (right end).

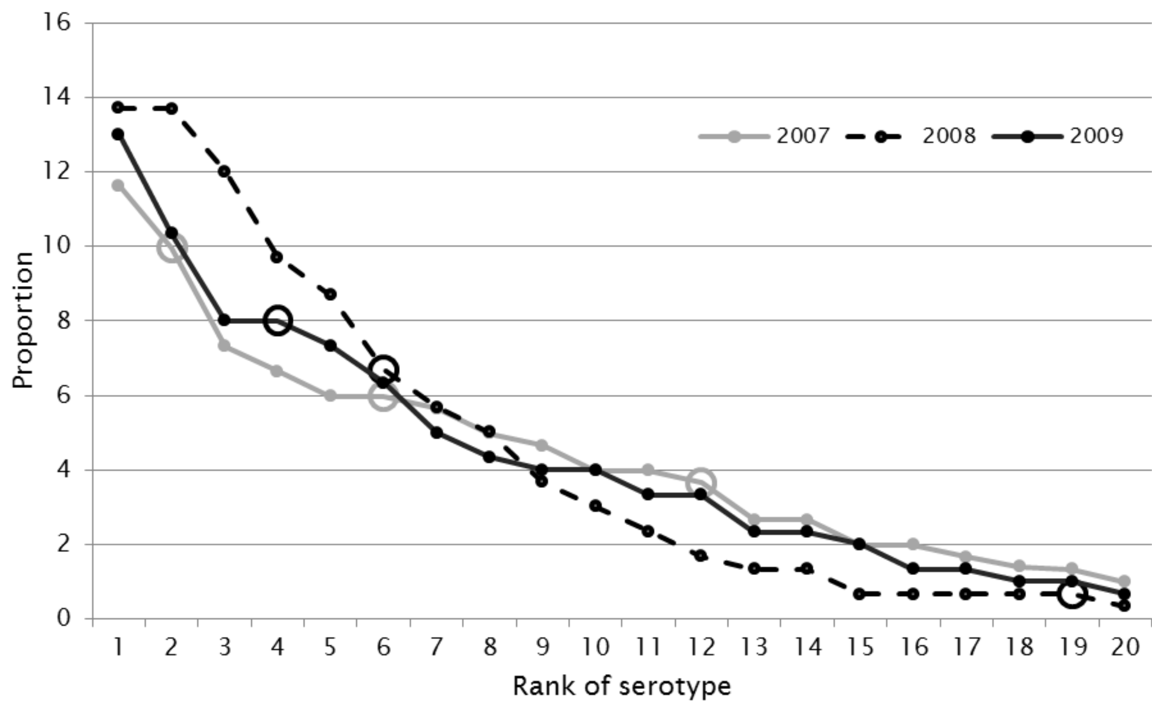


Figure 2.3. Ranked frequency distribution curves for the commonest 20 serotypes in each year.

PCV7 serotypes are shown as open symbols. To permit comparison with 2007 data when they were not distinguished and with previous publications using this approach, data for serotypes 6A and 6C and for 15B and 15C were combined

2.5 Discussion

The impact of PCV programmes depends heavily upon indirect effects due to changes in nasal colonisation and transmission between immunised children and their contacts. These changes may differ substantially from one place to another. As plans progress to roll out PCVs globally into more diverse settings, there is a pressing need to understand the main determinants of the size, shape and duration of the population effects of these immunisation programmes, to permit more accurate predictions and better informed choices about future vaccine formulations to be made.

Several other groups have documented changes in the ecology of Sp serotypes carried by children over time during the era of PCV7 use in their communities. In Massachusetts, USA and Southampton, UK, where immunisation rates rose rapidly to high levels and were then maintained, overall carriage rates were little changed, despite apparent fluctuations, and PCV7 serotypes rapidly disappeared to be replaced by others (239, 244, 245). In Nice, France, where

vaccine uptake, as in Portugal, was more progressive, an overall fall in pneumococcal carriage from 54% in 1999 to 45.2% in 2008 was documented (242). While all of these studies, including ours, suffer from the inherent limitations of repeated cross-sectional surveys, they nevertheless document trends in particular communities over time.

The data reported here are from children among whom immunisation rates rose during the study period to greater than 80%, but this was in the context of somewhat lower and falling overall vaccine coverage in the community.

The carriage rates of pneumococcus reported in two DCCs studies performed in Lisbon in 2001 and 2006 did not show any overall decrease (64.9% and 68.7% respectively) (249). This contrasts with a decline from 61% to 51% in Coimbra, between 2007 to 2009. The 2001 study from Lisbon reports circulation of all PCV7 serotypes apart from serotype 4 and the proportion of all pneumococci isolated that were PCV7 serotypes fell from 53% to 11% between 2001 and 2006 although only serotypes 4 and 9V were completely absent in the latter dataset, indicating clearly that extensive serotype replacement in carriage occurred in Portugal over this period (249). The first study in Coimbra, performed in 2007, 6 years after PCV7 was first used, demonstrated continuing circulation of serotypes 6B, 14, 19F and 23F but not 4, 9V or 18C (75). The new data presented here document the disappearance of PCV7 serotypes 6B and 23F and the near-disappearance of serotype 14, while serotype 19F persisted with no sign of diminution.

The rates of disappearance of different serotypes do appear to differ in other reports. In particular other studies have drawn attention to persistence of 19F (126, 243) and even in reports where this phenomenon is not so obvious, careful examination of datasets reveals that this serotype tends to be among the last of the PCV7 serotypes to remain in circulation (246). A study to estimate the vaccine efficacy against acquisition of specific serotypes using previously published trials found no statistically significant efficacy of PCV7 against vaccine serotypes 19F and 14 (313). Another study involving toddlers attending DCCs in Israel, conducted to document the effect of a 9-valent pneumococcal conjugate vaccine on the carriage rate, showed significant protection against serotypes 6B, 9V, 14 and 23F but not against 19F (236). Immunogenicity studies do not suggest that serotype 19F capsule or other relatively persistent serotypes in PCVs induce smaller IgG responses (201) and mucosal IgG and IgA responses in children primed with PCV7 and boosted with polysaccharide vaccine (PPV23),

show that 19F capsular antigen primes for mucosal memory responses of both these antibody isotypes that are similar in size to the other vaccine antigens (195). The main determinant of rate of elimination may instead be the quantity of capsule expressed, which is an inverse function of the metabolic cost of generating the necessary sugar repeats and which is high for serotype 19F and relatively high for the other vaccine serotypes whose prevalence is slower to diminish (60). In the context of moderate vaccine usage, it appears that the fall in prevalence of such serotypes is prolonged and that serotype 19F may circulate for longer or indefinitely.

It is less clear why, in this setting, the eventual disappearance of all the more persistent vaccine serotypes apart form 19F has not been accompanied by their replacement with emerging non-PCV7 serotypes over this period and has instead resulted in an approximately 10% fall in overall carriage rates, with the ratio of PCV7 serotypes to other serotypes staying roughly constant at 8.2-8.7%. This could prove to be a temporary phenomenon and ongoing surveillance will clarify this. Alternatively it may be that, at the population level, gradual and more modest rises in vaccine-induced mucosal immunity result in new equilibria between pneumococcal strains and other colonising bacterial species at least where the disappearance of relatively more encapsulated and persistent serotypes is concerned.

Recent reports from Massachusetts and the UK show a trend towards greater diversity among colonising serotypes as PCV7 serotypes disappear and replacement occurs at least during the first 2-4 years following vaccine introduction (245, 246). The samples collected in Coimbra span a period 6-8 years from the first use of PCV7 in Portugal and suggest a diversity that is consistently close to the highest seen in these other studies and not changing progressively over time, despite some year to year variation. Such comparisons between these studies need to be made recognising that in Massachusetts and England samples were taken from children attending primary care surgeries, whereas our samples were collected in nurseries in which, if anything, higher transmission rates between subjects and thus less diversity might be expected. This is consistent either with a more persistent diversity-enhancing effect of slower, lower level vaccine use, or a fundamentally different and more diverse pneumococcal population structure in Portugal than in the UK or Eastern USA from the outset. Further analyses of data from earlier and our current Portuguese studies may help clarify this.

With the advent of higher valency vaccines, among the many non-PCV7 serotype isolates, those that are included in the new vaccines are of particular interest. Some serotypes which cause significant numbers of cases of invasive disease are rarely seen in most colonisation studies, like serotypes 1 and 5 (79). In this context, the occasional detection of serotype 1 in this study is of some interest. Also of note is the apparent emergence of serotype 3, a somewhat anomalous serotype with a large capsule (60), and it will be of interest to observe, in due course, trends in colonisation in 2010, 2011 and beyond when PCV13, which includes serotype 3, has been in use and whether any cases of invasive serotype 3 disease are seen there or elsewhere in Portugal during the same period.

In this study, a high proportion of pneumococcal isolates were non-typeable both by Quellung and the multiplex PCR reaction compared, for example, to the study in Southampton, UK (244). A previous study in Portugal in a similar population that investigated non-typeable strains found that they had a diverse genetic background (314). As MLST and PFGE have not been done on these isolates to date, we are not certain whether their population structure has remained stable over the study period. These non-typeable strains continue to be an intriguing sub-group which warrant further investigation.

In conclusion, this study underlines the complexity of pneumococcal ecology and emphasises the fact that the effects of different approaches to implementation of conjugate vaccination exhibited in different environments may be more difficult to predict than we hope and expect. Further prospective studies of the nasopharyngeal colonisation trends of pneumococcus, including its relationships to other bacterial species and intercurrent respiratory viral infections are warranted.

Acknowledgements

Children and parents for their participation; nurses and staff of the DCCs for their dedicated work in this study; staff of the Microbiology Laboratory of Centro Hospitalar de Coimbra for isolation of pneumococci from the nasopharyngeal samples in 2008.

The study was partially supported by an unrestricted, investigator-initiated grant from Pfizer, Portugal. The funder had no role in study design, data collection and analysis or preparation of the manuscript.

2.6 Appendix to Chapter 2

In order to evaluate continuing trends in pneumococcal nasopharyngeal colonisation, in February-March 2010 we swabbed again the nasopharynges of 586 children attending the same DCCs in Coimbra. The previously described clinical and demographic questionnaire, methodologies of sample collection, storage (frozen at -80°C in STGG broth) and pneumococcal culture (briefly samples were inoculated onto blood agar plates and incubated at 37ºC in 5% CO_2 for 24 hours; *S. pneumoniae* was identified by morphology, optochin sensitivity, gentamicin resistance and bile solubility and isolates stored at −80ºC), were used.

PCV10 was licensed in Portugal in April 2009 and only a few children received it in the period between then and March 2010. For this reason only PCV7 types are considered to be vaccine types in this analysis, which permits comparison with previous data. A microarray based method for serotyping (see Chapter 1, section 1.1.6.1) was introduced in 2010. Since this method may detect serotypes present at low abundance that would be missed using Quellung reaction serotyping of a single cultured colony from each child, to permit comparison with earlier datasets, initially the sole or predominant serotype is considered in this analysis. The D index is used to access trends in diversity. Data on sole and co-colonisation with different serotypes are also presented.

Clinical and demographic characteristics of this group of children were similar to the previous years: median age was 41.5 months (range 6.3–74.5 months), 326 (56%) were boys, 11.8% had received antibiotics in the previous month, 86.5% had received at least one dose of PCV7 or PCV10, 36.3% had one or more siblings <6 years and 26.5% had smokers at home.

S. pneumoniae colonisation rate was 58.5%, showing a slight increase compared to the previous two years and being now closer to the rate found in 2007. This was due in part to an increase in non-PCV7 types but mostly due to an increase in the PVC7 serotype 19F (Figure 2.4.). Serotype 18C was detected in one child at an abundance of 91% alongside serotype 11D but no other PCV7 vaccine types were detected, in line with their reduction and disappearance observed in previous years. Although there was a net increase in non-vaccine types, among them some increased (6C, 16F, 21) while others decreased (23B, 16C). There was no obvious rise in serotypes 3, 7F and 19A, contained in PCV13, the use of which was only just starting at the time of this sample. Other non-vaccine types had fluctuations as seen in previous years but did not show obvious trends.

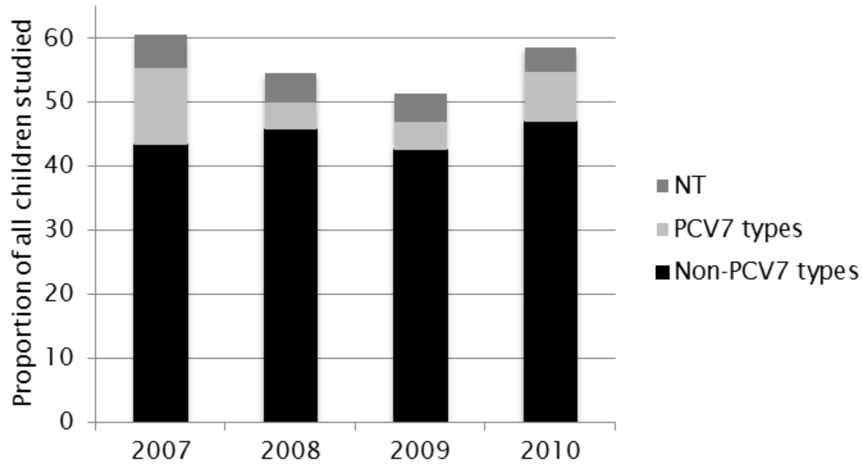


Figure 2.4. Proportions of all children studied carrying pneumococcus from 2007 to 2010.

Bars are subdivided into vaccine types (VT – 4, 6B, 9V, 14, 18C, 19F and 23F), non-vaccine types (NVT – all others) and non-typeable isolates (NT)

The number of different predominant or sole serotypes and non-typeables identified was 27, the same as in 2007 and 2009 having been 26 in 2008. The corresponding D index was 0.9334, having been 0.9366 in 2007, 0.9220 in 2008 and 0.9405 in 2009, reconfirming some fluctuation but no progressive trends over the 4 years of the study. The ranked frequency distribution curve is presented in Figure 2.5.

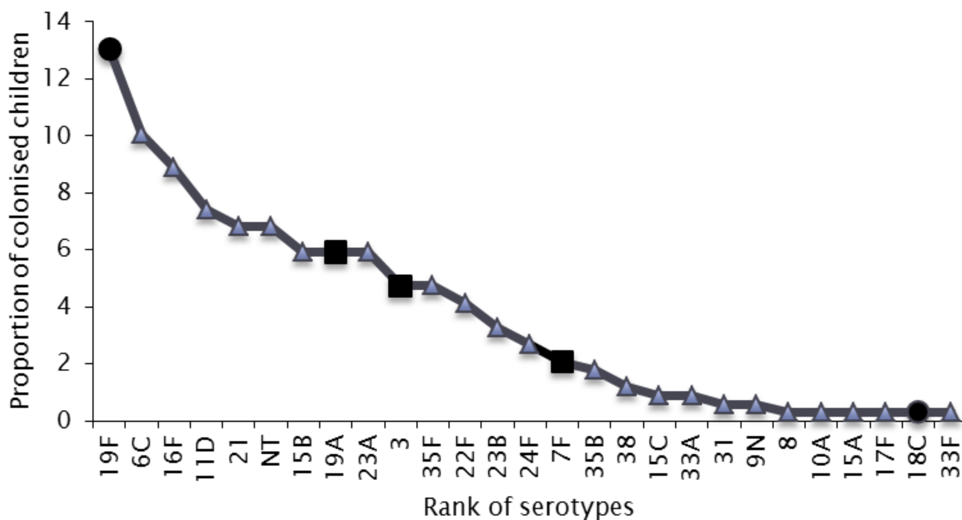


Figure 2.5. Rank frequency distribution of the only or predominant serotype detected in the 2010 samples.

Triangle – non-vaccine types and non-typeable; square – PCV7 types; circle – PCV13 types

The use of microarray serotyping permitted detection of co-colonisation with more than one encapsulated serotype or non-typeable pneumococcus in 103 children. Figure 2.6 shows the frequency distribution of all the serotypes and non-typeables detected and demonstrates that, with the exception of a small number which were only rarely found, all serotypes occurred both in isolation and in association with others although no particular combinations were seen more frequently than would be expected by chance. Non-typeables were more frequently found in co-colonisation.

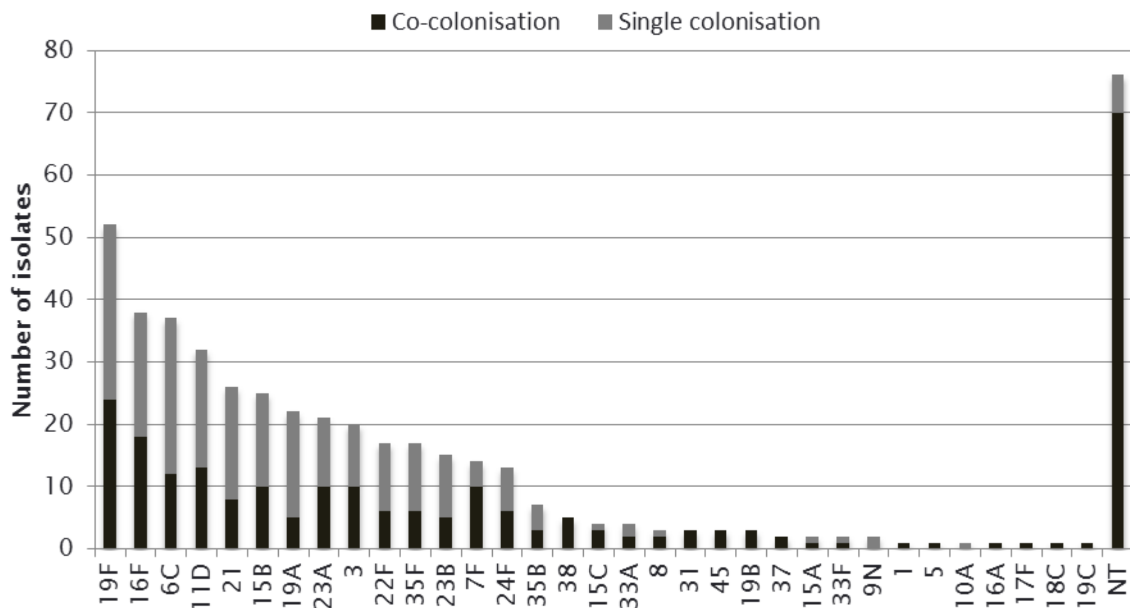


Figure 2.6. Individual serotype and non-typeable distribution of single and co-colonised samples.

Regarding the relative abundance of each strain in co-colonised children, in all but three, the predominant strain represented more than 50% of the DNA found in the sample (Figure 2.7.).

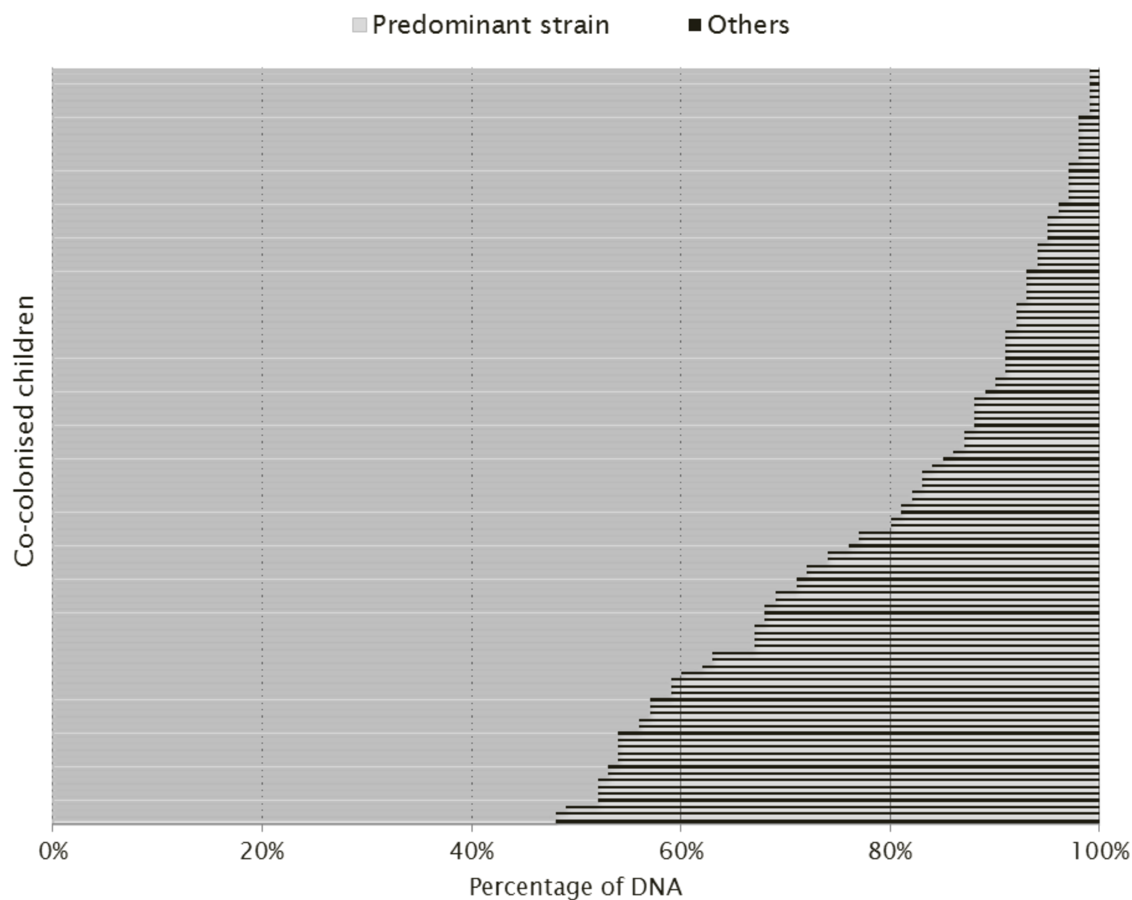


Figure 2.7. Percentage of DNA of the predominant strain (grey line) and the other strain(s) (black line) in co-colonised children.
Each line represents a child

In conclusion, there was a net increase in colonisation partly due to increase in non-vaccine types but mostly due to an increase in colonisation with serotype 19F. This will be described in detail in the following chapter. The diversity remained high without a progressive trend. The introduction of PCV13 in 2010 is likely to drive further changes in pneumococcal ecology rendering further studies important for the understanding of what remains an evolving situation.

3. Resurgence of serotype 19F carriage in pre- school children in Portugal in the context of continuing moderate conjugate pneumococcal vaccine uptake

Fernanda Rodrigues[1], Begonia Morales-Aza[2], Rachel Holland[2], Katherine Gould[3], Jason Hinds[3], Guilherme Gonçalves[4], Luís Januário[1], Adam Finn[2]

[1]Emergency Service & Infectious Diseases Unit Hospital Pediátrico de Coimbra, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal; [2]School of Social and Community Medicine, University of Bristol, Bristol, UK; [3]Division of Clinical Sciences, St. George's, University of London, London, UK; [4]Instituto de Ciências Médicas Abel Salazar, Universidade do Porto, Porto, Portugal

Clin Infect Dis. 2013;57(3):473-4

Reprinted from *Clin Infect Dis*, vol. 57, Issue 3, Fernanda Rodrigues, Begonia Morales-Aza B, Rachel Holland, Katherine Gould, Jason Hinds, Guilherme Gonçalves, Luís Januário, Adam Finn. Resurgence of serotype 19F carriage in preschool children in Portugal in the context of continuing moderate conjugate pneumococcal vaccine uptake, pages 473-4, Copyright 2013, with permission from Oxford University Press. All rights reserved.

Reduction to near extinction of pneumococcal (*Streptococcus pneumoniae* (Sp)) vaccine serotype nasal colonization has been reported over a period of around 5-7 years of pneumococcal conjugate vaccine (PCV) use (239, 245).

PCVs have been used in private practice in Portugal since 2001. They are not included in the universal national immunization program. After licensure of the 7-valent vaccine (PCV7, Wyeth), estimated coverage, based on sales information, increased from 32% in 2002 to 65% in 2005 and 79% in 2007, then fell slightly to 75% in 2008 (oral communication, Pfizer). In April 2009, the 10-valent vaccine (PCV10, GSK) became available and some infants received it. In January 2010, the 13-valent vaccine (PCV13, Pfizer) replaced PCV7 and is the vaccine which has been used almost exclusively since then (estimated coverage in 2010 and 2011 was approximately 65%; oral communication, Pfizer). Both schedules (2+1 and 3+1) have been used.

Previously, we reported carriage rates by serotype in cross-sectional studies performed annually in pre-school children attending day care centers (DCCs) in Coimbra, a city in the central region of Portugal, between 2007 and 2009 (75, 315). Among the children studied, the proportion who had received at least one dose of PCV7 vaccine showed a significant upward trend over the 3 years (76.5%, 82.4% and 84%) (315). The proportion of PCV7 serotypes (by Quellung reaction) showed a significant downward trend over time apart from 19F, which persisted, detected in 4.1% of all children studied in 2009 (315). Studies from other countries suggest that 19F is slower to disappear than other PCV types (126, 243).

In February-March 2010 we swabbed the nasopharynges of 586 children, attending the same urban DCCs in Coimbra. The study was approved by the Ethics Committee of Centro Hospitalar de Coimbra. Parents or guardians provided written informed consent for their child to participate.

The mean age was 41.5 months (standard deviation, 18.1; range 6.3-74.5); 326 (56%) were male, and 507 (86.5%) children had received at least one dose of PCV7 or PCV10. Among those aged ≥18 months, 78 (15.2%) and 338 (65.6%) had completed either a full 2+1 or 3+1 schedule, respectively. Nasopharyngeal swabs were inoculated into Skim-milk tryptone glucose glycerol broth and stored at -80°C prior to culture. Standard microbiological techniques for the isolation and identification of Sp were used as described previously (316). The Sp carriage rate was 58.5% (343/586). Molecular serotyping was undertaken, using a microarray based method to determine *cps* gene content from genomic DNA hybridization,

capable of detecting multiple serotypes in a single sample (123). Excluding non-typable (NT) signals (n=7 NT only, n=76 NT with other serotypes), which, using this methodology can include non-Sp streptococci, 73 children (12.5%) carried more than one Sp serotype. A total of 11.3% had PCV7/10 vaccine serotypes: 19F 8.9% (52); 7F 2.2% (13, including 2 with 19F); 1, 5 and 18C (1 each). Serotype 19F was more commonly detected than any other serotype. There was sole colonization by 19F in 34 children (5.8%) and by 19F with other serotype(s) in 18 (3.1%), among whom in 11 (1.9%) 19F was both the predominant serotype and represented >50% of the bacterial DNA detected in the sample (Figure 3.1.). Accordingly 45 children (7.7%) carried 19F as the only or the predominant serotype, a clear rise.

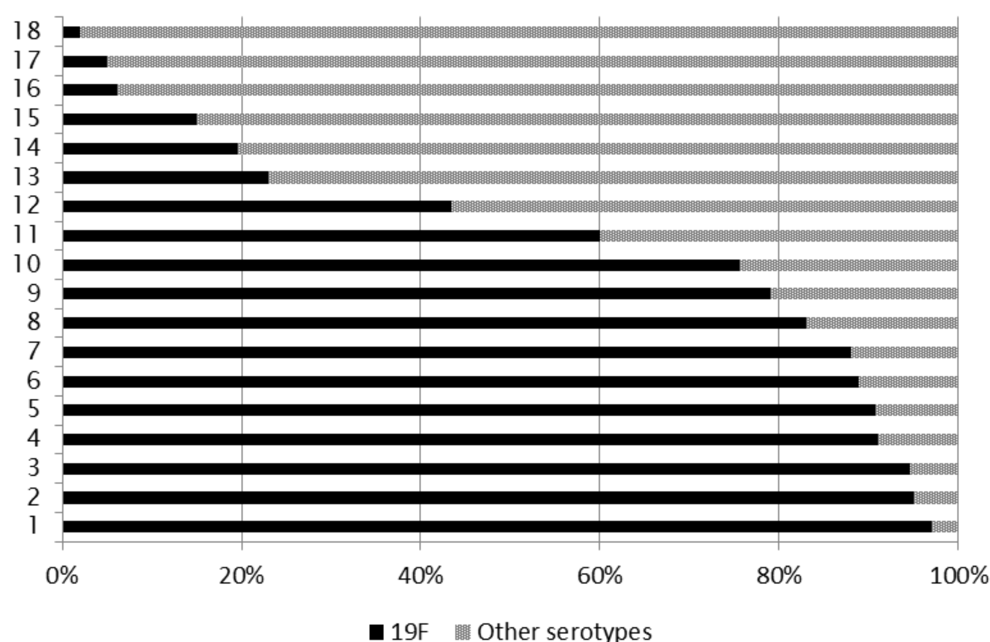


Figure 3.1. Percentage of serotype 19F DNA in 18 children co-colonized with 19F and at least 1 other serotype.

Twenty cases of invasive disease due to 19F were reported to the national surveillance scheme, from 2006 to 2012, with 6 cases occurring between July 2011 and June 2012 (317).

Serotype 19F, covered by all 3 PCVs, has emerged as the most commonly carried encapsulated pneumococcus in this DCC population. Higher coverage than is at present being achieved in Portugal may be necessary to control it at the population level.

4. Relationships between rhinitis symptoms, respiratory viral infections and nasopharyngeal colonization with *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* in children attending daycare

Fernanda Rodrigues[1], Dona Foster[2], Emily Nicoli[3], Caroline Trotter[3], Barry Vipond[4], Peter Muir[4], Guilherme Gonçalves[5], Luís Januário[1], Adam Finn[6]

[1]Infectious Diseases Unit and Emergency Service, Centro de Investigação e Formação Clinica, Hospital Pediátrico de Coimbra, Centro Hospitalar e Universitário de Coimbra, Faculdade de Medicina, Universidade de Coimbra, Coimbra, Portugal; [2]Nuffield Department of Medicine, University of Oxford, Oxford, UK; [3]School of Social and Community Medicine, University of Bristol, Bristol, UK; [4]Health Protection Agency Virology Laboratory, Bristol, UK; [5]Instituto de Ciências Médicas Abel Salazar, Universidade do Porto, Porto, Portugal; [6]Bristol Children's Vaccine Centre, School of Clinical Sciences and Cellular and Molecular Medicine, University of Bristol, Bristol, UK

Pediatr Infect Dis J. 2013 Mar;32(3):227-232.

Reprinted from *Ped Infect Dis J*, vol. 32, Issue 3, Fernanda Rodrigues, Dona Foster, Emily Nicoli, Caroline Trotter, Barry Vipond, Peter Muir, Guilherme Gonçalves, Luís Januário, Adam Finn. Relationships between rhinitis symptoms, respiratory viral infections and nasopharyngeal colonization with *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* in children attending daycare, pages 227-232, Copyright 2013, with permission from Wolters Kluwer Health. All rights reserved.

4.1 Abstract

Background: Nasal bacterial colonization is often dubbed "asymptomatic". We hypothesized that rhinitis, common in pre-school children, is associated with bacterial colonization and that respiratory viruses, which cause rhinitis, interact with bacteria in ways which promote transmission.

Methods: Five hundred eighty-five children (4.2–73.6 months) attending daycare had clinical information, a rhinitis score and nasal swabs collected in February 2009. Swabs in soya tryptone glucose glicerine broth were cultured for *Streptococcus pneumoniae* (Sp), *Haemophilus influenzae* (Hi) and *Staphylococcus aureus* (Sa) and analyzed by real-time polymerase chain reaction for respiratory viruses, both semi-quantitatively.

Results: Rhinitis symptoms, carriage of Sp and Hi and viral detection fell, while Sa carriage rates rose with age. Significant, age-independent associations between rhinitis symptoms and detection of Hi ($p<0.033$) and Hi colonization density ($p<0.027$) were observed. Of the 42% with detected viruses, most (78%) had picornavirus. There was a significant age-independent association between viral detection (and viral load, picornavirus infection and picornaviral load) and detection of Sp ($p=0.020$, 0.035, 0.005, 0.014) and between viral detection and viral load and Sp colonization density ($p=0.024$, 0.028).

Conclusions: Hi may promote its own transmission by inducing or amplifying rhinitis in children. There is a close quantitative relationship between respiratory viral detection, including picornavirus detection and Sp colonization. These findings have implications for understanding disease pathogenesis and formulating prevention strategies using vaccines.

Keywords: children, nasopharyngeal colonization, microbial interactions, rhinitis symptoms, transmission

4.2 Introduction

The nasopharynx is thought to be the source for many pediatric mucosal and invasive bacterial infections, including otitis media, pneumonia, septicemia and meningitis (8, 89, 94, 96). Nasopharyngeal colonization is affected by environmental and host factors, and microbial characteristics. Changes in nasopharyngeal ecology are occurring in many populations as a consequence of selection pressure due to widespread use of childhood conjugate vaccines which induce not only systemic but also mucosal specific immune responses to bacterial capsular antigens (195, 318).

Streptococcus pneumoniae (Sp), non-typeable *Haemophilus influenzae* (Hi) and *Staphylococcus aureus* (Sa) are frequent nasal colonizers of young children (18, 19, 268). Cross sectional studies show changes in frequency of detection of these species with age, with rates characteristically peaking in the first or second years of life for the former 2, whereas Sa carriage rates decrease from significant rates during the first weeks or months of life and then progressively rise during the pre-school years (18, 19, 25, 70, 265, 268, 274). For Sp, which has been most widely studied, there are also marked differences in carriage prevalence between populations (18, 63, 148, 162, 319). It is often suggested that the decline in carriage rates of Sp and Hi with increasing age in children may be due to maturation of mucosal immune responses, acquisition of specific immunity or both (320, 321). Other environmental alterations, such as competitive or synergistic interactions with other microbial species or progressive changes in the mucosal microenvironment could also explain observed trends with age, including observed rises in Sa carriage rates. None of these potential mechanisms are mutually exclusive and any combination of them, along with changing frequency and density of exposure over time as children get older, may influence carriage rates of all these organisms.

Different species occupying the same niche are often assumed to be competitors. Studies have shown evidence of a reciprocal relationship between Sa and Sp in the human nasopharynx suggestive of mutually inhibitory or other competitive effects (17, 25, 70, 268, 322). Experiments in mice and *in vitro* suggest that competitive interactions between Sp and *Haemophilus* spp. may exist, either directly, or indirectly through subversion of host responses (323). However, different species may not influence each other and synergistic relationships may even exist between them (19, 23, 24, 268, 324). In addition,

intercurrent viral infections may predispose to bacterial carriage or disease (24, 27, 101).

Since causing disease neither augments survival in the host nor transmission of these bacteria, it may be an accidental side-effect of their normally benign lifestyle. Proinflammatory, proadhesive or proinvasive gene products dubbed "virulence factors" because of their apparent disease-promoting effects (325, 326), may have been selected because they also improve colonization or transmission success. Intercurrent rhinitis represents a significant transmission opportunity. Perhaps nasal colonization is not asymptomatic, as it is commonly described (8, 17, 327). Maybe it is associated with rhinorrhea either because the bacteria induce host secretions and inflammatory responses or because they respond to rhinitis induced by respiratory viral infections by proliferating, or both.

To track changes in pneumococcal serotype carriage in pre-school children attending daycare, we began cross-sectional studies every winter in Coimbra, central Portugal in 2007 (75, 315). In 2009, cultures for Hi and Sa and PCR for respiratory viruses were also performed and a score for intercurrent rhinitis was recorded for each child.

4.3 Methods

4.3.1 Study population

Healthy children aged 4.2 to 73.6 months were recruited in 8 urban daycare centers (DCCs) in Coimbra, a city in the central region of Portugal, during February 2009.

4.3.2 Study approval and consent

The study was approved by the Ethics Committee of Hospital Pediátrico de Coimbra, Centro Hospitalar de Coimbra. Parents or guardians provided written informed consent for their child to participate.

4.3.3 Clinical data

A week before sample collection, parents were given a questionnaire on their child's age, sex, use of antibiotics in the preceding month and immunization history for pneumococcal conjugate vaccines (PCVs) (number of doses and dates). At the time of sample collection, a member of the research

team asked the carer of the child for a "symptoms of nasal outflow tally" (SNOT) score. A single score for severity of nasal discharge, blockage and of sneezing was made as follows: 0 - none; 1 - mild; 2 - moderate; 3 – severe. The approach is based on a previously described scoring system used for allergic rhinitis (328). However, in practice, it was found that for infectious rhinitis in pre-school children, who cannot usually report nasal blockage and in whom sneezing is a comparatively unusual symptom, the score primarily reflected the current and recent level of nasal discharge.

4.3.4 Nasopharyngeal swab collection

A single, fine dry tip, flexible plastic shaft, rayon bud swab (Medical Wire & Equipment, Corsham, Wilshire, UK) was passed horizontally into 1 nostril of the child until resistance was felt, rotated along the axis of the shaft, withdrawn and broken off into a 2-mL cryovial containing 1.5-mL soya tryptone glucose glycerine broth, as previously described (329). Vials were stored at minus 80˚C until batched culture was performed.

4.3.5 Bacterial culture

Swabs were thawed on ice, vortexed and 50μL of the resultant broth inoculated onto 5% Columbia blood agar and Chocolate agar plates (E & O Laboratories Ltd, Burnhouse, Bonnybridge, UK) with a gentamicin disc at a concentration 10μg (BD BBL Sensi-Disc, Oxford, UK) added to the Columbia plate. The cultures were incubated at 37°C in 5% carbon dioxide for 24 hours and then examined, with the density score for the presence of Sp, Hi and Sa recorded.

Identification of each pathogen was based upon standard microbiological techniques as follows: presumptive Sp were streaked onto a Columbia blood plate with an optochin disc (5.0μg of ethylhydrocupreine hydrochloride per disc supplied as BBL™ Taxo™ Discs for Differentiation of Pneumococci) and were considered positive if the size of the zone of inhibition was >14mm. Potential Hi isolates were identified using V (nicotinamide adenine dinucleotide, NAD) and XV (haemim and NAD) discs (BBL Taxo Differentiation Discs for *Haemophilus* spp., UK) on nutrient agar (E & O, UK), incubated for 24 hours in 5% carbon dioxide at 37°C. An isolate was confirmed as Hi if growth occurred only around the XV disc. Potential Sa identification was undertaken using DNase agar (E & O Laboratories Ltd, Burnhouse, Bonnybridge, UK) with a positive test indicated by a clear zone surrounding the growth on the plate after flooding with 1 N hydrochloric acid.

Further confirmation was obtained using the Staphurex latex agglutination test according to the standard protocol (Oxoid, Basingstoke, Hamshire, UK).

For each bacterial species, density was scored as follows: 0 = not detected; 1 = 1–5 colonies/50µL broth; 2 = >5–20; 3 = >20–50; 4 = >50–100; 5 = >100.

4.3.6 Viral PCR

Respiratory viral nucleic acid was detected for influenza A and B viruses, respiratory syncytial virus (RSV) types A and B, human metapneumoviruses, parainfluenzaviruses types 1-3, human rhinoviruses, enteroviruses, human adenoviruses and human bocavirus using established methodology. Total nucleic acid was recovered from 200µL aliquots of swab material in STGG broth using a Kingfisher 96 magnetic particle processor (Thermo Scientific) and eluted in 60µL sterile water. Viral DNA was amplified using real time PCR assays, and viral RNA using two-step reverse transcription-real time PCR assays. Complementary DNA (cDNA) synthesis was performed in 25µL volumes using 100 units MMLV reverse transcriptase (Promega) and 0.5mg/mL random hexamers for 30 minutes at 37°C followed by 10 minutes at 95°C. Real time PCR was performed in 20µL reactions consisting of ABI Fast Universal Master Mix (Applied Biosystems) with 5µL total nucleic acid extract or cDNA and primers and probes to detect influenza A (IFA) and B (IFB) viruses (330), respiratory syncytial virus types A and B (RSV) (Table 1), human metapneumoviruses (HMPV) (331), parainfluenzavirus (PIV) types 1-3 (Table 4.1.), human rhinoviruses (RV) (332), enteroviruses (EV) (333), human adenoviruses (ADV) (334), and human bocavirus (HBoV) (335). A two temperature thermal cycling protocol (50 cycles of 95°C denaturation and 60°C annealing/extension) in an ABI 7500 Fast processor (Applied Biosystems) was used.

Table 4.1. Primers and probes for RSV and PIF real time PCR.

Virus/Primer/Probe	**Sequence and Label (5'- 3')**	**Gene Target (Accession no.)**
Respiratory Syncytial Viruses		
RSVA-Forward	gtgcagggcaagtgatgttac	NP Gene (U39661) (AF013254)
RSVA-Reverse	cacccaatttttgggcatattc	
RSVB-Forward	ttcagggcaagtaatgctaagatg	
RSVB-Reverse	cctcccaacttctgtgcatactc	
RSV-Probe	NED-acaacttgttccatttctgc-MGB	

Parainfluenza viruses		
PIV1-Forward	acagatgaaattttcaagtgctactttagt	L Gene (NC003461)
PIV1-Reverse	gcctcttttaatgccatattatcattaga	
PIV1-Probe	NED-atggtaataaatcgactcgct-MGB	
PIV2-Forward	ctatgaaaaccatttacctaagtgatgga	HN Gene (AF533012)
PIV2-Reverse	cctccyggtatrgcagtgactgaa	
PIF2-Probe	VIC-tcaatcgcaaaagct-MGB	
PIV3-Forward	acagtggatcagattgggtcaat	NP Gene (FJ455842)
PIV3-Reverse	atggttgtgaggtcatttctgct	
PIV3-Probe	FAM-cggtctcaacagagct-MGB	

Results were reported as Cycle threshold (Ct) values, determined as the amplification cycle number when fluorescence become detectable. The Ct value is inversely related to the viral load, and a Ct value of ≤35 was regarded as evidence of infection for the purposes of this study. A categorical variable was also generated for viral load score as follows: 0 = not detected after 50 cycles; 1 = detected after 45-50 cycles; 2 = 40-45; 3 = 35-40; 4 = 30-35; 5 = 25-30; 6 = <25 cycles. Enterovirus and rhinovirus group-specific PCR tests which target conserved regions of the viral genome do not reliably differentiate between these virus groups due to their genetic similarity. In common with several other recent studies, we therefore classified samples testing positive with enterovirus and/or rhinovirus assays as picornavirus positive.

Assays were performed in a clinical virology laboratory, with appropriate positive controls and alongside routine samples among which positives for all viruses under investigation here were routinely detected.

4.3.7 Statistical analysis

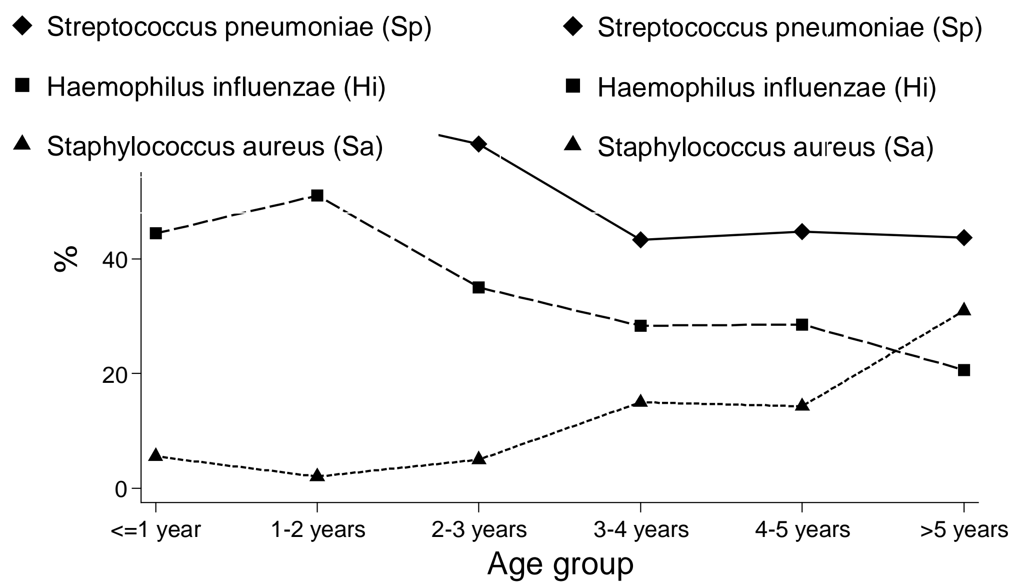
Pearson's chi-squared test for independence and odds ratios (ORs) were calculated for the binary variables of interest. Additionally multivariate logistic regression was performed followed by likelihood-ratio tests to analyze the associations while adjusting for covariates. Finally, analysis of variance for multiple linear regression was applied to categorical variables and covariates.

All analyses were carried out with STATA version 11.2 (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP).

4.4 Results

4.4.1 Characteristics of the study group

Nasopharyngeal swabs were obtained from 585 children of whom 307 (52.5%) were male. The mean (median) age was 41.2 (41.3) months (standard deviation 18.8, range 4.2 – 73.6) and age distribution is shown in the Table within Figure 4.1. and reflects the local demographic of DCC attendance. The use of an antibiotic within 1 month prior to study enrolment was reported for 106 (18.1%) children and receipt of at least 1 dose of 7-valent PCV (PCV7) in 84%.

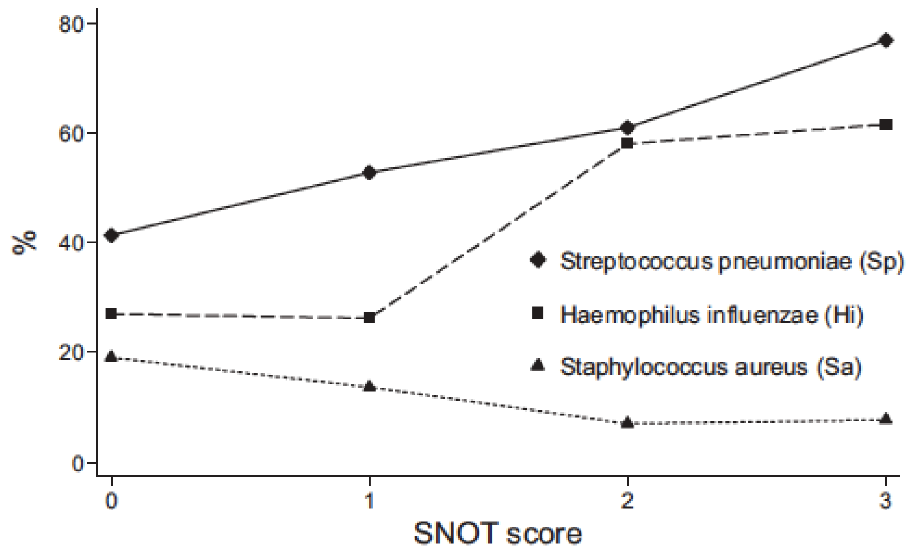


N	36	98	100	120	105	126
N with SNOT score	35	97	96	114	105	126
% with symptoms	91.4	92.8	91.7	68.4	45.7	38.1
Mean (SD) SNOT score	1.26 (0.61)	1.49 (0.74)	1.26 (0.68)	0.81 (0.65)	0.50 (0.57)	0.45 (0.63)
% with virus	86.1	72.5	54.0	40.8	20.0	15.9

Figure 4.1. Age distribution of detected bacterial carriage in the study population.
Table shows frequency of symptoms, mean SNOT score and rates of viral nucleic acid detection for different ages. N indicates number

SNOT scores were obtained for 573 (98.0%) and of these 384 (67.0%) presented with symptoms (Table within Figure 4.1.). Distribution of symptom severity for each bacterial colonization is shown in the table within Figure 4.2. In

most symptomatic children, symptoms were mild, although nearly 1 in 5 had moderate or severe rhinitis. Symptoms were much commoner in younger children with progressive falls in both frequency and mean severity of symptoms after the age of 2 (Table within Figure 4.1.).



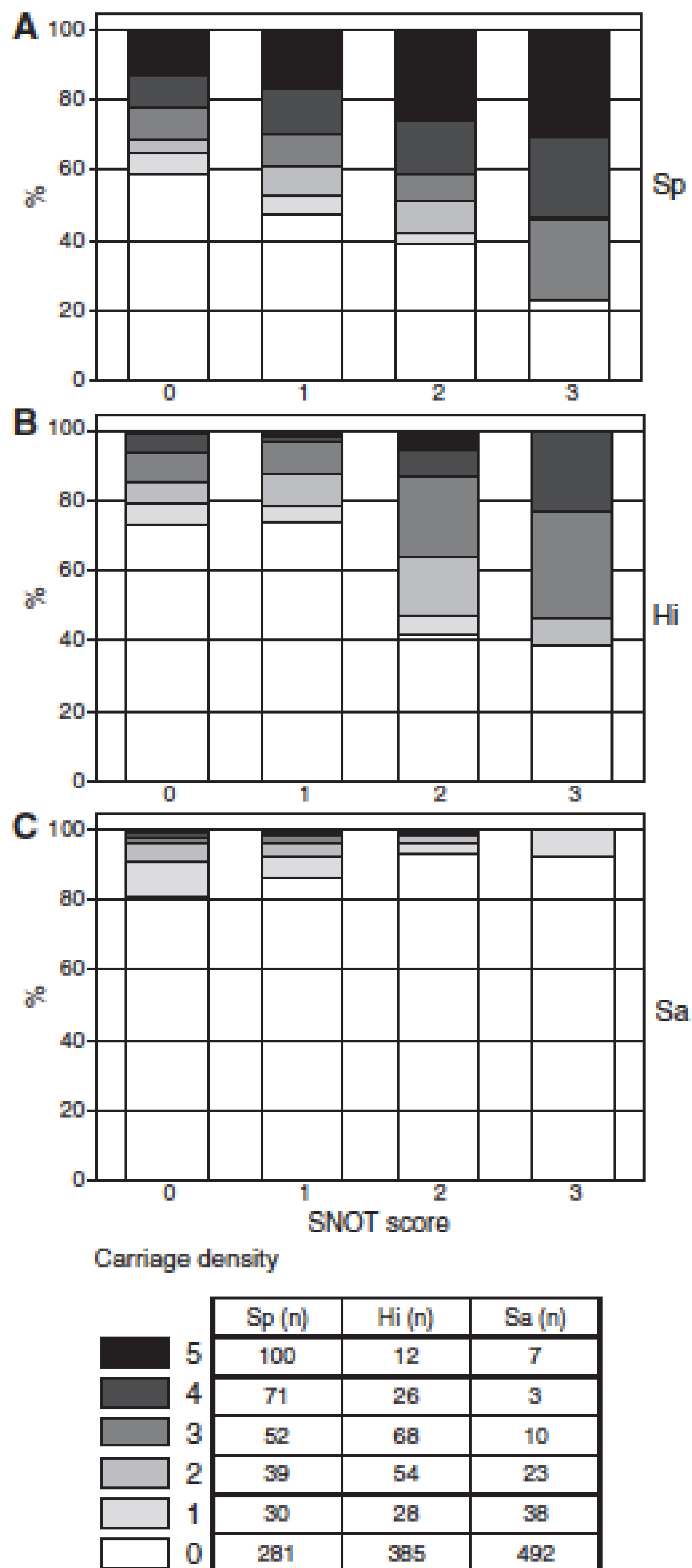
Total	189	271	100	13
Sp	78	143	61	10
Hi	51	71	58	8
Sa	36	37	7	1

Figure 4.2. Proportions of children with each SNOT score who were nasally colonized with Sp, Hi and Sa.
Absolute numbers are shown in the table

4.4.2 Bacterial carriage

Culture results were obtained for all 585 children. Overall the carriage rate was highest for Sp (51.3%, 300), intermediate for Hi (32.7%, 191) and lowest for Sa (13.9%, 81) with this hierarchy persisting in all age groups apart from the 5 year olds (Figure 4.1.). The carriage rates varied with age, with highest rates for both Sp and Hi and the lowest for Sa in the 1-year olds, then falling for Sp and Hi and rising progressively with age for Sa (ORs and *P* values for these age associations were Sp: 0.81, 0.001; Hi: 0.76, <0.001 and Sa: 1.82, <0.001).

The percentages of those colonized with Sp, Hi and Sa at each of the different densities are shown in Figures 4.3. A-C, respectively.



Carriage density	Sp (n)	Hi (n)	Sa (n)
5	100	12	7
4	71	26	3
3	52	68	10
2	39	54	23
1	30	28	38
0	281	385	492

Figure 4.3. Relationships between SNOT score and density of colonization for (A) Sp, (B) Hi and (C) Sa.

Absolute numbers are shown in the table

Sp carriage was most commonly of high, Hi of intermediate and Sa of low density. There was a slight preponderance of Sa carriage among males (61.7%) but this was found to be of weak significance ($P = 0.07$). Carriage of Sp and Hi appeared to be independent of gender. No evidence of an association between colonization with any of the 3 bacterial species and prior antibiotic use or between pneumococcal carriage and previous PCV7 administration was found.

There was no significant association between colonization with Sp and Sa ($P = 0.28$) and little evidence of a positive association between Sp and Hi ($P = 0.08$). By chi-squared test for independence there was a negative association between Hi and Sa with an OR of 0.42 (95% confidence interval: 0.24-0.76).

Analysis of the relationships between densities of the 3 species by ANOVA produced similar findings (significant for Hi and Sa only, $P = 0.02$). However, when age was taken into account in the analysis, no significant associations between any of the 3 species were found.

4.4.3 Association between rhinitis symptoms and bacterial carriage

SNOT scores were obtained and recorded for 98% (573) of the children. Colonization with each of the 3 bacterial species was strongly-positively (Sp and Hi) and negatively (Sa) associated with SNOT scores ($P = 0.002$, 0.001 and 0.04 by chi-squared analysis, respectively). However, there was also a highly significant inverse relationship between SNOT score and increasing age ($P < 0.001$, OR 0.46; 95% confidence interval: 0.39-0.53). Given the strong associations between bacterial colonization and age presented above, the relationship between SNOT score and colonization with each of the 3 bacterial species was assessed taking age into account.

A significant association was found for Hi ($P = 0.003$); however for Sp the evidence was weak ($P = 0.06$) and there was no evidence of an association with SNOT score for Sa ($P = 0.62$). The apparent association between Hi and rhinitis symptoms was investigated further by evaluating SNOT score against Hi colonization density, again taking age into account, and was again found to be significant ($P = 0.027$) while adjustment to include the effects of other variables including prior vaccination, antibiotic use, gender and exposure to smoking produced no significant alterations to the results.

4.4.4 Viral nucleic acid detection

Two hundred forty-six (42.1%) of the children had nucleic acid detected from 1 or more respiratory viruses. Despite being a wintertime cross sectional sample, there were no children with RSV infection and only 1 (0.2%) with influenza (type B). The most commonly detected viruses were picornaviruses (32.3%) of which the majority (27.5%) were presumed to be rhinoviruses based on the relative cycle threshold values of the enterovirus and rhinovirus PCR assays. Other viruses detected included bocavirus (8.4%), adenovirus (4.0%), parainfluenza type 3 (2.4%) and human metapneumovirus (2.2%). There was a strong association between age and viral nucleic acid detection (table within Figure 4.1.) with the odds of detection falling markedly with increasing age (OR = 0.5; 95% confidence interval: 0.44-0.57) with closely similar values for analysis by picornavirus detection alone. Although, by chi-squared, there was a significant association between Sp and Hi (positive) and Sa (negative) colonization and respiratory viral detection (P< 0.001, P= 0.037, P< 0.001, respectively), using a multiple regression model, taking age into account, a significant positive association between respiratory viral nucleic acid detection (P= 0.020) and detection of Sp was observed (picornavirus only P= 0.005), but no evidence of significance was seen for the other 2 bacterial species (Hi P= 0.85; Sa P= 0.18). Likewise, multiple regression analysis showed a significant association between viral nucleic acid detection and Sp bacterial colonisation density (P= 0.024; although picornavirus only, P= 0.08). Using PCR cycle threshold to estimate respiratory viral load, both the presence and density of Sp was associated with overall viral density (P= 0.035, 0.028 respectively) (picornavirus only, 0.014, 0.078).

4.5 Discussion

This study was conducted in a population of preschool children attending daycare and so likely to be experiencing close contact and high infection transmission rates amongst their peers. Two thirds of them had some symptoms of rhinitis at the time of study and such symptoms were near universal among the younger children. We investigated whether such symptoms and whether respiratory viral infection, generally presumed to be responsible for most wintertime rhinitis in children, were associated with bacterial nasal colonization and found evidence of a significant and independent positive association between colonization with Hi and symptoms of rhinitis and an equally robust and

quantitative association between colonization with Sp and respiratory viral nucleic acid detection – predominantly with picornaviruses. Both of these observations make biological sense in as much as generation and exploitation of transmission opportunities are important for survival of both these bacterial species, but that they were each found, individually, for different bacterial species was unexpected. Studies assessing any association between nasal bacterial colonization and symptoms are scarce. Sleeman et al. (182) reported an association between acquisition of Sp by newborn infants or their family members and general practitioner consultation for infection by the study infants in a prospective cohort study. Ours is the first study to measure rhinitis symptoms directly alongside colonization and, in this sample at least, it appears that Hi, rather than Sp or Sa colonization is independently associated with nasal discharge. A study of this design cannot establish causality or, if it exists, whether the bacteria are inducing symptoms, the symptoms are promoting bacterial growth or both. However, there is good evidence showing that Hi is intimately involved in the triggering and regulation of human inflammatory responses (336), and it is certainly plausible that by causing, amplifying or prolonging rhinitis, the bacterium might increase its chances of transmission from child to child. While, in principle, it is conceivable that bacterial density is unchanged but culture is somehow rendered more sensitive from secretions obtained from children with rhinitis, this possible explanation for our findings seems unlikely, particularly since a significant association was only seen for Hi and neither Sp nor Sa.

Our observation of an association between not only the presence of respiratory virus, specifically picornavirus, and Sp but also between viral load and bacterial colonization density extends related findings from other recent studies in distinct populations and samples. One study from Australia, including indigenous children, showed associations between rhinovirus infection and colonization with Sp, Hi and *Moraxella catarrhalis* (24). A study from Vietnam using PCR to detect nasopharyngeal bacteria and viruses showed higher Sp load in the presence of viral nucleic acid detection with rhinovirus, influenza A and respiratory syncytial virus in children with radiographic pneumonia (184). Peltola et al. (337) recently showed a temporal association between periods of rhinovirus activity and rates of invasive Sp disease in pre-school children in Finland. Taken together these findings are consistent with the hypothesis that Sp proliferates, thus becoming more easily detectable both by culture and PCR, in the presence of

rhinovirus infection, which is very common in young children. This may facilitate transmission and result, on occasion, in disease.

Several previous studies have explored the possibility that colonization with one bacterial species might impact on the chances of successful simultaneous colonization with another. In particular there has been much interest in the possibility of a competitive relationship between Sp and Sa, driven perhaps by their reciprocal age distribution patterns and by interest in the possibility that changes in Sp colonization patterns due to conjugate vaccine use could result in altered Sa ecology and disease. Some studies done when, unlike this one, PCV7 serotype carriage predominated suggested negative associations between those serotypes and Sa (25, 70, 274, 322), although concerns that Sa carriage rates would consequently rise over time have not been borne out (265). Our study failed to demonstrate any associations between the 3 bacterial species studied that were independent of age. Given the important relationships that exist between age and bacterial and viral infection, rhinitis and immunological experience and maturation, it is clear that any conclusions concerning interaction between colonizing bacteria should take age into account (268) and that other papers which do not do so should be treated with caution. Conversely, it is likely that in any given sample of children studied, differences, for example, in the incidence of specific viral infections at the time of study could have major impact on bacterial ecology. For example the complete absence of RSV and almost complete absence of influenza from the children reported in this study, both of which are consistent with available local and national epidemiological data for the annual epidemics of these two viruses which peaked early in 2008-09, could have impacted not only the levels of bacterial colonization but also the relationships between colonizing bacteria.

Most previous studies of bacterial colonization of the nose simply document the presence or absence of the organism (17, 19). The availability of quantitative PCR provides a rapid and technically robust means of measuring the number of copies of one or more specific bacterial genes but has the potential flaw that DNA from non-viable bacteria will be detected (184). We used a semiquantitative culture technique which permitted exploration of relationships between colonization, symptoms and viral nucleic acid detection to be conducted in more depth and which adds confidence to interpretations of associations by providing an additional dimension of dose-response.

That conjugate pneumococcal vaccine induces mucosal antibody responses (195) and that widespread use of the vaccine results in disappearance of vaccine serotypes (245, 315, 319) are both well known. Nevertheless, it is unsurprising that no association between prior vaccination and colonization status was found in this study. Carriage status in the day care setting is likely, primarily, to be affected by exposure to organisms from other children, and pneumococcal serotypes, which become scarcer following widespread vaccine use are, to a large extent, rapidly replaced by others.

This study provides new insights into the relationships between nasal bacterial colonization, intercurrent respiratory viral infection and symptoms of rhinitis in children attending daycare, which have implications for disease pathogenesis and prevention.

Acknowledgments

Children and parents for their participation; nurses and staff of the daycare centers for their dedicated work in this study. We thank Malcolm Guiver, Health Protection Agency Public Health Laboratory Manchester, for providing sequences of RSV and PIV primers and probes, and Ruby Ahmed and Jonathan Hubb, Health Protection Agency Public Health Laboratory for PCR testing.

5. Multiple *Streptococcus pneumoniae* serotypes in aural discharge from children with acute otitis media with spontaneous otorrhea

Fernanda Rodrigues[1,2], Begonia Morales-Aza[3], Katy ME Turner[4], Paulina Sikora[3], Katherine Gould[5], Jason Hinds[5], Guilherme Gonçalves[2], Luís Januário[1], Adam Finn[3]

[1]Infectious Diseases Unit and Emergency Service, Hospital Pediátrico de Coimbra, Centro Hospitalar e Universitário de Coimbra, Coimbra; [2]Instituto de Ciências Médicas Abel Salazar, Universidade do Porto, Porto, Portugal
[3]Bristol Children's Vaccine Centre, Schools of Clinical Sciences and Cellular and Molecular Medicine, University of Bristol, Bristol, UK
[4]School of Social and Community Medicine, University of Bristol, Bristol, UK
[5]Bacterial Microarray Group, St. George's University of London, London, UK

J Clin Microbiol. 2013 Oct;51(10):3409-11

Reprinted from *J Clin Microbiol*, vol. 51, Issue 10, Fernanda Rodrigues, Begonia Morales-Aza, Katy ME Turner, Paulina Sikora, Katherine Gould, Jason Hinds, Guilherme Gonçalves, Luís Januário, Adam Finn. Multiple *Streptococcus pneumoniae* serotypes in aural discharge from children with acute otitis media with spontaneous otorrhea, pages 3409-3411, Copyright 2013, with permission from American Society for Microbiology. All rights reserved.

Abstract

Among 55 children with cultures positive for acute otitis media with spontaneous otorrhea, 28 (51%) had cultures positive for aural *Streptococcus pneumoniae*, and in 10 of these, two distinct strains were detected, in which five had pairs of starins which were both capsule-bearing serotypes. Such cases were more likely to have cultures positive for other otopathogens than those with only one pneumococcus present.

Keywords: Children, acute otitis media, spontaneous otorrhea, multiple *S. pneumoniae* serotypes

The most common bacteria causing acute otitis media (AOM) in children are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pyogenes*. Spontaneous otorrhea can complicate AOM, and *S. pyogenes* may be found in higher and *H. influenzae* and *M. catarrhalis* in lower percentages among such patients than among those with AOM and intact tympanic membranes (302).

Nasopharyngeal colonization by potential middle-ear pathogens is presumed to precede AOM. There is an association between nasal bacterial load and the presence and severity of ear disease (300), and aural *S. pneumoniae* shows a close genetic relatedness with its nasopharyngeal counterparts (297).

Understanding of the etiopathogenesis of AOM is increasing. Viral and bacterial causation are no longer seen as alternatives. Intercurrent respiratory viral infections may render the middle ear susceptible to symptomatic infection with bacteria that normally colonize the nasopharynx. Ruohola et al. suggest that the majority of acute middle ear infections in children are due to bacterial and viral coinfection (304).

More than one bacterial species can simultaneously infect the middle ear in AOM (302, 304). Multibacterial species biofilm formation may be involved in chronic recurrent otitis media pathogenesis, perhaps explaining the demonstrated effectiveness of conjugate pneumococcal vaccines against AOM but not recurrent disease (338).

Conventional *S. pneumoniae* culture and serotyping methodologies underestimate multiple-serotype carriage. Molecular serotyping improves detection of multiple serotypes and determines the relative abundance of each (123, 125) but has not previously been applied to the middle ear.

The pneumococcal conjugate vaccine (PCV) became available in Portugal in 2001 but has not been included in the national immunization program. Coverage from private market sales data was around 65% in 2011 following a peak of around 79% in 2007 (oral communication, Pfizer). Since 2010, 13-valent PCV (PCV13) has been used predominantly.

Tympanocentesis is not routinely performed in the investigation and management of AOM in Portugal. To obtain data on the etiology of AOM, we studied children with AOM with spontaneous otorrhea (AOMSO). We hypothesized that just as simultaneous nasal colonization with multiple pneumococcal serotypes and strains occurs (125), this may also be the case in the ear.

The study was conducted at Coimbra Children's Hospital, a 120-bed tertiary care center in central Portugal, with more than 60,000 emergency service (ES) visits each year. It was approved by the hospital ethics committee. Parents or guardians provided written informed consent.

Children (aged 0-13 years) with AOMSO, defined as a history of acute onset signs and symptoms of middle ear inflammation, with presence of spontaneous otorrhea not due to acute otitis externa, who visited ES between December 2010 and July 2011were studied prospectively. Disease onset was defined as time of first symptom (fever and/or ear pain and/or otorrhea). Children with recurrent AOM or previous ear, nose, and throat surgery were included. Demographic and clinical data were recorded, and paired swabs taken from the nasopharynx and aural discharge. No prior external ear canal toilet nor aspiration through the perforation were performed. Swabs were stored at -80ºC in skim-milk tryptone glucose glycerol (STGG) (Oxoid, Basingstoke, UK) broth until batched analysis by semi-quantitative bacterial culture within 18 months. No routine cultures were performed.

S. pneumoniae, *H. influenzae*, *M. catarrhalis*, and *S. pyogenes* were considered true AOM pathogens. *Staphylococcus aureus* was excluded, as it may be a contaminant from the skin or external ear canal. Standard microbiological techniques were used for isolation and identification of *S. pneumoniae* and *H. influenzae*, as described previously (316). Additionally *M. catarrhalis* and *S. pyogenes* were cultured and identified using standard procedures (briefly, using Columbia blood agar supplemented with 5% defibrinated horse blood and streptoccocal selective plates with colistin sulphate and oxolinic acid (COBA, Oxoid Limited, Basingstoke, United Kingdom), respectively). *M. catarrhalis* identity was confirmed by cytochrome *c* oxidase test (Pro-Lab Diagnostics; Merseyside, United Kingdom) and presence of acetate esterase activity (Indoxyl Strip test, Sigma-Aldrich, Dorset, United Kingdom). S*. pyogenes* identity was based on β-haemolysis and detection of pyrrolidonyl peptidase activity (Pyrase Strip test; Sigma-Aldrich).

Molecular serotyping was undertaken on all *S. pneumoniae* culture-positive aural samples and nasal samples from the same patients, using a microarray-based method to determine serotype from *cps* gene content from genomic DNA hybridization (123). Detection of nontypeables, in the presence of one or more other *S. pneumoniae* serotypes, represented either true unencapsulated *S. pneumoniae* and/or closely related *Streptococcus* spp.

We used a chi-square test to check for significance of associations with STATA 12.0.

Over 5 months, 113 children with AOMSO were studied (113 aural swabs and 108 nasal swabs). The median age was 27 months (range, 3 to 158 months), and 62 (54.8%) were boys. Fourteen (12.4%) were receiving antibiotics at the time of swabbing, and 52 (46.0%) had received them in the previous month. Forty (35.4%) had smoking parents and 100 (88.5%) attended nursery or school. The median/mean duration of disease was 1/2.5 days (range, 0 to 14): 0-3 days in 89 children (78.8%), 4 to 7 days in 15 children (13.3%), and 8 to14 days in 9 children (8.0%). Previous history of AOM and/or ear surgery was recorded in 17 (14.9%) cases. Regarding vaccination history, 85/112 (75.9%) had received at least one dose of *S. pneumoniae* conjugate vaccine (Prevenar 7 or 13 and/or Synflorix).

Fifty-five (48.7%) children were culture positive for bacteria from aural discharge, and among these cultures, *S. pneumoniae* was present in 50.9% (28) (Figure 5.1.), *S. pyogenes* in 30.9% (17), *M. catarrhalis* in 27.3% (15), and *H. influenzae* in 20.0% (11). Fourteen children (25.4%) had two or more otopathogen species: *S. pneumoniae* and *M. catarrhalis* in 5; *S. pneumoniae* and *H. influenzae* in 4; *M. catarrhalis* and *S. pyogenes* in 2, *M. catarrhalis* and *H. influenzae* in 1; *S. pneumoniae*, *H. influenzae*, and *S. pyogenes* in 1 and *S. pneumoniae*, *H. influenzae*, *S. pyogenes*, and *M. catarrhalis* in 1.

Of the 28 children with *S. pneumoniae*-positive culture from aural discharge (median age 32.5 months, range of 5 to 125 months), five had *S. pneumoniae* in the ear only and 23 both in the ear and nose (one had nontypeable *S. pneumonaie* detected in the nose by PCR and array only and not by culture). In 10 (36%) cases, two distinct streptococcal strains were identified in the aural sample, often with one predominating (Figure 5.1.). Five of these were pairs of capsule-bearing serotypes (Figure 5.1.). Sixteen different serotypes were found in the ear: 6 PCV13 vaccine types (the most frequent being 19A and 14), and 10 nonvaccine types (the most frequent being 10A) (Figure 5.1.).

In the 23 children who had *S. pneumoniae* in both sites, individual serotypes could be found either in the ear, or in the nose, but all 23 had at least one serotype that was found simultaneously in both places (Figure 5.1.). Cases with multiple aural streptococcal strains did not differ noticeably in age from the group with a single strain (median/mean ages 37.5/35.9 and 32.5/38.6 months, respectively).

Seven of the 10 cases who had multiple aural streptococcal strains also had other bacterial species isolated from the ear: *M. catarrhalis* in five and *H. influenzae* in three. This occurred in only four of the 18 cases with only a single aural streptococcal strain (chi-square= 6.23, *P*= 0.0125) (Figure 5.1.).

	Bacterial species				S. pneumoniae serotypes and non-typables																
Subject number	S. pneumoniae	M. catarrhalis	H. influenzae	S. pyogenes	3*	6C	7F*	9V**	10A	11D	14**	15B	16F	19A*	19F**	23A	23B	24F	33F	45	Non-typable
1																					
2																					
3																					
4												83									
5																					
6																					
7						97															
8																					
9																					
10										47											
11																					
12														59							
13																					
14																					
15																					
16															94						
17																					
18																					
19																					
20																					
21																					
22										85											
23																					
24																					
25													86								
26																				21	
27								98													
28										6											

Figure 5.1. Microbiological characteristics of 28 children with acute otitis media with spontaneous otorrhea who were culture positive for pneumococcus.

Subject numbers of children on antibiotics are left justified. Culture results are shown on the left and microarray serotyping results on the right. The percentage of capsular locus DNA in the ear is shown to the right of the first of each pair of aural strains in subjects where two were detected. PCV13 serotypes are indicated with an asterisk and PCV7/13 serotypes with two. Black box, ear and nose; patterned box, ear; gray box, nose

This is the first report of the microbial etiology of AOMSO in Portugal. Among this group of children, the duration of disease from the first acute symptom or symptoms to enrollment in the study lasted less than 3 days for the majority, and only four children with aural *S. pneumoniae* had more than 7 days of illness. *S. pneumoniae* was the predominant bacterium and, as reported by others (302), we found that *S. pyogenes* was identified in a higher percentage than usually reported in patients with AOM with intact tympanic membranes.

The proportion of cases from whom aural bacteria were successfully cultured in this study was relatively low. Brook and Gober reported that by culturing both the otorrhea fluid and middle-ear fluid obtained by needle aspiration, 28% additional pathogens were identified (301). Use of PCR detection may also increase bacterial detection rates (338), particularly in children who have received antibiotics. We did not include *S. aureus* in our analysis, as it is often assumed to be a contaminant in studies of AOM. Nevertheless, it was frequently cultured both from ear and nose in these children.

Although detection of more than one bacterial species in aural samples was as frequent in this AOMSO series as in others, by using molecular serotyping, we were also able to show that multiple *S. pneumoniae* serotypes were sometimes present in the ear. Although the clinical significance, if any, is uncertain, in such cases, serotypes that are at relatively low density may be underrecognized as otopathogens when conventional serotyping methods are used.

Children with multiple aural *S. pneumoniae* serotypes were more likely to have multiple bacterial species present as well. There is increasing evidence that AOM and recurrent AOM lie on a spectrum of disease whose pathogenesis varies according to the microenvironment that has developed in the middle ear with increasing chronicity of disease. Initially AOM may represent penetration of the middle ear by a single bacterial strain, facilitated by preceding viral infection. However, this may evolve into a more complex picture as other nose-colonizing bacterial strains and species join the process, perhaps no longer necessarily virus driven and more closely resembling the multi-bacterial environment of the nasopharynx. One study suggests evolution over time with different species detected in successive samples (303). Contributions from the flora of the external auditory canal may also become relevant once the tympanic membrane has been breached. If combinations of pneumococcal strains persist for longer in the ear than the nose, this might provide additional opportunities for horizontal gene

exchange. Some of these hypotheses can be tested in future studies in this patient group.

Acknowledgments

We thank children and their families for their participation. We also thank the study nurses.

6. Nasopharyngeal pneumococcus is neither commoner nor more abundant in children with acute otitis media with spontaneous otorrhoea than healthy children; but other otopathogens may have lower density

Fernanda Rodrigues[1,2], Begonia Morales-Aza[3], Paulina Sikora[3], Guilherme Gonçalves[2], Luís Januário[1], Adam Finn[3]

[1]Infectious Diseases Unit and Emergency Service, Hospital Pediátrico de Coimbra, Centro Hospitalar e Universitário de Coimbra, Coimbra; [2]Instituto de Ciências Médicas Abel Salazar, Universidade do Porto, Porto, Portugal
[3]Bristol Children's Vaccine Centre, Schools of Clinical Sciences and Cellular and Molecular Medicine, University of Bristol, Bristol, UK

Manuscript in preparation

6.1 Abstract

Background and aims: Studies comparing rates and/or densities of *S. pneumoniae* colonisation in children with respiratory infections and in health are conflicting. We compared patterns of nasopharyngeal colonisation in healthy children attending daycare centres (DCCs) with those in children with acute otitis media with spontaneous otorrhoea (AOMSO).

Methods: In February-March 2011 we swabbed 515 children in DCCs and 107 with AOMSO. Nasopharyngeal swabs were stored at -80°C in STGG broth and cultured using standard techniques. *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. aureus* and *S. pyogenes* were identified and densities assessed by scoring numbers of colonies (0= not detected; 1= 1–5 colonies/50µL broth; 2= >5–20; 3= >20–50; 4= >50–100; 5= >100).

Results: 80% of the children with AOMSO attended nurseries (p=<0.001). By univariate analysis, rates of colonisation and mean densities did not differ between the two groups apart from *M. catarrhalis* which had lower density in AOMSO. By multivariate analysis (adjusting for age), colonisation densities for both *H. influenzae* and *M. catarrhalis* were lower in AOMSO. The mean number of bacterial species identified was similar in the two groups (1.7 versus1.8; p=0.674).

Conclusions: Children with AOMSO did not have higher rates or densities of nasal *S. pneumoniae* but significantly lower densities of both *H. influenzae* and *M. catarrhalis* were seen. This relative imbalance between species in otitis may point to ecological conditions associated with disease.

Keywords: nasopharyngeal colonisation, rates, density, acute otitis media

6.2 Introduction

Many children experience nasopharyngeal carriage of bacterial pathogens that in some will progress to AOM. A child must be colonised in order both to develop disease and to transmit the organism to others, but it is not clear by how much infectiousness varies between children. It seems plausible that among children with higher rates and density of colonisation there may be a higher likelihood of developing disease or of onward transmission or both. Although several studies have shown higher rates and/or densities of *S. pneumoniae* nasopharyngeal colonisation in children with respiratory infections than in health (4, 148, 183, 270, 298, 339), others have failed to find any such differences (185, 299).

A Finnish study that followed 329 children from the age of 2 to 24 months, found higher proportions of nasopharyngeal positive samples for pneumococcus during AOM (49%) and in particular during AOM confirmed as pneumococcal (by myringotomy), compared to children with respiratory infection without concurrent AOM (35%) or during health (21%) whereas non-pneumococcal AOM was not associated with higher carriage rates than respiratory infection without concurrent AOM (148). Pneumococcal carriage prevalence during health and respiratory infection without AOM increased with age over the course of the study while in contrast, during AOM, the rates were high, regardless of age. In a 5 year prospective study in five suburban paediatric practices in the USA, Xu et al. (270) compared colonisation rates in nasopharyngeal and oropharyngeal samples from 320 children aged 6 to 24 months during healthy and at AOM visits confirmed by tympanocentesis. Colonisation rates for *S. pneumoniae, H. influenzae* and *M. catarrhalis* at healthy and AOM visits were 30.3% and 52.7%, 11.7% and 47.9% and 36.3% and 43.4% respectively although colonisation rates for *S. pneumoniae* when neither *H. influenzae* nor *M. catarrhalis* were present, did not differ significantly (14.2% versus 14.4%; p=0.93). Polymicrobial colonisation was significantly less common at healthy visits than at AOM visits. Another American study performed in 294 children aged between 6 and 35 months during 709 episodes of upper respiratory tract infections, conducted over a 5 year period, showed that the same three bacterial species either alone or in combination were more likely to be isolated from the nasopharynx of children with AOM than from those without after controlling for breast feeding, daycare attendance, cigarette smoke exposure and number of PCV7 doses (340). An Australian study compared

nasal bacterial load of the same three respiratory pathogens using quantitative measures (semi-quantitative bacterial culture and real-time quantitative PCR) in nasal swabs from 52 non-Aboriginal children, aged 18 to 36 months attending urban DCCs and from 59 Aboriginal children of the same age among whom the prevalence of each respiratory pathogen and of suppurative OM is much higher. Nasal bacterial load was significantly higher among Aboriginal children and was significantly associated with the presence and severity of ear disease (300).

Levin et al. (183) showed that patients with pneumonia in China, aged between 2 and 60 months, were more likely to be colonised with Hib (7.3% vs 1.9%) and *S. pneumoniae* (44.8% vs. 38.8%) than control patients who had no indication of respiratory tract disease. For pneumococcus this difference achieved statistical significance when the data were adjusted for possible confounding factors such as age, day-care attendance, the presence of other children in the household and recent antibiotic use. Anh et al. (186) reported that carriage rates among 91 children aged <5 years with radiological pneumonia enrolled over 2 years in Vietnam, were higher than among 70 healthy children attending DCC (19.8% vs 10.0% for *S. pneumoniae*, 26.3% vs 5.7% for *H. influenzae* and 7.7% vs 1.4% for *M. catarrhalis*). The frequency of intense growth of these potential pathogens measured by quantitative culture was also higher in the pneumonia group.

However other investigators have reported similar nasopharyngeal prevalence rates both in healthy and in ill children. In a 2 year study in the USA, nasopharyngeal specimens were taken at scheduled monthly intervals as well as at the times of episodes of AOM from 62 children attending DCCs. During AOM episodes, pneumococcus was not found significantly more frequently than among the scheduled cultures (185). Another USA study involving 73 children aged between 5 months and 14 years, either with AOM, uncomplicated upper respiratory infection or who were healthy, compared their nasopharyngeal flora using a semi-quantitative method. There were no significant differences between nasopharyngeal density of bacterial species isolated among the different clinical categories. However, there were significant differences in carriage rates in some cases: *M. catarrhalis* was significantly more likely to be present in children without bacteriologically confirmed otitis media as compared with children with confirmed otitis media while the reverse relationship was found for *H. influenzae*. *S. pneumoniae* was found frequently but did not vary between the groups with different clinical syndromes (299).

A Vietnamese study compared nasopharyngeal carriage rates and densities (measured by PCR) of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* between 214 children less than 5 years of age with radiologically confirmed pneumonia and 350 healthy controls who were free of fever and signs or symptoms of acute respiratory infections. The cases were recruited from 16 communes in the study area, over more than 1 year, whereas healthy controls were randomly selected from only 2 of the 16 communes, in January, a cool month. The proportion of children carrying *S. pneumoniae* was higher in healthy controls compared to children with radiologically confirmed pneumonia (50.3% versus 38.7%; p=0.004) and the same was found for *M. catarrhalis* (58% versus 28.1%; p<0.0001). However, the median bacterial density of *S. pneumoniae* in children with radiologically confirmed pneumonia was significantly higher than in healthy controls, and even higher if pneumonia with viral coinfection, but no clinically useful cut-offs could be defined for the diagnosis of paediatric pneumonia. In contrast, *H. influenzae* and *M. catarrhalis* detected in children with radiologically confirmed pneumonia did not have significantly different density from healthy controls. The detection of any combination of the three bacteria was more frequent in the healthy controls (43.4%) than in children with pneumonia (32.5%; p=0.005) (184).

Some of these studies included only small numbers of children, and/or had poor seasonal matching between groups. The age range and distribution studied, sample collection and microbiological methods used also varied between studies. The somewhat contradictory results may be affected by these factors and others may also be at play so that further studies need to be done in order to understand these relationships more fully.

We hypothesised both that pneumococcal colonisation may be commoner among children with otitis than among healthy children and that those with otitis who are colonised may have higher colonisation densities than colonised healthy children. Such associations, although they might be non-causal, may contribute to disease pathogenesis. For example, high nasopharyngeal colonisation density might increase the probability of bacterial spread along the Eustachian tube into the ear and consequent development of otitis.

6.3 Methods

In February-March 2011 we swabbed again the nasopharynges of children attending the same DCCs in Coimbra. The group of children with otitis, previously described in Chapter 5, included 113 children seen at the Emergency Service of the paediatric hospital, with AOMSO, observed during the winter 2010-11 who had a nasopharyngeal sample collected. For this analysis, six children with AOMSO with more than eight days of duration of disease were excluded in order to avoid any risk of dilution of transient effects. Before children were enrolled, written informed consent was obtained. The protocol for this study was approved by the Ethics Committee of the hospital.

The chi-squared test was used to study the association between qualitative independent variables. The Mann-Whitney test was computed to study differences regarding a quantitative variable between two study groups. Multiple logistic regression was used to evaluate which factors contribute to explain colonisation, its density and the number of bacterial species. All statistical tests were two-sided and a p value <0.05 was considered significant.

The same clinical and demographic questionnaire, methodologies of sample collection and storage, bacterial culture techniques and semi-quantitative density measures were used as described previously (see Chapters 2, 4 and 5).

6.4 Results

In all, 107 children with AOMSO and 515 children attending DCCs were included in the study. Characteristics of the two groups are presented in Table 6.1. There were significant differences between the two groups in age, DCC attendance, recent antibiotic use, presence of siblings less than 6 years old, exposure to tobacco smoke and previous pneumococcal conjugate immunisation.

Table 6.1. Characteristics of children with acute otitis media with spontaneous otorrhoea and children in day care centres.

Risk factor	AOMSO (n=107)	DCCs (n= 515)	p- value
Gender (male)	58/107 (54.2%)	277/515 (53.8%)	0.937
Mean age (months) (median; range)	37.5 (28; 2-158)	39.1 (39; 5-72)	0.002
DCC attendance (%)	85/106 (80.2%)	515/515 (100%)	<0.001

Recent antibiotic use No (%)	52/104 (50%)	84/510 (16.5%)	<0.001
Passive smoking (%)	38/103 (36.9%)	140/511 (27.4%)	0.053
Siblings <6 years (%)	20/102 (19.6%)	156/459 (34%)	0.005
Received PCV (%)	79/105 (75.2%)	447/511 (87.5%)	0.001

PCV: Pneumococcal conjugate vaccine; **AOMSO**: acute otitis with spontaneous otorrhoea; **DCCs**: day care centres

By univariate analysis, *S. pneumoniae* colonisation was slightly more common in children in DCCs (60.6%) than among those presenting with AOMSO (56.3%) although this difference was not statistically significant (p=0.419). The colonisation rates in the two groups were similar for the other bacterial species studied, with slight, non-significant differences in both directions observed (Table 6.2.). With regard to bacterial density scores among those colonised, for *S. pneumoniae* they were almost identical for the two groups while for the other species they tended to be slightly higher on average in children in DCC (apart from *S. pyogenes*) although these differences did not achieve statistical significance except in the case of *M. catarrhalis.* This species was the most commonly detected in both groups. The mean number of bacterial species identified was also similar in the two groups (Table 6.2.).

Table 6.2. Colonisation rates and mean density scores of culture-positive children for *S. pneumoniae, H. influenzae, M. catarrhalis S. aureus* and *S. pyogenes* and mean number of bacterial species detected in univariate analysis.

	AOMSO (n=107)	**DCC (n=515)**	**p- value**
Rate of colonisation			
S. pneumoniae	58 (56.3%)	312 (60.6%)	0.419
H. influenzae	21 (20.4%)	88 (17.1%)	0.422
M. catarrhalis	65 (63.1%)	345 (67%)	0.446
S. aureus	17 (16.5%)	75 (14.6%)	0.613
S. pyogenes	15 (14.6%)	85 (16.5%)	0.625
Mean density (for densities ≥1)			
S. pneumoniae	4.1	4.2	0.641
H. influenzae	3.9	4.3	0.067
M. catarrhalis	3.9	4.2	0.024

S. aureus	2.4	3.0	0.146
S. pyogenes	3.3	3	0.468
Mean (median) number of bacterial species	1.7 (2)	1.8 (2)	0.674

AOMSO: acute otitis with spontaneous otorrhoea; **DCCs**: day care centres

Multivariate analysis using logistic regression was undertaken to take into account the potentially confounding effects of the various factors shown in Table 6.1. The odds of colonisation with *S. pneumoniae* and *M. catarrhalis* decrease with age (p=0.001 for *S. pneumoniae*, p<0.001 for *M. catarrhalis*) and for *S. aureus* increases with age (p<0.001). Rates of detection of *S. pneumoniae* as well as all other bacterial species including *M. catarrhalis* are not significantly different in AOMSO compared to DCCs (Table 6.3.).

Table 6.3. Colonisation rates for *S. pneumoniae, H. influenzae, M. catarrhalis, S. aureus* and *S. pyogenes* in multivariate analysis.

	OR[a]	**95% CI**	**p- value**
S. pneumoniae	1.07	0.62-1.82	0.818
H. influenzae	0.80	0.42-1.53	0.496
M. catarrhalis	1.12	0.62-2.01	0.713
S. aureus	0.85	0.40-1.81	0.676
S. pyogenes	1.21	0.58-2.52	0.607

OR: Odds ratio; **CI:** confidence interval
[a]Odds ratio calculated for children in DCCs versus children with AOMSO

When analysing colonisation densities, since the proportion of children with scores less than 4 was always small, logistic regressions were performed considering the following binary variable: 0 if density score is less than 5; 1 if score is 5. *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* colonisation densities were also significantly associated with age (p=0.055; 0.010; 0.022, respectively), with older children having lower densities (OR=0.874, CI=0.762-1.003; OR=0.654, CI=0.474-0.903; OR=0.848, CI=0.736-0.976, respectively). *S. aureus* colonisation density was significantly associated with pneumococcal vaccination (p=0.021) with vaccinated children having lower density (OR=0.170, CI=0.04-0.77). Colonisation densities for *S. pneumoniae*, *S. aureus* and *S. pyogenes* remained

similar between the two groups but densities for *H. influenzae* and *M. catarrhalis* were lower in children with AOMSO (Table 6.4.).

Table 6.4. Colonisation density for *S. pneumoniae, H. influenzae, M. catarrhalis, S. aureus* and *S. pyogenes* in multivariate analysis.

	OR[a]	**95% CI**	**p- value**
S. pneumoniae	0.92	0.45-1.87	0.812
H. influenzae	4.08	1.17-14.25	0.028
M. catarrhalis	2.01	1.13-3.59	0.018
S. aureus	1.85	0.27-12.76	0.534
S. pyogenes	0.36	0.08-1.54	0.168

OR: Odds ratio; **CI:** confidence interval
[a]Odds ratio calculated for the risk of a colonisation density score of 5 for children in DCCs versus children with AOMSO

Logistic regression analysis showed that the number of bacterial species detected in each child tended to fall with age ($p<0.001$). However, it also showed that the total number of bacterial species detected was not significantly different between the two groups ($p=0.959$).

6.5 Discussion

Our study, which included children living in the same area and samples collected during the same period, shows that, in univariate analysis, rates and densities of colonisation with *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* were not higher among AOMSO patients than among children in DCCs. The same was found for the number of bacterial species detected in colonisation. Data were adjusted for potential confounders (Table 6.1). Factors which were significantly different between the two groups and which were associated with differences in rates or densities of colonisation or both were included in the relevant regression equation in each case.

Unexpectedly children who had received pneumococcal conjugate vaccination and who were colonised with *S. aureus* were found to have lower density of colonisation than unvaccinated children. No obvious biological explanation for this observation is evident, in particular because differences in total carriage rates for *S. pneumoniae* are not seen in this population between immunised and unimmunised children (OR=1.29, CI:0.78-2.14; p=0.325) as

indirect transmission effects of vaccine use seem to be largely complete. Accordingly this finding requires reconfirmation before being investigated in more depth.

Although colonisation rates and densities of *S. pneumoniae* were similar between the two groups, and colonisation rates for *H. influenzae* and *M. catarrhalis* were likewise similar, the observed densities for the latter two species were lower in children with AOMSO. These differences could be offering insights relevant to pathogenesis of otitis.

As expected, the results of this study have both similarities to and differences from those of the heterogeneous previously published studies summarised above. Clearly, discussion comparing the findings of studies needs to take into account the differences in epidemiology and methodologies. It is interesting that carriage rates for *S. pneumoniae* in our AOMSO group are very high compared to other studies and that those in our DCC group are much higher than reported in similar studies. This raises the question whether the conflicting results of previous publications comparing ill with healthy children may be influenced to a great extent by the nasopharyngeal ecology of the healthy controls. In our case, the controls were a DCC population with very high colonisation rates as well as high presence of rhinitis symptoms, presumably reflecting the very high transmission rates in that setting. Our findings suggest that high carriage rates and densities do not, *per se*, predict disease on an individual level and that choice of controls may influence the conclusions of studies of this nature.

Although carriage rates and densities are related they are not the same. Increasing density from a very low level to a higher level may pass a threshold allowing colonisation to become detectable and thus counted and, when it reaches still higher levels, might also increase the risk of migration into the middle ear. It may also be possible to have two populations with similar rates of colonisation but one having much higher densities than the other or different balances between species. While it is possible that disease and marked increase in transmission rates occur as children reach density scores of 4 or 5, it is also possible that such changes only occur at much higher bacterial densities, all of which would be designated as score 5 using this methodology. Accordingly, use of alternative methodologies for bacterial detection capable of distinguishing between density of colonisation at much higher levels, such as species-specific real time quantitative PCR may permit clearer elucidation of the relationships

between density of colonisation and disease. It is also possible that children with similar bacterial densities vary in their infectiousness and predisposition to disease due to differences in the bacterial gene expression phenotype. Finally, differences in colonisation density which could contribute to pathogenesis may, of themselves be insufficient, requiring additional factors such as new bacterial acquisition, viral infection or certain kinds of host response to be present for disease to develop.

A novel concept, though, is the idea of changes in relative colonisation density between detected bacterial species. While our studies show that in health there are children in DCCs with no nasal bacteria detectable by culture or colonised with only one species, the large majority has multiple bacterial species detected. Our study shows that, taken as a group, children with AOMSO who have these bacterial species in their noses do not have higher rates or densities of *S. pneumoniae* but do have significantly lower densities of the two other species *H. influenzae* and *M. catarrhalis*. Upon these observations we can speculate that there may be a dynamic balance between bacterial species in which disease sometimes occurs at times of change or imbalance. The association of this relative imbalance between species in otitis may be pointing to ecological conditions in which disease is more likely to occur. This could occur following acquisition of a new strain, it could be affected by intercurrent viral infection and other circumstances and factors such as changes in the host innate and specific immune responses could help bring it about. Syrjanen et al. (35) showed that the majority of *S. pneumoniae* associated AOM were due to newly acquired strains, rather than the ones found in health, suggesting that a new acquisition may contribute to altering an existing balance. However, it is not possible to attribute the direction of causality in associations observed in cross sectional studies of this nature. Nevertheless they can be used to construct new hypotheses that can then be addressed in future studies or reanalaysis of existing datasets.

In conclusion we found similarly high colonisation rates and densities for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, in health and disease. Colonisation densities for *H. influenzae* and *M. catarrhalis* were lower, on average, in disease, suggesting that a relative imbalance between species in otitis may point to ecological conditions associated with disease. The potential role of commensals in this process remains unclear and warrants further study.

7. General conclusions

Streptococcus pneumoniae is a common asymptomatic resident of the human nasopharynx and is also an important aetiological agent of meningitis, sepsis, bacteraemia, pneumonia and otitis media. During its residence in the nasopharynx, pneumococcus commonly shares this anatomical and physiological niche with other strains and with several other bacterial and viral inhabitants. Colonisation of the nasopharynx is the source of transmission between individuals and a necessary but not sufficient pre-condition for development of pneumococcal disease. Pneumococcal conjugate vaccines are now known to impact on disease largely through their effects on colonisation and transmission.

The studies presented in this thesis contribute to a better understanding of the effect of PCV use on nasopharyngeal colonisation and of the microbial interactions that may alter the nature of pneumococcal colonisation and its potential to be transmitted to another host or to progress to disease, in preschool children.

Recognising the importance of monitoring pneumococcal nasopharyngeal colonisation in Portugal, particularly given the pattern of vaccine usage – in contrast to most other countries with either near universal uptake or virtually no vaccine use – we commenced a programme of surveillance in a group of DCCs in Coimbra. We have conducted cross sectional surveys involving collection of nasopharyngeal swabs and clinical and demographic data in between 500 and 600 children, each year in February-March. A standard approach has been taken towards subject recruitment and data collection over the years. For the most part, the same nurseries have been studied each year and at roughly the same time of year. Approximately the same number of children, of the same age range, have been recruited and the same demographic and clinical dataset collected including age, sex, current respiratory symptoms, vaccination history, recent antibiotic use, exposure to tobacco smoke and number of siblings <6 years of age. In contrast, the sampling and laboratory methodology has evolved to keep pace with advances in the field. Whereas, at the outset, we took swabs and plated them promptly in the local laboratory onto selective media agar plates to identify pneumococcus and then forwarded these isolates to another laboratory for serotyping using the conventional Quellung reaction methodology, we have progressed to taking swabs into enrichment broth (STGG) which can be stored frozen for subsequent batched analysis by culture not only for pneumococcus but also for other bacterial species that colonise the nasopharynx, and later included in our studies. This methodology has been proven to be as good as direct plating,

saves time and increases scientific yield. We have also moved to a new molecular microarray serotyping methodology that permits detection and quantification of multiple pneumococcal serotypes in a single sample.

As expected from the methodology used, the demographic of our series of cross sectional studies remains very constant over the years. The immunisation rate among this sample of children attending inner city private DCCs is, unsurprisingly, higher and more homogeneous than that known to exist in the wider community, rising steadily to around 87.5% who had received at least one dose of PCVs in 2011.

We have made several important observations. First we have charted the decline of most PCV7 types in this population. In the first part of our study, six years after PCV introduction in Portugal, it became clear that we were observing reduction of the vaccine types that were detectable in this population. The combination of moderately high vaccine uptake among children attending these nurseries, in the context of somewhat more modest general vaccine usage in the paediatric population at large, was sufficient to bring about effective population immunity. However, the impact of the vaccine on the different vaccine serotypes varied, so that marked differences in their rate of disappearance were seen: some serotypes (4, 9V and 18C) had already disappeared by the time this study began whereas we have documented a failure of serotype 19F to decline. It is possible that the lower selection pressure of the moderately high vaccine uptake in Portugal, in contrast with the higher vaccine rates in other well studied populations in the USA (239, 245) and UK (246), may have permitted such inter-serotype differences to be observed more clearly. These findings coincided with observations by Weinberger et al. (60), who raised an hypothesis to explain them, based on the capsular morphology, size and susceptibility to neutrophilic killing, predicting that serotype 19F, with a very large capsule, would be hard to dislodge. Lipsitch et al. (62) also demonstrated that 19F is a highly fit serotype with a long time to clearance and low susceptibility to competition. Vaccine efficacy studies found no statistically significant efficacy of PCV7 against acquisition of vaccine serotypes 19F and 14 and a study to evaluate the effect of a 9-valent conjugate vaccine on carriage, did not show significant protection against 19F (236, 313). The most recent sample set for which we currently have complete serotyping results was collected in 2010 and shows an unexpected and surprising rise in the proportion of children carrying serotype 19F, that became the most frequently carried serotype in this population. Although we used

microarray serotyping with enhanced sensitivity for detection of multiple serotypes in the same sample, even when children in whom 19F was a minority serotype (<50% of the DNA of the sample) are excluded, the upward trend remains obvious. Other studies have drawn attention to persistence of 19F (126, 243), but a resurgence of this kind has not previously been reported in countries using PCVs. This could be a local phenomenon. It is also possible that we are describing, in real life, one of the scenarios about re-emergence of serotypes, proposed by Cobey and Lipstich (67) in their detailed model of pneumococcal carriage ecology recently published and described in section 1.6.5. Nevertheless 19F is among the PCV7 serotypes still causing invasive disease both locally in the central region and nationally in Portugal (317) and so further monitoring is certainly needed. The practical implications for Portugal and for other countries of these observations are that 19F requires special attention in future surveillance and the need for effective control may influence future policy decisions. In Portugal today, this is of particular importance because it is possible that serotype 19F or other vaccine types may increase if economic pressures drive down immunisation rates. A randomized double-blind trial to compare immunogenicity and efficacy of PCV7 and PCV13 in reducing nasopharyngeal colonisation showed that the latter resulted in lower acquisition and prevalence of nasopharyngeal colonisation than PCV7 for 19F (341). If confirmed, this unexpected finding could mean that the rise in 19F carriage we have documented may already have been reversed. Therefore it will be of great interest to track any such changes following the introduction of PCV13 in 2010.

Another recent observation, also predicted by mathematical models (67), is that PCV usage results in a temporary increase in serotype carriage diversity in the paediatric population that then trends back towards the original level over several years, the vaccine serotypes having been replaced by others (245). The data used to support this compelling concept have been somewhat fragmentary, comparing different types of nasopharyngeal samples (healthy children and children with AOM) and samples from different studies. Our data, derived from of very consistently collected samples, spanning a period 6–8 years after the first use of PCV7 in Portugal, suggests a diversity that has been continuously close to the highest seen in other studies (245, 246) and, despite some year to year variation, not changing progressively over time. Since we do not have pre-vaccine data, this could reflect either that any changes in diversity were already complete by the time of our first study or that the PCV7-induced changes were still evolving

in this community at that time, a difference that might be due in part to the different pattern of vaccine use in Portugal. Understanding better how nasopharyngeal bacterial populations change and rebalance themselves under vaccine-induced selection pressure will help to shape the future of this and related immunisation programmes.

With the microarray pneumococcal serotyping method applied to nasopharyngeal samples of children attending DCCs and to a group of children with AOMSO, we have shown that multiple capsular serotypes and strains could be detected in the same child, both in the nasopharynx and in the ear. This method is an important tool to recognise a pool available for genetic exchange and to identify pneumococcal strains that may be circulating at low abundance. The apparent disappearance of some vaccine serotypes may sometimes represent low-level persistence such that any reductions in vaccine coverage could result in rapid re-emergence of detectable colonisation and disease. The more frequent serotypes were consistently found both as single-serotype and in co-colonisation.

Pneumococcus was the bacterium most frequently found the ear of children with AOMSO. Of the children with *S. pneumoniae* positive culture from aural discharge, the majority had pneumococcus also in the nose and, in this group, at least one serotype was found simultaneously in both places confirming a strong correlation between the nasopharyngeal flora and middle ear infections.

Since nasopharyngeal colonisation precedes disease and is a source of transmission, it is important to understand interactions between microorganisms in the nasopharynx and other factors that may be associated with both, especially in pre-school children who have high carriage rates and close contact with each other. Accordingly we explored carriage and density of pneumococcus alongside other bacterial species, intercurrent viral infection and the association of these factors with the presence of rhinitis. It turned out that the children studied early in 2009 were sampled after the end of both the wintertime respiratory syncytial virus and influenza virus epidemics that year and these two viruses were virtually undetected. Like viral detection and bacterial colonisation with pneumococcus and *H. influenzae* (but not *S. aureus*), rhinitis was commonest in the first 2 years of life and rates fell rapidly after the third birthday. The effects of vaccines upon bacterial colonisation and transmission take place upon a background of many influences. Age is clearly an important correlate of colonisation, of susceptibility to infectious diseases and of propensity to transmit them. Immunological immaturity and naïveté and the social habits of the young all change with age. We

have shown a significant positive association, using multiple regression analysis, between rhinitis and nasopharyngeal colonisation with *H. influenzae* and between respiratory viral detection and the rates and density of colonisation with pneumococcus, independently of age. By collecting quantitative clinical data on rhinitis while conducting a carriage study we have been able to contribute to a better understanding of how microbes influence each other and the human host. Our findings also suggest that, contrary to the common assertion that bacterial colonisation of the nasopharynx is "asymptomatic", *H. influenzae* colonisation may be independently associated with rhinitis. Since effects on microbial transmission are critical for vaccine effectiveness, understanding the underlying mechanisms that determine how successfully transmission happens will contribute to design of vaccines and immunisation programmes in the future. The observation in our study that pneumococcal colonisation rates and densities were associated with the presence of rhinovirus and rhinovirus viral load in the absence of any association with increased symptoms of rhinitis, raises questions about the underlying mechanisms driving this association. Rhinovirus persists in children long after the symptoms caused by the infection have abated. It is possible, perhaps, that, as observed with influenza virus infection in ferrets by McCullers et al. (342), the presence of rhinovirus enhances the chance of successful pneumococcal acquisition perhaps through some effect on mucosal epithelium or by alteration of the immune response diminishing the ability of the host to clear pneumococcus. Although due to the cross-sectional design of these studies, it remains unclear whether these reflect a true cause-effect relationship, and if so, in what direction these effects occur, it makes biological sense either that, in combination with viral infections, bacteria might proliferate in response to rhinitis or might induce, amplify or prolong rhinitis symptoms resulting in increased chances of successful transmission to another host. Such phenomena might also play a role in causation of disease including pneumonia and otitis media. Longitudinal studies during health and disease are needed to better understand the sequence of the observed effects.

Several studies have shown higher rates and/or densities of *S. pneumoniae* nasopharyngeal colonisation in children with respiratory infections than in health but others have failed to find such differences. In 2011 we compared patterns of pneumococcal nasopharyngeal colonisation between healthy children in DCCs and children with AOMSO, showing that, after data were adjusted for potential confounders, colonisation rates and densities for *S. pneumoniae* were similar

between the two groups but colonisation densities for *H. influenzae* and *M. catarrhalis* were lower in children with AOMSO, suggesting that there may be an equilibrium between bacterial species and disease may occur at times of imbalance.

There are some limitations in our studies. Bacterial species were identified using conventional culture methods. The use of PCR for bacterial detection instead of conventional culture, may have increased detection rates, especially in children who were on antibiotics and is likely to become more widely used in future studies. Second we analysed bacterial density using a semiquantitative scoring system that does not distinguish between high and very high levels of colonisation. The use of PCR for bacterial load definition may also overcome this limitation. Finally we restricted our investigations to the bacterial pathogens that are generally considered to be the major contributors to respiratory disease in childhood. However there are other pathogenic and commensal bacterial species and how they fit into the picture and relate to pathogenesis is an important topic for research.

These studies provide a foundation for future work.

Published studies on nasopharyngeal co-colonisation with different pneumococcal serotypes and strains are few and a more extensive analysis of our results, exploring the relationship between pneumococci and other bacterial species is planned. As discussed above, it is now important to collect further samples and clinical data to evaluate the impact of the PCV13 vaccine, which has replaced PCV7. In addition, if evidence emerging from countries introducing universal influenza vaccines in childhood suggests this is cost-effective and given results from our studies and those of others indicating that associations exist between viral infections and bacterial colonisation and transmission, there are important studies to be done evaluating how flu vaccine in children affects nasopharyngeal bacterial ecology at the individual and the population levels.

In addition to the development and use of the single gene PCR-detection methods discussed above, to use alongside and, potentially, to replace culture-based microbial detection, the possibility of studying the nasopharyngeal microbiota by sequencing of bacterial 16S rRNA gene hypervariable regions (3) is rapidly becoming both feasible and affordable.

With improved case definitions and more sophisticated sampling and microbiology techniques designed to allow not only detection of viable microbes

and their DNA footprint but also levels of microbial gene expression and of host inflammatory and immune response gene expression as well, there is potential to conduct not only further cross sectional surveys in healthy and sick children but also longitudinal and interventional studies designed to elucidate the dynamics of respiratory tract infections at the individual and population level. Such studies are vital both in the understanding of disease pathogenesis, current health interventions and rational and cost-effective design of the interventions of the future.

References

1. Watson K, Carville K, Bowman J, et al. 2006. Upper respiratory tract bacterial carriage in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia. Pediatr Infect Dis J 25 (9):782-790.
2. Laufer AS, Metlay JP, Gent JF, et al. 2011. Microbial communities of the upper respiratory tract and otitis media in children. MBio 2 (1):e00245-00210.
3. Bogaert D, Keijser B, Huse S, et al. 2011. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. PLoS One 6 (2):e17035.
4. Faden H, Duffy L, Wasielewski R, et al. 1997. Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. J Infect Dis 175 (6):1440-1445.
5. Ghaffar F, Friedland IR, McCracken GH, Jr. 1999. Dynamics of nasopharyngeal colonization by Streptococcus pneumoniae. Pediatr Infect Dis J 18 (7):638-646.
6. Faden H, Duffy L, Williams A, et al. 1995. Epidemiology of nasopharyngeal colonization with nontypeable Haemophilus influenzae in the first 2 years of life. J Infect Dis 172 (1):132-135.
7. Faden H, Harabuchi Y, Hong JJ. 1994. Epidemiology of Moraxella catarrhalis in children during the first 2 years of life: relationship to otitis media. J Infect Dis 169 (6):1312-1317.
8. Bogaert D, De Groot R, Hermans PW. 2004. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis 4 (3):144-154.
9. Blaser MJ, Falkow S. 2009. What are the consequences of the disappearing human microbiota? Nat Rev Microbiol 7 (12):887-894.
10. Garcia-Garcia ML, Calvo C, Pozo F, et al. 2008. Human bocavirus detection in nasopharyngeal aspirates of children without clinical symptoms of respiratory infection. Pediatr Infect Dis J 27 (4):358-360.
11. Winther B, Alper CM, Mandel EM, et al. 2007. Temporal relationships between colds, upper respiratory viruses detected by polymerase chain reaction, and otitis media in young children followed through a typical cold season. Pediatrics 119 (6):1069-1075.
12. Winther B, Hayden FG, Hendley JO. 2006. Picornavirus infections in children diagnosed by RT-PCR during longitudinal surveillance with weekly sampling: Association with symptomatic illness and effect of season. J Med Virol 78 (5):644-650.
13. van der Zalm MM, van Ewijk BE, Wilbrink B, et al. 2009. Respiratory pathogens in children with and without respiratory symptoms. J Pediatr 154 (3):396-400, 400 e391.
14. Mathisen M, Strand TA, Valentiner-Branth P, et al. 2010. Respiratory viruses in nepalese children with and without pneumonia: a case-control study. Pediatr Infect Dis J 29 (8):731-735.

15. Jansen RR, Wieringa J, Koekkoek SM, et al. 2011. Frequent detection of respiratory viruses without symptoms: toward defining clinically relevant cutoff values. J Clin Microbiol 49 (7):2631-2636.
16. Fry AM, Lu X, Olsen SJ, et al. 2011. Human rhinovirus infections in rural Thailand: epidemiological evidence for rhinovirus as both pathogen and bystander. PLoS One 6 (3):e17780.
17. Pettigrew MM, Gent JF, Revai K, et al. 2008. Microbial interactions during upper respiratory tract infections. Emerg Infect Dis 14 (10):1584-1591.
18. Garcia-Rodriguez JA, Fresnadillo Martinez MJ. 2002. Dynamics of nasopharyngeal colonization by potential respiratory pathogens. J Antimicrob Chemother 50 Suppl S2:59-73.
19. Jourdain S, Smeesters PR, Denis O, et al. 2011. Differences in nasopharyngeal bacterial carriage in preschool children from different socio-economic origins. Clin Microbiol Infect 17 (6):907-914.
20. Varon E, Levy C, De La Rocque F, et al. 2000. Impact of antimicrobial therapy on nasopharyngeal carriage of Streptococcus pneumoniae, Haemophilus influenzae, and Branhamella catarrhalis in children with respiratory tract infections. Clin Infect Dis 31 (2):477-481.
21. Ekdahl K, Ahlinder I, Hansson HB, et al. 1997. Duration of nasopharyngeal carriage of penicillin-resistant Streptococcus pneumoniae: experiences from the South Swedish Pneumococcal Intervention Project. Clin Infect Dis 25 (5):1113-1117.
22. Huang SS, Finkelstein JA, Lipsitch M. 2005. Modeling community- and individual-level effects of child-care center attendance on pneumococcal carriage. Clin Infect Dis 40 (9):1215-1222.
23. Madhi SA, Adrian P, Kuwanda L, et al. 2007. Long-term effect of pneumococcal conjugate vaccine on nasopharyngeal colonization by Streptococcus pneumoniae--and associated interactions with Staphylococcus aureus and Haemophilus influenzae colonization--in HIV-Infected and HIV-uninfected children. J Infect Dis 196 (11):1662-1666.
24. Jacoby P, Watson K, Bowman J, et al. 2007. Modelling the co-occurrence of Streptococcus pneumoniae with other bacterial and viral pathogens in the upper respiratory tract. Vaccine 25 (13):2458-2464.
25. Regev-Yochay G, Dagan R, Raz M, et al. 2004. Association between carriage of Streptococcus pneumoniae and Staphylococcus aureus in Children. JAMA 292 (6):716-720.
26. McNally LM, Jeena PM, Gajee K, et al. 2006. Lack of association between the nasopharyngeal carriage of Streptococcus pneumoniae and Staphylococcus aureus in HIV-1-infected South African children. J Infect Dis 194 (3):385-390.
27. Pettigrew MM, Gent JF, Pyles RB, et al. 2011. Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection. J Clin Microbiol 49 (11):3750-3755.
28. Brogden KA, Guthmiller JM, Taylor CE. 2005. Human polymicrobial infections. Lancet 365 (9455):253-255.
29. Simell B, Auranen K, Kayhty H, et al. 2012. The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines 11 (7):841-855.
30. Davis S, Deloria-Knoll M, Kassa HT, et al. 2013. Impact of pneumococcal conjugate vaccines on nasopharyngeal carriage and invasive disease among unvaccinated people: Review of evidence on indirect effects. Vaccine.
31. Egere U, Townend J, Roca A, et al. 2012. Indirect effect of 7-valent pneumococcal conjugate vaccine on pneumococcal carriage in newborns in rural Gambia: a randomised controlled trial. PLoS One 7 (11):e49143.

32. Weinberger DM, Malley R, Lipsitch M. 2011. Serotype replacement in disease after pneumococcal vaccination. Lancet 378 (9807):1962-1973.
33. Weiser JN. 2010. The pneumococcus: why a commensal misbehaves. J Mol Med (Berl) 88 (2):97-102.
34. Shak JR, Vidal JE, Klugman KP. 2013. Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. Trends Microbiol 21 (3):129-135.
35. Syrjanen RK, Auranen KJ, Leino TM, et al. 2005. Pneumococcal acute otitis media in relation to pneumococcal nasopharyngeal carriage. Pediatr Infect Dis J 24 (9):801-806.
36. Todar K. 2008-12. Todar's Online Textbook of Bacteriology. *In* Todar K (ed.). University of Wisconsin, Madison, Wisconsin.
37. Bentley SD, Aanensen DM, Mavroidi A, et al. 2006. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet 2 (3):e31.
38. Jin P, Kong F, Xiao M, et al. 2009. First report of putative Streptococcus pneumoniae serotype 6D among nasopharyngeal isolates from Fijian children. J Infect Dis 200 (9):1375-1380.
39. Park IH, Pritchard DG, Cartee R, et al. 2007. Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. J Clin Microbiol 45 (4):1225-1233.
40. Calix JJ, Nahm MH. 2010. A new pneumococcal serotype, 11E, has a variably inactivated wcjE gene. J Infect Dis 202 (1):29-38.
41. Poolman J, Kriz P, Feron C, et al. 2009. Pneumococcal serotype 3 otitis media, limited effect of polysaccharide conjugate immunisation and strain characteristics. Vaccine 27 (24):3213-3222.
42. Llull D, Lopez R, Garcia E. 2001. Genetic bases and medical relevance of capsular polysaccharide biosynthesis in pathogenic streptococci. Curr Mol Med 1 (4):475-491.
43. Scott JR, Hinds J, Gould KA, et al. 2012. Nontypeable pneumococcal isolates among navajo and white mountain apache communities: are these really a cause of invasive disease? J Infect Dis 206 (1):73-80.
44. Hausdorff WP. 2002. Invasive pneumococcal disease in children: geographic and temporal variations in incidence and serotype distribution. Eur J Pediatr 161 Suppl 2:S135-139.
45. Whitney CG, Farley MM, Hadler J, et al. 2003. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N Engl J Med 348 (18):1737-1746.
46. O'Brien KL, Wolfson LJ, Watt JP, et al. 2009. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. Lancet 374 (9693):893-902.
47. Spinale JM, Ruebner RL, Kaplan BS, et al. 2013. Update on Streptococcus pneumoniae associated hemolytic uremic syndrome. Curr Opin Pediatr 25 (2):203-208.
48. Burman LA, Norrby R, Trollfors B. 1985. Invasive pneumococcal infections: incidence, predisposing factors, and prognosis. Rev Infect Dis 7 (2):133-142.
49. Lipsky BA, Boyko EJ, Inui TS, et al. 1986. Risk factors for acquiring pneumococcal infections. Arch Intern Med 146 (11):2179-2185.
50. Gessner BD, Ussery XT, Parkinson AJ, et al. 1995. Risk factors for invasive disease caused by Streptococcus pneumoniae among Alaska native children younger than two years of age. Pediatr Infect Dis J 14 (2):123-128.
51. Takala AK, Jero J, Kela E, et al. 1995. Risk factors for primary invasive pneumococcal disease among children in Finland. JAMA 273 (11):859-864.

52. Cherian T, Steinhoff MC, Harrison LH, et al. 1994. A cluster of invasive pneumococcal disease in young children in child care. JAMA 271 (9):695-697.
53. Nuorti JP, Butler JC, Crutcher JM, et al. 1998. An outbreak of multidrug-resistant pneumococcal pneumonia and bacteremia among unvaccinated nursing home residents. N Engl J Med 338 (26):1861-1868.
54. Gleich S, Morad Y, Echague R, et al. 2000. Streptococcus pneumoniae serotype 4 outbreak in a home for the aged: report and review of recent outbreaks. Infect Control Hosp Epidemiol 21 (11):711-717.
55. Hausdorff WP, Bryant J, Paradiso PR, et al. 2000. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clin Infect Dis 30 (1):100-121.
56. Hausdorff WP, Bryant J, Kloek C, et al. 2000. The contribution of specific pneumococcal serogroups to different disease manifestations: implications for conjugate vaccine formulation and use, part II. Clin Infect Dis 30 (1):122-140.
57. Brueggemann AB, Peto TE, Crook DW, et al. 2004. Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children. J Infect Dis 190 (7):1203-1211.
58. Syrogiannopoulos GA, Katopodis GD, Grivea IN, et al. 2002. Antimicrobial use and serotype distribution of nasopharyngeal Streptococcus pneumoniae isolates recovered from Greek children younger than 2 years old. Clin Infect Dis 35 (10):1174-1182.
59. Smith T, Lehmann D, Montgomery J, et al. 1993. Acquisition and invasiveness of different serotypes of Streptococcus pneumoniae in young children. Epidemiol Infect 111 (1):27-39.
60. Weinberger DM, Trzcinski K, Lu YJ, et al. 2009. Pneumococcal capsular polysaccharide structure predicts serotype prevalence. PLoS Pathog 5 (6):e1000476.
61. Kadioglu A, Weiser JN, Paton JC, et al. 2008. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 6 (4):288-301.
62. Lipsitch M, Abdullahi O, D'Amour A, et al. 2012. Estimating rates of carriage acquisition and clearance and competitive ability for pneumococcal serotypes in Kenya with a Markov transition model. Epidemiology 23 (4):510-519.
63. Hill PC, Cheung YB, Akisanya A, et al. 2008. Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian infants: a longitudinal study. Clin Infect Dis 46 (6):807-814.
64. Goldblatt D, Hussain M, Andrews N, et al. 2005. Antibody responses to nasopharyngeal carriage of Streptococcus pneumoniae in adults: a longitudinal household study. J Infect Dis 192 (3):387-393.
65. Weinberger DM, Dagan R, Givon-Lavi N, et al. 2008. Epidemiologic evidence for serotype-specific acquired immunity to pneumococcal carriage. J Infect Dis 197 (11):1511-1518.
66. Granat SM, Ollgren J, Herva E, et al. 2009. Epidemiological evidence for serotype-independent acquired immunity to pneumococcal carriage. J Infect Dis 200 (1):99-106.
67. Cobey S, Lipsitch M. 2012. Niche and neutral effects of acquired immunity permit coexistence of pneumococcal serotypes. Science 335 (6074):1376-1380.
68. Hogberg L, Geli P, Ringberg H, et al. 2007. Age- and serogroup-related differences in observed durations of nasopharyngeal carriage of penicillin-resistant pneumococci. J Clin Microbiol 45 (3):948-952.

69. Abdullahi O, Nyiro J, Lewa P, et al. 2008. The descriptive epidemiology of Streptococcus pneumoniae and Haemophilus influenzae nasopharyngeal carriage in children and adults in Kilifi district, Kenya. Pediatr Infect Dis J 27 (1):59-64.
70. Bogaert D, van Belkum A, Sluijter M, et al. 2004. Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children. Lancet 363 (9424):1871-1872.
71. Feikin DR, Klugman KP. 2002. Historical changes in pneumococcal serogroup distribution: implications for the era of pneumococcal conjugate vaccines. Clin Infect Dis 35 (5):547-555.
72. Feikin DR, Davis M, Nwanyanwu OC, et al. 2003. Antibiotic resistance and serotype distribution of Streptococcus pneumoniae colonizing rural Malawian children. Pediatr Infect Dis J 22 (6):564-567.
73. Scott JR, Millar EV, Lipsitch M, et al. 2012. Impact of more than a decade of pneumococcal conjugate vaccine use on carriage and invasive potential in Native American communities. J Infect Dis 205 (2):280-288.
74. Hanage WP, Huang SS, Lipsitch M, et al. 2007. Diversity and antibiotic resistance among nonvaccine serotypes of Streptococcus pneumoniae carriage isolates in the post-heptavalent conjugate vaccine era. J Infect Dis 195 (3):347-352.
75. Rodrigues F, Nunes S, Sa-Leao R, et al. 2009. Streptococcus pneumoniae nasopharyngeal carriage in children attending day-care centers in the central region of Portugal, in the era of 7-valent pneumococcal conjugate vaccine. Microb Drug Resist 15 (4):269-277.
76. Rodgers GL, Arguedas A, Cohen R, et al. 2009. Global serotype distribution among Streptococcus pneumoniae isolates causing otitis media in children: potential implications for pneumococcal conjugate vaccines. Vaccine 27 (29):3802-3810.
77. Hanage WP, Auranen K, Syrjanen R, et al. 2004. Ability of pneumococcal serotypes and clones to cause acute otitis media: implications for the prevention of otitis media by conjugate vaccines. Infect Immun 72 (1):76-81.
78. Greenberg D, Givon-Lavi N, Newman N, et al. 2011. Nasopharyngeal carriage of individual Streptococcus pneumoniae serotypes during pediatric pneumonia as a means to estimate serotype disease potential. Pediatr Infect Dis J 30 (3):227-233.
79. Hausdorff WP, Feikin DR, Klugman KP. 2005. Epidemiological differences among pneumococcal serotypes. Lancet Infect Dis 5 (2):83-93.
80. Moore MR. 2009. Rethinking replacement and resistance. J Infect Dis 199 (6):771-773.
81. Moore MR, Whitney CG. 2008. Emergence of nonvaccine serotypes following introduction of pneumococcal conjugate vaccine: cause and effect? Clin Infect Dis 46 (2):183-185.
82. Fenoll A, Granizo JJ, Aguilar L, et al. 2009. Temporal trends of invasive Streptococcus pneumoniae serotypes and antimicrobial resistance patterns in Spain from 1979 to 2007. J Clin Microbiol 47 (4):1012-1020.
83. Harboe ZB, Benfield TL, Valentiner-Branth P, et al. 2010. Temporal trends in invasive pneumococcal disease and pneumococcal serotypes over 7 decades. Clin Infect Dis 50 (3):329-337.
84. Berron S, Fenoll A, Ortega M, et al. 2005. Analysis of the genetic structure of nontypeable pneumococcal strains isolated from conjunctiva. J Clin Microbiol 43 (4):1694-1698.
85. Buck JM, Lexau C, Shapiro M, et al. 2006. A community outbreak of conjunctivitis caused by nontypeable Streptococcus pneumoniae in Minnesota. Pediatr Infect Dis J 25 (10):906-911.

86. Hathaway LJ, Stutzmann Meier P, Battig P, et al. 2004. A homologue of aliB is found in the capsule region of nonencapsulated Streptococcus pneumoniae. J Bacteriol 186 (12):3721-3729.
87. Weatherholtz R, Millar EV, Moulton LH, et al. 2010. Invasive pneumococcal disease a decade after pneumococcal conjugate vaccine use in an American Indian population at high risk for disease. Clin Infect Dis 50 (9):1238-1246.
88. Garcia L. 2010. Clinical Microbiology Procedures Handbook, 3rd ed. ASM, Washington D.C.
89. Murphy TF, Faden H, Bakaletz LO, et al. 2009. Nontypeable Haemophilus influenzae as a pathogen in children. Pediatr Infect Dis J 28 (1):43-48.
90. MacNeil JR, Cohn AC, Farley M, et al. 2011. Current epidemiology and trends in invasive Haemophilus influenzae disease--United States, 1989-2008. Clin Infect Dis 53 (12):1230-1236.
91. Bender JM, Cox CM, Mottice S, et al. 2010. Invasive Haemophilus influenzae disease in Utah children: an 11-year population-based study in the era of conjugate vaccine. Clin Infect Dis 50 (7):e41-46.
92. Health Protection Agency. Identification of Moraxella species and Morphologically Similar Organisms. UK Standards for Microbiology Investigations. ID 11 Issue 2.3. 2012. Available at: http://www.hpa.org.uk/SMI/pdf. Accessed 25 April, Access 2012.
93. Health Protection Agency. Identification of Staphylococcus species, Micrococcus species and Rothia species. UK Standards for Microbiology Investigations. ID 7 Issue 2.2. 2011. Available at: http://www.hpa.org.uk/SMI/pdf. Accessed 25 April, Access 2011.
94. Wertheim HF, Melles DC, Vos MC, et al. 2005. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis 5 (12):751-762.
95. Luzar MA, Coles GA, Faller B, et al. 1990. Staphylococcus aureus nasal carriage and infection in patients on continuous ambulatory peritoneal dialysis. N Engl J Med 322 (8):505-509.
96. von Eiff C, Becker K, Machka K, et al. 2001. Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group. N Engl J Med 344 (1):11-16.
97. Wertheim HF, Vos MC, Ott A, et al. 2004. Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers. Lancet 364 (9435):703-705.
98. Heikkinen T, Jarvinen A. 2003. The common cold. Lancet 361 (9351):51-59.
99. Pappas DE, Hendley JO, Hayden FG, et al. 2008. Symptom profile of common colds in school-aged children. Pediatr Infect Dis J 27 (1):8-11.
100. Longtin J, Bastien M, Gilca R, et al. 2008. Human bocavirus infections in hospitalized children and adults. Emerg Infect Dis 14 (2):217-221.
101. Moore HC, Jacoby P, Taylor A, et al. 2010. The interaction between respiratory viruses and pathogenic bacteria in the upper respiratory tract of asymptomatic Aboriginal and non-Aboriginal children. Pediatr Infect Dis J 29 (6):540-545.
102. Wiertsema SP, Chidlow GR, Kirkham LA, et al. 2011. High detection rates of nucleic acids of a wide range of respiratory viruses in the nasopharynx and the middle ear of children with a history of recurrent acute otitis media. J Med Virol 83 (11):2008-2017.
103. van den Bergh MR, Biesbroek G, Rossen JW, et al. 2012. Associations between pathogens in the upper respiratory tract of young children: interplay between viruses and bacteria. PLoS One 7 (10):e47711.

104. Blomqvist S, Roivainen M, Puhakka T, et al. 2002. Virological and serological analysis of rhinovirus infections during the first two years of life in a cohort of children. J Med Virol 66 (2):263-268.
105. Mackay IM, Arden KE, Nitsche A. 2002. Real-time PCR in virology. Nucleic Acids Res 30 (6):1292-1305.
106. Singleton RJ, Bulkow LR, Miernyk K, et al. 2010. Viral respiratory infections in hospitalized and community control children in Alaska. J Med Virol 82 (7):1282-1290.
107. Thavagnanam S, Christie SN, Doherty GM, et al. 2010. Respiratory viral infection in lower airways of asymptomatic children. Acta Paediatr 99 (3):394-398.
108. O'Brien KL, Nohynek H. 2003. Report from a WHO Working Group: standard method for detecting upper respiratory carriage of Streptococcus pneumoniae. Pediatr Infect Dis J 22 (2):e1-11.
109. O'Brien KL, Nohynek H. 2003. Report from a WHO working group: standard method for detecting upper respiratory carriage of Streptococcus pneumoniae. Pediatr Infect Dis J 22 (2):133-140.
110. O'Brien KL, Bronsdon MA, Dagan R, et al. 2001. Evaluation of a medium (STGG) for transport and optimal recovery of Streptococcus pneumoniae from nasopharyngeal secretions collected during field studies. J Clin Microbiol 39 (3):1021-1024.
111. Charalambous BM, Batt SL, Peek AC, et al. 2003. Quantitative validation of media for transportation and storage of Streptococcus pneumoniae. J Clin Microbiol 41 (12):5551-5556.
112. Stubbs E, McKinnon M, Shelby-James T, et al. Methodological aspects of pneumococcal carriage studies. In: 2nd International Symposium on Pneumococci and Pneumococcal Diseases. SunCity, South Africa 2000.
113. Turner P, Po L, Turner C, et al. 2011. Detection of respiratory viruses by PCR assay of nasopharyngeal swabs stored in skim milk-tryptone-glucose-glycerol transport medium. J Clin Microbiol 49 (6):2311-2313.
114. Sorensen UB. 1993. Typing of pneumococci by using 12 pooled antisera. J Clin Microbiol 31 (8):2097-2100.
115. Griffith F. 1928. The Significance of Pneumococcal Types. J Hyg (Lond) 27 (2):113-159.
116. Hodges RG, Mac LC, Bernhard WG. 1946. Epidemic pneumococcal pneumonia; pneumococcal carrier studies. Am J Hyg 44 (2):207-230.
117. Kaltoft MS, Skov Sorensen UB, Slotved HC, et al. 2008. An easy method for detection of nasopharyngeal carriage of multiple Streptococcus pneumoniae serotypes. J Microbiol Methods 75 (3):540-544.
118. da Gloria Carvalho M, Pimenta FC, Jackson D, et al. 2010. Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J Clin Microbiol 48 (5):1611-1618.
119. Bronsdon MA, O'Brien KL, Facklam RR, et al. 2004. Immunoblot method to detect Streptococcus pneumoniae and identify multiple serotypes from nasopharyngeal secretions. J Clin Microbiol 42 (4):1596-1600.
120. Rivera-Olivero IA, Blommaart M, Bogaert D, et al. 2009. Multiplex PCR reveals a high rate of nasopharyngeal pneumococcal 7-valent conjugate vaccine serotypes co-colonizing indigenous Warao children in Venezuela. J Med Microbiol 58 (Pt 5):584-587.
121. Antonio M, Hakeem I, Sankareh K, et al. 2009. Evaluation of sequential multiplex PCR for direct detection of multiple serotypes of Streptococcus pneumoniae from nasopharyngeal secretions. J Med Microbiol 58 (Pt 3):296-302.

122. Brugger SD, Hathaway LJ, Muhlemann K. 2009. Detection of Streptococcus pneumoniae strain cocolonization in the nasopharynx. J Clin Microbiol 47 (6):1750-1756.
123. Turner P, Hinds J, Turner C, et al. 2011. Improved detection of nasopharyngeal cocolonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. J Clin Microbiol 49 (5):1784-1789.
124. Gratten M, Montgomery J, Gerega G, et al. 1989. Multiple colonization of the upper respiratory tract of Papua New Guinea children with Haemophilus influenzae and Streptococcus pneumoniae. Southeast Asian J Trop Med Public Health 20 (4):501-509.
125. Brugger SD, Frey P, Aebi S, et al. 2010. Multiple colonization with S. pneumoniae before and after introduction of the seven-valent conjugated pneumococcal polysaccharide vaccine. PLoS One 5 (7):e11638.
126. Vestrheim DF, Hoiby EA, Aaberge IS, et al. 2010. Impact of a pneumococcal conjugate vaccination program on carriage among children in Norway. Clin Vaccine Immunol 17 (3):325-334.
127. Hinds J, Gould K, Witney A, et al. Investigation of serotype diversity, non-typeable isolates and nasopharyngeal co-colonization by microarray-based molecular serotyping of Streptococcus pneumoniae. In: 7th International Symposium on Pneumococci and Pneumococcal Diseases. Tel Aviv, Israel 2010.
128. Gould K, Pond M, Baldry S, et al. Evaluation and optimization of methods to facilitate direct analysis of nasopharyngeal swabs by microarray-based molecular serotyping. In: 7th International Symposium on Pneumococci and Pneumococcal Diseases. Tel Aviv, Israel 2010.
129. Valente C, Hinds J, Pinto F, et al. 2012. Decrease in pneumococcal co-colonization following vaccination with the seven-valent pneumococcal conjugate vaccine. PLoS One 7 (1):e30235.
130. Hussain M, Melegaro A, Pebody RG, et al. 2005. A longitudinal household study of Streptococcus pneumoniae nasopharyngeal carriage in a UK setting. Epidemiol Infect 133 (5):891-898.
131. Sa-Leao R, Nunes S, Brito-Avo A, et al. 2008. High rates of transmission of and colonization by Streptococcus pneumoniae and Haemophilus influenzae within a day care center revealed in a longitudinal study. J Clin Microbiol 46 (1):225-234.
132. Vives M, Garcia ME, Saenz P, et al. 1997. Nasopharyngeal colonization in Costa Rican children during the first year of life. Pediatr Infect Dis J 16 (9):852-858.
133. Tigoi CC, Gatakaa H, Karani A, et al. 2012. Rates of acquisition of pneumococcal colonization and transmission probabilities, by serotype, among newborn infants in Kilifi District, Kenya. Clin Infect Dis 55 (2):180-188.
134. Turner P, Turner C, Jankhot A, et al. 2012. A longitudinal study of Streptococcus pneumoniae carriage in a cohort of infants and their mothers on the Thailand-Myanmar border. PLoS One 7 (5):e38271.
135. Sleeman KL, Griffiths D, Shackley F, et al. 2006. Capsular serotype-specific attack rates and duration of carriage of Streptococcus pneumoniae in a population of children. J Infect Dis 194 (5):682-688.
136. Abdullahi O, Karani A, Tigoi CC, et al. 2012. Rates of acquisition and clearance of pneumococcal serotypes in the nasopharynges of children in Kilifi District, Kenya. J Infect Dis 206 (7):1020-1029.
137. Faden H, Duffy L, Williams A, et al. 1996. Epidemiology of nasopharyngeal colonization with nontypeable Haemophilus influenzae in the first two years of life. Acta Otolaryngol Suppl 523:128-129.

138. Christenson B, Sylvan SP, Noreen B. 1997. Carriage of multiresistant Streptococcus pneumoniae among children attending day-care centres in the Stockholm area. Scand J Infect Dis 29 (6):555-558.
139. De Lencastre H, Kristinsson KG, Brito-Avo A, et al. 1999. Carriage of respiratory tract pathogens and molecular epidemiology of Streptococcus pneumoniae colonization in healthy children attending day care centers in Lisbon, Portugal. Microb Drug Resist 5 (1):19-29.
140. Syrogiannopoulos GA, Grivea IN, Katopodis GD, et al. 2000. Carriage of antibiotic-resistant Streptococcus pneumoniae in Greek infants and toddlers. Eur J Clin Microbiol Infect Dis 19 (4):288-293.
141. Bogaert D, Engelen MN, Timmers-Reker AJ, et al. 2001. Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study. J Clin Microbiol 39 (9):3316-3320.
142. Givon-Lavi N, Dagan R, Fraser D, et al. 1999. Marked differences in pneumococcal carriage and resistance patterns between day care centers located within a small area. Clin Infect Dis 29 (5):1274-1280.
143. Lee NY, Song JH, Kim S, et al. 2001. Carriage of antibiotic-resistant pneumococci among Asian children: a multinational surveillance by the Asian Network for Surveillance of Resistant Pathogens (ANSORP). Clin Infect Dis 32 (10):1463-1469.
144. Soewignjo S, Gessner BD, Sutanto A, et al. 2001. Streptococcus pneumoniae nasopharyngeal carriage prevalence, serotype distribution, and resistance patterns among children on Lombok Island, Indonesia. Clin Infect Dis 32 (7):1039-1043.
145. Jebaraj R, Cherian T, Raghupathy P, et al. 1999. Nasopharyngeal colonization of infants in southern India with Streptococcus pneumoniae. Epidemiol Infect 123 (3):383-388.
146. Joloba ML, Bajaksouzian S, Palavecino E, et al. 2001. High prevalence of carriage of antibiotic-resistant Streptococcus pneumoniae in children in Kampala Uganda. Int J Antimicrob Agents 17 (5):395-400.
147. Adegbola RA, Obaro SK, Biney E, et al. 2001. Evaluation of Binax now Streptococcus pneumoniae urinary antigen test in children in a community with a high carriage rate of pneumococcus. Pediatr Infect Dis J 20 (7):718-719.
148. Syrjanen RK, Kilpi TM, Kaijalainen TH, et al. 2001. Nasopharyngeal carriage of Streptococcus pneumoniae in Finnish children younger than 2 years old. J Infect Dis 184 (4):451-459.
149. Bogaert D, Sluijter M, Toom NL, et al. 2006. Dynamics of pneumococcal colonization in healthy Dutch children. Microbiology 152 (Pt 2):377-385.
150. Roca A, Hill PC, Townend J, et al. 2011. Effects of community-wide vaccination with PCV-7 on pneumococcal nasopharyngeal carriage in the Gambia: a cluster-randomized trial. PLoS Med 8 (10):e1001107.
151. Adetifa IM, Antonio M, Okoromah CA, et al. 2012. Pre-vaccination nasopharyngeal pneumococcal carriage in a Nigerian population: epidemiology and population biology. PLoS One 7 (1):e30548.
152. Jousimies-Somer HR, Savolainen S, Ylikoski JS. 1989. Comparison of the nasal bacterial floras in two groups of healthy subjects and in patients with acute maxillary sinusitis. J Clin Microbiol 27 (12):2736-2743.
153. Gunnarsson RK, Holm SE, Soderstrom M. 1998. The prevalence of potential pathogenic bacteria in nasopharyngeal samples from healthy children and adults. Scand J Prim Health Care 16 (1):13-17.
154. Musher DM, Groover JE, Reichler MR, et al. 1997. Emergence of antibody to capsular polysaccharides of Streptococcus pneumoniae during outbreaks of

pneumonia: association with nasopharyngeal colonization. Clin Infect Dis 24 (3):441-446.

155. Gray BM, Converse GM, 3rd, Huhta N, et al. 1981. Epidemiologic studies of Streptococcus pneumoniae in infants: antibody response to nasopharyngeal carriage of types 3, 19, and 23. J Infect Dis 144 (4):312-318.

156. Lindberg K, Freijd A, Rynnel-Dagoo B, et al. 1993. Anti pneumococcal antibody activity in nasopharyngeal secretions in healthy adults and children. Acta Otolaryngol 113 (5):673-678.

157. Lebon A, Labout JA, Verbrugh HA, et al. 2008. Dynamics and determinants of Staphylococcus aureus carriage in infancy: the Generation R Study. J Clin Microbiol 46 (10):3517-3521.

158. Lebon A, Moll HA, Tavakol M, et al. 2010. Correlation of bacterial colonization status between mother and child: the Generation R Study. J Clin Microbiol 48 (3):960-962.

159. Nouwen JL, Ott A, Kluytmans-Vandenbergh MF, et al. 2004. Predicting the Staphylococcus aureus nasal carrier state: derivation and validation of a "culture rule". Clin Infect Dis 39 (6):806-811.

160. Armstrong-Esther CA. 1976. Carriage patterns of Staphylococcus aureus in a healthy non-hospital population of adults and children. Ann Hum Biol 3 (3):221-227.

161. Pelton SI, Dagan R, Gaines BM, et al. 2003. Pneumococcal conjugate vaccines: proceedings from an interactive symposium at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy. Vaccine 21 (15):1562-1571.

162. Millar EV, O'Brien KL, Zell ER, et al. 2009. Nasopharyngeal carriage of Streptococcus pneumoniae in Navajo and White Mountain Apache children before the introduction of pneumococcal conjugate vaccine. Pediatr Infect Dis J 28 (8):711-716.

163. Principi N, Marchisio P, Schito GC, et al. 1999. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group. Pediatr Infect Dis J 18 (6):517-523.

164. Coles CL, Kanungo R, Rahmathullah L, et al. 2001. Pneumococcal nasopharyngeal colonization in young South Indian infants. Pediatr Infect Dis J 20 (3):289-295.

165. Petrosillo N, Pantosti A, Bordi E, et al. 2002. Prevalence, determinants, and molecular epidemiology of Streptococcus pneumoniae isolates colonizing the nasopharynx of healthy children in Rome. Eur J Clin Microbiol Infect Dis 21 (3):181-188.

166. Kulkarni R, Rampersaud R, Aguilar JL, et al. 2010. Cigarette smoke inhibits airway epithelial cell innate immune responses to bacteria. Infect Immun 78 (5):2146-2152.

167. Peacock SJ, Justice A, Griffiths D, et al. 2003. Determinants of acquisition and carriage of Staphylococcus aureus in infancy. J Clin Microbiol 41 (12):5718-5725.

168. Dunais B, Pradier C, Carsenti H, et al. 2003. Influence of child care on nasopharyngeal carriage of Streptococcus pneumoniae and Haemophilus influenzae. Pediatr Infect Dis J 22 (7):589-592.

169. Yagupsky P, Porat N, Fraser D, et al. 1998. Acquisition, carriage, and transmission of pneumococci with decreased antibiotic susceptibility in young children attending a day care facility in southern Israel. J Infect Dis 177 (4):1003-1012.

170. Leino T, Hoti F, Syrjanen R, et al. 2008. Clustering of serotypes in a longitudinal study of Streptococcus pneumoniae carriage in three day care centres. BMC Infect Dis 8:173.

171. Tonnaer EL, Hafmans TG, Van Kuppevelt TH, et al. 2006. Involvement of glycosaminoglycans in the attachment of pneumococci to nasopharyngeal epithelial cells. Microbes Infect 8 (2):316-322.
172. Bluestone CD. 1996. Pathogenesis of otitis media: role of eustachian tube. Pediatr Infect Dis J 15 (4):281-291.
173. Murphy TF, Bakaletz LO, Smeesters PR. 2009. Microbial interactions in the respiratory tract. Pediatr Infect Dis J 28 (10 Suppl):S121-126.
174. Mook-Kanamori BB, Geldhoff M, van der Poll T, et al. 2011. Pathogenesis and pathophysiology of pneumococcal meningitis. Clin Microbiol Rev 24 (3):557-591.
175. Mastro TD, Nomani NK, Ishaq Z, et al. 1993. Use of nasopharyngeal isolates of Streptococcus pneumoniae and Haemophilus influenzae from children in Pakistan for surveillance for antimicrobial resistance. Pediatr Infect Dis J 12 (10):824-830.
176. Lloyd-Evans N, O'Dempsey TJ, Baldeh I, et al. 1996. Nasopharyngeal carriage of pneumococci in Gambian children and in their families. Pediatr Infect Dis J 15 (10):866-871.
177. Peltola VT, Boyd KL, McAuley JL, et al. 2006. Bacterial sinusitis and otitis media following influenza virus infection in ferrets. Infect Immun 74 (5):2562-2567.
178. Kadioglu A, Gingles NA, Grattan K, et al. 2000. Host cellular immune response to pneumococcal lung infection in mice. Infect Immun 68 (2):492-501.
179. Ogunniyi AD, LeMessurier KS, Graham RM, et al. 2007. Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of Streptococcus pneumoniae D39 in a mouse model. Infect Immun 75 (4):1843-1851.
180. Gray BM, Converse GM, 3rd, Dillon HC, Jr. 1980. Epidemiologic studies of Streptococcus pneumoniae in infants: acquisition, carriage, and infection during the first 24 months of life. J Infect Dis 142 (6):923-933.
181. Syrjanen RK, Herva EE, Makela PH, et al. 2006. The value of nasopharyngeal culture in predicting the etiology of acute otitis media in children less than two years of age. Pediatr Infect Dis J 25 (11):1032-1036.
182. Sleeman KL, Daniels L, Gardiner M, et al. 2005. Acquisition of Streptococcus pneumoniae and nonspecific morbidity in infants and their families: a cohort study. Pediatr Infect Dis J 24 (2):121-127.
183. Levine OS, Liu G, Garman RL, et al. 2000. Haemophilus influenzae type b and Streptococcus pneumoniae as causes of pneumonia among children in Beijing, China. Emerg Infect Dis 6 (2):165-170.
184. Vu HT, Yoshida LM, Suzuki M, et al. 2011. Association between nasopharyngeal load of Streptococcus pneumoniae, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. Pediatr Infect Dis J 30 (1):11-18.
185. Prellner K, Christensen P, Hovelius B, et al. 1984. Nasopharyngeal carriage of bacteria in otitis-prone and non-otitis-prone children in day-care centres. Acta Otolaryngol 98 (3-4):343-350.
186. Anh DD, Huong Ple T, Watanabe K, et al. 2007. Increased rates of intense nasopharyngeal bacterial colonization of Vietnamese children with radiological pneumonia. Tohoku J Exp Med 213 (2):167-172.
187. Albrich WC, Madhi SA, Adrian PV, et al. 2012. Use of a rapid test of pneumococcal colonization density to diagnose pneumococcal pneumonia. Clin Infect Dis 54 (5):601-609.
188. Givon-Lavi N, Fraser D, Porat N, et al. 2002. Spread of Streptococcus pneumoniae and antibiotic-resistant S. pneumoniae from day-care center attendees to their younger siblings. J Infect Dis 186 (11):1608-1614.

189. Reichler MR, Rakovsky J, Slacikova M, et al. 1996. Spread of multidrug-resistant Streptococcus pneumoniae among hospitalized children in Slovakia. J Infect Dis 173 (2):374-379.
190. Hoge CW, Reichler MR, Dominguez EA, et al. 1994. An epidemic of pneumococcal disease in an overcrowded, inadequately ventilated jail. N Engl J Med 331 (10):643-648.
191. Leiberman A, Dagan R, Leibovitz E, et al. 1999. The bacteriology of the nasopharynx in childhood. Int J Pediatr Otorhinolaryngol 49 Suppl 1:S151-153.
192. Macleod CM, Hodges RG, Heidelberger M, et al. 1945. Prevention of Pneumococcal Pneumonia by Immunization with Specific Capsular Polysaccharides. J Exp Med 82 (6):445-465.
193. Koskela M, Leinonen M, Haiva VM, et al. 1986. First and second dose antibody responses to pneumococcal polysaccharide vaccine in infants. Pediatr Infect Dis 5 (1):45-50.
194. Douglas RM, Miles HB. 1984. Vaccination against Streptococcus pneumoniae in childhood: lack of demonstrable benefit in young Australian children. J Infect Dis 149 (6):861-869.
195. Choo S, Zhang Q, Seymour L, et al. 2000. Primary and booster salivary antibody responses to a 7-valent pneumococcal conjugate vaccine in infants. J Infect Dis 182 (4):1260-1263.
196. Douglas RM, Hansman D, Miles HB, et al. 1986. Pneumococcal carriage and type-specific antibody. Failure of a 14-valent vaccine to reduce carriage in healthy children. Am J Dis Child 140 (11):1183-1185.
197. Herva E, Luotonen J, Timonen M, et al. 1980. The effect of polyvalent pneumococcal polysaccharide vaccine on nasopharyngeal and nasal carriage of Streptococcus pneumoniae. Scand J Infect Dis 12 (2):97-100.
198. Stein KE. 1992. Thymus-independent and thymus-dependent responses to polysaccharide antigens. J Infect Dis 165 Suppl 1:S49-52.
199. Black S, Shinefield H, Fireman B, et al. 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr Infect Dis J 19 (3):187-195.
200. O'Brien KL, Moulton LH, Reid R, et al. 2003. Efficacy and safety of seven-valent conjugate pneumococcal vaccine in American Indian children: group randomised trial. Lancet 362 (9381):355-361.
201. Choo S, Seymour L, Morris R, et al. 2000. Immunogenicity and reactogenicity of a pneumococcal conjugate vaccine administered combined with a haemophilus influenzae type B conjugate vaccine in United Kingdom infants. Pediatr Infect Dis J 19 (9):854-862.
202. McIntosh ED, Fritzell B, Fletcher MA. 2007. Burden of paediatric invasive pneumococcal disease in Europe, 2005. Epidemiol Infect 135 (4):644-656.
203. Butler JC, Breiman RF, Lipman HB, et al. 1995. Serotype distribution of Streptococcus pneumoniae infections among preschool children in the United States, 1978-1994: implications for development of a conjugate vaccine. J Infect Dis 171 (4):885-889.
204. Serrano I, Ramirez M, Melo-Cristino J. 2004. Invasive Streptococcus pneumoniae from Portugal: implications for vaccination and antimicrobial therapy. Clin Microbiol Infect 10 (7):652-656.
205. WHO Tech Rep Ser. 2005. World Health Organization. Pneumococcal conjugate vaccines. Recommendations for production and control of pneumococcal conjugate vaccines. Tech Rep Ser 927.

206. Vesikari T, Wysocki J, Chevallier B, et al. 2009. Immunogenicity of the 10-valent pneumococcal non-typeable Haemophilus influenzae protein D conjugate vaccine (PHiD-CV) compared to the licensed 7vCRM vaccine. Pediatr Infect Dis J 28 (4 Suppl):S66-76.
207. Chevallier B, Vesikari T, Brzostek J, et al. 2009. Safety and reactogenicity of the 10-valent pneumococcal non-typeable Haemophilus influenzae protein D conjugate vaccine (PHiD-CV) when coadministered with routine childhood vaccines. Pediatr Infect Dis J 28 (4 Suppl):S109-118.
208. Prymula R, Peeters P, Chrobok V, et al. 2006. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable Haemophilus influenzae: a randomised double-blind efficacy study. Lancet 367 (9512):740-748.
209. Snape MD, Klinger CL, Daniels ED, et al. 2010. Immunogenicity and reactogenicity of a 13-valent-pneumococcal conjugate vaccine administered at 2, 4, and 12 months of age: a double-blind randomized active-controlled trial. Pediatr Infect Dis J 29 (12):e80-90.
210. Pilishvili T, Lexau C, Farley MM, et al. 2010. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J Infect Dis 201 (1):32-41.
211. Bettinger JA, Scheifele DW, Kellner JD, et al. 2010. The effect of routine vaccination on invasive pneumococcal infections in Canadian children, Immunization Monitoring Program, Active 2000-2007. Vaccine 28 (9):2130-2136.
212. Rodenburg GD, de Greeff SC, Jansen AG, et al. 2010. Effects of pneumococcal conjugate vaccine 2 years after its introduction, the Netherlands. Emerg Infect Dis 16 (5):816-823.
213. Harboe ZB, Valentiner-Branth P, Benfield TL, et al. 2010. Early effectiveness of heptavalent conjugate pneumococcal vaccination on invasive pneumococcal disease after the introduction in the Danish Childhood Immunization Programme. Vaccine 28 (14):2642-2647.
214. Rosen JB, Thomas AR, Lexau CA, et al. 2011. Geographic variation in invasive pneumococcal disease following pneumococcal conjugate vaccine introduction in the United States. Clin Infect Dis 53 (2):137-143.
215. Simonsen L, Taylor RJ, Young-Xu Y, et al. 2011. Impact of pneumococcal conjugate vaccination of infants on pneumonia and influenza hospitalization and mortality in all age groups in the United States. MBio 2 (1):e00309-00310.
216. Weinberger DM, Givon-Lavi N, Shemer-Avni Y, et al. 2013. Influence of pneumococcal vaccines and respiratory syncytial virus on alveolar pneumonia, Israel. Emerg Infect Dis 19 (7):1084-1091.
217. Afonso ET, Minamisava R, Bierrenbach AL, et al. 2013. Effect of 10-valent pneumococcal vaccine on pneumonia among children, Brazil. Emerg Infect Dis 19 (4):589-597.
218. Elemraid MA, Rushton SP, Shirley MD, et al. 2013. Impact of the 7-valent pneumococcal conjugate vaccine on the incidence of childhood pneumonia. Epidemiol Infect 141 (8):1697-1704.
219. Stamboulidis K, Chatzaki D, Poulakou G, et al. 2011. The impact of the heptavalent pneumococcal conjugate vaccine on the epidemiology of acute otitis media complicated by otorrhea. Pediatr Infect Dis J 30 (7):551-555.
220. Alonso M, Marimon JM, Ercibengoa M, et al. 2013. Dynamics of Streptococcus pneumoniae serotypes causing acute otitis media isolated from children with spontaneous middle-ear drainage over a 12-year period (1999-2010) in a region of northern Spain. PLoS One 8 (1):e54333.

221. Block SL, Hedrick J, Harrison CJ, et al. 2004. Community-wide vaccination with the heptavalent pneumococcal conjugate significantly alters the microbiology of acute otitis media. Pediatr Infect Dis J 23 (9):829-833.
222. Taylor S, Marchisio P, Vergison A, et al. 2012. Impact of pneumococcal conjugate vaccination on otitis media: a systematic review. Clin Infect Dis 54 (12):1765-1773.
223. Queirós L, Vieira M, Frutuoso A. Cobertura pela vacina pnemocócica conjugada heptavalente nas coortes de nascimento de 2001 a 2005 na região Norte 2007. Available at: http://portal.arsnorte.minsaude.pt/portal/page/portal/ARSNorte/Conte%C3%BAdos/Sa%C3%BAde P%C3%BAblica Conteudos/Relatorio 2008 Vacina Prevenar.pdf Accessed 16 August, Access 2007.
224. Aguiar SI, Serrano I, Pinto FR, et al. 2008. Changes in Streptococcus pneumoniae serotypes causing invasive disease with non-universal vaccination coverage of the seven-valent conjugate vaccine. Clin Microbiol Infect 14 (9):835-843.
225. Aguiar SI, Brito MJ, Goncalo-Marques J, et al. 2010. Serotypes 1, 7F and 19A became the leading causes of pediatric invasive pneumococcal infections in Portugal after 7 years of heptavalent conjugate vaccine use. Vaccine 28 (32):5167-5173.
226. Kauppi M, Eskola J, Kayhty H. 1995. Anti-capsular polysaccharide antibody concentrations in saliva after immunization with Haemophilus influenzae type b conjugate vaccines. Pediatr Infect Dis J 14 (4):286-294.
227. Heath PT, McVernon J. 2002. The UK Hib vaccine experience. Arch Dis Child 86 (6):396-399.
228. Finn A, Heath P. 2005. Conjugate vaccines. Arch Dis Child 90 (7):667-669.
229. Obaro SK, Adegbola RA, Banya WA, et al. 1996. Carriage of pneumococci after pneumococcal vaccination. Lancet 348 (9022):271-272.
230. Lipsitch M. 1997. Vaccination against colonizing bacteria with multiple serotypes. Proc Natl Acad Sci U S A 94 (12):6571-6576.
231. Klugman KP, Madhi SA, Huebner RE, et al. 2003. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. N Engl J Med 349 (14):1341-1348.
232. Mbelle N, Huebner RE, Wasas AD, et al. 1999. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. J Infect Dis 180 (4):1171-1176.
233. Cheung YB, Zaman SM, Nsekpong ED, et al. 2009. Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian children who participated in a 9-valent pneumococcal conjugate vaccine trial and in their younger siblings. Pediatr Infect Dis J 28 (11):990-995.
234. O'Brien KL, Millar EV, Zell ER, et al. 2007. Effect of pneumococcal conjugate vaccine on nasopharyngeal colonization among immunized and unimmunized children in a community-randomized trial. J Infect Dis 196 (8):1211-1220.
235. Prymula R, Kriz P, Kaliskova E, et al. 2009. Effect of vaccination with pneumococcal capsular polysaccharides conjugated to Haemophilus influenzae-derived protein D on nasopharyngeal carriage of Streptococcus pneumoniae and H. influenzae in children under 2 years of age. Vaccine 28 (1):71-78.
236. Dagan R, Givon-Lavi N, Zamir O, et al. 2002. Reduction of nasopharyngeal carriage of Streptococcus pneumoniae after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. J Infect Dis 185 (7):927-936.
237. Dagan R, Givon-Lavi N, Fraser D, et al. 2005. Serum serotype-specific pneumococcal anticapsular immunoglobulin g concentrations after immunization

with a 9-valent conjugate pneumococcal vaccine correlate with nasopharyngeal acquisition of pneumococcus. J Infect Dis 192 (3):367-376.

238. van Gils EJ, Veenhoven RH, Hak E, et al. 2009. Effect of reduced-dose schedules with 7-valent pneumococcal conjugate vaccine on nasopharyngeal pneumococcal carriage in children: a randomized controlled trial. JAMA 302 (2):159-167.

239. Huang SS, Hinrichsen VL, Stevenson AE, et al. 2009. Continued impact of pneumococcal conjugate vaccine on carriage in young children. Pediatrics 124 (1):e1-11.

240. Ghaffar F, Barton T, Lozano J, et al. 2004. Effect of the 7-valent pneumococcal conjugate vaccine on nasopharyngeal colonization by Streptococcus pneumoniae in the first 2 years of life. Clin Infect Dis 39 (7):930-938.

241. Sharma D, Baughman W, Holst A, et al. 2013. Pneumococcal carriage and invasive disease in children before introduction of the 13-valent conjugate vaccine: comparison with the era before 7-valent conjugate vaccine. Pediatr Infect Dis J 32 (2):e45-53.

242. Dunais B, Bruno-Bazureault P, Carsenti-Dellamonica H, et al. 2011. A decade-long surveillance of nasopharyngeal colonisation with Streptococcus pneumoniae among children attending day-care centres in south-eastern France: 1999-2008. Eur J Clin Microbiol Infect Dis 30 (7):837-843.

243. Grivea IN, Tsantouli AG, Michoula AN, et al. 2011. Dynamics of Streptococcus pneumoniae nasopharyngeal carriage with high heptavalent pneumococcal conjugate vaccine coverage in Central Greece. Vaccine 29 (48):8882-8887.

244. Tocheva AS, Jefferies JM, Rubery H, et al. 2011. Declining serotype coverage of new pneumococcal conjugate vaccines relating to the carriage of Streptococcus pneumoniae in young children. Vaccine 29 (26):4400-4404.

245. Hanage WP, Finkelstein JA, Huang SS, et al. 2010. Evidence that pneumococcal serotype replacement in Massachusetts following conjugate vaccination is now complete. Epidemics 2 (2):80-84.

246. Flasche S, Van Hoek AJ, Sheasby E, et al. 2011. Effect of pneumococcal conjugate vaccination on serotype-specific carriage and invasive disease in England: a cross-sectional study. PLoS Med 8 (4):e1001017.

247. Nunes S, Sa-Leao R, Carrico J, et al. 2005. Trends in drug resistance, serotypes, and molecular types of Streptococcus pneumoniae colonizing preschool-age children attending day care centers in Lisbon, Portugal: a summary of 4 years of annual surveillance. J Clin Microbiol 43 (3):1285-1293.

248. Frazao N, Brito-Avo A, Simas C, et al. 2005. Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of Streptococcus pneumoniae in healthy children attending day-care centers in Lisbon. Pediatr Infect Dis J 24 (3):243-252.

249. Sa-Leao R, Nunes S, Brito-Avo A, et al. 2009. Changes in pneumococcal serotypes and antibiotypes carried by vaccinated and unvaccinated day-care centre attendees in Portugal, a country with widespread use of the seven-valent pneumococcal conjugate vaccine. Clin Microbiol Infect 15 (11):1002-1007.

250. Regev-Yochay G, Trzcinski K, Thompson CM, et al. 2006. Interference between Streptococcus pneumoniae and Staphylococcus aureus: In vitro hydrogen peroxide-mediated killing by Streptococcus pneumoniae. J Bacteriol 188 (13):4996-5001.

251. Pericone CD, Overweg K, Hermans PW, et al. 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract. Infect Immun 68 (7):3990-3997.

252. Cundell DR, Gerard NP, Gerard C, et al. 1995. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. Nature 377 (6548):435-438.
253. Weiser JN, Shchepetov M, Chong ST. 1997. Decoration of lipopolysaccharide with phosphorylcholine: a phase-variable characteristic of Haemophilus influenzae. Infect Immun 65 (3):943-950.
254. Shakhnovich EA, King SJ, Weiser JN. 2002. Neuraminidase expressed by Streptococcus pneumoniae desialylates the lipopolysaccharide of Neisseria meningitidis and Haemophilus influenzae: a paradigm for interbacterial competition among pathogens of the human respiratory tract. Infect Immun 70 (12):7161-7164.
255. Tan TT, Morgelin M, Forsgren A, et al. 2007. Haemophilus influenzae survival during complement-mediated attacks is promoted by Moraxella catarrhalis outer membrane vesicles. J Infect Dis 195 (11):1661-1670.
256. Bosch AA, Biesbroek G, Trzcinski K, et al. 2013. Viral and bacterial interactions in the upper respiratory tract. PLoS Pathog 9 (1):e1003057.
257. Munoz-Elias EJ, Marcano J, Camilli A. 2008. Isolation of Streptococcus pneumoniae biofilm mutants and their characterization during nasopharyngeal colonization. Infect Immun 76 (11):5049-5061.
258. Iwase T, Uehara Y, Shinji H, et al. 2010. Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. Nature 465 (7296):346-349.
259. Jurcisek JA, Bookwalter JE, Baker BD, et al. 2007. The PilA protein of non-typeable Haemophilus influenzae plays a role in biofilm formation, adherence to epithelial cells and colonization of the mammalian upper respiratory tract. Mol Microbiol 65 (5):1288-1299.
260. Armbruster CE, Hong W, Pang B, et al. 2010. Indirect pathogenicity of Haemophilus influenzae and Moraxella catarrhalis in polymicrobial otitis media occurs via interspecies quorum signaling. MBio 1 (3).
261. Allegrucci M, Hu FZ, Shen K, et al. 2006. Phenotypic characterization of Streptococcus pneumoniae biofilm development. J Bacteriol 188 (7):2325-2335.
262. Domenech M, Garcia E, Prieto A, et al. 2013. Insight into the composition of the intercellular matrix of Streptococcus pneumoniae biofilms. Environ Microbiol 15 (2):502-516.
263. Weimer KE, Armbruster CE, Juneau RA, et al. 2010. Coinfection with Haemophilus influenzae promotes pneumococcal biofilm formation during experimental otitis media and impedes the progression of pneumococcal disease. J Infect Dis 202 (7):1068-1075.
264. Weimer KE, Juneau RA, Murrah KA, et al. 2011. Divergent mechanisms for passive pneumococcal resistance to beta-lactam antibiotics in the presence of Haemophilus influenzae. J Infect Dis 203 (4):549-555.
265. Lee GM, Huang SS, Rifas-Shiman SL, et al. 2009. Epidemiology and risk factors for Staphylococcus aureus colonization in children in the post-PCV7 era. BMC Infect Dis 9:110.
266. Dunne EM, Manning J, Russell FM, et al. 2012. Effect of pneumococcal vaccination on nasopharyngeal carriage of Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, and Staphylococcus aureus in Fijian children. J Clin Microbiol 50 (3):1034-1038.
267. Bae S, Yu JY, Lee K, et al. 2012. Nasal colonization by four potential respiratory bacteria in healthy children attending kindergarten or elementary school in Seoul, Korea. J Med Microbiol 61 (Pt 5):678-685.

268. Kwambana BA, Barer MR, Bottomley C, et al. 2011. Early acquisition and high nasopharyngeal co-colonisation by Streptococcus pneumoniae and three respiratory pathogens amongst Gambian new-borns and infants. BMC Infect Dis 11:175.
269. Chien YW, Vidal JE, Grijalva CG, et al. 2013. Density interactions among Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus in the nasopharynx of young Peruvian children. Pediatr Infect Dis J 32 (1):72-77.
270. Xu Q, Almudervar A, Casey JR, et al. 2012. Nasopharyngeal bacterial interactions in children. Emerg Infect Dis 18 (11):1738-1745.
271. Zemlickova H, Urbaskova P, Adamkova V, et al. 2006. Characteristics of Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis and Staphylococcus aureus isolated from the nasopharynx of healthy children attending day-care centres in the Czech Republic. Epidemiol Infect 134 (6):1179-1187.
272. Quintero B, Araque M, van der Gaast-de Jongh C, et al. 2011. Epidemiology of Streptococcus pneumoniae and Staphylococcus aureus colonization in healthy Venezuelan children. Eur J Clin Microbiol Infect Dis 30 (1):7-19.
273. Verhaegh SJ, Snippe ML, Levy F, et al. 2011. Colonization of healthy children by Moraxella catarrhalis is characterized by genotype heterogeneity, virulence gene diversity and co-colonization with Haemophilus influenzae. Microbiology 157 (Pt 1):169-178.
274. van Gils EJ, Hak E, Veenhoven RH, et al. 2011. Effect of seven-valent pneumococcal conjugate vaccine on Staphylococcus aureus colonisation in a randomised controlled trial. PLoS One 6 (6):e20229.
275. Dunne EM, Smith-Vaughan HC, Robins-Browne RM, et al. 2013. Nasopharyngeal microbial interactions in the era of pneumococcal conjugate vaccination. Vaccine 31 (19):2333-2342.
276. Spijkerman J, Prevaes SM, van Gils EJ, et al. 2012. Long-term effects of pneumococcal conjugate vaccine on nasopharyngeal carriage of S. pneumoniae, S. aureus, H. influenzae and M. catarrhalis. PLoS One 7 (6):e39730.
277. van Gils EJ, Veenhoven RH, Rodenburg GD, et al. 2011. Effect of 7-valent pneumococcal conjugate vaccine on nasopharyngeal carriage with Haemophilus influenzae and Moraxella catarrhalis in a randomized controlled trial. Vaccine 29 (44):7595-7598.
278. Kuo CY, Hwang KP, Hsieh YC, et al. 2011. Nasopharyngeal carriage of Streptococcus pneumoniae in Taiwan before and after the introduction of a conjugate vaccine. Vaccine 29 (32):5171-5177.
279. Prymula R, Hanovcova I, Splino M, et al. 2011. Impact of the 10-valent pneumococcal non-typeable Haemophilus influenzae Protein D conjugate vaccine (PHiD-CV) on bacterial nasopharyngeal carriage. Vaccine 29 (10):1959-1967.
280. Ho PL, Chiu SS, Chan MY, et al. 2012. Molecular epidemiology and nasal carriage of Staphylococcus aureus and methicillin-resistant S. aureus among young children attending day care centers and kindergartens in Hong Kong. The Journal of infection 64 (5):500-506.
281. Dukers-Muijrers NH, Stobberingh E, Beisser P, et al. 2012. Nasal carriage of Streptococcus pneumoniae serotypes and Staphylococcus aureus in Streptococcus pneumoniae-vaccinated and non-vaccinated young children. Epidemiol Infect:1-8.
282. McCullers JA. 2006. Insights into the interaction between influenza virus and pneumococcus. Clin Microbiol Rev 19 (3):571-582.

283. Pitkaranta A, Roivainen M, Blomgren K, et al. 2006. Presence of viral and bacterial pathogens in the nasopharynx of otitis-prone children. A prospective study. Int J Pediatr Otorhinolaryngol 70 (4):647-654.
284. Chonmaitree T, Revai K, Grady JJ, et al. 2008. Viral upper respiratory tract infection and otitis media complication in young children. Clin Infect Dis 46 (6):815-823.
285. Revai K, Dobbs LA, Nair S, et al. 2007. Incidence of acute otitis media and sinusitis complicating upper respiratory tract infection: the effect of age. Pediatrics 119 (6):e1408-1412.
286. Klugman KP, Chien YW, Madhi SA. 2009. Pneumococcal pneumonia and influenza: a deadly combination. Vaccine 27 Suppl 3:C9-C14.
287. Hament JM, Aerts PC, Fleer A, et al. 2004. Enhanced adherence of Streptococcus pneumoniae to human epithelial cells infected with respiratory syncytial virus. Pediatr Res 55 (6):972-978.
288. Sajjan US, Jia Y, Newcomb DC, et al. 2006. H. influenzae potentiates airway epithelial cell responses to rhinovirus by increasing ICAM-1 and TLR3 expression. FASEB J 20 (12):2121-2123.
289. Verkaik NJ, Nguyen DT, de Vogel CP, et al. 2011. Streptococcus pneumoniae exposure is associated with human metapneumovirus seroconversion and increased susceptibility to in vitro HMPV infection. Clin Microbiol Infect 17 (12):1840-1844.
290. Madhi SA, Klugman KP. 2004. A role for Streptococcus pneumoniae in virus-associated pneumonia. Nat Med 10 (8):811-813.
291. Vergison A, Dagan R, Arguedas A, et al. 2010. Otitis media and its consequences: beyond the earache. Lancet Infect Dis 10 (3):195-203.
292. Vergison A. 2008. Microbiology of otitis media: a moving target. Vaccine 26 Suppl 7:G5-10.
293. Teele DW, Klein JO, Rosner B. 1989. Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. J Infect Dis 160 (1):83-94.
294. Gehanno P, Lenoir G, Barry B, et al. 1996. Evaluation of nasopharyngeal cultures for bacteriologic assessment of acute otitis media in children. Pediatr Infect Dis J 15 (4):329-332.
295. Faden H, Stanievich J, Brodsky L, et al. 1990. Changes in nasopharyngeal flora during otitis media of childhood. Pediatr Infect Dis J 9 (9):623-626.
296. Groothuis JR, Thompson J, Wright PF. 1986. Correlation of nasopharyngeal and conjunctival cultures with middle ear fluid cultures in otitis media. A prospective study. Clin Pediatr (Phila) 25 (2):85-88.
297. Tonnaer EL, Rijkers GT, Meis JF, et al. 2005. Genetic relatedness between pneumococcal populations originating from the nasopharynx, adenoid, and tympanic cavity of children with otitis media. J Clin Microbiol 43 (7):3140-3144.
298. Faden H, Waz MJ, Bernstein JM, et al. 1991. Nasopharyngeal flora in the first three years of life in normal and otitis-prone children. Ann Otol Rhinol Laryngol 100 (8):612-615.
299. Long SS, Henretig FM, Teter MJ, et al. 1983. Nasopharyngeal flora and acute otitis media. Infect Immun 41 (3):987-991.
300. Smith-Vaughan H, Byun R, Nadkarni M, et al. 2006. Measuring nasal bacterial load and its association with otitis media. BMC Ear Nose Throat Disord 6:10.
301. Brook I, Gober AE. 2000. Reliability of the microbiology of spontaneously draining acute otitis media in children. Pediatr Infect Dis J 19 (6):571-573.

302. Leibovitz E, Serebro M, Givon-Lavi N, et al. 2009. Epidemiologic and microbiologic characteristics of culture-positive spontaneous otorrhea in children with acute otitis media. Pediatr Infect Dis J 28 (5):381-384.
303. Ruohola A, Meurman O, Nikkari S, et al. 2007. The dynamics of bacteria in the middle ear during the course of acute otitis media with tympanostomy tube otorrhea. Pediatr Infect Dis J 26 (10):892-896.
304. Ruohola A, Meurman O, Nikkari S, et al. 2006. Microbiology of acute otitis media in children with tympanostomy tubes: prevalences of bacteria and viruses. Clin Infect Dis 43 (11):1417-1422.
305. Broides A, Dagan R, Greenberg D, et al. 2009. Acute otitis media caused by Moraxella catarrhalis: epidemiologic and clinical characteristics. Clin Infect Dis 49 (11):1641-1647.
306. Dagan R, Leibovitz E, Greenberg D, et al. 2013. Mixed Pneumococcal-Nontypeable Haemophilus influenzae Otitis Media Is a Distinct Clinical Entity With Unique Epidemiologic Characteristics and Pneumococcal Serotype Distribution. J Infect Dis.
307. The NHS Information Centre, Public Health Indicators and Population Statistics Team. NHS Immunisation Statistics England 2009–10 [ic.nhs.uk web site] 2010. Available at: http://www.ic.nhs.uk/webfiles/publications/003HealthLifestyles /immstats0910/Immunisations Bulletin 2009-10.pdf. Accessed 6 December, Access 2010.
308. Gaudelus J, Jean-Verdier B, Cohen R. 2011. Evolution de la couverture vaccinale du vaccine pneumococcique conjugué de 2006 à 2010: analyse des carnets de santé. Méd Enf 31:187-190.
309. Pai R, Gertz RE, Beall B. 2006. Sequential multiplex PCR approach for determining capsular serotypes of Streptococcus pneumoniae isolates. J Clin Microbiol 44 (1):124-131.
310. Park IH, Park S, Hollingshead SK, et al. 2007. Genetic basis for the new pneumococcal serotype, 6C. Infect Immun 75 (9):4482-4489.
311. Ubukata K, Asahi Y, Yamane A, et al. 1996. Combinational Detection of Autolysin and Penicillin-Binding Protein 2B Genes of *Streptococcus pneumoniae* by PCR. J Clin Micro 34 (3):592-596.
312. Simpson EH. 1949. Measurement of diversity. Nature 163:688.
313. Rinta-Kokko H, Dagan R, Givon-Lavi N, et al. 2009. Estimation of vaccine efficacy against acquisition of pneumococcal carriage. Vaccine 27 (29):3831-3837.
314. Sa-Leao R, Simoes AS, Nunes S, et al. 2006. Identification, prevalence and population structure of non-typable Streptococcus pneumoniae in carriage samples isolated from preschoolers attending day-care centres. Microbiology 152 (Pt 2):367-376.
315. Rodrigues F, Foster D, Caramelo F, et al. 2012. Progressive changes in pneumococcal carriage in children attending daycare in Portugal after 6 years of gradual conjugate vaccine introduction show falls in most residual vaccine serotypes but no net replacement or trends in diversity. Vaccine 30 (26):3951-3956.
316. Rodrigues F, Foster D, Nicoli E, et al. 2013. Relationships Between Rhinitis Symptoms, Respiratory Viral Infections and Nasopharyngeal Colonization With Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus in Children Attending Daycare. Pediatr Infect Dis J 32 (3):227-232.
317. Grupo de Estudo de Doença Pneumocócica da Sociedade de Infecciologia Pediátrica/SPP. Dados Nacionais DIP 2010–2012 2012. Available at:

http://www.spp.pt/UserFiles/file/Protocolos/Doenca_Invasiva_Pneumococica_2010_2012_2.pdf. Accessed 16 August, Access 2012.

318. Zhang Q, Pettitt E, Burkinshaw R, et al. 2002. Mucosal immune responses to meningococcal conjugate polysaccharide vaccines in infants. Pediatr Infect Dis J 21 (3):209-213.
319. Klugman KP. 2001. Efficacy of pneumococcal conjugate vaccines and their effect on carriage and antimicrobial resistance. Lancet Infect Dis 1 (2):85-91.
320. Lipsitch M, Whitney CG, Zell E, et al. 2005. Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? PLoS Med 2 (1):e15.
321. Zhang Q, Bernatoniene J, Bagrade L, et al. 2006. Serum and mucosal antibody responses to pneumococcal protein antigens in children: relationships with carriage status. Eur J Immunol 36 (1):46-57.
322. Regev-Yochay G, Bogaert D, Malley R, et al. 2008. Does pneumococcal conjugate vaccine influence Staphylococcus aureus carriage in children? Clin Infect Dis 47 (2):289-291; author reply 291-282.
323. Lysenko ES, Lijek RS, Brown SP, et al. 2010. Within-host competition drives selection for the capsule virulence determinant of Streptococcus pneumoniae. Curr Biol 20 (13):1222-1226.
324. Mackenzie GA, Leach AJ, Carapetis JR, et al. 2010. Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. BMC Infect Dis 10:304.
325. Bernatoniene J, Zhang Q, Dogan S, et al. 2008. Induction of CC and CXC chemokines in human antigen-presenting dendritic cells by the pneumococcal proteins pneumolysin and CbpA, and the role played by toll-like receptor 4, NF-kappaB, and mitogen-activated protein kinases. J Infect Dis 198 (12):1823-1833.
326. Mitchell AM, Mitchell TJ. 2010. Streptococcus pneumoniae: virulence factors and variation. Clin Microbiol Infect 16 (5):411-418.
327. Sulikowska A, Grzesiowski P, Sadowy E, et al. 2004. Characteristics of Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis isolated from the nasopharynges of asymptomatic children and molecular analysis of S. pneumoniae and H. influenzae strain replacement in the nasopharynx. J Clin Microbiol 42 (9):3942-3949.
328. Prenner BM, Chervinsky P, Hampel FC, Jr., et al. 1996. Double-strength beclomethasone dipropionate (84 micrograms/spray) aqueous nasal spray in the treatment of seasonal allergic rhinitis. J Allergy Clin Immunol 98 (2):302-308.
329. Lakshman R, Murdoch C, Race G, et al. 2003. Pneumococcal nasopharyngeal carriage in children following heptavalent pneumococcal conjugate vaccination in infancy. Arch Dis Child 88 (3):211-214.
330. Health Protection Agency Standard Method vSOP25. Real Time Quadriplex PCR for the Detection of Influenza 2006. Available at: http://www.hpa-standardmethods.org.uk/wg_virology.asp. Accessed 25 April, Access 2006.
331. Maertzdorf J, Wang CK, Brown JB, et al. 2004. Real-time reverse transcriptase PCR assay for detection of human metapneumoviruses from all known genetic lineages. J Clin Microbiol 42 (3):981-986.
332. Scheltinga SA, Templeton KE, Beersma MF, et al. 2005. Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR. J Clin Virol 33 (4):306-311.

333. Corless CE, Guiver M, Borrow R, et al. 2002. Development and evaluation of a 'real-time' RT-PCR for the detection of enterovirus and parechovirus RNA in CSF and throat swab samples. J Med Virol 67 (4):555-562.
334. Heim A, Ebnet C, Harste G, et al. 2003. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J Med Virol 70 (2):228-239.
335. Lu X, Chittaganpitch M, Olsen SJ, et al. 2006. Real-time PCR assays for detection of bocavirus in human specimens. J Clin Microbiol 44 (9):3231-3235.
336. Wang WY, Hyang Lim J, Li JD. 2012. Synergistic and feedback signaling mechanisms in the regulation of inflammation in respiratory infections. Cell Mol Immunol.
337. Peltola V, Heikkinen T, Ruuskanen O, et al. 2011. Temporal association between rhinovirus circulation in the community and invasive pneumococcal disease in children. Pediatr Infect Dis J 30 (6):456-461.
338. Veenhoven R, Bogaert D, Uiterwaal C, et al. 2003. Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. Lancet 361 (9376):2189-2195.
339. Mogdasy MC, Camou T, Fajardo C, et al. 1992. Colonizing and invasive strains of Streptococcus pneumoniae in Uruguayan children: type distribution and patterns of antibiotic resistance. Pediatr Infect Dis J 11 (8):648-652.
340. Revai K, Mamidi D, Chonmaitree T. 2008. Association of nasopharyngeal bacterial colonization during upper respiratory tract infection and the development of acute otitis media. Clin Infect Dis 46 (4):e34-37.
341. Dagan R, Patterson S, Juergens C, et al. 2013. Comparative Immunogenicity and Efficacy of 13-Valent and 7-Valent Pneumococcal Conjugate Vaccines in Reducing Nasopharyngeal Colonization: A Randomized Double-Blind Trial. Clin Infect Dis.
342. McCullers JA, McAuley JL, Browall S, et al. 2010. Influenza enhances susceptibility to natural acquisition of and disease due to Streptococcus pneumoniae in ferrets. J Infect Dis 202 (8):1287-1295.