Streptococcus pneumoniae Carriage and Effect of Vaccination on Prevalence, Serotypes and Antibiotic Resistance

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Streptococcus pneumoniae Carriage and Effect of Vaccination on Prevalence, Serotypes and Antibiotic Resistance

Streptococcus pneumoniae kolonisatie en effect
van vaccinatie op prevalentie, serotypen en antibioticaresistentie

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Ter nagedachtenis aan mijn vader…

CONTENTS

1 General introduction, aim and outline of this thesis

Streptococcus pneumoniae, commonly known as pneumococcus, is generally an asymptomatic colonizer of the upper respiratory tract. Pneumococcal colonization is the first step towards infection[1]. *S. pneumoniae c*an induce several infections such as meningitis, pneumonia, sepsis and otitis-media. Understanding pneumococcal carriage is the key to understand and prevent pneumococcal diseases.

History of identification of *S. pneumoniae*

1n 1875, Klebs was the first who described *Streptococcus pneumoniae* as non-motile "monads" when microscopically examining lung fluids from pneumonia patients[2]. Few years later Sternberg and Pasteur inoculated rabbits with saliva from patients with pneumococcal infections and showed the pathogenicity of *S. pneumoniae*[2]. In 1884, Gram described an "indispensable strain" slightly elongated, dark aniline-gentian violet colored (Gram positive) cocci in fluid of lung tissue[3]. In 1886, Fraenkle was the first who presented a complete description of *S. pneumoniae* and its etiological relationship with pneumonia in humans[2]. In 1900, Neufeld discovered that bile dissolves the cell wall of this microorganism. Two years later, he described a specific reaction with pneumococcal anti-sera i.e. a microscopic visible agglutination, the so-called Quellung reaction. This reaction would later be used as a pneumococcal typing method[2]. Thanks to experiments on *S. pneumoniae*, by Griffith in 1928, i.e. the transformation of a non-encapsulated strain in a capsulated one, DNA was identified as a source of genetic information[4]. In 1944, Avery confirmed these findings. In 1943, Mørch reported that low concentrations of optochin could be used to differentiate pneumococcal strains (optochin susceptible) from other streptococci (optochin resistant)[5].

Taxonomy of *S. pneumoniae*.

S. pneumoniae is a Gram positive, catalase negative, facultative anaerobic bacterium that is microscopically typically arranged in pairs. It belongs to the order of Lactobacillus, the family Streptococcacea and genus Streptococcus[6].

S. pneumoniae is a member of the mitis group of streptococci including, *Streptococcus pseudopneumoniae*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus infantis*, *Streptococcus sanguinis*, *Streptococcus gordonii*, *Streptococcus parasanguinis*, *Streptococcus cristatus*, *Streptococcus peroris*, *Streptococcus australis*, and *Streptococcus sinensis*[7]. *S. pneumoniae* is genetically very close to *S. mitis, S. oralis* and *S. pseudopneumoniae* sharing 99% similarity of 16 rRNA sequences with these species[8,9].

From carriage to disease

Pneumococcal colonization is the first step towards dissemination and infection[1]. The human nasopharynx is the predominant ecological niche for *S. pneumoniae*[10]. Colonization rates are associated with age, crowding (day-care centers), smoking, seasonality, recent viral respiratory infection, socioeconomic and environmental factors, and mucosal immunity[1].

Pneumococcal carriage is acquired during the first months of life. In industrialized countries, the highest carriage prevalence (60%-80%) is in children younger than 3 years old (Figure 1). The prevalence decreases during childhood and declines further to 10-20% in adults[1]. Transmission of pneumococci occurs through aerosols and spread of contaminated oral fluids. Pneumococcal carriage can progress to otitis media via local spread from the upper respiratory tract, to pneumonia via aspiration or to invasive pneumococcal disease when pneumococci invade the bloodstream[1] (figure 2).

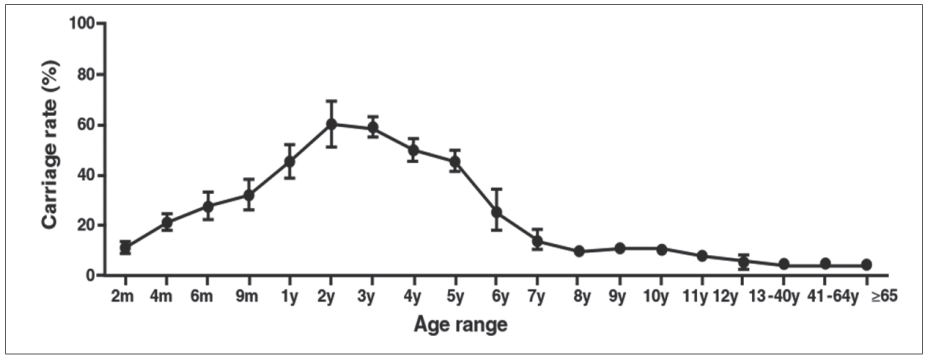


Figure 1: Pneumococcal carriage prevalence by age range in industrialized countries. Figure adapted from Ferreira et al[11]

Detection of *S. pneumoniae,* identification and serotype determination

Detection

To study the carriage rate of *S. pneumoniae* in the upper respiratory tract several sampling methods have been described. Rapola et al. found in Finnish children with acute respiratory infection no significant difference in pneumococcal isolation rate using nasal or nasopharyngeal swabs[13]. Also, Carville et al. found the same pneumococcal prevalence in nasopharyngeal aspirates compared with nasal swabs in 41 Aboriginal and non-Aboriginal children carriers younger than two years[14]. Abdullahi et al., however, obtained in Kenyan children carriers a higher yield using nasopharyngeal washes compared to nasopharyngeal swabs[15]. In adults with a respiratory infection, the detection rates of *S. pneumoniae* using nasopharyngeal swabs, nasopharyngeal or nasal washes were similar[16,17]. To standardize the different sampling methods and to compare the results of the different carriage studies the World Health Organization (WHO) recommends the use of nasopharyngeal swabs for sample collection[18]. However, for large epidemiological studies taking nasopharyngeal swabs is not feasible due to the possible intolerance of this sampling method by the participants[18].

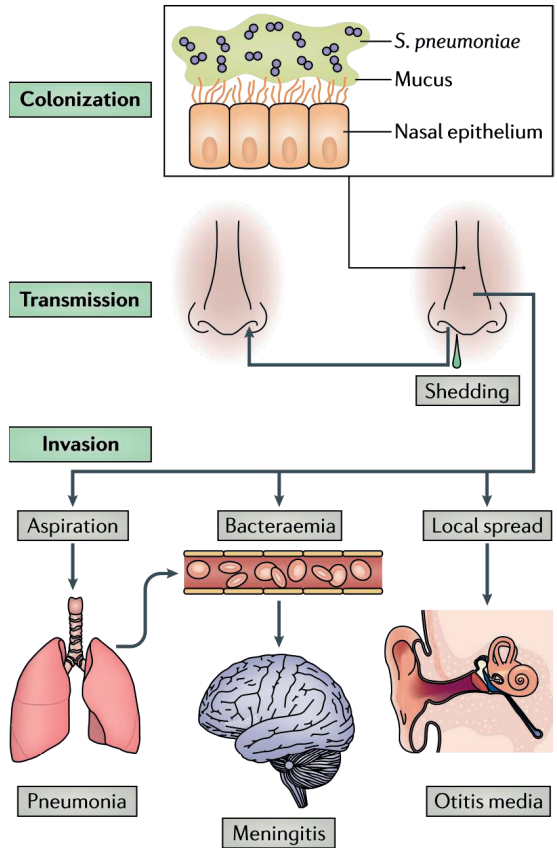


Figure 2: Life cycle of S. pneumoniae and pathogenesis of pneumococcal disease. Figure from Weiser et al[12]

Identification

For the laboratory identification of *S. pneumoniae* and to distinguish this species from other α-hemolytic streptococci conventionally colony morphology (mucoid colonies or colonies with central depression), susceptibility to optochin and solubility in bile salt are used[19]. However, pneumococci with atypical morphology as well resistance to optochin and insolubility in bile salt have been reported, making identification of *S. pneumoniae* and differentiation between α-hemolytic streptococci difficult[8,19]. To overcome this problem, other methods have been developed and implemented such as the Polymerase Chain Reaction (PCR) targeting genes encoding pneumococcal virulence factors, such as *Ply*, *LytA*, *PsaA* and *PBP*. However, PCR did not solve the misidentification issue since also other members of the viridans group harbour genes encoding similar pneumococcal virulence factors[20]. Matrix Assisted Laser Desorption/Ionization Time-of-Flight spectrometry (MALDI TOF MS) emerged as a rapid and cost-effective method for identification of bacteria in routine laboratories[21,22]. Despite the usefulness of this method to accurately identify different bacte-

rial species, especially gram negative rods, it remains not optimal to discriminate between viridans group streptococci[23,24], since these bacteria share more than 99% of nucleotides sequences of the 16S rRNA genes[9]. An easy to perform method to distinguish *S. pneumoniae* from other alfa-hemolytic streptococci is urgently needed.

Serotyping

The conventional method for pneumococcal serotyping is the Quellung reaction. This method uses serotype-specific monoclonal antibodies[25]. When no capsular swelling is visible, the strain is considered as non-typable. Non-typable pneumococci have been associated with infections such as conjunctivitis, acute otitis media and acute exacerbations in patients with chronic obstructive pulmonary disease[26-28]. Major drawbacks of serotyping are that the method is time consuming, the interpretation subjective and the antisera expensive[29,30]. To solve this problem a sequence based serotyping method, Capsular Sequence Typing (CST), has been developed. This method is based on the amplification and sequencing of a fragment of the capsular gene *wzh*, a regulatory gene required in the capsule biosynthesis and conserved among different serotypes. After amplification of this gene using different sets of primers, sequencing allows the assessment of various serotypes[31]. This method has been shown to be highly useful for serotyping of pneumococcal strains[31].

Pneumococcal molecular typing:

In the last decades, molecular typing became an important element of bacterial epidemiolocal studies. Pneumococcal molecular typing allowed to have a better insight into clonal spread during epidemics, infections pathways and transmission, and populations changes after vaccines implementation[32]. Different molecular typing methods were developed over the years[33]. Next to PCR based typing methods targeting a single genetic region such as virulence factors or antibiotic resistance genes, other DNA fragment-based typing methods such as Pulsed Field Gel Electrophoresis (PFGE)[34], Random amplified polymorphic DNA (RAPD)[35] and Amplification Fragment Length Polymorphism (AFLP)[36] were in use. In PFGE, genomic DNA is digested with restriction enzymes yielding a limited and separable umber of high molecular weight DNA fragments. These fragments are separated in an electric field. The patterns obtained from different isolates are compared[34]. RAPD assays are based on the use of short random sequence primers, 9 to 10 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures. The annealing of the RAPD primers within a few kilobases of each other results in a PCR product with a molecular length corresponding to the distance between the two primers. The separation of the amplification products by agarose gel electrophoresis yields a pattern of bands which is characteristic of the particular bacterial strain[37]. AFLP is based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digestion. Bacterial DNA is digested with two different enzymes. The restriction fragments

are then ligated to linkers containing each restriction site and a sequence homologous to a PCR primer binding site[38,39]. The selective nucleotides present in the PCR primers allow an amplification of only a subset of the genomic restriction fragments. the electrophoretic separation of amplification products yields a characteristic pattern of the particular bacterial strain[35].

Since the implementation of genome sequencing, other DNA sequences-based methods appeared, providing new methods like Multilocus Sequence Typing (MLST)[40]. MLST is based on the DNA sequencing of multiple housekeeping genes to uncover allelic variants in conserved genes of bacterial species. Based on differences in sequences of each gene, distinct alleles are assigned. A sequence type (ST) is assigned to each isolate according the combination of alleles at each locus (the allelic profile). The progress in technology from Sanger sequencing to next-generation sequencing (NGS) has revolutionized bacterial typing. The broad use of whole genome sequencing due to technological progress and decease of costs, allowed the improvement of existing method such as MLST and the development of core genome MLST (cgMLST)[41]. Core genome MLST (cgMLST) combines the discriminatory power of classical MLST with the extensive genetic data derived from WGS[42,43]. One of the advantages of cgMLST compared to the conventional MLST is the exploitation of hundreds of gene targets of the entire bacterial genome, increasing the resolution of the analyses[44-46].

Pneumococcal virulence

The conversion of colonization to invasive disease requires an inflammatory cascade that changes the type and number of target receptors facilitating, bacterial penetration of the host tissue and resulting in progression to localized or invasive diseases. Specific pneumococcal strains have the capacity to cause invasive disease due to the presence of several virulence factors[1,12], of which the most important one is the polysaccharide capsule[10,47]. This capsule inhibits the phagocytosis of *S. pneumoniae* by macrophages by interacting with the phagocytic cell receptors[48,49]. The high heterogenicity of the pneumococcal capsular polysaccharides resulted in the use of capsular types for the typing of pneumococci. To date, more than 93 different capsular types (serotypes) are identified[50]. The serotypes play a role in the ability of *S. pneumoniae* to resist phagocytosis and to trigger the humoral immune response[10]. The polysaccharide capsule prevents the Fc region of IgG and the complement component iC3b on the bacterial cell from interacting with their receptors on the phagocytic cells[51].

Other pneumococcal virulence factors are Pneumolysin (Ply), Choline-Binding Proteins (CBP), divalent metal-ion-binding proteins, LPXTG-anchored proteins (leucine-proline-unknown-threonine-glycine motif) and pili.

Pneumolysin plays a role in invasive infections such as meningitis, pneumonia and bacteremia, by its ability to activate complement and its lytic activity[52,53]. CBP's include Pneumococcal Surface Protein A (PspA), Pneumococcal Surface Protein C (PspC) and Autolysin

A (LytA). PspA inhibits the complement-binding mediated opsonization[54]. PspC binds the polymeric immunoglobulin receptor responsible for transporting IgA. It also inhibits opsonization by inhibiting C3b formation through the alternative complement pathway[53]. LytA plays a role in releasing other virulence factors such as pneumolysin, inflammatory peptidoglycans and teichoic acids from lysed bacterial cell[55] . Divalent metal-ion-binding proteins include, among others, pneumococcal surface antigen A (PsaA), pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA). PsaA inhibits the complement-binding mediated opsonization[54]. PiaA and PiuA are involved in the iron uptake and play a role in development of pneumonia and bacteremia[56]. Several pneumococcal proteins are anchored by an LPXTG motif, including hyaluronidase and neuraminidase[47]. Hyaluronidase degrades the hyaluronic acid of the extracellular matrix acid, allowing the colonization and spread of bacteria[57]. Neuraminidase cleaves the N-acetylneuraminic acid from glycolipids, lipoproteins and oligosaccharides on the cell surfaces[58]. It plays a role in colonization and development of otitis media[59]. Up to twenty pneumococcal proteins, such as neuraminidase, using the mechanism of N-acetylneuraminic acid cleavage are known[60-62]. Pili mediate the adherence of streptococci to the respiratory epithelial cell and stimulate the production of cytokines[63,64].

Pneumococcal carriership

Although the primary aim of vaccination is to prevent the burden of pneumococcal infections, vaccination also influences pneumococcal colonization[12]. The decrease of colonization may lead to a decrease of pneumococcal horizontal spread[1]. In addition, vaccination may reduce pneumococcal disease in non-vaccinated individuals secondary to increased herd immunity[65]. However, a negative effect of vaccination is the selection of non-vaccine serotypes in carriage and IPD[66]. In addition to its role in bacterial infections, pneumococcal carriage is a potential reservoir of antibiotic resistance determinants[67]. Monitoring antibiotic resistance rates in the community is important to develop guidelines and strategies to control antibiotic resistance. Furthermore, studying pneumococcal carriage allows to study the effect of vaccination on serotype distribution in the community and to identify the emergence of non-vaccine serotypes. The identification of the serotypes in pneumococcal carriage is the first step in developing new vaccines or modifying existing vaccines. Finally, studies on pneumococcal carriage will improve insight in the interactions between different species next to *S. pneumoniae* in the upper respiratory tract such as *Staphylococcus aureus*, *Hemophilus influenzae* and *Moraxella catarrhalis* and the effect of these interactions on pneumococcal invasiveness and antibiotic resistance.

Pneumococcal vaccination

To date two vaccine types are available worldwide: a pneumococcal polysaccharide vaccine (PPV 23) and a pneumococcal conjugate vaccine (PCV 7, 10 and 13). The major difference

between these two types is that in PPV plain polysaccharide is used, whereas in PCV each polysaccharide is conjugated to a protein carrier. Conjugation of the polysaccharides with a carrier protein induce a better immune response due to the involvement of T-cell mediated pathway[68]. Since 1983, the 23-valent PPV is available which includes the serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. This vaccine is T-cell independent and therefore not immunogenic in children under the age of 2 years and only recommended for use in elderly and children older than 2 years[69-71]. In 2001, PCVs were first introduced in Europe. The first PCV was the 7-valent vaccine PCV7 (*Prevenar, Pfizer)* containing the serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. Other PCVs are since then been introduced. In 2008 PCV10 (Synflorix, *GlaxoSmithKline*) was licensed in Canada, Europe and Australia, followed in 2010 by PCV13. PCV10 is now the most widely used PCV. It contains three additional serotypes (1, 5 and 7A) compared to PCV7. PCV13 (Prevnar, Pfizer) includes three additional serotypes (3, 6B and 19F) compared to PCV10. Vaccination with PCVs not only results in protection of the immunized persons but also of non-immunized individuals when herd immunity is high enough. In 2017, the worldwide pneumococcal vaccination coverage rate in children of one year of age who have received three doses of a PCV, was estimated to be around 44%[72]. This coverage percentage is below the cut off, estimated at two third of children younger than 5 years, sufficient to induce herd immunity[73]. In Europe, this rate was as high as 70% in the same year[72].

Since 2000, many countries have included pneumococcal vaccination in their national immunization program (NIP). NIP schedules vary widely between countries with respect to modality of implementation (mandatory, recommended or risk-based vaccination), vaccine type, doses, vaccination age, and the presence or absence of a catch-up campaign for older children. Next to the NIP, pneumococcal vaccination is recommended in some countries in elderly persons or individuals who belong to specific risk groups (table 1)[74].

The introduction of PCVs in the NIP was associated with a decrease in carriage and related IPD as well as in antibiotic resistance of vaccine serotypes but resulted in an increase in prevalence of infections of antibiotic resistant non-vaccine serotypes due to serotypes replacement[75]. Post PCV introduction studies such as those of PCV7, reported the efficiency of these vaccines to reduce the prevalence of pneumococcal carriage and invasive pneumococcal diseases[76-79].

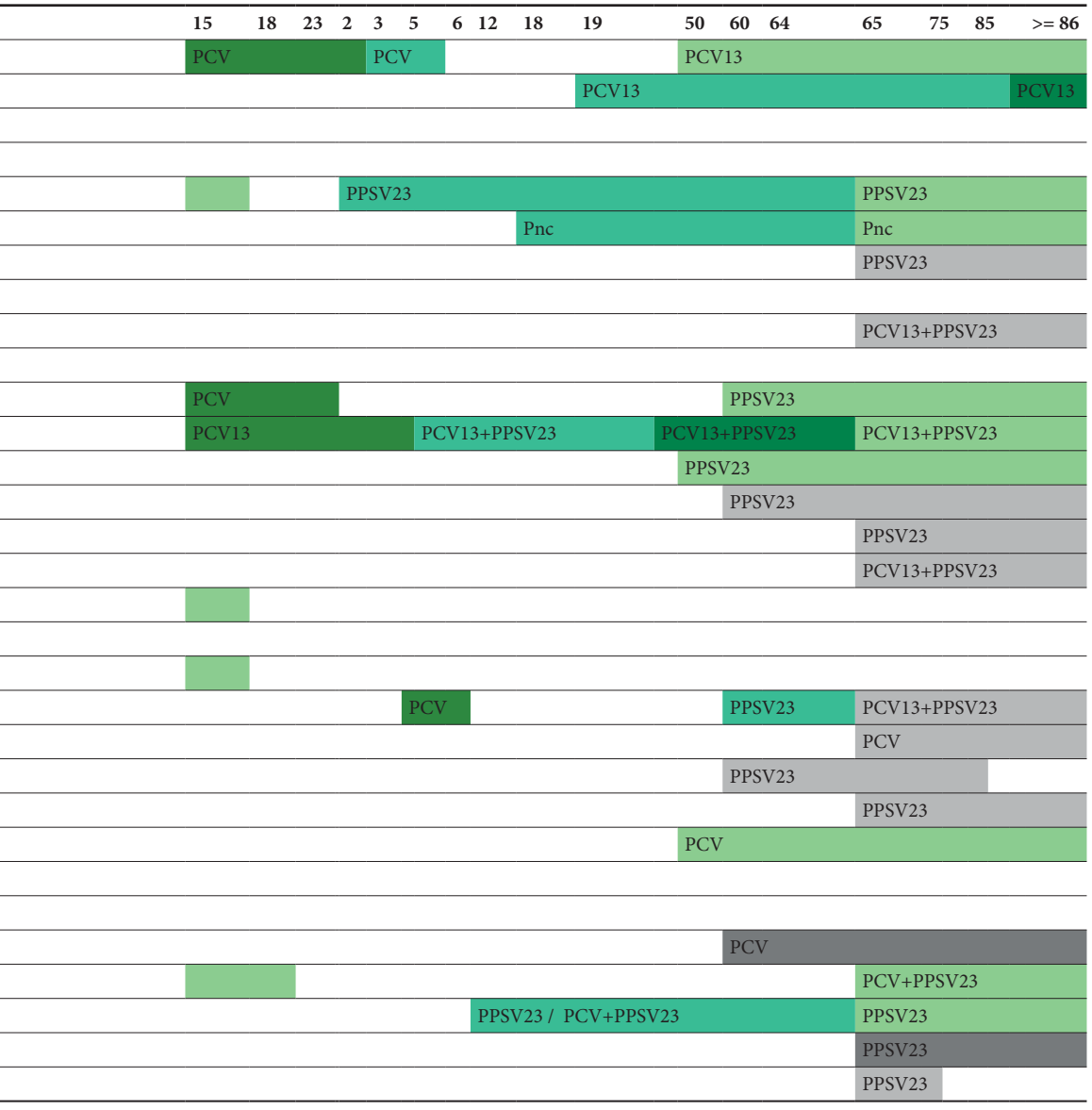
Antibiotic resistance

In 1977, the first report of an antibiotic resistant epidemic pneumococcal strain came from South Africa. The strains were penicillin non-susceptible (MIC 2-8 µg/ml), and resistant to macrolides, chloramphenicol and tetracycline[80]. The rising antibiotic resistance is mainly due to the selective pressure associated with the widespread use of antibiotics[81,82]. Worldwide, 80%-90% of the antibiotics used in humans are prescribed in general practice patients. The levels of antibiotic consumption are significantly associated with antibiotic resistance[83].

Table 1: Overview of recommended pneumococcal vaccination in European countries (in 2018), adapted from ECDC data[74]

	Months									
	2	3	4	5	6	10	11	12	13	14
Austria		PCV	PCV		PCV			PCV		
Belgium	PCV		PCV					PCV		
Bulgaria	PCV	PCV	PCV					PCV		
Croatia	PCV		PCV					PCV		
Cyprus	PCV		PCV					PCV		
Czech Republic		PCV		PCV			PCV			
Denmark		PCV13		PCV13				PCV13		
Estonia										
Finland		PCV10		PCV10				PCV10		
France	PCV		PCV				PCV			
Germany	PCV		PCV				PCV			
Greece	PCV13		PCV13		PCV13			PCV13		
Hungary	PCV13		PCV13					PCV13		
Iceland		PCV10		PCV10				PCV10		
Ireland	PCV				PCV				PCV	
Italy		PCV		PCV			PCV			
Latvia	PCV		PCV					PCV		
Liechtenstein	PCV13		PCV13					PCV13		
Lithuania	PCV		PCV					PCV		
Luxembourg	PCV		PCV					PCV		
Malta										
Netherlands		PCV		PCV			PCV			
Norway		PCV13		PCV13			PCV13			
Poland	PCV		PCV						PCV	
Portugal	PCV13		PCV13					PCV13		
Romania	PCV		PCV				PCV			
Slovakia	PCV		PCV			PCV				
Slovenia		PCV		PCV			PCV			
Spain	PCV		PCV				PCV			
Sweden		PCV		PCV				PCV		
United Kingdom		PCV13						PCV13		

General recommendation
Catch-up (e.g. if previous doses missed)
Recommendation for specific groups only


15 18 23 2 3 5 6 12 18 19 50 60 64 65 75 85 >= 86
PCV
PCV
PCV13
PCV13
PCV13
PPSV23
PPSV23
Pnc
Pnc
PPSV23
PCV13+PPSV23
PCV
PPSV23
PCV13
PCV13+PPSV23
PCV13+PPSV23
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Several mechanisms of antibiotic resistance have been described[84]. Acquisition of foreign genetic material (plasmids/ transposons) encoding for resistance is the most prevalent one. Tetracycline resistance is conferred by the acquisition of *letM* or *letO* genes, genes that are associated with the highly mobile conjugative transposons Tn916-TN1545 and transposons Tn5253 and Tn3872[85]. *letM* and *letO* encodes *LetM* and *LetO* proteins responsible for the protection of the bacterial 30S ribosomal subunit against antibiotic binding[86]. Resistance to beta-lactam agents is due to structural changes of one or more Penicillin-Binding Proteins (PBP's), key proteins in the cell wall synthesis[87]. Resistance to quinolones is mostly due to point mutations in the bacterial genome i.e. in the subunit of DNA topoisomerase IV and DNA gyrase[88]. This mutation has been reported to confer simultaneous resistance to macrolides and chloramphenicol [89]. The most frequent mechanism for macrolide resistance especially in Europe is modification of the target site mediated by the demethylation of the adenine residue at position 2058 on the 25S rRNA by a *ermB* gene encoded methylase[90]. Another macrolide resistance mechanism is mediated by the acquisition of *mef* gene[91,92].

Multi drug resistant pneumococci (MDR), defined as resistant to three or more different antibiotic classes, were first identified in children. MDR strains are predominantly associated with pediatric serotypes i.e. 6A, 6B, 9V, 14, 19A, 19F and 23F all of which except serotype 6A are included in PCV7[93-95]. The introduction of molecular typing methods by late 1990's, revealed that the worldwide increase of MDR strains was linked to the spread of a small number of MDR clones[96,97].

According to the European Antimicrobial Resistance Surveillance Network (EARS-Net) report of 2018, the prevalence of penicillin non-susceptible *S. pneumoniae* (PNSP) was below 10% in most northern European countries and up to 40% in eastern and southern Europe. Similarly, a high variability in the prevalence of macrolide resistance was observed ranging between 2.5% in Denmark to 32.3% in Romania[98] (figure 3). In the last few years, a significant increase in the prevalence of PNSP was noted in the Netherlands and France (1.8% in 2015 to 3.2% in 2018 and 22.9% to 29.1%, respectively)[98].

The effectiveness of antibiotics to treat infections decreased in the past years resulting among others in increased morbidity and mortality in patients with resistant bacterial infections and increasing costs of treatment[99]. To keep antibiotics effective both in hospitals and in the community monitoring of antibiotic use and resistance rate is needed. This includes monitoring the (appropriateness of) antibiotic prescription by collecting data concerning antibiotic use[100,101], implementation of an antibiotic stewardship program and infection prevention and control guidelines and measure the effect of interventions[102]. In addition, education of healthcare professionals and the public explaining the consequences of inappropriate prescription and use of antibiotics is important[103] as is promoting vaccination[99]. Finally, rapid diagnostic tests to early detect antibiotic resistant strains will contribute to a reduction of unnecessary broad-spectrum antibiotic to the expense of narrow spectrum agents[104-106]. In the Netherlands, a national surveillance system ISIS-AR (Infectieziekten

Surveillance Informatie Systeem-Antibiotica Resistentie) is in place to collect and to publish the data on antibiotic use and resistance yearly in Nethmap (https://swab.nl/nl/nethmap).

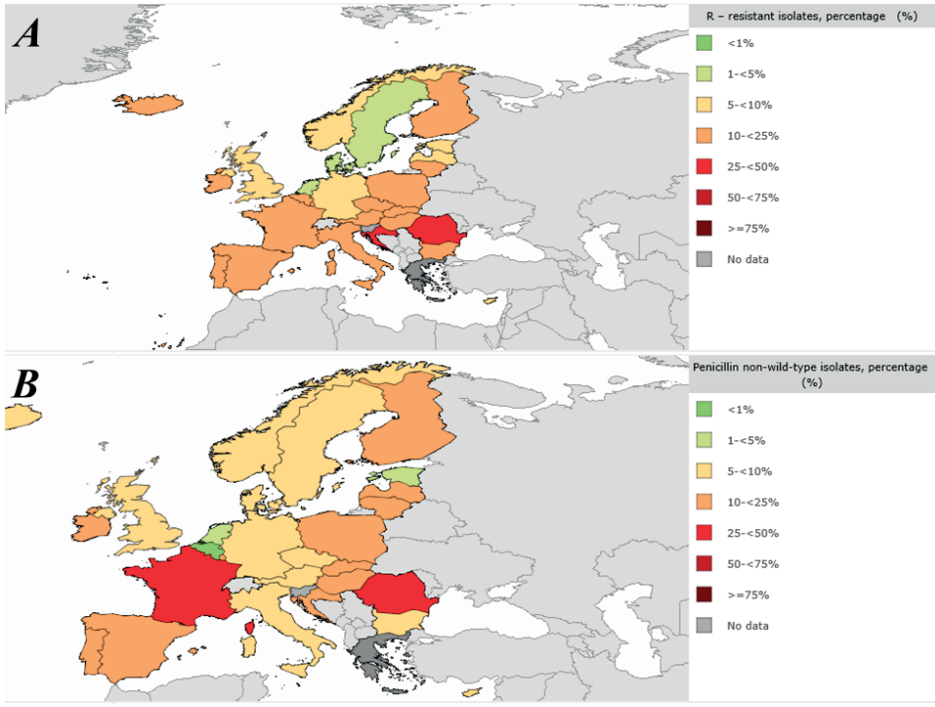

Figure 3: Proportion of non-susceptibility for macrolides (A) and penicillin (B) in invasive pneumococcal isolates in Europe in 2018[98].

Effect of vaccination on *S. pneumoniae* serotypes causing disease

Four years after the introduction of PCV7 in the United States a significant increase of invasive pneumococcal disease caused by serotype 19A (not included in PCV7) was shown[75]. Data from 2007 and 2008 (prior to the implementation of PCV13) showed that 78% to 97% of PNSP related IPD was caused by PCV13 serotypes but not PCV7[107]. In Portugal, introduction of PCV7 into a day care center have shown an emergence of antibiotic resistant non-vaccine serotypes. No significant difference in antibiotic resistance was found between children attending that day care center and unvaccinated children in a control day care center[108]. In Spain, the proportion of non-PCV7 serotypes among PNSP related IPD increased between 2001 and 2008, the period after the introduction of PCV7 and prior to the implementation of PCV13, from 12% to 49.5%[109]. The introduction of PCV was associated with a decrease in carriage and related IPD as well as in antibiotic resistance of vaccine serotypes but resulted in an increase in prevalence of infections and antibiotic resistance of non-vaccine serotypes[75].

As shown in table 1, a large variation in vaccination strategies, including, vaccine type, implementations of a catch-up schedule, used vaccine types, years of inclusion of the pneumococcal vaccination in national immunization programs, exists between the European countries. Studies after the introduction of PCVs reported the efficiency of these vaccines to reduce pneumococcal carriage and to prevent invasive pneumococcal diseases by vaccine serotypes[110]. PCVs confer direct protection by immunization of vaccinated persons and indirectly to non-vaccinated ones by herd immunity[110-112]. However, non-PCV serotypes have emerged among asymptomatic carriers resulting in a global increase of invasive pneumococcal disease (IPD) due to these serotypes[113,114]. Few years after the introduction of PCV13, emergence of serotypes 23B and 15A (not included in the vaccine) was reported, suggesting that serotype replacement is ongoing[115]. In Europe, the prevalence of non-vaccine serotypes is diverse for different reasons[116]. Reports suggest that the vaccines implemented may affect non-vaccine types in IPD[117,118]. Monitoring the circulating non-vaccine serotypes is an important step toward a formulation of new vaccines.

Interspecies interactions and effect of vaccination

S. pneumoniae belongs next to *Staphylococcus aureus*, *Moraxella catarrhalis* and *Hemophilus influenzae* to the normal microbiota of the upper respiratory tract[67]. Pneumococci have the genetic properties to coexist, to act synergistically with other bacteria such as M. *catarrhalis* and *H. influenzae*, and to inhibit the growth of other bacteria such as *S. aureus.* Possible mechanisms playing a role in the growth inhibition of *S. aureus* by *S. pneumoniae* are production of hydrogen peroxide of *S. pneumoniae*[119-121], and the presence of pili of *S. pneumoniae,* which mediates an immune response and the CD4+ T-cell mediated bacterial cross reactivity[121,122].

In addition to the direct interaction between *S. pneumoniae* and other microbes of the upper respiratory tract, several studies described an increase in *S. aureus* carriage and infections in children after pneumococcal vaccination[78,123]. However, this effect was only of short duration after implementation of PCV vaccine[120,124]. It is unclear whether the effect depends on the type of vaccine used and what the effect is on the other microbiota.

Outlines of this thesis:

Streptococcus pneumoniae is an important inhabitant of the microbiome of the upper respiratory tract. The microbiome is also an important source of antibiotic resistant genes. Colonization of *S. pneumoniae* in the upper respiratory tract is the first step towards dissemination of the microbe to develop an infection. The presence of antibiotic resistance genes in the microbiome can spread to susceptible inhabitants of the microbiome and contribute to the development of antibiotic resistant infections.

Knowledge concerning the serotypes and the antibiotic susceptibility of *S. pneumoniae* in the upper respiratory tract form the base for the choice of the vaccines used in the

National immunisation program (NIP) and for the empiric therapy of respiratory tract infections. As there is great variation in the NIP in the different European countries as well as in antibiotic use, variation in antibiotic resistance is to be expected. Apart from the serotypes and the antibiotic resistance, an important first step is the correct identification of the *S. pneumoniae*.

The objectives of this thesis are to determine the prevalence of pneumococcal carriage and antibiotic resistance, and the distribution of pneumococcal serotypes in healthy individuals older than 4 years in Europe in relation to the vaccination policy.

Pneumococcal identification remains one of the drawbacks in studying *S. pneumoniae* due to the lack of sensitivity and specificity of the most current methods. **Part I** focused on identification of the *S. pneumoniae*. **Chapter 2** describes different identification methods and algorithms used in routine microbiological laboratories including optochin susceptibility and bile solubility, an algorithm defined by the World Health Organization (WHO) and a PCR targeting the virulence factor, CpsA. A signal sequence signature was used as a "reference method". In **chapter 3**, the MALDI-TOF performance was compared with the "gold standard", using the identification database used routinely. Spectral analysis was also performed in order to find specific spectral peaks of *S. pneumoniae* and to improve the identification performance of this method. Colonization is the first step towards pneumococcal infection. In addition, colonizing *S. pneumoniae* represents a potential niche of antibiotic resistance determinants.

In **Part II**, the prevalence of pneumococcal carriage and antibiotic resistance in outpatients was studied (**Chapter 4**). The introduction of pneumococcal vaccines has led to a decrease of vaccine types' prevalence. However, non-vaccine serotypes emerged as a cause of colonization as well as infections. Some serotypes were linked to antibiotic resistance. In **chapter 5**, the relationship between serotypes and antibiotic resistance was discuss in relation to different National Immunization Programs (NIP) in the participating countries including vaccine type, vaccination schedule, year of vaccine introduction and catch-up campaign. The upper respiratory tract is colonized by different microorganisms. In **Part III**, the co-colonization of *S. pneumoniae* and *S. aureus* in the upper respiratory tract in relation to serotypes of *S. pneumoniae* was highlighted (**Chaper 6**). Finally, in **Chapter 7** a summary of the results presented in this thesis are discussed and recommendations concerning future research are given.

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2 Evaluation of phenotypic and molecular methods for identification of *Streptococcus pneumoniae*

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AIM

Streptococcus pneumoniae is a major respiratory pathogen. It can colonize the human nasopharynx[1-4], causing various infections such as otitis media, sinusitis, pneumoniae, septicemia and meningitis[5]. *S. pneumoniae* is a member of *Streptococcus mitis* group, which further includes *S. mitis*, *S. oralis*, *S. pseudopneumoniae*, *S. sanguins*, *S. parasanguinis*, *S. gordonii*, *S. austalis*, *S. oligofermantans*, *S. cristatus*, *S. infantis*, *S. sinensis* and *S. peroris*[6,7]. Accurate laboratory tests to differentiate *S. pneumoniae* from other *S. mitis* group streptococci, therefore, remain of crucial importance. Several tests are currently used for the identification of *S. pneumoniae* based on phenotypic or genotypic characteristics, but these tests remain unable to correctly identify this microorganism[8]. The most common tests to identify *S. pneumoniae* are colony morphology, optochin susceptibility and bile solubility. The diagnostic procedure of *S. pneumoniae* generally begins with recognizing typical colonies with a central depression or mucoid with α-hemolysis. Nevertheless, positive optochin susceptibility and bile solubility test results can be positive in *S. pneumoniae* with atypical morphology[9], leading to misidentification of bacteria. Until now, optochin susceptibility has been considered as a reliable method to distinguish *S. pneumoniae* from other members of the mitis group[10]. However, many reports have shown that this test often yields an ambiguous interpretation[10] and the results depend on the inoculum size of the bacteria used[11]. In recent years, several studies have reported the emergence of optochin-resistant *S. pneumoniae*[12] and optochin susceptibility of some genetically related mitis group members such as *S. pseudopneumoniae*[8].

In the last decade, molecular-based tests were developed as an alternative to overcome the low sensitivity of conventional tests. Several polymerase chain reaction (PCR)-based tests became available using specific primer-targeting genes that encode for different pneumococcal virulence factors, such as *lytA*, *ply*, *PsaA*[13-17], or repetitive genomic regions and rRNA[18,19]. Furthermore, these tests can cross-react with genetically related streptococci, such as *S. mitis* and *S. pseudopneumoniae*[8,14,20]. In this study, we compared three conventional tests used in routine microbiology (colony morphology, optochin susceptibility and bile solubility), one molecular-based method targeting a virulence factor (*CpsA*) and an identification algorithm (WHO/CDC algorithm). We then assessed the sensitivity and specificity of each method in order to identify the most accurate method to identify *S. pneumoniae*. For this aim, strains isolated as part of the APRES study, an EU-funded study among general practitioners in nine European countries, were used[21].

MATERIALS & METHODS

Bacterial strains

Bacterial strains were isolated from nasal swabs taken by general practitioner (GP) patients in the period between November 2010 and August 2011 in nine European countries, in other words, Austria, Belgium, Croatia, France, Hungary, Spain, Sweden, The Netherlands and the UK as described by van Bijnen *et al.*[21]. After collection, swabs were sent to a national laboratory within 48 h, except for France. Swabs collected in this country were sent to our laboratory. Isolation and identification were carried out using standardized protocols[21] and included inoculation on blood agar plates without and with gentamicin (Mediaproducts BV, Groningen, Netherlands). After identification, using optochin susceptibility test on small α-hemolytic colonies[21], the isolates were kept frozen in skimmed milk and sent batchwise to our laboratory.

Colony morphology

Colony morphology was examined after overnight incubation in 5% CO2 on 5% sheep blood agar (Oxoïd, The Netherlands). Three categories were defined: button (colonies showed a central depression), mucoid or other (colonies with α-hemolysis, other than button or mucoid)[9].

Optochin susceptibility

Optochin susceptibility was performed in CO2 atmosphere for all isolates. Strains were inoculated on 5% sheep blood agar and incubated at 37°C in 5% CO2. This test was performed by using 5 μg optochin disks (Oxoïd, The Netherlands). The optochin disc was placed on the first streak of the wire loop on the agar plate as described by Facklam[22]. After 18–24 h, the zone of inhibition in millimetres was measured. An inhibition zone less than 14 mm was considered as indicative of optochin resistance[22].

Bile solubility

Bile solubility was tested qualitatively. A drop (40 μl) of 2% sodium deoxycholate (Merck, Germany) was put on 18–24 h colonies on sheep blood agar. After incubation for 30 min at 37°C, disappearance or flattening of colonies was interpreted as a positive test result[23].

PCR targeting *CpsA* gene (*CpsA* PCR)

The *CpsA* PCR was performed as described by Park et al.[24]. DNA amplification was achieved with an initial cycle of 10 s at 95°C followed by 45 cycles of 15 s at 95°C of denaturation and 1 min at 60°C of annealing and elongation using 7900 Real-Time system (Applied Biosystems, The Netherlands). Data were analyzed by using SDS software, v1.4 (Applied Biosystems, The Netherlands).

Gold standard as described by Scholz *et al.*

The analysis of *S. pneumoniae* specific sequence signature was used as 'gold standard'. This method was performed as described by Scholz *et al.*[25]. Briefly, a 286-bp fragment of the 16S rRNA was amplified from α-hemolytic isolates.

PCR-positive isolates were digested and analysed by agarose gel electrophoresis. Due to practical constraints, this test was performed on a selection of isolates. The selection was made as follows: the collection of isolates was grouped into 22 subgroups based on the results of morphology, optochin susceptibility, bile solubility and *CpsA* PCR. When a subgroup included more than 25 isolates, 3 isolates per subgroup, per country were tested. If the subgroup contained less than 25 isolates, all isolates were tested (Table 1). In total, 259 isolates were tested for the *S. pneumoniae*-specific sequence signature. The obtained results were extrapolated to the entire collection (Table 1).

Table 1. Overview of morphology, optochin susceptibility, bile solubility and *CpsA* polymerase chain reaction results and the number of isolates used for the gold standard performance.

Morphology	Optochin	Bile solubility	*CpsA* polymerase chain reaction	Total number of isolates n = 1339	Isolates for sequence signature n = 259	Sequence signature results (positive/negative) n = 259
Atypical	Resistant	Negative	Negative	207	20	(0/20)
			Positive	1	1	(1/0)
		Positive	Negative	11	11	(7/4)
			Positive	16	16	(16/0)
		Ambiguous	Negative	1	1	(0/1)
	Sensitive	Negative	Negative	116	25	(25/0)
			Positive	5	5	(2/3)
		Positive	Negative	27	25	(20/5)
			Positive	81	12	(12/0)
		Ambiguous	Negative	1	1	(0/1)
Button	Resistant	Negative	Negative	26	16	(0/16)
			Positive	1	1	(0/1)
		Positive	Negative	1	0	(0/0)
			Positive	136	24	(24/0)
	Sensitive	Negative	Negative	11	8	(0/8)
			Positive	8	8	(8/0)
		Positive	Negative	18	18	(17/1)
			Positive	593	28	(28/0)
Mucoid	Resistant	Negative	Negative	3	2	(0/2)
		Positive	Positive	12	12	(12/0)
	Sensitive	Positive	Positive	63	24	(24/0)
		Ambiguous	Negative	1	1	(1/0)

WHO/CDC algorithm

Results were interpreted by using the WHO/CDC algorithm described by Centers for Disease Control and Prevention (National Center for Infectious Diseases and WHO [CDC/WHO])[22]. According to the CDC/WHO, α-hemolytic isolates are considered as *S. pneumoniae* if they are optochin susceptible (optochin ≥14 mm) or if they are optochin resistant (9 mm ≤ optochin ≤ 13 mm) and are bile soluble. Isolates with optochin susceptibility less than 9 mm are always considered as not *S. pneumoniae*.

Evaluation of the identification methods

Sensitivity and specificity of colony morphology, optochin susceptibility, *CpsA* PCR and the WHO/CDC algorithm were determined by using the online tool medcalc[26]. The sensitivity of a method was calculated as the proportion of true-positive results among the total number of positive results and specificity as the proportion of the true-negative results among the total negative results. To compare the performance of these methods, p-value was calculated by using the online tool epitools[27]. p-value <0.05 was considered significant.

RESULTS

We received 1371 isolates as putative *S. pneumoniae* (α-hemolytic isolates). After subculture, 12 isolates were not α-hemolytic, and 20 isolates did not show any growth. In total, 1339 morphologically different α-hemolytic colonies were included for analysis.

Morphology, optochin, bile solubility & *CpsA* PCR test results

Of the 1339 isolates, 59.3% (n = 794) showed colonies with central depression (button), 5.9% (n = 79) were mucoid and 34.8% (n = 466) had an atypical morphology (not central depression or mucoid). Overall, 31.0% (n = 415) of all isolates were optochin resistant (optochin <14 mm), and 69.0% (n = 924) were optochin susceptible. The results of bile solubility test showed 71.5% (n = 958) bile soluble and 28.2% (n = 378) bile insoluble isolates. 0.2% (n = 3) yielded ambiguous results. When regarding *CpsA* PCR, 68.4% (n = 916) had positive and 31.6% (n = 423) had negative results (Table 1).

Gold standard

Using the gold standard, 9 of the 22 selection subgroups (comprising 911 isolates, 68.0%) were *S. pneumoniae*. Three subgroups, containing 0.3% (n = 4), contained no *S. pneumoniae* α-hemolytic isolates. Ten subgroups, representing 31.6% of the isolates (n = 423), included both *S. pneumoniae* and no *S. pneumoniae* α-hemolytic isolates (Table 1). In total, 72.2% of all isolates were identified as *S. pneumoniae* (n = 967).

WHO/CDC algorithm

According to the WHO/CDC algorithm, 1068 (79.8%) isolates were identified as *S. pneumoniae* and 271 (20.2%) were not *S. pneumoniae*.

Sensitivity & specificity assessment & comparison of identification methods

Table 2 and 3 show the sensitivity and specificity of morphology, optochin, bile solubility, *CpsA* PCR tests and the WHO/CDC algorithm. With a sensitivity of 97.9% (95% CI: 96.8–98.7), is bile solubility the most sensitive test. *CpsA* PCR has highest specificity with 98.4% (95% CI: 96.3–99.4%). The lowest sensitivity was noted for optochin testing with 81.7% (95% CI: 79.1–84.1). With a specificity of 61.7% (95% CI: 56.2–66.9), the WHO/CDC algorithm was the less specific.

Table 2. Sensitivity of morphology, optochin susceptibility, bile solubility and *CpsA* PCR when used singly to identify *S. pneumoniae*, and WHO/CDC algorithm.

Identification method	Sensitivity† % (95% CI)	p-value			
		Colony morphology	Optochin susceptibility	Bile solubility	*CpsA* PCR
Morphology	86.4 (84.0-88.5)	–			
Optochin susceptibility	81.7 (79.1-84.1)	0.0009			
Bile solubility	97.9 (96.8-98.7)	0.0000	0.0000		
CpsA PCR	94.1 (92.4-95.5)	<0.0001	0.0000	<0.0001	
WHO/CDC algorithm	96.3 (94.9-97.4)	0.0000	0.0000	0.0136	0.0077

† Sensitivity was calculated as the proportion of true positive results among the total positive results.
PCR: Polymerase chain reaction.
$p > 0.05$ is considered as statistically significant. The null hypothesis that sample proportions are equal is rejected.

Table 3. Specificity of morphology, optochin susceptibility, bile solubility and *CpsA* polymerase chain reaction when used singly to identify *S. pneumoniae*, and WHO/CDC algorithm.

Identification method	Specificity† % **(95% CI)**	**p-value**			
		Colony morphology	**Optochin susceptibility**	**Bile solubility**	***CpsA* PCR**
Morphology	89.8 (86.3-92.7)				
Optochin susceptibility	64.0 (58.9-68.9)	0.0000			
Bile solubility	96.8 (94.4-98.3)	<0.0001	0.0000		
CpsA PCR	98.4 (96.3-99.4)	0.0000	0.0000	0.0068	
WHO/CDC algorithm	61.7 (56.2-66.9)	0.0000	0.2181	0.0000	0.0000

Boldface type indicates statistical significance.
† Specificity was calculated as the proportion of true negative results among the total negative results.
$p > 0.05$ is considered as statistically significant. The null hypothesis that sample proportions are equal is rejected.
ND: Not determinate

DISCUSSION

This study assessed the sensitivity and specificity of different phenotypic, biochemical and genotypic methods used alone and in combination (the WHO/CDC algorithm) to identify *S. pneumoniae* isolates provided by laboratories, participating in a multicenter study. This study allowed a comparison between phenotypic, biochemical and genotypic methods to identify *S. pneumoniae*, using a large collection issued from different countries. The sequence signature was used as gold standard. This method is based on a detection of a *S. pneumoniae*-specific punctual nucleotide conversion. This method keeps the high specificity and the sensitivity of MLSA[28] and resolves the problems due to lack of sensitivity and specificity of phenotypic methods and homologous recombination of 16S rRNA-based methods[25]. Using this method, 967 *S. pneumoniae* were identified. However, none of morphology, optochin susceptibility testing, bile solubility and *CpsA* PCR, used separately was able to correctly identify *S. pneumoniae*. 16S rRNA gene sequencing has been considered as unsuitable for the identification of *S. pneumoniae* because of the high genetic similarity between mitis group members[8]. However, recent reports have shown that internal species-specific signature sequences of 16S rRNA gene can be easily used to distinguish between these species[29]. The sensitivity and specificity of the optochin susceptibility test were low as also described by Richter *et al.*[9]. This can be explained on the one hand by an increase in the prevalence of optochin resistance[30], which was in our study as high as 18.3% (n = 177). On the other hand, the susceptibility to optochin of other members of the viridans group is increasing[31]. This may lead to misidentification of *S. pneumoniae* and an incorrect discrimination between mitis group streptococci, causing a wrong estimation of antimicrobial resistance[32]. Viridans streptococci are, unlike *S. pneumoniae*, only pathogenic in sterile sites.

Therefore, misidentification of these bacteria as *S. pneumoniae* may cause an overestimation of the pneumococcal disease burden[33]. When compared with viridans streptococci, resistance of *S. pneumoniae* for some antimicrobial agents is reduced. Wrong identification of viridans streptococci as *S. pneumoniae* may lead to an overestimation of pneumococcal resistance for antibiotics[32,33]. Verhelst *et al.*[34] have compared five genotypic methods for the identification of optochinresistant *S. pneumoniae*. These authors have described the optochin susceptibility test as the standard method for identification of *S. pneumoniae* among α-hemolytic streptococci. Moreover, Kellogg *et al.* have compared several diagnostic methods on 99 clinical *S. pneumoniae* isolates and reported that optochin susceptibility testing has a high sensitivity and specificity[35]. These findings are in contrast with our results showing a high optochin resistance that contributes to the low specificity of this method (Table 3). In the case of an ambiguous optochin susceptibility test result, the bile solubility test is used, but disadvantages of this test are frequent nonconclusive results and the ability to crossreact with other mitis group streptococci[36,37]. Despite these findings, our results have shown that this method is the most accurate method to use in the daily microbiological routine laboratories due to its ease and low workload but also to the high sensitivity and specificity. The detection of the capsular biosynthesis gene *CpsA* was described as a reliable method to identify *S. pneumoniae*[24,38]. However, some reports have shown the presence of this gene in other mitis group streptococci[39]. Other researchers have reported the lack of this gene in serotypes 25A and 35[28].

In recent years, matrix-assisted laser desorption ionization-time-of-flight MS (MALDI-TOF MS) has emerged as a new rapid method and an eventual alternative to the phonotypical methods of bacterial identification. Wessels et al.[40] have found discrepancies comparing biochemical (optochin susceptibility and bile solubility) and genotypic methods (*lytA* gene and sequence analysis of *tuf* and *rpoB* genes), and MALDI-TOF MS for identification of *S. pneumoniae*. Moreover, at present, the available databases of the MALDI-TOF are not optimal. Recent reports have shown that available MALDI-TOF MS systems erroneously identify *S. pneumoniae* and mitis streptococci[41]. However, Werno et al.[42] have shown *S. pneumoniae*-specific peak profiles by using MALDI-TOF MS. These profiles may differentiate this bacterium from other mitis group streptococci, suggesting that MALDI-TOF might be an alternative to better identify *S. pneumoniae*. Nevertheless, this study was carried out only on 38 invasive isolates. A larger study is needed before making conclusions. The identification performance of the currently used methods to identify *S. pneumoniae* showed a large variation, especially in specificity. Moreover, the high rate of optochin resistance in mitis group streptococci represents an extra challenge for the identification accuracy. We have demonstrated the failure of optochin susceptibility *S. pneumoniae* identification algorithms. If biochemical methods yield uncertain results, PCR-targeting specific genes may be an option for a definitive conclusion. The high rate of optochin-resistant *S. pneumoniae*

might justify the necessity of a close monitoring in order to evaluate the usefulness of the optochin susceptibility testing.

CONCLUSION

Our results demonstrated the necessity to review the *S. pneumoniae* identification methods. Awaiting the development of a new strategy for the identification of *S. pneumoniae*, bile solubility is the most accurate method among the currently used methods. Moreover, a close monitoring might be necessary in order to evaluate the usefulness of optochin susceptibility testing.

FUTURE PERSPECTIVE

We demonstrated the failure of the optochin susceptibility for the identification of *S. pneumoniae* and the necessity of a new strategy. This strategy should avoid optochin susceptibility testing. Awaiting new accurate methods, bile solubility should be used as an identification test in the routine microbiological laboratories.

EXECUTIVE SUMMARY

- *Streptococcus pneumoniae* is a major respiratory pathogen. It can colonize the human nasopharynx causing various infections such as otitis media, sinusitis, pneumoniae, septicemia and meningitis.
- A comparison was made between phenotypic, biochemical and genotypic methods to identify *S. pneumoniae*, using a large collection of colonizing isolates issued from different countries.
- Individual use of morphology, optochin susceptibility testing, bile solubility and *CpsA* polymerase chain reaction were able to correctly identify *S. pneumoniae*.
- The results of morphology, optochin and bile solubility testing were also interpreted by using the WHO/CDC algorithm described by Centers for Disease Control and Prevention. Using this algorithm, only 79.8% of the isolates were identified as *S. pneumoniae*.
- The sensitivity and specificity of the optochin susceptibility test were low (81.7% and 64.0%, respectively). This can be explained by the increase in the prevalence of optochin resistance, which was in our study as high as 18.3%.

- The susceptibility to optochin of other members of the viridans group is increasing. This may lead to misidentification of *S. pneumoniae* and an incorrect discrimination between mitis group streptococci, causing a wrong estimation of antimicrobial resistance.
- Misidentification of other members of the viridans group as *S. pneumoniae* may cause an overestimation of the pneumococcal disease burden.
- The identification performance of the currently used methods to identify *S. pneumoniae* showed a large variation, especially in specificity.
- The high rate of optochin-resistant *S. pneumoniae* might justify the necessity of a close monitoring in order to evaluate the usefulness of the optochin susceptibility testing.

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3

Differentiation between *Streptococcus pneumoniae* and other viridans group streptococci by matrix-assisted laser desorption/ionization time of flight mass spectrometry

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ABSTRACT

Objectives

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is becoming the method of choice for bacterial identification. However, correct identification by MALDI-TOF of closely related microorganisms such as viridans streptococci is still cumbersome, especially in the identification of *S. pneumoniae*. By making use of additional spectra peaks for *S. pneumoniae* and other viridans group streptococci (VGS). We re-identified viridans streptococci that had been identified and characterized by molecular and phenotypic techniques by MALDI-TOF.

Methods

VGS isolates (n = 579), 496 *S. pneumoniae* and 83 non-*S. pneumoniae* were analyzed using MALDI-TOF MS and the sensitivity and specificity of MALDI-TOF MS was assessed. Hereafter, mass spectra analysis was performed. Presumptive identification of proteins represented by discriminatory peaks was performed by molecular weight matching and the corresponding nucleotides sequences against different protein databases.

Results

Using the Bruker reference library, 495 of 496 *S. pneumoniae* isolates were identified as *S. pneumoniae* and one isolate was identified as non-*S. pneumoniae*. Of the 83 non-*S. pneumoniae* isolates, 37 were correctly identified as non-*S. pneumoniae*, and 46 isolates as *S. pneumoniae*. The sensitivity of the MALDI-TOF MS was 99.8% (95% confidence interval (CI) 98.9–100) and the specificity was 44.6% (95% CI 33.7–55.9). Eight spectra peaks were mostly present in one category (*S. pneumoniae* or other VGS) and absent in the other category and inversely. Two spectra peaks of these (m/z 3420 and 3436) were selected by logistic regression to generate three identification profiles. These profiles could differentiate between *S. pneumoniae* and other VGS with high sensitivity and specificity (99.4% and 98.8%, respectively).

Conclusions

Spectral peaks analysis based identification is a powerful tool to differentiate *S. pneumoniae* from other VGS species with high specificity and sensitivity and is a useful method for pneumococcal identification in carriage studies. More research is needed to further confirm our findings. Extrapolation of these results to clinical strains need to be deeply investigated.

INTRODUCTION

Streptococcus pneumoniae, *Streptococcus oralis*, *Streptococcus mitis* and *Streptococcus pseudopneumoniae* are closely related species of viridans group streptococci (VGS). VGS belong to the indigenous microbiota in the different tracts of the human body and can be associated with infections such as endocarditis and sepsis[1,2]. Additionally, *S. pneumoniae* is associated with otitis media, pneumonia and meningitis[3].

Epidemiological studies on *S. pneumoniae* may have different aims, for example identifying the effect of pneumococcal vaccination on pneumococcal carriage, assessing the prevalence of antimicrobial resistance in colonizing strains or understanding the interaction between *S. pneumoniae* and other colonizers of the upper respiratory tract. Such studies are often complicated by the misidentification of this bacterium. In general, identification of VGS species is based on phenotypical and biochemical methods such as colony morphology, optochin susceptibility and bile solubility. However, these methods lack sensitivity and specificity, and are time consuming[4-8].

In the last decades, new molecular methods have been introduced which specifically detect *S. pneumoniae*, such as nucleic acid-based identification using different genes encoding *S. pneumoniae* virulence factors like capsular polysaccharide A (*CpsA*), pneumolysin (*ply*), autolysin (*lytA*) and pneumococcal surface antigen A (psa). Although these methods are rapid and more reliable than phenotypical and biochemical methods[5], cross reactions between other VGS species occur[4,9,10]. Also, sequence analysis of the 16S RNA gene cannot discriminate *S. pneumoniae* from other VGS species due to the high similarity in nucleic acid composition (>99%) of these species[11,12]. Recently, direct bacterial profiling using matrix associated laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) has been introduced as an alternative for phenotypic and genotypic bacterial identification especially for Gram-negative rods[12,13]. This method is based on analysis of the protein mass spectra of bacterial isolates. However, using the current MALDI-TOF databases misidentification of *S. pneumoniae* has been reported[14,15].

In this study, we investigated the accuracy of MALDI-TOF MS for identification of carriage of *S. pneumoniae* strains. For this study, we tested the previously characterized pneumococcal and the other VGS strains from our strain collection, obtained from healthy participants from nine European countries[16] by MALDI-TOF MS using the Bruker library database. Additionally, the same isolates were used to determine the accuracy of the adapted MALDI-TOF MS identification software by adding additional marker peaks to distinguish *S. pneumoniae* from other VGS.

MATERIALS AND METHODS

Bacterial strains collection and identification

VGS isolates (n = 579), 496 *S. pneumoniae* and 83 non-*S. pneumoniae* were derived from samples collected as part of 'The appropriateness of prescribing antimicrobial agents in primary health care in Europe with respect to antimicrobial resistance' (APRES) study[17]. Strain identification was performed using molecular and phenotypic methods as described by Yahiaoui et al.[16].

MALDI -TOF mass spectroscopy

Bacterial colonies grown overnight on Columbia sheep blood agar (bioTRADING, The Netherlands) at 37°C and 5% CO2 were used for ethanol formic acid extraction according to the MALDI Biotyper protocol (Bruker Daltonics GmbH, Bremen, Germany)[18]. MALDI-TOF MS identification was performed in duplicate on Microflex LT Bruker MALDI-TOF MS using flexControl v3.4 software (Bruker Daltonics, Germany), using the reference library v.4.0.0.1, within a range from 2000 to 20 000 Da. A constant tolerance of 0.5, a linear tolerance of 500 and a peak detection rate of 1 were used. The score value was calculated by the Biotyper software according to the manufacturer's recommendation: highly probable identification at species level (score ≥2.30), secure genus and probable species level (score 2.00–2.29), probable genus level identification (1.70–1.99) and unreliable identification (score <1.70).

Mass spectra analysis

Mass spectra analysis was performed using Bionumerics v7.6, (Applied Maths NV, Sint-Martens-Latem, Belgium). All spectra were checked manually on quality. Spectra with the best quality of duplicate analysis were used for further analysis. After smoothing and baseline subtraction, mass spectra were exported to SPSS software (PASW software package 19.0) (IBM, US) as binary data (peak present or absent) and compared for the presence or the absence in *S. pneumoniae* or non-pneumococcal isolates. An arbitrary percentage of 70% of spectra present in one category (*S. pneumoniae* or other VGS) and absent in the other was retained for further analysis. All isolates were grouped by retained spectra peaks and compared with the identified species. Spectral peaks which could differentiate between *S. pneumoniae* and non-pneumococcal strains were subject for further statistical analysis.

Sensitivity and specificity assessment

The sensitivity of MALDI-TOF MS was calculated as the proportion of true positive results among the total number of positive results and specificity as the proportion of the true negative results among the total number of negative results.

Biomarkers spectra peak discovery

Presumptive identification of proteins represented by discriminatory peaks was performed by molecular weight matching against the European Bioinformatics Institute (EMBL-EBI), the Swiss Institute of Bioinformatics (SIB) and the Protein Information Resource (PIR) database (www.uniprot.org). The molecular weight of biomarker protein was derived from the corresponding marker peak's m/z considering simple protonation (m/z – 1), double protonation (2 × m/z – 2), methionine loss (m/z – 1 + 131 and 2 × m/z – 2 + 131) and deacetylation of the N-terminus (m/z – 1 – 42 and 2 × m/z – 2 – 42).

Presumptive protein sequences were matched against the National Center for Biotechnology Information, U.S. National Library of Medicine (NCBI) proteins database (proteins Blast), and the corresponding nucleotides sequences against the NCBI nucleotides sequences database (Nucleotides Blast) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

To generate identification profiles based on highly discriminatory spectral peaks, a model was constructed by forward logistic regression using the PASW software package 19.0 (IBM, US). The model fit was assessed using Hosmer-Lemeshow test. A p value of >0.1 is considered as a good fit.

RESULTS

MALDI-TOF MS identification

In total 579 isolates, 496 *S. pneumoniae* and 83 non-pneumococcal isolates were analysed. Of the 496 *S. pneumoniae* isolates, 495 isolates (99.8%) were identified as *S. pneumoniae* with scores between 1.9 and 2.5 and one isolate (0.2%) was identified as *S. oralis* with a score of 2.1. Of the 83 non-*S. pneumoniae* isolates, 34 were identified as *S. mitis* (41.0%, score 2.0–2.5), three isolates as *S. oralis* (3.6%, score 2.2–2.3) and 46 isolates (55.4%, score 2.1–2.4) as *S. pneumoniae* (Table 1).

Table 1. Identification scores of matrix-assisted laser desorption/ionization time of flight mass of the *Streptococcus pneumoniae* and other viridans group streptococci strains

Score	*S. pneumoniae* strains (n = 496) identified as			Other VGS strains (n = 83) identified as		
	S. pneumoniae	*S. mitis*	*S. oralis*	*S. pneumoniae*	*S. mitis*	*S. oralis*
1.9–2.0	15	0	0	0	1	0
2.0–2.3	322	0	1	17	13	1
>2.3	158	0	0	29	20	2

Mass spectra analysis

Three hundred and twenty-eight different spectral peaks were detected, ranging between m/z 2000 and m/z 10 629. Seven peaks were present in at least 70% of the isolates of one category (*S. pneumoniae* or other VGS) and absent in at least 70% of the second category (Table 2).

Table 2. Presence and absence of discriminatory peaks in *Streptococcus pneumoniae* (n = 496) and other viridans group streptococci (n = 83) i.e. *S. mitis*

m/z	***S. pneumoniae***	**Other VSG**
	N = 496 (%)	**N = 8 3 (%)**
2624	1 (0.2)	59 (71,1)
3420	3 (0.6)	82 (98.8)
3436	429 (86.5)	0 (0,0)
5252	1 (0.2)	60 (72.3)
6840	3 (0,6)	82 (98.8)
7509	114 (23.0)	71 (85.5)

Six of these spectra peaks were specific for non-pneumococcal strains (Table 2). Peaks with m/z 3420 and m/z 6840 (both present in 3/496 *S. pneumoniae* isolates and 82/83 non-pneumococcal isolates) were the most discriminatory between *S. pneumoniae* and other VGS. These peaks could differentiate *S. pneumoniae* from other VGS with high sensitivity and specificity (99.8% and 96.5%, respectively). The spectra peak with m/z 3436 was present in 429/496 *S. pneumoniae* isolates and absent in 83 non-pneumococcal isolates (Table 2).

Using logistic regression, two spectra peaks (m/z 3420 and 3436) were selected to generate a model for an optimal differentiation of *S. pneumoniae* and VGS (please see supplementary material). Three MALDI profiles based on these spectra peaks allowed a correct identification of 493/496 *S. pneumoniae* isolates and 82/83 non-*S. pneumoniae* isolates (Table 3). The sensitivity and specificity of the spectra peaks analysis were 99.4% and 98.8%, respectively.

Table 3. Matrix-assisted laser desorption/ionization identification profiles using peaks selected by logistic regression

m/z value		**Profile**	**Strains**	**Number of strains**	**Correct identification**
3420	**3436**			**N = 579**	**N (%)**
-	-	Profile 1	*S. pneumoniae*	65	64 (98.5)
-	+	Profile 2	*S. pneumoniae*	429	429 (100)
+	-	Profile 3	Other VGS	85	82 (96.5)

Of the four discordant (misidentified) identifications, additional conventional tests were performed and the results are shown in Table 4. No explanation of MALDI-TOF MS misidentification can be found. When m/z 3420 peaks were compared between all isolates, a small shift in peak/spectrum was noticed in isolate 66 where the peak was detected at m/z 3422.

Table 4. Conventional identification of the misidentified isolates using spectral peak analysis

Isolate number	Strain	Morphology	Optochin (mm)	Bile solubility	cpsA PCR	Profile
66	Other VGS	Button	11	negative	negative	1
218	*S. pneumoniae*	Button	6	positive	positive	3
234	*S. pneumoniae*	Button	14	positive	positive	3
338	*S. pneumoniae*	Button	9	positive	positive	3

Button, colonies with α-haemolysis, presenting central depression.

Biomarkers spectra peak discovery

Table 5 shows the presumptive proteins corresponding to the most discriminatory peaks. One presumptive protein corresponded to each of the spectral peaks m/z 2624 (Uncharacterized protein), m/z 3420 (chlorohydrolase), m/z 5252 (N-ethyl ammeline chlorohydrolase) and m/z 6840 (chlorohydrolase), and three to m/z 3435 (MarR family transcriptional regulator and chlorohydrolase). No conclusive presumptive protein was found for spectral peak m/z 7509. Zooming into the nucleotide sequences of presumptive proteins corresponding to spectral peaks m/z 2426 and m/z 5252 shows that their nucleotides sequences share 41.0% of similarity. These shared nucleotides were identical for 90.2%. Furthermore, the presumptive proteins corresponding to spectral peaks m/z 3420 and m/z 6840 shared 65% of their nucleotides. These nucleotides sequences were identical for 88.6%.

Table 5. Presumptive proteins corresponding to the discriminatory spectral peaks

Mass spectrum (Da)		**6840**	**3420**	**5252**	**2624**	**3436**	**3436**	**3436**
Presumptive protein		Chlorohydrolase	Chlorohydrolase	N-ethyl ammeline chlorohydrolase	Uncharacterized protein	MarR family transcriptional regulator	MarR family transcriptional regulator	Chlorohydrolase
Mass (Da)		6289	3569	5246	2689	3436	3436	3435
NCBI Blast protein (first record)	Protein	Chlorohydrolase	Chlorohydrolase	Uncharacterized protein	Chlorohydrolase	MarR family transcriptional regulator	MarR family transcriptional regulator	Chlorohydrolase
	Microorganism	*Streptococcus pseudopneumoniae*	*Streptococcus mitis*	*Streptococcus mitis*	*Streptococcus mitis*	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*
	Identity (%)	96	92.9	88.2	100	96.6	96.6	92.9
NCBI Blast protein (second record)	Protein	Chlorohydrolase	Chlorohydrolase	N-ethylammeline chlorohydrolase	Uncharacterized protein	Transcriptional regulator, MarR family domain protein	MarR family protein	Chlorohydrolase
	Microorganism	*Streptococcus pneumoniae*	*Streptococcus* pseudopneumoniae	*Streptococcus* oralis	*Streptococcus sp.*	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*	*Streptococcus mitis*
	Identity (%)	96	92,9	79,2	100	96,6	100	92,9
Nucleotide Blast (first record)	Microorganism	*Streptococcus pneumoniae*	*Streptococcus mitis*	*Streptococcus mitis*	*Streptococcus mitis*	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*
	Identity (%)	96.7	100	94.93	100	98.89	100	100
Nucleotide Blast (second record)	Microorganism	*Streptococcus pneumoniae*	*Streptococcus mitis*	*Streptococcus mitis*	*Streptococcus gordonii*	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*
	Identity (%)	97.2	94.8	93.53	98.72	98.89	100	100

DISCUSSION

In this study, 579 isolates were analysed using the MALDI-TOF MS Bruker identification reference database. The generated mass spectra peaks were analysed, and the most discriminatory spectra peaks able to discriminate *S. pneumoniae* from other VGS species were selected.

Our results showed that MALDI-TOF MS identification based on the Bruker reference database had a high sensitivity (99.8%) and a low specificity (43.6%). The analysis of the generated mass spectra revealed two spectral peaks, m/z 3420 and m/z 3436, which solely highly discriminate between *S. pneumoniae* and other VGS species, namely *S. mitis* with a sensitivity and specificity of 99.8% and 96.5%, respectively.

The strength of this study was that the analyses were performed on a large well-identified collection, which allows reliable conclusions to be drawn. Moreover, our isolates were derived from a cross-sectional study in different European countries. This suggests that our results might also be applicable in other countries. However, some weaknesses must be recognized. Our collection consisted of colonizing strains. Whether our conclusions also apply for clinical isolates needs to be elucidated. Furthermore, this study was performed on more *S. pneumoniae* strains than non-pneumococcal strains. This was because isolates were screened in the country of sampling and only suspected *S. pneumoniae* isolates were sent to the central laboratory for further identification and analysis[16].

MALDI-TOF MS based on reference database has been generally accepted as a new rapid method for bacterial identification as an alternative to the phenotypical and biochemical methods in use at present. However, the available MALDI-TOF data base systems is described to erroneously identify *S. pneumoniae* and other VGS species[12,19].

In our study, MALDI-TOF MS identification based on Bruker database lacks specificity (47 of 83 non-pneumococcal isolates were identified as *S. pneumoniae*), but showed a high sensitivity (99.8%), These results were in concordance with the findings of Werno et al.[19], Chen et al.[20] and Ikryannikova et al.[21]. The persistence of misidentification and classification of VGS members despite the continuous update of reference databases might be explained by low phylogenetic resolution of MALDI-TOF MS systems due to the small subset of microbial proteome (1%) represented in the whole cell spectra[22,23].

Sixteen isolates had a score <2.0 (1.80–1.99). According to Bruker guidelines, these isolates should be identified at genus level. Identification at species level might be low confident. In our study, all 16 isolates were correctly identified (15 *S. pneumoniae* and one *S. mitis*).

Peak analysis was used for confirmation of MALDI TOF MS identification. In this study we found six spectral peaks not described before (m/z 3420, m/z 3436, m/z 5252, m/z 6840 and m/z 7509) present in at least 70% of one of the two categories and absent in the second (*S. pneumoniae* or other VGS). None of the peaks reported by Ikrynnikova et al.[21] or Chen

et al.[20] was found in this study. However, our results were in agreement with the peaks of m/z 2625 and m/z 5253 reported by Werno et al.[19] as specific for *S. mitis* and *S. oralis*, and m/z 6839.7 described by Marin et al.[23] as specific for *S. mitis*. In our study, spectral peak m/z 6955 was significantly more frequent in non-pneumococcal strains. It was found in one of 496 pneumococcal and 16 of the 83 non-pneumococcal strains ($p < 0.0001$). However, this spectral peak did not meet our criteria of presence in 70% in one strain category and absence in the other, and was therefore not selected in the analysis. Marin et al. described a peak of m/z 5298 as specific for *S. oralis*. The difference in Daltons (Da) between this peak of m/z 5252 and the peak found in this study is 46 Da. This may suggest that both peaks correspond to the same protein but acylated (gain of 42 Da) in the study of Marin et al. and deacylated in our case.

Using logistic regression, three identification profile-based model were generated using two spectral peaks. These profiles had a sensitivity and a specificity close to 100%. The biomarkers search has shown presumptive discriminatory proteins for *S. pneumoniae* (m/z 3436) and other VGS (m/z 2624, m/z 3420 and m/z 5252 and m/z 6840). Both presumptive proteins corresponding to m/z 2624.14 and m/z 5252, on the one hand, and m/z 3420 and m/z 6840, on the other hand, share significant nucleotides sequence similarity. This suggest that these spectral peaks correspond to the same protein, with single protonation in m/z 2624 and m/z 3420 and double protonation in m/z 5252 and m/z 6840.

In conclusion, reference database-based identification of *S. pneumoniae* has shown a high sensitivity and a low specificity to differentiate between VGS, that is *S. mitis* group including *S. pneumoniae*. Spectral peaks analysis-based identification has shown to be a powerful tool to differentiate *S. pneumoniae* from other VSG species i.e. *S. mitis* group, and a useful method for pneumococcal identification in pneumococcal carriage studies. More research is needed in order to further confirm our findings. Extrapolation of these results to clinical strains need to be deeply investigated.

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4 Prevalence and antibiotic resistance of commensal *Streptococcus pneumoniae* in nine European countries

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ABSTRACT

The human microbiota represents an important reservoir of antibiotic resistance. Moreover, the majority of antibiotics are prescribed in primary care. For this reason, we assessed the prevalence and antibiotic resistance of nasal carriage strains of *Streptococcus pneumoniae*, the most prevalent bacterial causative agent of community-acquired respiratory tract infections, in outpatients in nine European countries. Nasal swabs were collected between October 2010 and May 2011, from 32,770 patients, recruited by general practices in nine European countries. Overall prevalence of *S. pneumoniae* nasal carriage in the nine countries was 2.9%. The carriage was higher in men (3.7%) than in women (2.7%). Children (4–9 years) had a higher carriage prevalence (27.2%) compared with those older than 10 years (1.9%). The highest resistance observed was to cefaclor. The highest prevalence of multidrug resistance was found in Spain and the lowest prevalence was observed in Sweden.

INTRODUCTION

Streptococcus pneumoniae is an important causative agent of community-acquired pneumonia, otitis media, bacteremia and meningitis, especially in immunocompromised patients, young children and elderly individuals[1]. The upper respiratory tract is the main ecological niche of *S. pneumoniae*[2]. The colonization rates range, depending on age, between 3 and 60%[3,4] and depend on geographic area, age, genetic background and socioeconomic status of the host[1].

The introduction of pneumococcal conjugate vaccines has led to a major decrease in pneumococcal carriage and the prevalence of pneumococcal-related diseases[5]. Depending on the vaccine used, emergence of new colonizing serotypes has been observed that have replaced the vaccine serotypes[6,7]. Many host, environment and microbiological factors play a role in the increase of susceptibility to carry *S. pneumoniae.* One example of the microbiological factors is coinfection with influenza virus. Several reports have observed pneumococcal transmission via contact of a nonpneumococcal carrier with infected respiratory secretion of an influenza infected pneumococcal carrier[8], or by person-to-person contact[9].

Most population studies conducted thus far have focused on nasopharyngeal carriage in children[10], on the prevalence of invasive isolates[1,5,7] and on the effectiveness of vaccination strategies[6,11-14]. Pneumococcal carriage in adults has hardly been described, despite the fact that colonization is essential to understand bacterial epidemiology and to assess the effectiveness of preventive strategies[15].

During the last few decades, antimicrobial drug resistance has increased worldwide[16]. The human microbiota is generally considered as the main reservoir for antibiotic resistant genes and resistant microorganisms[17,18]. The acquisition of antibiotic resistance in this environment is enhanced by the enormous potential of genomic exchange and mutations[19].

One of the aims of the APRES study, was to assess the prevalence and antibiotic resistance of nasal carriage of *S. aureus* and *S. pneumoniae* in general practice patients and to compare antibiotic resistance patterns of these isolates[20] with different antibiotic prescription policies. The results of *S. aureus* analyses have been described before[21]. Here, we report the results of the prevalence and antibiotic resistance of *S. pneumoniae* carriage in the participating countries.

MATERIALS & METHODS

Patient population

The study design was described previously by van Bijnen *et al.*[20]. In short, 20 general practitioners (GP) were recruited per country, by national GP networks in nine European countries (Austria, Belgium, Croatia, France Hungary, Spain, Sweden, The Netherlands and

the UK), and aimed to each provide 200 nasal swabs, from patients older than 4 years, with the exception of the UK where patients were 18 years or older (due to ethical committee constraints). To be included in the study, patients should have visited the practice for a non-infectious condition, not used antibiotics nor been hospitalized in the 3 months preceding the swab collection. Immunocompromised patients, as well as nursing home residents, were excluded. Included patients were sampled consecutively.

Information of the participating patients was derived from a questionnaire and included age, gender, profession (livestock farming, working in healthcare or in day care/nursery school), the number of GP visits during the last year, living with children younger than 5 years (other than the index patient), suffering from a chronic skin condition and vaccination against pneumococci and/or influenza (season 2009–2010 and/or 2010–2011). Consent and information of children were obtained from the parents or the legal representative. The questionnaire did not differentiate between 2009 and 2010 seasonal and pandemic vaccines. Information on pneumococcal and influenza vaccination policy in each participating country was obtained from a literature search.

Prior to the start of the study, GPs received a protocol for sampling the nasal swabs[21]. Isolation and identification of *S. pneumoniae* was performed in a national microbiological laboratory in each participating country except for France. These swabs were analyzed in the microbiological laboratory of Maastricht University Medical Center (MUMC), The Netherlands), because no laboratory in France was prepared to perform these analyses. All (putative) pneumococci isolated at the national laboratories were kept frozen in skimmed milk at -80°C and sent at the end of the study period on dry ice to the laboratory in Maastricht for antibiotic susceptibility testing.

The antibiotic resistance of the isolates was performed with microdilution for cefuroxime, cefaclor, ceftazidime, clarithromycin, clindamycin, ciprofloxacin, moxifloxacin, penicillin, tetracycline and trimethroprim-sulfamethoxazole. The control strain was *S. pneumoniae* ATCC 49619. Invalid results due to nonhomogenous growth were repeated twice. The method used was in accordance with EUCAST guidelines and EUCAST epidemiological cutoffs were used as resistance breakpoints[22].

Patients with incomplete demographic information or laboratory data, as well as erroneous samples (no bacterial growth noted at laboratory analysis), were excluded from the analyses.

Statistical analysis

Results were analysed using the PASW software package 19.0. A p-value of <0.05 was considered as statistically significant.

To determine pneumococcal carriage determinants odd ratios (OR)s were calculated. OR was defined as the cross-product ratio of the numbers shown in the two-by-two contingency table of flu vaccination, pneumococcal vaccination, occupation, having a chronic skin disease and living with other young children. The total number of patients who answered the specific question was used as the denominator.

To account for nonrandom clustering of our data at family level and to control whether age and gender affect the pneumococcal carriage prevalence, a three-level multilevel logistic regression model (country, GP and patient) was estimated using MLN-Software for N-level analysis package.

Multidrug resistance (MDR) was defined as being resistant to three or more classes of antibiotics.

The statistical analysis was made on two age groups: <10 years and ≥10 years. This cut off was used to differentiate young children versus the older ones and adults as after 10 years of age pneumococcal carriage was stable[1].

Role of the funding source

The sponsor of this study had no role in study design, data collection, data analysis data interpretation or writing of this report. The corresponding author had full access to all the data on the study and had final responsibility for the submission for publication, following agreement from all authors.

RESULTS

The number of participants ranged between 3132 in Belgium and 4017 in Hungary, resulting in a total of 32,770 patients recruited **(Table 1)**. Patients who did not meet the inclusion criteria because of their demographic characteristics, or incorrect sampling were excluded from the analysis[21] resulting in a total of 32,161 patients available for the data analysis.

Prevalence of nasal carriage

The overall crude prevalence of *S. pneumoniae* nasal carriage was 2.9% (n = 937), and ranged from 1.2% in Austria (n = 38) and the UK (n = 36) to 4.4% in France (n = 170).

The prevalence of *S. pneumoniae* carriage was higher in men (3.7%; 95% CI: 3.4–4.0) than in women (2.7%; 95% CI: 2.1–2.5). Children (4–9 years) had a higher carriage prevalence (27.2%; 95% CI: 24.7–29.6) compared with those older than 10 years (1.9%; 1.8–2.1). The highest carriage rate in the 4–9year-old was in Spain (24.0%) followed by France (22.2%) **(Table 2)**.

Table 1. Baseline characteristics of the participating patients.

Country	Total patient population (n)	Excluded from analysis			Included (n)	Women (% of total)	Age distribution (% of total)				
		Age (n)	Mismatch (n)	Incorrect sampling (n)			4–9	10–17	18–29	30–60	>60
Austria	3380	4	56	11	3309	56.6	1.2	2.2	15.9	50.6	30.0
Belgium	3132	6	101	0	3025	54.4	1.3	3.1	8.2	41.0	46.5
Croatia	4013	1	35	25	3952	59.5	6.0	8.3	9.2	38.9	37.5
France	3870	0	12	1	3857	55	3.4	4.7	11.5	45.4	35.1
Hungary	4017	3	17	165	3832	55.1	8.6	15.7	9.6	36.8	29.4
The Netherlands	3873	14	12	0	3847	57.2	3.2	5.3	11.2	46.1	34.2
Spain	4001	0	11	21	3969	58.4	4.9	5.9	9.2	41.5	38.5
Sweden	3273	33	26	0	3214	57.5	5.4	5.4	9.7	37.9	41.7
UK	3211	3	52	0	3156	59.2	0.0	0.0	14.8	46.4	38.9
Total	32,770	64	322	223	32,161	57	3.9	5.8	10.9	42.6	36.7

Table 2. *Streptococcus pneumoniae* nasal carriage prevalence.

Country	Total swabs (n)	Pneumococcal prevalence			
		Unadjusted (years)		Adjusted (age, gender and GP); (years)	
		4–9 (%)	>10 (%)	4–9 (%)	>10 (%)
Austria	3380	21.5	1.4	13.8	1.3
		(13.7–32.2)	(1.0–2.1)	(8.6–21.5)	(0.8–1.7)
Belgium	3132	20.0	1.5	12.8	1.3
		(12.8–29.8)	(1.0–2.3)	(8.0–19.8)	(0.9–1.9)
Croatia	4013	23.1	2.1	14.9	1.8
		(16.3–31.6)	(1.4–3.1)	(10.0–21.4)	(1.2–2.6)
France	3870	32.0	3.4	22.2	2.8
		(23.6–41.8)	(2.3–5.0)	(15.7–30.5)	(1.9–4.0)
Hungary	4017	24.8	1.5	17.3	1.2
		(18.0–33.1)	(1.0–2.3)	(12.0–24.3)	(0.8–1.7)
The Netherlands	3873	27.5	2.5	16.9	2.1
		(19.6–37.0)	(1.7–3.6)	(11.6–23.9)	(1.4–2.9)
Spain	4001	38.6	2.9	24.0	2.2
		(29.5–48.7)	(2.0–4.2)	(16.9–32.9)	(1.5–3.2)
Sweden	3273	27.4	2.0	15.3	1.5
		(19.8–36.5)	(1.3–2.9)	(10.5–21.7)	(1.0–2.3)
UK	3211	No data†	1.8	No data†	1.2
			(1.0–2.2)	(0.8–1.8)	

†All participants were ≥18 years.
GP: General practitioner.

Vaccination rates

Immunization strategies against *S. pneumoniae* and seasonal influenza and pandemic influenza A virus differed between the nine participating countries. Variation in pneumococcal immunization included vaccination strategy (recommended or voluntary, universal or risk groups only), vaccine type, vaccination schedule, administrated doses and targeted groups.

Seasonal influenza and pandemic Flu A immunization differed in targeted groups and clinical risk indications. Spain had the highest overall pneumococcal vaccination rate with 29.2% (46.4% in children younger than 9 years, and 28.3% in adolescent and adults), whereas Croatia had the lowest one with an overall rate of 0.7% (3.2% in patients younger than 9 years and 0.6% in patients of 10 years or older). France had the highest vaccination rate in children <9 years old (46.7%). Overall, 11.6% of all participating patients were vaccinated **(Table 3)**.

Table 3. Pneumococcal vaccination status in participating patients.

Country	Patients with known vaccination status			Vaccinated patients		
	Total (n)	4–9 years (n)	>10 years (n)	4–9 years % (95% CI)	>10 years % (95% CI)	Total % (95% CI)
Austria	2426	34	2392	8.8 (–0.7–18.3)	7.9 (6.8–9)	7.9 (6.8–9)
Belgium	2942	36	2906	11.1 (0.8–21.4)	16.8 (15.4–18.2)	16.7 (15.4–18)
Croatia	3778	216	3562	3.2 (0.9–5.6)	0.6 (0.4–0.9)	0.7 (0.4–1)
France	3552	122	3430	46.7 (37.9–55.6)	4.1 (3.4–4.8)	5.5 (4.8–6.3)
Hungary	3595	313	3282	7.7 (4.8–10.7)	3.4 (2.8–4)	3.7 (3–4.3)
The Netherlands	3080	97	2983	14.4 (7.4–21.4)	2.5 (1.9–3.1)	2.9 (2.3–3.5)
Spain	3800	192	3608	46.4 (39.4–53.5)	28.3 (26.8–29.8)	29.2 (27.8–30.7)
Sweden	2708	155	2553	5.2 (1.7–8.7)	11.5 (10.3–12.7)	11.2 (10–12.4)
UK	2713	No data†	2713	No data†	28.3 (26.6–30)	28.3 (26.6–30)
Total	28,594	1165	27,449	17.7 (15.5–19.9)	11.3 (10.9–11.7)	11.6 (11.2–12.0)

†All participants were ≥18 years.

Determinants of nasal carriage

Living with children younger than 5 years of age (or other children if the individual is a child <5 years old), resulted in a four-fold increased risk to carry *S. pneumoniae* (OR: 3.92; 3.41–4.50). In all participating countries, patients vaccinated against *S. pneumoniae* had a (nonsignificant) higher prevalence of pneumococcal carriage. The carriage prevalence among the vaccinated group was 3.5%, whereas in the unvaccinated patients this was 2.9% (OR: 1.2; 0.99–1.48). Seasonal and pandemic influenza A vaccination had no effect on the nasal pneumococcal carriage.

Antibiotic resistance

Antibiotic resistance results were available for 928 (99.0%) of 937 *S. pneumoniae* isolates. Resistance to cefaclor was most common: overall 52.3% and ranging between 30.6% in the UK and 77.3% in Belgium. No resistance was observed for moxifloxacin and ciprofloxacin. Tetracycline showed the highest intercountry variability ranging between 1.9% in Sweden and 29.9% in Spain **(Table 4)**.

The most frequently used antibiotics in outpatients were penicillin, macrolides and tetracycline[14] [14]. These antibiotics showed a resistance that ranged between 3.9% (Sweden) and 31.7% (Spain) for penicillin, 1.9% (Sweden) and 29.3% (Spain) for clarithromycin, and 1.9% (Sweden) and 29.9% (Spain) for tetracycline **(Table 5)**.

Table 4. Lowest and highest prevalence of antibiotic resistance of commensal *S. pneumoniae.*

Antimicrobial agent	Lowest antimicrobial resistance		Highest antimicrobial resistance	
	Prevalence % (95% CI)	Country	Prevalence % (95% CI)	Country
Cefaclor	30.6 (15.6–45.7)	UK	77.3 (64.9–89.7)	Belgium
Cefuroxime	3.9 (0.5–7.2)	The Netherlands	31.1 (24.1–38.1)	Spain
Ceftazidime	2.9 (0.0–6.1)	Sweden	24.6 (17.3–31.9)	Croatia
Clarythromycin	1.9 (0.0–4.5)	Sweden	29.3 (22.4–36.4)	Spain
Clindamycin	1.9 (0.0–4.5)	Sweden	27.5 (20.7–34.3)	Spain
Penicillin	3.9 (0.2–7.6)	Sweden	31.7 (24.6–36.8)	Spain
Tetracyclin	1.9 (0.0–4.5)	Sweden	29.9 (23–36.8)	Spain
Trimethroprim/ sulfothoxazole	1.9 (0.0–4.5)	Sweden	28.7 (21.8–35.6)	Spain

No resistance was found to moxiflxacin and ciprofloxacin

Multidrug resistance

Spain had the highest prevalence of MDR (resistance for three or more classes), in other words, 50.4%, (n = 63). The lowest percentage was observed in Sweden with 3.8% (n = 2). Thirty isolates were resistant to six classes, of which ten were from Croatia. Overall, 27 of these 30 isolates were resistant to all tested antibiotics, except for moxifloxacin and ciprofloxacin and three were resistant to all tested antibiotics. The most prevalent MDR was resistance to five classes (n = 70). The most common combination was resistance to cephalosporines (cefaclor, cefuroxime and ceftazidime), macrolides fluoroquinolone, penicillin and tetracycline (n = 40). Most of these isolates were isolated in France (n = 17) and Spain (n = 16).

Table 5. Antibiotic resistance prevalence in the nine participating countries.

Country	n	Cefaclor % (95% CI)	Cefuroxime % (95% CI)	Ceftazidime % (95% CI)	Clarythromycin % (95% CI)	Clindamycin % (95% CI)	Penicillin % (95% CI)	Tetracycline % (95% CI)	Trimethroprim-Sulfothoxazole % (95% CI)
Austria	38	36.8	7.9	7.9	10.5	2.6	7.9	2.6	5.3
		(21.5–52.1)	(−0.7–16.5)	(−0.7–16.5)	(0.8–20.3)	(−2.5–7.7)	(−0.7–16.5)	(−2.5–7.7)	(−1.8–12.4)
Belgium	44	77.3	25	22.7	22.7	18.2	25	20.5	20.5
		(64.9–89.7)	(12.2–37.8)	(10.3–35)	(10.3–35)	(6.8–29.6)	(12.2–37.8)	(8.6–32.4)	(8.6–32.4)
Croatia	134	59.7	26.9	24.6	20.9	15.7	22.4	16.4	15.7
		(51.4–68.0)	(19.4–34.4)	(17.3–31.9)	(14.0–27.8)	(9.5–21.9)	(15.3–29.5)	(10.1–22.7)	(9.5–21.9)
France	170	54.1	25.9	21.8	24.1	21.2	22.9	20.0	8.8
		(46.6–61.6)	(19.3–32.5)	(15.6–28)	(17.7–30.5)	(15–27.3)	(16.6–29.2)	(14–26)	(4.5–13)
Hungary	116	56.0	29.3	22.4	15.5	9.5	26.7	13.8	25.9
		(47.0–65.0)	(21.0–37.6)	(14.8–30.0)	(8.9–22.1)	(4.2–14.8)	(18.7–34.8)	(7.5–20.1)	(17.9–33.9)
The Netherlands	129	34.1	3.9	3.9	3.9	3.1	4.7	3.1	7.0
		(25.9–42.3)	(0.5–7.2)	(0.5–7.2)	(0.5–7.2)	(0.1–6.1)	(1.1–8.4)	(0.1–6.1)	(2.6–11.4)
Spain	167	59.9	31.1	24.0	29.3	27.5	31.7	29.9	28.7
		(52.5–67.4)	(24.1–38.1)	(17.5–30.5)	(22.4–36.4)	(20.7–34.3)	(24.6–38.8)	(23.0–36.8)	(21.8–35.6)
Sweden	103	48.5	5.8	2.9	1.9	1.9	3.9	1.9	1.9
		(38.9–58.2)	(1.3–10.3)	(0.0–6.1)	(0.0–4.5)	(0.0–4.5)	(0.2–7.6)	(0.0–4.5)	(0.0–4.5)
UK	36	30.6	13.9	13.9	13.9	11.1	13.9	11.1	13.9
		(15.6–45.7)	(2.6–25.2)	(2.6–25.2)	(2.6–25.2)	(0.9–21.4)	(2.6–25.2)	(0.8–21.4)	(2.6–25.2)
Total	931	52.6	20.9	17.3	17.3	14.2	19.4	15.2	15.0
		(49.1–55.5)	(18.3–23.5)	(14.9–19.7)	(14.9–19.7)	(12.0–16.3)	(16.9–21.9)	(12.9–17.5)	(12.7–17.3)

No resistance was found to moxiflxacin and ciprofloxacin.

DISCUSSION

In this study, nasal samples from more than 32,000 outpatients from nine European countries were collected and analysed. The overall *S. pneumoniae* carriage prevalence was 2.9% and resistance to cefaclor was the most common among the isolates with 52.3%. Of all resistant isolates, 19.4% were no susceptible to penicillin. MDR was observed in 33.9%, ranging from 3.8% in Sweden to 50.4% in Spain.

To the best of our knowledge, our study is the first to compare the prevalence and antibiotic resistance of commensal *S. pneumoniae* in healthy outpatients aged 4+ in several European countries. For this aim, existing GP networks were involved for patient recruitment. In addition, standardized and validated protocols were used to prevent inter laboratory differences.

Our study has some limitations. For logistic reasons, in other words, feasibility to perform a study in nine different countries with 20 different general practitioners we choose to take nasal swabs instead of nasopharyngeal ones. In all swabs, even in the nasal swabs from France that were sent to Maastricht, the prevalence was in accordance to the expected one[23]. Furthermore, based on these observations, we may conclude that taken nasal swabs do not influence the results negatively. Another limitation is that, due to ethical constraints, the lower age limit for eight countries was 4 years of age, for the UK 18 years. This hampers the assessment of pneumococcal carriage and the antibiotic resistance in the lowest age group (<4 years). Finally, we did not collect clinical data, such as among others comorbidities and recent hospitalization.

A wide variation in pneumococcal carriage prevalence was found among the participating countries. One of the major factors that influence pneumococcal colonization is the vaccination strategy, including vaccine type, whether vaccine was given to all children >2 years, universal or restricted to clinical risk groups only, such as patients suffering from chronic respiratory disease or cardiovascular disease (risk based), if the implementation of a vaccine was followed by a catch-up pneumococcal vaccination and the number of doses given. The high carriage prevalence in Sweden might be explained by the late introduction of vaccination to the national immunization program (2009). The introduction of the vaccine without a catch-up campaign[24] does not allow individuals who exceeded the immunization age to still be vaccinated. In Croatia and Spain pneumococcal vaccine was given only to patients belonging to specific risk groups. This strategy reduces the number of vaccinated individuals and might increase the number of colonized patients. In Belgium, Croatia and The Netherlands, only PCV-7 vaccine is used[24]. The emergence of nonvaccine serotypes after the introduction of this vaccine (replacement phenomenon) might explain the high prevalence of pneumococcal carriage in these countries[5].

No significant difference was noticed between carriage prevalence in vaccinated and unvaccinated individuals. Our results support earlier studies with comparable outcomes.

In a carriage study carried on 683 subjects from 217 households in England and Wales, van Hoek *et al.* found no change in pneumococcal carriage and a decrease of PCV7 vaccine serotypes transmission in vaccinated and unvaccinated individuals 6 years after the introduction of PCV7 vaccine, compared with carriage prevalence before the introduction of this vaccine[25]. In another carriage study, performed by Principi *et al.*, enrolling 2076 children and adolescents from Italy, it was found that pneumococcal prevalence was higher in vaccinated individuals than in unvaccinated ones[26]. Moreover, they found that the prevalence of vaccine serotypes was comparable to this nonvaccine serotypes, suggesting no role of serotypes replacement. Taken together with these results, our results might be explained by the emergence of none-vaccine serotypes that cocirculated but were suppressed by vaccine serotypes. Another explanation is the possibility that the protection effect of vaccination against pneumococcal carriage might decrease over the time, allowing a re-emergence of vaccine serotypes in vaccinated patients. In two separate mice model studies, researchers suggested that influenza virus infection enhanced the carriage of *S. pneumoniae* by an increase in pneumococcal load in carriers and the increase of susceptibility in influenza infected cohoused subjects[27,28]. This suggests that influenza vaccination, by reducing the spread of infected droplets, might reduce pneumococcal transmission and, consequently, pneumococcal carriage. However, our data did not confirm this suggestion.

In this study, a large variability was found between the participating countries in resistance prevalence to the commonly used antimicrobial agents in outpatients (penicillin, macrolide and tetracycline). Belgium, France, Spain and Croatia that in 2010 had a penicillin consumption of 16.3, 15.6, 12.6 and 9.5 defined daily dose (DDD) per 1000 inhabitants per day, respectively[29], had the highest rate of antibiotic resistance to this antibiotic class. The Netherlands, with a penicillin consumption of 4.4 DDD per 1000 inhabitants per day[29], had one of the lowest penicillin resistance. In Sweden, despite the higher rate of penicillin consumption compared with The Netherlands (7.1 DDD per 1000 inhabitants per day)[29], the resistance rate to this agent was the lowest. This might be explained by the use in Sweden of narrow spectrum penicillins (more than 60% of the used penicillins). Furthermore, our data showed a high resistance to clarithromycin in Croatia. The consumption of this agent represents, with roxithromycin, more than 30% of the macrolides, lincosamides and streptogramin use in Croatia[30].

Differences in antibiotic consumption between the participating countries might not be the only explanation of the resistance rate variations. In fact, Spain with tetracycline consumption of 0.7 DDD per 1000 inhabitants per day[29], has the highest resistance rate, whereas Sweden with a more than four-fold consumption (3.3 DDD per 1000 inhabitants per day)[29], had the lowest resistance prevalence. These results underscore that other factors than antibiotic pressure, socioeconomic, behavioural and cultural determinants and differences in healthcare policies among the participating countries[31], as well as clonal spread of

resistant microorganisms[32] and antibiotic cross-resistance between members of antibiotic classes[33] might influence antibiotic resistance prevalence.

Our study highlights the prevalence of antibiotic resistant *S. pneumoniae* carriage strains in the outpatient setting in nine European countries. These data are important to make a rational empiric choice and to create antibiotic prescription guidelines for the treatment of *S. pneumoniae* by GPs (i.e., the outpatient setting). As the majority of human antibiotic use is prescribed outside healthcare settings[16,34], optimal use of antibiotics based on actual antibiotic resistance data is important to support the control of antibiotic resistance problem. Professionals involved in the set-up/reviewing of existing national antibiotic prescription guidelines have to take into account the data presented of the country involved.

CONCLUSION

A low *S. pneumoniae* nasal carriage prevalence was assessed in general practice patients in nine European countries. A large variability in antibiotic resistance was noted among the participating countries, with the highest overall resistance in Spain and the lowest in Sweden.

EXECUTIVE SUMMARY

- During the last few decades, antimicrobial drug resistance has increased worldwide.
- The prevalence of *Streptococcus pneumoniae* nasal carriage was assessed across nine European countries (2.9%).
- Pneumococcal carriage prevalence was higher in children (4–9 years old [27.2%]) than in individuals older than 10 years (1.9%).
- A large variation was found in antibiotic resistance across the participating countries.
- Resistance to penicillin was ranged between 3.9% in Sweden and 31.7% in Spain.
- A large variation was found in multidrug resistance prevalence. The highest was found in Spain and the lowest in Sweden.

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5 Distribution of serotypes and patterns of antimicrobial resistance among commensal *Streptococcus pneumoniae* in nine European countries

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ABSTRACT:

Streptococcus pneumoniae is a commensal of the human upper respiratory tract and a major cause of morbidity and mortality worldwide. This paper presents the distribution of serotypes and antimicrobial resistance in commensal *S. pneumoniae* strains cultured from healthy carriers older than four years of age in nine European countries.

Methods:

Nasal swabs from healthy persons (age between 4 and 107 years old) were obtained by general practitioners from each country from November 2010 to August 2011. Swabs were cultured for *S. pneumoniae* using a standardized protocol. Antibiotic resistance was determined for isolated *S. pneumoniae* by broth microdilution. Capsular sequencing typing was used to identify serotypes, followed by serotype-specific PCR assays in case of ambiguous results.

Results:

Thirty-two thousand one hundred sixty-one nasal swabs were collected from which 937 *S. pneumoniae* were isolated. A large variation in serotype distribution and antimicrobial resistant serotypes across the participating countries was observed. Pneumococcal vaccination was associated with a higher risk of pneumococcal colonization and antimicrobial resistance independently of country and vaccine used, either conjugate vaccine or PPV 23).

Conclusions:

Serotype 11A was the most common in carriage followed by serotypes 23A and 19A. The serotypes showing the highest resistance to penicillin were 14 followed by 19A. Serotype 15A showed the highest proportion of multidrug resistance.

BACKGROUND

Streptococcus pneumoniae (pneumococcus) is a commensal of the human upper respiratory tract[1] and a major cause of morbidity and mortality worldwide. Pneumococcal disease has various manifestations including otitis media, pneumonia, septicemia and meningitis[2]. Incidence of the disease is highest at extremities of life: in very young children and in the elderly. Based on capsular polysaccharide chemistry and immunogenicity, over 90 distinct capsular types (serotypes) have been identified so far[3]. Two types of pneumococcal vaccines are commercially available: a pneumococcal polysaccharide vaccine (PPV) and pneumococcal conjugate vaccines (PCVs). Currently used PPV (Pneumovax 23) was first introduced in 1983 and by targeting 23 pneumococcal serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9 N, 9 V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F) has valency broader than any PCV. However, unlike PCVs, PPV is not effective in very young children. Three different PCV's are in use. The seven-valent vaccine (Prevnar, PCV7) was introduced in 2000, followed in 2009 by ten-valent (Synflorix, PCV10) and in 2010 by thirteen-valent (Prevenar13, PCV13) vaccines. PCV7 comprises serotypes 4, 6B, 9 V, 14, 18C, 19F and 23F, PCV10 includes additional serotypes 1, 5 and 7F, and PCV13 contains extra serotypes 3, 6A and 19A. The introduction of PCVs into infant immunization programs has led to a major decrease in vaccine serotype (VT) disease in vaccinated children but to a various degree also resulted in herd effects across whole populations[4]. These indirect effects in unvaccinated individuals were caused by reduction of VT strain carriage in young children who are the main reservoir and main transmitters of pneumococci[5,6]. PCVs may also contribute to a decrease in overall incidence of antimicrobial resistant pneumococcal disease[7]. This effect was augmented by the fact that VT strains were usually more resistant to antibiotics compared to non-vaccine (NVT) strains[7]. However, long term benefits of PCVs were eroded by the emergence of (multidrug-resistant) NVTs in carriage and in disease, the so-called vaccine-induced serotype replacement[8]. There are differences in the timing of vaccine introduction, vaccination policies and vaccine coverage among countries that implemented PPV and PCVs into their National Immunization Programs (NIPs)[9]. This could result in differences in direct and herd effects, in serotype replacement and in resistance to anti-pneumococcal drugs in strains circulating in carriage and causing disease. By the end of 2012, 26 of the 53 countries of the European region vaccinated infants with PCVs in their NIPs[9]. In 2011, the PCVs coverage in infants' vaccination (after administration of a minimum of 3 doses of vaccine by the age of 2 years) was more than 90% in these 26 European countries[9]. To our knowledge, there are no studies that evaluated the serotype distribution in commensal pneumococcal populations and its relationship with antimicrobial resistance across European countries as well as the impact of pneumococcal vaccination in a multicentre surveillance on pneumococcal carriage. In this report, we assessed serotypes and resistance to antimicrobial agents of *S. pneumoniae* strains cultured from healthy carriers older than

four years of age in nine European countries that differed in the timing of pneumococcal vaccines introduction, vaccines schedule and coverage, vaccines used (PCVs and PPV) and presence of catch-up campaigns. Results were analysed for impact of pneumococcal vaccination (immunization status) on individual person level and for effects that could be linked to differences in pneumococcal vaccination programs.

METHODS

Study design

S. pneumoniae strains were cultured from samples collected from November 2010 to August 2011, as a part of the 'The Appropriateness of prescribing antimicrobial agents in primary health care in Europe with respect to antimicrobial resistance' (APRES) study as described by van Bijnen et al.[10]. Briefly, general practitioners (GP) from Austria, Belgium, Croatia, France Hungary, Spain, Sweden, the Netherlands and the United Kingdom (9 countries, 20 GPs per country), were each asked to provide nasal swabs from 200 healthy persons (with no history of antibiotic therapy or hospitalization in the previous three months), older than 4 years (except for UK, where for ethical reasons patients were older than 18 years). Within 48 h after collection swabs were transported to each national laboratory for further processing, with the exception of samples collected in France that were all sent to the Dutch national laboratory at Maastricht University Medical Centre (MUMC). On arrival to diagnostic labs, samples were cultured for *S. pneumoniae* using a standardized protocol[10]. Putative *S. pneumoniae* isolates from all participating countries were sent to the MUMC in skimmed milk at -80 °C for further analysis. All participants provided written informed participatory consent and in the case of children aged less than 16, their parents or guardians provided written informed participatory consent on their behalf. All methods were approved by named institutional committee and were performed in accordance with relevant guidelines and regulations.

Capsular sequence typing (CST)

CST was performed in the National Institute for Public Health and the Environment, Bilthoven, The Netherlands as previously described by Elberse et al.[11]. Briefly, culture of *S. pneumoniae* in Brain Heart Infusion broth with 0,5% yeast extract, incubated overnight at 37 °C and 5% CO2, was heated at 95 °C for 10 min and used as a DNA template in PCR to amplify a fragment of the capsular wzh gene. Amplicons were sequenced by BaseClear BV, Leiden, The Netherlands. Sequences generated were assembled, edited and trimmed using Bionumerics v6.1 (Applied Maths, Sint-Maartens-Latem, Belgium) and assigned a capsular sequence type (CT) using the CST database (https://www.rivm.nl/mpf/typingtool/spn/). CT is a composite assignment, in which the first part represents the serotype assessed

by conventional serotyping (Quellung) followed by the number representing a consecutive wzh allele identified among strains of a given serotype[11]. When an allele not yet recorded in CST database was found, serotype of an isolate was determined by Quellung method at the Netherlands National Reference Laboratory for Bacterial Meningitis (NRBM), Amsterdam, the Netherlands. For statistical analysis feasibility, isolates were grouped by serotype and not by individual CTs. For CTs associated with a single serotype (61 of the 79 CST types found in this study), grouping was based on the first part of the CST assignment. For CTs represented by multiple serotypes, particularly those concerning vaccine serotypes, additional PCR and/or PCR-sequencing assays were performed as follows. For CTs representing serogroup 6 isolates, distinction between 6A/B and 6C/D was made using primers specific for wciNbeta, after which 6A and 6B were distinguished by PCR sequencing of wciP as described[12]. For CTs 15B-01, 15C-01, 22F-01, 23F-01, and 24F-01, the appropriate serotype-specific primers from the protocol of the CDC for multiplex PCR serotype deduction (http://www.cdc.gov/streplab/pcr.html) were used in single PCR. For isolates of CT 34–01, PCR-sequencing of the wzg gene was performed to distinguish between serotype 17 and 34 isolates. Finally, distinction between CT 25F-02-associated serotypes 25A/F and 38 was achieved by PCR-amplification of the wcyV gene. When no gene was amplified with the used primers and no CST could not be assigned, isolates were considered as not typable. Primers used for these additional PCR assays are shown in Additional file 1: Table S5.

Antimicrobial susceptibility

All isolates were tested for susceptibility to ceftazidime, clarithromycin, clindamycin, penicillin, tetracycline and trimethoprim-sulfamethoxazole. Minimal inhibitory concentrations of these drugs were assessed by broth microdilution method in accordance with EUCAST guidelines and EUCAST epidemiological cut-offs were applied as breakpoints[13]. Multidrug resistance was defined as resistance to three or more classes of antimicrobial agents.

Data analysis

In order to study the effect of vaccination on pneumococcal carriage and antimicrobial resistance, a multilevel logistic regression was performed. To account for non-random clustering of our data at family level and to control whether age and gender affects pneumococcal carriage prevalence, a 3-level multilevel logistic regression model (country, GP and patient) was estimated using MLWIN software package. Statistical analysis was performed using the PASW software package 19.0, with p-value < 0.05 considered statistically significant.

RESULTS

Participants and bacterial strains

Table 1 shows the demographic background of the participating individuals. In total, 31,625 individuals were recruited, varying from 3969 in Spain to 3025 in Belgium. The proportion of males ranged between 39.9% in Croatia and 45.6% in Belgium. Working in the health-care sector ranged between 2.2% in Hungary to 16.5% in Sweden and working in nursery between 1% in Croatia to 4.1% in Sweden. Living with children < 5 years of age was between 10.1% in Belgium and 16.1% in Sweden. Among all participants, 937 were identified as pneumococcal carriers and ranged between170 in France to 36 in UK.

Table 1. Demographic overview of the participant individuals (in %)

	Austria	Belgium	Croatia	France	Hungary	Netherlands	UK	Spain	Sweden
Gender of patient									
Man	42.8	45.6	39.9	44.5	44.2	41.6	40.6	41.6	42.3
Women	55.8	53.9	58.5	54.5	54.2	55.7	58.8	58.4	57.2
Unknown	1.4	0.5	1.6	1.0	1.6	2.7	0.6	0.0	0.5
Age in year category									
4–9	1,2	1,3	6.0	3,3	8,5	3,2	0.0	4,9	5,4
10–17	2,2	3,1	8,2	4,7	15,6	5,3	0.0	5,9	5,4
18–29	15,8	8,2	9,2	11,5	9,6	11,1	14,7	9,2	9,6
30–60	50,2	40,9	38,8	45,2	36,5	45,7	46,3	41,5	37,7
>60	29,8	46,5	37,4	34,9	29,1	33,9	38,8	38,5	41,6
Job in Healthcare sector									
Yes	6	4,6	3,7	3,1	2,2	10,3	8,8	3,3	16,5
Job in nursery									
Yes	1,9	2,2	1	2,1	3,4	3	2,4	2	4,1
None from the above-mentioned jobs or unknown									
Yes	92,1	93,2	95,3	94,8	94,4	86,7	88,8	94,8	79,4
Do you live with children of 5 years and younger?									
Yes	11,8	10,1	13,6	14,2	11,1	13,7	13,9	14	16,1
No	87,1	89,9	85,6	85,2	86,2	84,6	85,2	82	82,8
Unknown	1,1	0	0,8	0,6	2,7	1,7	0,9	4	1,1

Pneumococcal vaccination policy in the participating countries

Seven of the nine participating countries had introduced a pneumococcal conjugate vaccine in their NIP before November 2010 (Additional file 1: Table S1a) and used PCV7 during one (Sweden) to six years (Austria) prior to study onset. The exceptions were Croatia and Spain, two countries that only have risk-based pneumococcal vaccination programs. France was

the only country with vaccination of risk group patients in the NIP in addition to infants. In Austria, Belgium, France and Sweden, PCV13 and PPV23 were used in adults and in risk populations (Additional file 1: Table S1b). In the Netherlands and Spain, only PPV23 was used in adult and on risk-based immunization. In Hungary, no recommendations were in place for vaccination of elderly and risk patients (Additional file 1: Table S1b).

Vaccination status among participants

Among all participants, 10.3% (n = 3316) were vaccinated, 79.0% (n = 25,404) were not, and 10.7% (n = 3441) had an unknown vaccination status. Among carriers, Spain had the highest percentage of vaccinated individuals (30.5%) and Croatia the lowest (0.7%) (Table 2). In all countries except Austria and Belgium, participants between 4 and 9 years old

Table 2. Vaccination status of participants per country

	Total participants population	**Vaccinated participants N (%)**	**Non-vaccinated participants N (%)**	**Participants with unknown status N (%)**	***S. pneumoniae* isolated N (%)**	**Vaccinated carriers %**
Austria	3309	193 (5.8)	2254 (68.1)	862 (26.1)	38 (1.1)	5.3
Belgium	3025	493 (16.3)	2475 (81.8)	57 (1.9)	44 (1.5)	15.9
Croatia	3952	27 (0.7)	3763 (95.2)	162 (4.1)	134 (3.4)	0.7
France	3857	197 (5.1)	3368 (87.3)	292 (7.6)	170 (4.4)	13.5
Hungary	3832	134 (3.5)	3484 (90.9)	214 (5.6)	116 (3.0)	5.2
Netherlands	3847	89 (2.3)	3012 (78.3)	746 (19.4)	129 (3.4)	6.2
Spain	3969	1109 (27.9)	2691 (67.8)	169 (4.3)	167 (4.2)	30.5
Sweden	3214	304 (9.5)	2411 (75.0)	499 (15.5)	103 (3.2)	6.8
UK	3156	770 (24.4)	1946 (61.7)	440 (13.9)	36 (1.1)	27.8
Total	32,161	3316 (10.3)	25,404 (79.0)	3441 (10.7)	937 (2.9)	12.3

were more frequently vaccinated than those older than 10 years ($p < 0.0001$; Additional file 1: Table S2). Serotype carriage in the participating countries Serotype 11A was the most common in carriage in the study population (n = 60) followed by serotypes 23A (n = 58), 19A (n = 52), 3 (n = 51), 6C (n = 44) and 23B (n = 39). All these serotypes were considered as non-PCV types since none was targeted by either PCV7 or PCV10 used in the study populations at the time of sample collection. These serotypes were followed by 19F (n = 38) and 23F (n = 37), targeted by all commercially available pneumococcal vaccines. Serotype 23F was the most frequent among *S. pneumoniae* carriage isolates in Croatia (n = 15 of 134, 11.2%) one of two countries without PCVs in NIP. Serotype 23F was along with serotype 11A (n = 11 of 103, 10.7% each) also most common in carriage in Sweden, country with shortest PCV immunization program at the time the study was conducted (Additional file 1: Table S1a and b). Serotype 6C was the most common in the Netherlands (n = 11 of 129,

8.5%) and Spain (n = 11 of 167, 6.6%). Serotype 3 (n = 6, 14.0%), 15A (n = 6, 12.8%), 11A (n = 18, 10.6%) and 10A (n = 5, 13.9%) were the most common in Austria, Belgium, France and UK, respectively. Serotypes 23A and 15 B/C (n = 9, 7.8%) were the most common in Hungary (Table 3 and Additional file 1: Table S3). Serotypes 17F and 22F, both targeted by

Table 3. Serotypes distribution by country (%). Serotypes listed in order from highest to lowest in frequency among all *S. pneumoniae* strains cultured in the study as reported in the last column

Serotype	Austria N = 38	Belgium N = 44	Croatia *N* = 134	France *N* = 170	Hungary *N* = 116	Netherlands *N* = 129	Spain *N* = 167	Sweden *N* = 103	UK *N* = 36	Total *N* = 937
11A	5.3	6.8	2.2	**10.6**	6.9	7.0	2.4	**10.7**	5.6	**6.4**
23A	2.6	2.3	6.7	7.6	**7.8**	4.7	4.8	7.8	8.3	6.2
19A	2.6	2.3	7.5	8.2	0.9	6.2	4.8	5.8	8.3	5.5
3	**15.8**	0.0	3.7	9.4	4.3	4.7	5.4	2.9	2.8	5.4
6C	5.3	4.5	3.0	5.3	0.9	**8.5**	**6.6**	1.9	5.6	4.7
NT	5.3	9.1	3.7	4.7	3.4	7.0	3.6	3.9	2.8	4.6
23B	5.3	6.8	0.0	7.1	0.0	7.0	5.4	1.9	5.6	4.2
19F	5.3	0.0	6.7	3.5	5.2	3.1	3.0	4.9	2.8	4.1
23F	5.3	0.0	**11.2**	0.0	0.9	2.3	1.2	**10.7**	8.3	3.9
15B/C	7.9	2.3	0.7	2.4	**7.8**	1.6	4.8	3.9	8.3	3.7
35B	2.6	2.3	2.2	5.3	6.9	1.6	3.6	1.9	5.6	3.6
35F	2.6	4.5	3.0	2.9	5.2	1.6	1.2	8.7	5.6	3.5
22F	2.6	4.5	1.5	4.7	2.6	3.9	2.4	4.9	5.6	3.4
6A	7.9	0.0	7.5	1.2	3.4	3.1	1.2	2.9	0.0	3.0
10A	0.0	2.3	0.0	1.2	6.0	4.7	4.2	0.0	**13.9**	3.0
6B	0.0	0.0	6.7	0.6	1.7	6.2	1.2	2.9	0.0	2.7
15A	2.6	**13.6**	0.7	3.5	0.0	1.6	3.6	1.9	0.0	2.6
17F	0.0	2.3	0.0	3.5	3.4	4.7	3.0	0.0	2.8	2.5
24F	0.0	4.5	0.0	2.9	5.2	0.8	4.2	0.0	0.0	2.2
18C	0.0	0.0	0.7	0.0	6.0	3.1	1.2	5.8	0.0	2.1
16F	0.0	2.3	0.7	0.6	4.3	0.8	5.4	1.0	0.0	2.0
7F	2.6	6.8	1.5	1.2	0.0	2.3	3.0	1.9	0.0	1.9
9 N	0.0	2.3	5.2	0.6	0.9	2.3	1.2	1.9	0.0	1.8
37	2.6	0.0	3.0	0.0	1.7	3.1	3.0	1.0	0.0	1.8
14	0.0	2.3	3.7	0.0	1.7	0.0	4.2	1.0	0.0	1.7
33F	0.0	0.0	0.0	2.4	0.9	2.3	1.8	3.9	0.0	1.6
34	0.0	0.0	2.2	0.0	3.4	0.8	1.8	1.9	0.0	1.4
21	0.0	0.0	0.0	3.5	0.9	0.0	3.0	0.0	2.8	1.4
31	0.0	9.1	0.7	1.2	2.6	0.0	1.2	0.0	0.0	1.3

UK United Kingdom; in bold: serotypes with the highest frequency in a country, *NT* not typable. Only serotypes represented by more than 10 strains among all *S. pneumoniae* strains cultured in the study are reported. Complete overview of serotypes is shown in Additional file 1: Table S3

PPV23 vaccine, were significantly associated with age older than 10 years (p = 0.03 and p = 0.01, respectively). Serotype 23F (PCV7 serotype) was significantly associated with age 4–9 years (p = 0.01). Other serotypes were not associated with any age category (Additional file 1: Table S4).

Vaccination effect on pneumococcal colonization and serotypes Among the pneumococcal carriers (all serotypes considered), 115 were vaccinated and 727 were not vaccinated, while the vaccination status was unknown for 95 individuals. Table 4 shows the effect of pneumococcal vaccination on pneumococcal carriage. Being vaccinated was associated with a higher risk of pneumococcal colonization. None of the variables i.e. vaccine regimen, the presence of a catch-up campaign, the year of the vaccine implementation, the vaccine type, or the extent of vaccination program had separately a significant effect on pneumococcal colonization. Of 937 pneumococcal strains cultured from carriers, 170 (18.1%) were of PCV10 types. Of these 149 (15.9% of the total number of strains) were of PCV7. There were significantly fewer carriers of PCV10 serotypes among vaccinated (11 of 115, 9,6%) compared to non-vaccinated individuals (138 of 727, 19.0%), p = 0.01 (Table 5). These findings in combination with Table 4 suggest that vaccinated individuals have more chance to be colonized by non-vaccine serotypes.

Table 4. Relationship between vaccine-related variables (number of doses, presence of catch up campaign, year of implementation, vaccine type and extent of vaccination program), and pneumococcal carriage in the participating individuals

Models	Reference variable	Comparative variables	OR*	*p* value**
Basic model:				
Model 1: vaccination	Vaccinated	Not vaccinated	0.70 (0.55–0.89)	0.00**
		Unknown vaccination status	0.76 (0.54–1.05)	0.10
Models after addition vaccination program characteristics separately:				
Model 2: vaccine dose	2 + 1[b]	3 + 1[a]	1.39 (0.66–2.92)	0.37
Model 3: catch up campaign conduction	Catch up campaign conducted[c]	No catch-up campaign conducted[d]	0.76 (0.54–1.05)	0.41
Model 4: period since the vaccine implementation	Implemented for one year	implemented for more than one year	1.05 (0.88–1.25)	0.53
Model 5: type of vaccine	PCV 7	PCV 7, PCV10 and PCV 13	1.00 (0.39–2.55)	0.98
		PCV 10 and PCV 13	0.80 (0.31–1.87)	0.65
Model 6: extent of vaccination program	risk based and universal [e]	Risk based [g]	0.57 (0.17–1.87)	0.36
		Universal [f]	0.43 (0.14–1.33)	0.14

*OR adjusted for countries, age and general practitioner; **$p < 0.05$ is significant

Table 5. distribution of vaccine and non-vaccine serotypes in vaccinated and non-vaccinated among the study population

Serotype carried	Vaccinated	Non-vaccinated	Unknown	Total
PCV7 serotypes [a]	9	121	19	149
PCV10-PCV7 serotypes [b]	2	17	2	21
PCV13-PCV10 serotypes [c]	15	103	13	131
No vaccine type or PCV serotypes [d]	0	2	0	2
Non-PCV13 vaccine types	88	477	61	626
PPV23 serotypes [f]	50	396	54	500
Non-PPV23 vaccine types	65	331	41	437
Not determinate	1	7	0	8

[a]serotypes 4, 6B, 9V, 14, 18C, 19F and 23F; [b] serotypes 1, 5 and 7F; [c] serotypes 3, 6A and 19A; [d] serotypes 18For 18C (the serotyping method is not able to differentiate between serotypes 18F(NVT) and 18C (VT)); [f] serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F

Table 6 shows the antimicrobial resistance per serotypes. The highest resistance proportion to ceftazidime and penicillin was noticed among serotype 14 strains (13 of 16, 81.3%). Serotype 14 was the most frequent serotype showing resistance to penicillin, followed by serotype 19A and 15A. Among serotypes with more than 10 isolates, paediatric serotypes (6B, 9 V, 14, 19F and 23F) were more resistant to antimicrobial agents than non-paediatric serotypes (1, 3, 4, 7F). The serotypes presenting the highest proportion of multidrug resistance were 15A followed by 19A and 14. Serotypes 6C, 23B, 15A, 19A, 6A and 19F were significantly more prevalent whereas serotypes 22F, 23A, 3 and 14 were significantly less prevalent in the multidrug resistant fraction compared to the total study collection (Table 6).

Vaccination effect on antimicrobial resistance carriage

Table 7 shows the effect of vaccination on carriage of antimicrobial resistant pneumococcal isolates. Being vaccinated enhanced carriage of isolates resistant to at least one of the tested antimicrobial agents (model 1, OR = 0.60, p = 0.03). After adding different vaccination program characteristics (vaccine dose, conduction of catch-up campaign, period since vaccine was implemented, vaccine type and extent of vaccination program) separately, none of these could explain alone the vaccination effect (models 2–6).

Table 6. Distribution of antimicrobial resistance by serotype. Only serotypes represented by more than 10 isolates are reported. Correlation between frequency of serotypes in study population and in MDR fraction is given in the last column

Serotypes	N (%)	Ceftazidime (%)	Clarithromycin (%)	Clindamycin (%)	Penicillin (%)	Tetracyclin (%)	Trimethropim/ sulfamethoxazole (%)	MDR Fraction N (%)	*p* value
11A	60 (6.4)	8.3	6.7	3.3	5.0	0.0	10.0	5 (8,3)	0.090
23A	58 (6.2)	0.0	19.0	19.0	1.7	19	24.1	2 (3,4)	0.007*
19A	52 (5.5)	67.3	53.8	50	67.3	55.8	30.8	32 (61,5)	< 0.001*
3	50 (5.3)	0.0	0.0	0.0	0.0	0.0	2.0	0 (0,0)	0.001*
6C/D	44 (4.7)	6.8	29.5	27.3	27.3	25.0	0.0	11 (25,0)	0.001*
23B	39 (4.2)	12.8	0.0	0.0	30.8	0.0	28.2	11 (28,2)	0.093
19F	37 (3.9)	32.4	56.8	48.6	37.8	54.1	18.9	14 (37,8)	0.001*
23F	37 (3.9)	13.5	16.2	10.8	16.2	10.8	16.2	6 (16,2)	0.934
15B/C	35 (3.7)	2.9	8.6	0.0	22.9	11.4	31.4	3 (8,6)	0.255
35B	34 (3.6)	38.2	8.8	5.9	38.2	2.9	5.9	3 (8,8)	0.283
35F	33 (3.5)	6.1	3.0	3.0	0.0	0.0	0.0	1 (3,0)	0.050
22F	32 (3.4)	0.0	0.0	0.0	0.0	0.0	0.0	0 (0,0)	0.017*
6A	28 (3.0)	39.3	39.3	10.7	39.3	10.7	25.0	10 (35,7)	0.015*
10A	28 (3.0)	7.1	10.7	7.1	7.1	10.7	10.7	2 (7,1)	0.243
6B	25 (2.7)	24.0	32.0	28.0	20.0	28.0	24.0	8 (32,0)	0.081
15A	24 (2.6)	58.3	62.5	62.5	62.5	50	8.3	15 (62,5)	< 0.001*
17F	23 (2.5)	8.7	4.3	4.3	8.7	4.3	0.0	1 (4,3)	0.172
24F	21 (2.2)	28.6	28.6	28.6	28.6	28.6	19.0	6 (28,6)	0.260
18C	20 (2.1)	0.0	0.0	0.0	0.0	0.0	10.0	0 (0,0)	0.079
16F	19 (2.0)	0.0	15.8	15.8	0.0	15.8	42.1	1 (5,3)	0.282
37	17 (1.8)	0.0	0.0	0.0	0.0	0.0	5.9	0 (0,0)	0.117

Table 6. Distribution of antimicrobial resistance by serotype. Only serotypes represented by more than 10 isolates are reported. Correlation between frequency of serotypes in study population and in MDR fraction is given in the last column (continued)

Serotypes	N (%)	Ceftazidime (%)	Clarithromycin (%)	Clindamycin (%)	Penicillin (%)	Tetracyclin (%)	Trimethropim/ sulfamethoxazole (%)	MDR Fraction N (%)	*p* value
7F	17 (1.8)	0.0	0.0	0.0	0.0	0.0	0.0	0 (0,0)	0.117
9 N	17 (1.8)	0.0	0.0	0.0	0.0	0.0	0.0	0 (0,0)	0.117
14	16 (1.7)	81.3	31.3	25.0	81.3	18.8	43.8	8 (50,0)	0.001*
33F	15 (1.6)	0.0	33.3	33.3	0.0	26.7	6.7	1 (6,7)	0.462
21	13 (1.4)	0.0	0.0	0.0	7.7	0.0	0.0	0 (0,0)	0.201
31	13 (1.4)	0.0	0.0	0.0	0.0	0.0	0.0	0 (0,0)	0.201
34	13 (1.4)	61.5	0.0	0.0	30.8	0.0	23.1	0 (0,0)	0.201

*$p < 0.05$ is significant; MDR = Multidrug resistance; In bold: serotypes presenting the most MDR isolates; underlined: highly resistance

Table 7. Effect of pneumococcal vaccination and vaccination variables (dose, conduction of catch up campaign, year of implementation, vaccine type and extent of vaccination program), on carriage of antimicrobial resistant pneumococcus (resistant to at least one antibiotic) in the participating individuals

Models	Reference variable	Comparative variables	OR*	*p* value**
Basic model:				
Model 1: vaccination	Vaccinated	Not vaccinated	0.60 (0.37–0.96)	0.03**
		Unknown vaccination status	0.63 (0.33–1.18)	0.15
Models after addition vaccination program characteristics separately:				
Model 2: vaccine dose	2 + 1 [b]	3+1 [a]	0.91 (0.35–2.32)	0.84
Model 3: catch up campaign conduction	Catch up campaign conducted [c]	No catch-up campaign conducted [d]	1.57 (0.65–3.79)	0.32
Model 4: period since vaccine implementation	Implemented for one year	implemented for more than one year	1.10 (0.90–1.34)	0.34
Model 5: vaccine type	PCV 10	PCV 7	1.23 (0.39–3.87)	0.72
		PCV 13	0.87 (0.27–2.72)	0.81
Model 6: extent of vaccination program	risk based and universal e	Universal [f]	0.69 (0.17–2.81)	0.61
		Risk based [g]	1.07 (0.24–4.69)	0.92

*$p < 0.05$ is significant

[a]) Austria, Hungary, Netherlands, Spain, [b]) Belgium, France, Hungary, Sweden, UK, [c]) Belgium, FR, Hungary, UK, [d]) Austria, Hungary, Netherlands, Spain, Sweden, [e]) France, [f]) Austria, Belgium, Hungary, Netherlands, Sweden, UK, [g]) Hungary, Spain

DISCUSSION

In this report, we assessed serotypes and resistance to antimicrobial agents of *S. pneumoniae* strains cultured from healthy carriers older than four years of age in nine European countries that differed in the timing of pneumococcal vaccines introduction. A large variation was found in serotype distribution in the participating countries and we observed difference in antimicrobial resistance including multidrug resistance, among these serotypes. The major finding was that pneumococcal vaccination was associated with a high risk of non PCV10 serotypes carriage. This study was carried out on a large and well documented population, covering different age groups. To eliminate intra-laboratory variations, all methods were conducted in a central laboratory per method (susceptibility testing in MUMC, molecular serotyping in RIVM and conventional serotyping using Quellung method in NRBM). These points allowed us to accurately address the goals of this study. However, drawbacks were differences in numbers of strains collected and tested per country with significantly fewer strains from UK, Austria and Belgium ($p < 0.0001$) compared to any other participating sites. This could limit the generalization of our findings to the entire population in those countries. Possible explanation for differences in prevalence of carriage could be differ-

ences in age of sampled individuals (e.g. no minors under age of 18 years were sampled in UK) or in patterns of social contacts with very young children: carriage rates in parents of young children are reported to be few-fold higher compared to childless adults[14]. This study cannot be used to assess the spread and antimicrobial resistance in *S. pneumoniae* among nonvaccinated persons. This would require a longitudinal study. Furthermore, many factors determine carriage and resistance (e.g. antibiotic use was exclusion criterium in this study, seasonal variations in pneumococcal carriage), so one needs to be careful with drawing general conclusion based on these results. For feasibility and costs considerations, capsular sequence typing was used in the primary method of serotyping. When highly frequent or vaccine CST's could not be differentiated, additional PCR's were performed. Conventional serotyping with type-specific sera was performed only when results generated with the CST method were equivocal. Our results support CST as a method alternative to the conventional Quellung serotyping in epidemiological studies. PCV-7 was licensed in Europe in 2001. Higher valency vaccines were introduced since then (PCV-10 and PCV-13 in 2009 and 2010, respectively). If our results represented the effects of any vaccine in the studied population, these should be likely indirect (herd) effects of PCV7, and without any contribution of PCV13 since this vaccine was implemented after our study onset. The most frequent serotypes among the carriage isolates were 11A, 19A, 3, 6C, 23A and 23B. All these serotypes were PPV vaccine or non-vaccine types.

The most prevalent PCV7 vaccine type was 19F. These results might be explained by a replacement in the serotypes carriage. Our results are in concordance with an earlier report have reporting the predominance of non-vaccine serotypes, among which serotype 11A, in 336 paediatric patients in Ireland[15]. In contrast to previous carriage studies[16-18] which reported a decrease in pneumococcal carriage after vaccine implementation, our analyses show a higher risk of pneumococcal colonization upon vaccination. Most of the studies dealing with the effect of vaccination on pneumococcal carriage were conducted in vaccinated children and within few years after vaccination[19-24]. This reflects probably the immediate immunity and indirect effect of vaccination on the population. Our findings might be explained by the emergence of nonvaccine serotypes that co-circulated but were suppressed by vaccine serotypes. Another possible explanation could be that the protective effect of vaccination against pneumococcal carriage might decrease over time, allowing a re-emergence of vaccine serotypes in vaccinated patients. These results are supported by Principi et al.,[25] who found that pneumococcal prevalence was higher in vaccinated individuals than in unvaccinated ones in a study enrolling 2076 children and adolescents from Italy. Our results have shown that pneumococcal vaccination was associated with an increase in the prevalence of pneumococcal antimicrobial resistance. This may be explained by the fact that a vaccination might facilitate the introduction of new pneumococcal serotypes, which are more resistant to antimicrobial agents[26] due to the replacement of vaccine serotypes[27]. A large variation in serotype distribution as well as in antimicrobial resistant se-

rotypes was observed in the participating countries. In some countries, some serotypes were associated to resistance (19A and 14 resistance penicillin and ceftazidime). This might be due to differences in antimicrobial agents use between the participating countries[28], clonal spread of resistant microorganisms and antimicrobial cross-resistance between members of antimicrobial agents' classes. This variation might justify the necessity of implementation of guidelines on antimicrobial agents use at country level.

Serotype 19A, a PCV-13 vaccine serotype, is the most frequent causative agent of invasive pneumococcal diseases[29]. In our study, this serotype was also one of the most frequent carriage isolates (5.5%, n = 52) and one of the most resistant serotypes to all antimicrobial agents tested. These results are in accordance with earlier reports[30]. Hackel et al., reported that serotype 15A was highly resistant to erythromycin and penicillin in clinical strains worldwide[31]. In our study, 15A representing 2.6% of all pneumococcal isolates (n = 24), was one of the most resistant serotypes to penicillin, ceftazidime, clarithromycin, clindamycin and tetracycline. Serotype 35B was, after 15A, the second most resistant non-vaccine serotype to penicillin and ceftazidime (38.2% of serotype 35B strains were resistant for both antibiotics). In the US, this serotype is becoming the dominating serotype in carriage and invasive pneumococcal disease. This is due to a clonal shift after the implementation of PCV7 and a spread of a β –lactam resistant clonal complex after the implementation of PCV13[8,32].

CONCLUSIONS

In conclusion, pneumococcal vaccination is associated with a higher risk of non PCV10 serotypes colonization and antimicrobial resistance independently of country and vaccine used. Serotypes 14 (PCV-7), 15A (non-vaccine serotype) and 19A (PCV-13) had the highest proportion of antimicrobial resistance and multidrug resistance. The emergence of new serotypes and related prevalence of antimicrobial resistance might justify at the short-term, a continuous evaluation and adjustment of available vaccines, in order to include newly emerged serotypes. At the long-term, the implementation of new vaccines that could cover all pneumococcal serotypes such as whole cell vaccines, might be helpful.

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SUPPLEMENTARY TABLES:

Table S1a: Infants vaccination in National Immunization Program in the nine participating countries [33-38]

Country	Vaccine type	Schedule	Extent of vaccination program	Catch up campaign	Year of implementation		
					PCV7	PCV10	PCV13
Austria	PCV7, PCV10 PCV13	3+1	Universal	No	2004	2010	2009
Belgium	PCV7, PCV13	2+1	Universal	Yes (2007)	2005		2011
Croatia	PCV7	3+1	Risk based	No	2006	-	-
France	PCV7, PCV13	2+1	Universal + risk-based	Yes (2010)	2006	-	2010
Hungary	PCV7, PCV13	2+1	Universal	Yes (2008)	2008	-	2010
Netherlands	PCV7, PCV10	3+1	Universal	No	2006	2011	
Spain	PCV7, PCV10, PCV13	3+1	Risk based	No	2001	2009	2010
Sweden	PCV7, PCV10 PCV13	2+1	Universal	No	2009	2009	2010
UK	PCV7 PCV13	2+1	Universal	Yes (2006)	2006		2010

Table S1b: Vaccination in elderly and risk patients in the nine participating countries [33-38].

Country	Vaccine type	Date of implementation of recommendation	Age based	Date of implementation PPV23
Austria	PCV13, PPV23	2014	≥50 years	2003
Belgium	PCV13, PPV23	2013	≥65 years	1993
Croatia	-	-	-	-
France	PCV13, PPV23	2013	No	-
Hungary	-	-	-	-
Netherlands	PPV23	-	-	-
Spain	PPV23	2001	-	1999
Sweden	PPV23	1994	≥65 years	1994
UK	PCV13, PPV23	1992	≥65 years	1992

Table S2: Association between age and vaccination status in (non-) vaccinated participants in participants with known age range

	4-9 years		Older than 10 years		Unknown age		p-value
	Vaccinated	Non-vaccinated	Vaccinated	Non-vaccinated	Vaccinated	Non-vaccinated	
Austria	3	31	189	2203	1	20	0.9028
Belgium	4	32	488	2442	1	1	0.507
Croatia	7	209	20	3542	0	12	< 0.0001*
France	57	65	139	3291	1	12	< 0.0001*
Spain	89	103	1020	2588	0	23	< 0.0001*
Netherlands	14	83	75	2909	0	20	< 0.0001*
Hungary	24	289	110	3172	0	0	0.0002*
Sweden	8	147	294	2259	2	5	0.0209*
UK	0	0	768	1945	2	1	nd
Total	206	959	3103	24351	7	94	p<0.0001*

*P<0.05 is significant; nd=not determined

Table S3: Serotypes distribution by country (%). Serotypes listed in order from highest to lowest in frequency among all *S. pneumoniae* strains cultured in the study as reported in the last column.

Serotype	Austria N=38	Belgium N=44	Croatia N=134	France N=170	Hungary N=116	Netherlands N=129	Spain N=167	Sweden N=103	UK N=36	Total N=937
11A	5.3	6.8	2.2	**10.6**	6.9	7.0	2.4	**10.7**	5.6	**6.4**
23A	2.6	2.3	6.7	7.6	**7.8**	4.7	4.8	7.8	8.3	6.2
19A	2.6	2.3	7.5	8.2	0.9	6.2	4.8	5.8	8.3	5.5
3	**15.8**	0.0	3.7	9.4	4.3	4.7	5.4	2.9	2.8	5.4
6C	5.3	4.5	3.0	5.3	0.9	**8.5**	**6.6**	1.9	5.6	4.7
NT	5.3	9.1	3.7	4.7	3.4	7.0	3.6	3.9	2.8	4.6
23B	5.3	6.8	0.0	7.1	0.0	7.0	5.4	1.9	5.6	4.2
19F	5.3	0.0	6.7	3.5	5.2	3.1	3.0	4.9	2.8	4.1
23F	5.3	0.0	**11.2**	0.0	0.9	2.3	1.2	**10.7**	8.3	3.9
15B/C	7.9	2.3	0.7	2.4	**7.8**	1.6	4.8	3.9	8.3	3.7
35B	2.6	2.3	2.2	5.3	6.9	1.6	3.6	1.9	5.6	3.6
35F	2.6	4.5	3.0	2.9	5.2	1.6	1.2	8.7	5.6	3.5
22F	2.6	4.5	1.5	4.7	2.6	3.9	2.4	4.9	5.6	3.4
6A	7.9	0.0	7.5	1.2	3.4	3.1	1.2	2.9	0.0	3.0
10A	0.0	2.3	0.0	1.2	6.0	4.7	4.2	0.0	**13.9**	3.0
6B	0.0	0.0	6.7	0.6	1.7	6.2	1.2	2.9	0.0	2.7
15A	2.6	**13.6**	0.7	3.5	0.0	1.6	3.6	1.9	0.0	2.6
17F	0.0	2.3	0.0	3.5	3.4	4.7	3.0	0.0	2.8	2.5

Table S3: Serotypes distribution by country (%). Serotypes listed in order from highest to lowest in frequency among all *S. pneumoniae* strains cultured in the study as reported in the last column. (continued)

Serotype	Austria N=38	Belgium N=44	Croatia N=134	France N=170	Hungary N=116	Netherlands N=129	Spain N=167	Sweden N=103	UK N=36	Total N=937
24F	0.0	4.5	0.0	2.9	5.2	0.8	4.2	0.0	0.0	2.2
18C	0.0	0.0	0.7	0.0	6.0	3.1	1.2	5.8	0.0	2.1
16F	0.0	2.3	0.7	0.6	4.3	0.8	5.4	1.0	0.0	2.0
7F	2.6	6.8	1.5	1.2	0.0	2.3	3.0	1.9	0.0	1.9
9N	0.0	2.3	5.2	0.6	0.9	2.3	1.2	1.9	0.0	1.8
37	2.6	0.0	3.0	0.0	1.7	3.1	3.0	1.0	0.0	1.8
14	0.0	2.3	3.7	0.0	1.7	0.0	4.2	1.0	0.0	1.7
33F	0.0	0.0	0.0	2.4	0.9	2.3	1.8	3.9	0.0	1.6
34	0.0	0.0	2.2	0.0	3.4	0.8	1.8	1.9	0.0	1.4
21	0.0	0.0	0.0	3.5	0.9	0.0	3.0	0.0	2.8	1.4
31	0.0	9.1	0.7	1.2	2.6	0.0	1.2	0.0	0.0	1.3
8	0.0	2.3	2.2	0.0	1.7	1.6	0.0	0.0	2.8	1.0
38	2.6	2.3	0.7	1.8	0.0	0.8	0.6	0.0	0.0	0.9
12F	2.6	2.3	0.0	1.2	0.9	0.0	0.6	0.0	0.0	0.6
28A	2.6	0.0	2.2	0.0	0.0	0.8	0.0	0.0	0.0	0.5
4	0.0	0.0	2.2	0.6	0.0	0.8	0.0	0.0	0.0	0.5
9V	0.0	0.0	0.7	0.6	0.0	0.0	0.6	1.9	0.0	0.5
13	0.0	0.0	0.7	0.0	0.9	0.0	1.8	0.0	0.0	0.5
1	0.0	0.0	0.7	0.0	0.0	0.0	1.2	0.0	0.0	0.3
20	0.0	0.0	0.0	1.2	0.0	0.0	0.6	0.0	0.0	0.3
42	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.2
18F or 18C	0.0	0.0	0.7	0.6	0.0	0.0	0.0	0.0	0.0	0.2
7C	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	2.8	0.2
10B	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.1
29	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
17A	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
15F	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
27	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.1
5	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.1
25F or 25A	0.0	2.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
35A/C	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.1

UK = United Kingdom; in bold: serotypes of the highest prevalence in a country; NT=not typable

Table S4: Serotypes distribution by age category (%). Serotypes listed in order from highest to lowest in frequency. Only serotypes represented by more than 10 strains among all *S. pneumoniae* strains cultured in the study are reported. Correlation between serotypes frequency in different age groups is shown in the last column.

Serotypes	N	4-9 years	10 years or older	Unknown age	p-value
11A	60	31.7	66.7	1.7	0.5442
23A	58	34.5	65.5	0.0	0.8206
19A	52	44.2	53.8	1.9	0.2606
3	51	39.2	60.8	0.0	0.8191
06C	44	34.1	63.6	2.3	0.9236
NT	43	23.3	76.7	0.0	0.6331
23B	39	46.2	51.3	2.6	0.2238
19F	38	44.7	55.3	0.0	0.3827
23F	37	56.8	43.2	0.0	0.0162*
15B/C	35	48.6	51.4	0.0	0.1929
35B	34	35.3	64.7	0.0	0.6125
35F	33	42.4	57.6	0.0	0.2522
22F	32	15.6	84.4	0.0	0.0196*
06A	28	35.7	64.3	0.0	0.9320
10A	28	42.9	57.1	0.0	0.6289
06B	25	28.0	68.0	4.0	0.5723
15A	24	25.0	70.8	4.2	0.3933
17F	23	13.0	87.0	0.0	0.0302*
24F	21	57.1	42.9	0.0	0.0830
18C	20	55.0	45.0	0.0	0.1393
16F	19	52.6	47.4	0.0	0.2256
07F	18	33.3	66.7	0.0	0.9558
09N	17	29.4	70.6	0.0	0.7053
37	17	52.9	47.1	0.0	0.2893
14	16	62.5	37.5	0.0	0.0581
33F	15	20.0	80.0	0.0	0.4599
21	13	38.5	61.5	0.0	0.8732
34	13	23.1	76.9	0.0	0.7831
31	12	25.0	75.0	0.0	0.2777

*) $P<0.05$ is significant; NT = not typable

Table S5 Primers used in this study.
For amplification, 20 µl PCR mixtures containing HotStarTaq mastermix (Qiagen, Hilden, Germany) and 10 pmol (*wciN* and *wciP*) or 25 pmol (all others) of each primer were used for 30 cycles of amplification under the following conditions: *wciN* and *wciP*, 1 min at 95°C, 30 sec at 58°C, and 1 min at 72°C; all others, 45 sec at 95°C, 45 sec at 54°C, and 1 min at 72°C.

Primer	Sequence	Target	Used for CT's	Reference
wciNbeta_F	TGCACAGTACTTTTGCAGGTGT	*wciNbeta*	06A-01, 06A-03, 06A-04, 06A-06, 06B-01, 06B-02, 06C-01	This study
wciNbeta_R	CGCCCACGCAATTCGCCATC			
wciP-up	ATGGTGAGAGATATTTGTCAC	*wciP*	06A-01, 06A-03, 06A-04, 06A-06, 06B-01, 06B-02	[12]
wciP-down	AGCATGATGGTATATAAGCC			
15B/C-f	TTGGAATTTTTTAATTAGTGGCTTACCTA	*wzy*	15B-01, 15C-01, 22F-01, 23F-01	CDC
15B/C-r	CATCCGCTTATTAATTGAAGTAATCTGAACC			
35A/35C/42-f	ATTACGACTCCTTATGTGACGCGCATA	*wzx*	15C-01	CDC
35A/35C/42-r	CCAATCCCAAGATATATGCAACTAGGTT			
35B-f	GATAAGTCTGTTGTGGAGACTTAAAAAGAATG	*wcrH*	15C-01	CDC
35B-r	CTTTCCAGATAATTACAGGTATTCCTGAAGCAAG			
17F-f	TTCGTGATGATAATTCCAATGATCAAACAAGAG	*wciP*	15C-01	CDC
17F-r	GATGTAACAAATTTGTAGCGACTAAGGTCTGC			
24F/24A/24B-f	GCTCCCTGCTATTGTAATCTTTAAAGAG	*wzy*	24F-01	CDC
24F/24A/24B-r	GTGTCTTTTATTGACTTTATCATAGGTCGG			
20-f	GAGCAAGAGTTTTTCACCTGACAGCGAGAAG	*wciL*	24F-01	CDC
20-r	CTAAATTCCTGTAATTTAGCTAAAACTCTTATC			
7C/7B/**40**-f	CTATCTCAGTCATCTATTGTTAAAGTTTACGACGGGA	*wcwL*	24F-01	CDC
7C/7B/**40**-r	GAACATAGATGTTGAGACATCTTTTGTAATTTC			
wzg-1_f	TGTGACAGCACCGACTGGGACT	*wzg*	34-01	This study
wzg-1_r	GTTGGTTGCGACCACGGTCACG			
ST38-F	TGCCAAGACACTTGGGGAAG	*wcyV*	25F-02	This study
ST38-R	TCAGCAGGCTTCTCTATCGTCT			

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6

S. pneumoniae and *S. aureus* interaction: possible reasons and implications for carriage and antibiotic resistance

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ABSTRACT:

We describe the in vivo effect of pneumococcal vaccination on pneumococcal and staphylococcal carriage in relation to the carriers age and vaccination status, and the possible effect of co-colonization of *S. pneumoniae* and *S. aureus* on antibiotic (non)susceptibility.

Materials and methods:

7622 nasal swabs were analysed. Of these 216 were positive for both *S. aureus* and *S. pneumoniae*. Pneumococcal capsular typing was performed for *S. pneumoniae* isolates.

Results:

Co-colonization was not associated with vaccine or non-vaccine pneumococcal serotypes. Only serotypes 3 and 10A, were significantly higher in co-colonization than in single colonization.

Conclusion:

We highlighted the interactions between co-colonizing isolates of *S. pneumoniae* and *S. aureus*. The effect of new pneumococcal vaccines on *S. aureus* colonization and the effect of co-colonization on antibiotic resistance need to be further investigated.

INTRODUCTION:

Streptococcus pneumoniae and *Staphylococcus aureus* are part of the respiratory microbiome of the upper respiratory tract (URT). Colonization with these species is inversely correlated, suggesting a natural competition between these bacterial species [1,2]. Stability of the microbiome is influenced by inter-bacterial and host-bacteria interactions and available nutritional resources[3]. Disruption of this stability is considered to play a role in the pathogenesis of upper respiratory (URT) and lower respiratory tract (LRT) infections[3].

In rats, *S. pneumoniae* elimination increased the likelihood of staphylococcal carriage and the risk of staphylococcal infections [4]. Moreover, both an increase in the prevalence of *S. aureus* related otitis media[5] and a temporarily increased *S. aureus* colonization in 12 months old children after use of PCV-7 vaccine has been reported[4]. In a dual-species biofilm on eukaryotic cell line and mouse co-colonization model, Reddinger et al. showed that *S. pneumoniae* inhibited *S. aureus* dispersion from biofilm and the subsequent development of staphylococcal infections[6]. Different mechanisms of interaction between *S. pneumoniae* and *S. aureus* have been suggested such as the bactericidal effect of *S. pneumoniae* on *S. aureus* by production of hydrogen peroxide, and an immune-mediated interaction elicited toward the pneumococcal pili[7,8]. Furthermore, *S. pneumoniae* and *S. aureus* may interact with other microorganisms such as *Moraxella catarrhalis*, *Haemophilus influenzae* and influenza virus via immune mediated cross-reactivity or pneumococcal pilus immune mediated reactivity [7] . However, the effect of pneumococcal vaccination on co-colonizing *S. pneumoniae* and *S. aureus* (i.e. pneumococcal and staphylococcal carriage) has not yet been studied.

The aim of this study was to describe the in vivo effect of pneumococcal vaccination on pneumococcal and staphylococcal carriage in relation to the carriers age and vaccination status. In addition, the possible effect of co-colonization of *S. pneumoniae* and *S. aureus* on antibiotic susceptibility was determined.

MATERIALS AND METHODS:

Bacterial strains:

Bacterial carriage strains were collected as part of 'The Appropriateness of prescribing antimicrobial agents in primary health care in Europe with respect to antimicrobial resistance' (APRES) study as described by Van Bijnen et al [9]. In short, 20 general practitioner's practices (GP) were recruited per country, by national GP networks in Austria, Belgium, Croatia, France Hungary, Spain, Sweden, The Netherlands and the UK.

Each GP practice aimed to provide 200 nasal swabs from patients older than 4 years, except in the UK where patients were 18 years or older (due to ethical committee constraints). To participate in the study, patients should have visited the practice for a noninfectious

condition, not have used antibiotics nor have been hospitalized in the 3 months preceding the swab collection. Immunocompromised patients, as well as nursing home residents were excluded. Bacterial identification and antimicrobial susceptibility testing

Identification and antimicrobial susceptibility testing of *S. aureus* and *S. pneumoniae* were performed in a central laboratory as described previously [10,11]. The antibiotic susceptibility of the isolates was determined for benzyl-penicillin, cefaclor, cefuroxime, ceftazidime, ciprofloxacin, clarithromycin, clindamycin, co-trimoxazole, moxifloxacin and tetracycline for *S. pneumoniae*, and azithromycin, benzyl-penicillin, ciprofloxacin, clindamycin, daptomycin, erythromycin, gentamicin, linezolid, oxacillin, tetracycline, co-trimoxazole and vancomycin for *S. aureus* [10,11]. The method used was in accordance with EUCAST guidelines and EUCAST epidemiological cut-offs were used as susceptibility breakpoints[12].

Pneumococcal capsular typing:

Pneumococcal capsular typing was done as described before with some modifications [13]. In short: *S. pneumoniae* was incubated overnight at 37°C and 5% CO_2 in Brain Heart Infusion broth with 0,5% yeast extract, and thereafter heated at 95°C for 10 minutes before being used as a DNA template in PCR to amplify a fragment of the capsular *wzh* gene. Amplicons were sequenced by BaseClear BV, Leiden, the Netherlands. Sequences generated were assembled, edited and trimmed using Bionumerics v6.1 (Applied Maths, Sint-Maartens-Latem, Belgium) and assigned a capsular sequence type (CST) using the CST database (http://www.rivm.nl/ mpf/spn/cst). Sequence type (CST) is a composite assignment, consisting of the serotype assessed by conventional serotyping (Quellung) and the number representing a consecutive *wzh* allele identified among strains of a given serotype[13]. When an allele was not yet recorded in the CST database, the serotype of the isolate was determined by the Quellung method at the National Reference Laboratory for Bacterial Meningitis (NRBM), Amsterdam, the Netherlands. For CT's representing serogroup 6 isolates, distinction between 6A/B and 6C/D was made using primers specific for *wciNbeta,* after which 6A and 6B were distinguished by PCR sequencing of *wciP* as described[14]. For CT's 15B-01, 15C-01, 22F-01, 23F-01, and 24F-01, the appropriate serotype-specific primers from the protocol of the CDC for multiplex PCR serotype deduction (http://www.cdc.gov/ streplab/pcr.html) were used in single PCR reactions. For isolates of CT 34-01, PCR-sequencing of the *wzg* gene was performed to distinguish between serotype 17 and 34 isolates. Finally, distinction between CT 25F-02-associated serotypes 25A/F and 38 was achieved by PCR-amplification of the *wcy* gene. When no gene was amplified with the used primers and no CST could be assigned, isolates were considered as not-typable.

Statistical analysis:

Statistical analysis was performed using PASW software package 19.0, with p-value <0.05 considered statistically significant.

For serotype assessment, isolates were grouped by serotype and not by individual CTs. For CT's associated with a single serotype (61 of the 79 CST types found in this study), grouping was based on the first part of the CST assignment.

Odd ratio (OR) were performed using the Medcalc software package 14.0, with p-value <0.05 considered statistically significant.

RESULTS:

Bacterial strains:

In total 28619 nasal swabs were analyzed of which 7622 proved positive. Of these 7622 swabs, 6685 grew *S. aureus,*721 *S. pneumoniae* and 216 both *S. aureus* and *S. pneumoniae*.

The age distribution of the participants colonized with *S. aureus* and *S. pneumoniae* is shown in table 1. *S. aureus* single colonization increased with age from 3.5% in children of 4-9 years to 27.6% in participants > 60 years. Pneumococcal colonization was highest among children of 4-9 years, declining with age in older children (10-17 years) and young adults (18-29 years) and increasing thereafter. Co-colonization was the same in all age groups and was 1% or less.

Table 1: Age distribution of the participants colonized % (N) with *S. aureus, S. pneumoniae* or both (N=7622).

Age in years	Positive strains N=7622		
	***S. aureus* single colonization % (N/7622)**	***S. pneumoniae* single colonization % (N/7622)**	**Co-colonization % (N/7622)**
not known	0.4 (29)	0.1 (5)	0.0 (0)
4-9	3.5 (266)	3.5 (268)	1.0 (74)
10-17	6.2 (473)	1.3 (99)	0.3 (25)
18-29	10.9 (829)	0.6 (48)	0.3 (20)
30-60	39.2 (2988)	2.4 (180)	0.9 (71)
>60	27.6 (2100)	1.6 (121)	0.3 (26)
Total	87.7 (6685)	9.5 (721)	2.8 (216)

Effect of pneumococcal vaccination on carriage of *S. pneumoniae* and *S. aureus*

During the study period the pneumococcal vaccines PCV7 and PVCV10 were in use. No significant relationship was found between vaccination and colonization of either *S. aureus* or *S. pneumoniae* (p value=0.095) (table 2).

Table 2: Single and co-colonization in vaccinated and not vaccinated individuals. Only individuals met known vaccination status (N=6758) were taken into account.

	Single colonization *S. pneumoniae* N=653 (%)	**Single colonization *S. aureus* N=5916 (%)**	**Co-colonization N=189 (%)**
Vaccinated (N=779)	86 (13)	664 (11.2)	29 (15.3)
Not vaccinated (N=5979)	567 (87)	5252 (88.8)	160 (84.7)

Co-colonization was not associated with vaccination with the pneumococcal serotypes of PCV7/ PCV10 vaccines or non-PCV7/PCV10 vaccines, p-value = 0.076 (table 3).

Table 3: Prevalence of pneumococcal serotypes of PCV 7 / 10 or non PCV 7 or 10 vaccine types in **s**ingle and co-colonization in vaccinated and not vaccinated individuals.

	Single or co-colonization	**PCV7/PCV10 serotypes N=149 (%)**	**Other than PCV7/PCV10 serotypes N=693 (%)**
Vaccinated	Single colonization *S. pneumoniae* (N=86)	9 (6.1)	77 (11.1)
	Co-colonization (N=29)	2 (1.3)	27 (3.9)
Not vaccinated	Single colonization *S. pneumoniae* (N=567)	111 (74.5)	456 (65.8)
	Co-colonization (N=160)	27 (18.1)	133 (19.2)

PCV= Pneumococcal Conjugate Vaccine; PCV7 serotypes: 4, 6B, 9V, 14, 18C, 19F and 23F; PCV10: PCV7 serotypes plus serotypes 1, 5 and 7F

As shown in table 4, *S. aureus* carriage tends to be higher in both vaccinated and non-vaccinated individuals > 60 years. No staphylococcal carriage rate differences (11.6% and 11.4%) were shown in vaccinated and not vaccinated individuals (OR =1.023, 95% CI 0.936-1.118). In vaccinated persons the *S. aureus* carriership was statistically significantly higher in individuals from age groups 10-17 (OR=0.4836, 95% CI 0.296-0.790) and 30-60 (OR=0.732, 95% CI 0.583-0.919).

Table 4: *S. aureus* carriers in vaccinated and not vaccinated individuals (N=28619)

Age	Is the individual vaccinated against *S. pneumoniae*?	Negative Swabs for *S. aureus* N=22538 (%)	Positive swabs for *S. aureus* N=6081 (%)	OR (95% CI)	P value
4-9	Yes	146 (0.6)	60 (1.0)	0.849 (0.608-1.185)	0.3352
	No	711 (3.2)	248 (4.1)		
10-17	Yes	40 (0.2)	29 (0.5)	0.484 (0.296-0.790)	0.0037*
	No	1215 (5.4)	426 (7.0)		
18-29	Yes	91 (0.4)	36 (0.6)	0.820 (0.552-1.218)	0.3252
	No	2081 (9.2)	675 (11.1)		
30-60	Yes	278 (1.2)	106 (1.7)	0.732 (0.583-0.919)	0.0073*
	No	9182 (40.7)	2562 (42.1)		
>60	Yes	2062 (9.1)	461 (7.6)	0.982 (0.875-1.102)	0.7583
	No	6732 (29.9)	1478 (24.3)		
Total	Yes	2617 (11.6)	692 (11.4)	1.023 (0.936-1.118)	0.6159
	No	19921 (88.4)	5389 (88.6)		

OR= Odd Ratio; 95% CI= confidence interval 95%; * p value<0.05 is considered as significant

Association between Pneumococcal serotypes and single (co-) colonization

Table 5 shows the pneumococcal serotypes distribution of 866 single and co-colonizing isolates. Only serotypes 3 and 10A, were significantly associated to co-colonization (p=0.039 and p≤0.005, respectively).

Table 5: Serotype distribution in 866 single and co-colonizing *S. pneumoniae* isolates. Only serotypes represented by more than 10 isolates are shown.

Serotype	**Total N=866**	**Single colonization N=658**	**Co-colonization N=208**	**P-value**
06C[1]	44	35	9	0.854
15A[1]	24	19	5	1.000
16F[1]	19	13	6	0.408
21[1]	13	8	5	0.190
23A[1]	58	40	18	0.147
23B[1]	39	33	6	0.331
24F[1]	21	18	3	0.438
31[1]	12	9	3	1.000
34[1]	12	10	2	1.000
35B[1]	34	25	9	0.678
35F[1]	33	24	9	0.532
37[1]	17	12	5	0.561

Table 5: Serotype distribution in 866 single and co-colonizing *S. pneumoniae* isolates. Only serotypes represented by more than 10 isolates are shown. (continued)

Serotype	Total N=866	Single colonization N=658	Co-colonization N=208	P-value
NT[1]	44	34	10	1.000
14 [2,5]	16	10	6	0.225
18C [2,5]	20	17	3	0.591
19F [2,5]	38	28	10	0.693
23F [2,5]	37	29	8	1.000
06B [2,5]	25	23	2	0.090
07F [3,5]	18	14	4	1.000
06A [4]	28	24	4	0.363
3 [4,5]	51	33	18	0.039*
19A [4,5]	52	39	13	0.737
09N [5]	17	13	4	1.000
10A [5]	28	15	13	0.005*
11A [5]	60	47	13	0.870
15B/C [5]	36	30	6	0.424
17F [5]	23	21	2	0.131
22F [5]	32	25	7	1.000
33F [5]	15	10	5	0.733

[1]) Non-vaccine serotype, [2]) PCV-7, [3]) PCV-10 minus PCV-7, [4]) PCV-13 minus PCV-10, [5]) PPV-23.
PCV-7: 7-valent polyvalent conjugate vaccine (including 4, 6B, 9V, 14, 18C, 19F and 23F),
PCV-10: 10-valent PCV (including 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F),
PCV-13: 13-valent PCV (including 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F),
PPV-23: pneumococcal polysaccharide vaccine (including, 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F)
*) significant p-value

Antibiotic susceptibility in single and co-colonization:

In *S. aureus*, antibiotic susceptibility ranged between 27.1% for benzyl-penicillin and 96.1% for trimethoprim-sulfamethoxazole in single colonization, and 18.1% for benzyl-penicillin and 100% for trimethoprim-sulfamethoxazole in co-colonization. Of the antibiotics tested, only benzyl-penicillin susceptibility was significantly higher in carriers < 30 years of age co-colonizing then in colonizing strains among carriers < 30 years of age (OR=1.7 (95% CI 1.2-2.3). (supplementary tables 1 and 3). In *S. pneumoniae*, antibiotic susceptibility ranged between 44.9% for cefaclor and 85.3% for clindamycin in single colonization, and 54.6% for cefaclor and 85.6% for tetracycline in co-colonization. Of the antibiotics tested, cefaclor susceptibility was significantly lower in patients co-colonized compared to those single colonized with *S. pneumoniae* (OR= 0.7 95% CI 0.5-0.9) among participants 30-60 years

of age. (OR=0.01, 95% CI 0.3-0.8), (supplementary table 3). In the other age groups, no differences were observed (supplementary table 3 and 4).

DISCUSSION:

In this study, co-colonization between *S. pneumoniae* and *S. aureus*, was found in 2.8% (216/7622) of all isolates. In all age groups the prevalence of co-colonization was less than 1.0%. Higher *S. aureus* carriage was found in vaccinated individuals from age groups 10-17 comparing to unvaccinated individuals. Serotypes 3A and 10A were more prevalent in co-colonizing than in single colonizing *S. pneumoniae*. No association was found between co-colonization and vaccination status of the participants, or whether the colonizing serotype was part of the vaccine in use. The prevalence of benzyl-penicillin non-susceptible *S. aureus* was higher in co-colonizing than in single isolates among carriers younger than 30 years of age. In *S. pneumoniae,* cefaclor non-susceptibility was significantly higher in single colonization than in co-colonizing strains in participants of 30-60 years of age.

The overall rate of co-colonization between *S. aureus* and *S. pneumoniae* was 2.8% of all carriers. The relative low rate might be explained by the complex mechanisms of interaction between the two bacteria including hydrogen peroxide production by *S. pneumoniae*, and elimination of *S. aureus* by *S. pneumoniae* mediated immune reaction[15]. Although the co-colonization rate is low, introduction of PCV might induce a disruption of the balance between the two bacteria and the decrease of their interaction due to the absence of the inverse association[15]. This might lead to an increase of co-colonization rate, which justify continuous research in order to better understand the mechanisms of interactions and their implications on infections and antibiotic non-susceptibility.

It has been reported that the introduction of pneumococcal conjugate vaccine led to an increase of *S. aureus* carriage and infections in children younger than 1 year due to the disappearance of the negative effect (inverse correlation) of the *S. pneumoniae* vaccine serotypes on *S. aureus*[4,16]. More recent studies in children younger than two years, showed that increase of *S. aureus* carriage and infection in vaccinates persons was of a short term after implementation of PCV vaccine [17,18]. Our results showed no overall significant difference in *S. aureus* carriage between vaccinated and not vaccinated individuals. However, higher *S. aureus* carriage was noticed in age groups 10-17 years. Our results might be explained by decrease of pneumococcal carriage in this vaccinated age group.

Two pneumococcal serotypes, serotype 3 and 10A, were more frequently present in persons with co-colonization. Both serotypes 3 and 10A are part of the PPV-23 vaccine used in risk population and not recommended for young children. The PPV-23 pneumococci are mostly non-pilliated serotypes [19]. In contrast to pilliated serotypes, non-pilliated serotypes are not able to enhance immune reactions inducing an inflammatory reaction

and elimination of *S. aureus* by the immunity system [15] . Regev-Yochay et al., reported that carriage of palliated serotypes was negatively correlated with *S. aureus* colonization, independently whether these serotypes were included in PCV7 vaccine. However, they found no association between non-piliated pneumococcal serotypes and *S. aureus* colonization[20]. Our findings might suggest that co-colonization is due to the absence of pili in the co-colonizing serotypes.

Several studies have described an inverse correlation between colonizing bacteria in the respiratory tract [21-23], especially, between *S. pneumoniae* and *S. aureus*[24-26]. This inverse correlation was not affected by the early humoral immune response against both bacteria. Expression of antibiotic resistance determinants is one of the bacterial survival strategies in a hostile environment. Studies on bacterial co-colonization have shown a role of the human host immune cells in adaptation of bacterial virulence strategies, including antibiotic resistance. A pairwise comparison of gene transcription of three multidrug resistant bacteria (*Enterobacter hormaechei*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*) were performed in co-colonization with each other, with a probiotic bacterium (*Lactobacillus reuteri), with a skin commensal (staphylococcus epidermis)* and in colonization of human host cells (cultured adult human fibroblast cells). The authors suggested that co-colonization may modulate the bacteria colonization and virulence of host cells[27]. Whether differences in expression of antibiotic resistance determinants in co-colonizing bacteria, could explain the increased prevalence of benzyl-penicillin non-susceptibility in *S. aureus* and of cefaclor susceptibility in *S. pneumoniae* in our study is unknown.

Strengths of this study were the integration of a large strain collection from carriers of all ages from nine European countries located mainly in Western Europe. However, drawback was that this study was a cross-sectional study. To better study the long-term effect of vaccination on *S. aureus* and *S. pneumoniae* carriage as well as on co-colonization, a longitudinal study is needed.

The interaction between *S. aureus* and *S. pneumoniae* might involve bacterial (pilus, hydrogen peroxide production) and host factors (immune response). The effect of implementation of new pneumococcal vaccines on *S. aureus* colonization, as also the effect of co-colonization on antibiotic resistance need to be further investigated.

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SUPPLEMENTARY TABLES:

Supplementary table 1: Antibiotic susceptibility (%) of single and co-colonizing *S. aureus*

Antibiotic	Susceptibility	Single colonization n (%) Total N=6685	Co-colonization n (%) Total N=216	OR (95% CI)	P-value
Azithromycin	Susceptible	6032 (90.2)	198 (91.7)	0.8 (0.5-1.4)	0.484
	Non-susceptible	653 (9.8)	18 (8.3)		
Ciprofloxacin	Susceptible	6544 (97.9)	211 (97.7)	1.0 (0.5-2.7)	0.837
	Non-susceptible	141 (2.1)	5 (2.3)		
Clindamycin	Susceptible	6124 (91.6)	199 (92.1)	0.9 (0.6-1.5)	0.785
	Non-susceptible	561 (8.4)	17 (7.9)		
Erythromycin	Susceptible	6052 (90.5)	199 (92.1)	0.8 (0.5-1.3)	0.429
	Non-susceptible	633 (9.5)	17 (7.9)		
Gentamicin	Susceptible	6634 (99.2)	215 (99.5)	0.6 (0.8-4.4)	0.620
	Non-susceptible	51 (0.8)	1 (0.5)		
Oxacillin	Susceptible	6598 (98.7)	214 (99.1)	0.7 (0.2-2.9)	0.632
	Non-susceptible	87 (1.3)	2 (0.9)		
Benzyl-penicillin	Susceptible	1813 (27.1)	39 (18.1)	1.7 (1.2-2.4)	**0.003***
	Non-susceptible	4872 (72.9)	177 (81.9)		
Tetracycline	Susceptible	6414 (95.9)	213 (98.6)	0.3 (0.1-1.1)	0.060
	Non-susceptible	271 (4.1)	3 (1.4)		
Trimethoprim-sulfamethoxazole	Susceptible	6661 (99.6)	216 (100.0)	0.6 (0.0-10.4)	0.745
	Non-susceptible	24 (0.4)	0 (0.0)		

*) significant P-value; All isolates were susceptible to linezolid and vancomycin; OR, Odd ratio; CI: confidence interval.

Supplementary table 2: Antibiotic susceptibility of single and co-colonizing *S. aureus* grouped by age

Antibiotic tested	Susceptibility	4-9		10-17		18-29		30-60		>60		Total	
		OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value
Azithromycin	susceptible (N=6032) / Non-susceptible (N=653)	0.6, (0.2-1.5)	0.27	0.8, (0.2-3.6)	0.80	1.2, (0.3-4.1)	0.80	0.6, (0.2-1.6)	0.27	1.4, (0.4-4.7)	0.58	0.8 (0.5-1.4)	0.484
Ciprofloxacin	susceptible (N=6544) / Non-susceptible (N=141)	3.6, (0.1-182.5)	0.50	3.7, (0.2-79.2)	0.40	1.2, (0.1-20.7)	0.90	2.2, (0.7-7.3)	0.17	2.6, (0.6-11.3)	0.18	1.0 (0.5-2.7)	0.837
Clindamycin	susceptible (N=6124) / Non-susceptible (N=561)	0.5, (0.2-1.5)	0.21	0.9, (0.2-4.1)	0.94	1.98, (0.6-6)	0.22	0.8, (0.3-2.1)	0.72	1.1, (0.2-4.5)	0.94	0.9 (0.6-1.5)	0.785
Erythromycin	susceptible (N=6052) / Non-susceptible (N=633)	0.5, (0.2-1.4)	0.17	0.9, (0.2-3.7)	0.83	1.2, (0.3-4.2)	0.77	0.6, (0.2-1.6)	0.30	1.5, (0.4-4.9)	0.54	0.8 (0.5-1.3)	0.429
Gentamicin	susceptible (N=6634) / Non-susceptible (N=51)	3.6, (0.2-58.7)	0.33	3.7, (0.2-79.1)	0.37	4.5, (0.2-85.9)	0.28	0.7, (0.0-11.2)	0.78	2.9, (0.2-50.4)	0.44	0.6 (0.8-4.4)	0.620

Supplementary table 2: Antibiotic susceptibility of single and co-colonizing *S. aureus* grouped by age (continued)

Antibiotic tested	Susceptibility	4-9		10-17		18-29		30-60		>60		Total	
		OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value
Oxacillin	susceptible (N=6598) / Non-susceptible (N=87)	3.6, (0.2-58.7)	0.33	3.7, (0.2-79.1)	0.37	3.1, (0.2-56.7)	0.42	0.4, (0.0-5.9)	0.46	3.8, (0.5-29.1)	0.17	0.7 (0.2-2.9)	0.632
Penicillin	susceptible (N=1813) / Non-susceptible (N=4872)	0.5, (0.3-0.9)	**0.03***	13.6, (0.8-225)	**0.02***	7.4, (1-55.8)	**0.02***	1.2, (0.7-2)	0.57	2.4, (0.8-6.9)	0.10	1.7 (1.2-2.4)	**0.003***
Tetracycline	susceptible (N=6414) / Non-susceptible (N=271)	1.5, (0.3-7.6)	0.66	0.5, (0.0-8.2)	0.61	0.7, (0.0-12.1)	0.81	0.3, (0.0-2.4)	0.28	0.4, (0.0-6.3)	0.49	0.3 (0.1-1.1)	0.060
Co-trimoxazole	susceptible (N=6661) / Non-susceptible (N=24)	1.2, (0.0-29.5)	0.91	6.2, (0.2-155.8)	0.27	3.7, (0.2-68.4)	0.38	1.7, (0.1-28.4)	0.72	7.2, (0.4-133.4)	0.18	0.6 (0.0-10.4)	0.745

*) significant p-value; All isolates were susceptible to linezolid and vancomycin; OR, Odd ratio; CI: confidence interval.

Supplementary table 3: Antibiotic susceptibility of 928 (%) single and co-colonizing *S. pneumoniae* strains

Antibiotic tested	Susceptibility	Single colonization n (%) N=712	Co-colonization n (%) N=216	OR (95%CI)	P-value
Cefaclor	Non-susceptible	392 (55.1)	98 (45.4)	0.7 (0.5-0.9)	0.013*
	Susceptible	320 (44.9)	118 (54.6)		
Ceftazidime	Non-susceptible	125 (17.6)	37 (17.1)	1.0 (0.6-1.5)	0.885
	Susceptible	587 (82.4)	179 (82.9)		
Cefuroxime	Non-susceptible	153 (21.5)	43 (19.9)	0.9 (0.6-1.3)	0.618
	Susceptible	559 (78.5)	173 (80.1)		
Clarithromycin	Non-susceptible	129 (18.1)	33 (15.3)	0.8 (0.5-1.2)	0.336
	Susceptible	583 (81.9)	183 (84.7)		
Clindamycin	Non-susceptible	105 (14.7)	28 (13.0)	0.9 (0.6-1.3)	0.518
	Susceptible	607 (85.3)	188 (87.0)		
Benzyl-penicillin	Non-susceptible	144 (20.2)	38 (17.6)	0.8 (0.6-1.3)	0.394
	Susceptible	568 (79.8)	178 (82.4)		
Tetracycline	Non-susceptible	111 (15.6)	31 (14.4)	0.9 (0.6-1.4)	0.658
	Susceptible	601 (84.4)	185 (85.6)		
Trimethoprim-sulfamethoxazole	Non-susceptible	107 (15.0)	34 (15.7)	1.1 (0.7-1.6)	0.798
	Susceptible	605 (85.0)	182 (84.3)		

*) significant p value. all isolates were susceptible to moxifloxacin and ciprofloxacin; OR: Odd-ratio; CI: confidence interval.

Supplementary table 4: Antibiotic susceptibility of single and co-colonizing *S. pneumoniae* grouped by age

Antibiotic tested	Susceptibility	4-9		10-17		18-29		30-60		>60		Total	
		OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value
Cefaclor	Susceptible N= (438)	1.1, (07-1.9)	0.64	0.5, (0.2-1.3)	0.15	1.1, (0.4-3.1)	0.87	0.5, (0.3-0.8)	**0.01***	0.5, (0.2-1.2)	0.14	0.7 (0.5-0.9)	**0.013***
	Non-susceptible N= (490)												
Ceftazidime	Susceptible N= (766)	1.2, (0.7-2.3)	0.49	0.8, (0.2-3.8)	0.76	1.3, (0.3-4.9)	0.71	0.7, (0.3-1.5)	0.37	0.7, (0.2-2.6)	0.61	1.0 (0.6-1.5)	0.885
	Non-susceptible N= (162)												
Cefuroxime	Susceptible N= (732)	1.1, (0.6-1.9)	0.84	0.5, (0.1-2.3)	0.36	1.1, (0.3-4.1)	0.87	0.8, (0.4-1.7)	0.59	1.0, (0.3-2.9)	0.97	0.9 (0.6-1.3)	0.618
	Non-susceptible (N=196)												
Clarithromycin	Susceptible (N =766)	0.7, (0.3-1.3)	0.24	0.8, (0.2-3.8)	0.76	0.8, (0.2-3.3)	0.74	0.9, (0.4-1.9)	0.80	1.1, (0.4-3.2)	0.87	0.8 (0.5-1.2)	0.336
	Non-susceptible (N= 162)												
Clindamycin	Susceptible (N =795)	0.8, (0.4-1.7)	0.54	0.4, (0-3.1)	0.34	0.5, (0.1-2.5)	0.39	1.3, (0.6-2.6)	0.56	0.9, (0.3-3.0)	0.91	0.9 (0.6-1.3)	0.518
	Non-susceptible (N =133)												

Supplementary table 4: Antibiotic susceptibility of single and co-colonizing *S. pneumoniae* grouped by age (continued)

Antibiotic tested	Susceptibility	4-9		10-17		18-29		30-60		>60		Total	
		OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value	OR, (95% CI)	P value
Penicillin	Susceptible (N =746)	1.0, (0.5-1.8)	1.00	0.6, (0.1-2.8)	0.49	1.3, (0.3-4.9)	0.71	0.7, (0.4-1.5)	0.42	0.6, (0.2-2.2)	0.43	0.8 (0.6-1.3)	0.394
	Non-susceptible (N =182)												
Tetracycline	Susceptible (N=786)	0.8, (0.4-1.7)	0.63	1.4, (0.3-5.5)	0.65	0.2, (0-1.7)	0.11	1.1, (0.5-2.4)	0.76	1.2, (0.4-3.4)	0.79	0.9 (0.6-1.4)	0.658
	Non-susceptible (N =142)												
Co-trimoxazole	Susceptible (N = 787)	0.8, (0.4-1.6)	0.49	1.4, (0.4-4.8)	0.59	0.8, (0.1-4.3)	0.79	1.2, (0.5-2.5)	0.67	2.0, (0.7-5.7)	0.19	1.1 (0.7-1.6)	0.798
	Non-susceptible (N =141)												

*) significant p-value. all isolates were susceptible to moxifloxacin and ciprofloxacin; OR: Odd-ration; CI: confidence interval.

7 Summarizing discussion

Streptococcus pneumoniae is generally a quiescent colonizer of the mucosal surface of the human upper respiratory tract[1]. Pneumococcal carriage is the first step towards different (invasive) infections [2]. Pneumococcal transmission occurs via person-to-person contact or via spread from contaminated surfaces and objects[3]. In a nutrient rich environment, *S. pneumoniae* can survive for days[4]. Local spread, aspiration or spread to the bloodstream may induce infections such as pneumonia, meningitis, sepsis and otitis media[2]. Up to 60% of young children carry this bacterium asymptomatically [5]. In 2000, worldwide 14 million episodes of severe, invasive pneumococcal) diseases (IPD) have been reported[6]. The yearly death is estimated at 1.6 million patients, including one million children under the age of 5 years[6]. In 2017, the World Health Organization (WHO) added *S. pneumoniae* to the twelve priority pathogens[3].

Bacterial identification is the cornerstone toward better insight in the prevalence of *S. pneumoniae* both in carriage as well as in infections. The lack of a "gold standard" makes bacterial identification one of the most important challenges to be met in order to get insight in the prevalence and pathogenesis of this bacterium. *S. pneumoniae* shares up to 99% of the rRNA gene nucleotide sequences with the most related members of the *S. mitis* group such as *S. oralis* and *S. mitis*[7]. Misidentification affects the presumed burden of pneumococcal diseases[8]. In addition, it may lead to an erroneous estimation of pneumococcal antimicrobial resistance since these closely related species are associated with high penicillin non-susceptibility[9]. Furthermore, lack of accurate identification followed by serotyping influences the effective formulation of pneumococcal vaccines, based on the most prevalent serotypes[9]. Alternative identification algorithms and methods were discussed in **Chapter 2** and **3**.

The high burden of pneumococcal diseases and the rising antimicrobial resistance underscore the relevance of prevention[3]. Pneumococcal vaccination (PCV) is the cornerstone in the prevention of invasive pneumococcal disease (IPD). The widespread use of PCV in the paediatric population significantly reduced the incidence of IPD caused by vaccine serotypes in both children and non-vaccinated adults, due to the so-called herd immunity[10]. In contrast, non-vaccine serotypes increased both among asymptomatic carriers, and as causative agent of IPD. Monitoring of emerging pneumococcal serotypes in commensal microbiota of carriers is an important tool to have insight into circulating serotypes and to subsequently adapt the serotypes of the current pneumococcal vaccine when necessary. In **Chapter 4**, serotype distribution, the emerging non-vaccine serotypes, and their antibiotic resistance patterns were discussed in relation to vaccination policies in the participating countries.

For the treatment of IPD, the rise of antibiotic resistant *S. pneumoniae* is a global concern, resulting in treatment failure, increased hospital stay, increased morbidity and even increased mortality[11]. The most available data concerning antibiotic resistance are collected in hospital settings. However, up to 90% of antibiotic medication in humans is

prescribed in outpatient settings[12]. There is an association between antibiotic resistance and use of antibiotic drugs[13-19]. A surveillance program on antibiotic use and resistance in primary healthcare, such as reported in Nethmap (https://swab.nl/nl/nethmap), gives valuable information. For years, penicillin was the antimicrobial agent of choice to treat pneumococcal infections[1]. However, worldwide an increase of pneumococcal resistance to several antimicrobial agents including penicillin has been reported necessitating other empiric antibiotic choices in the beginning of the 21th centry[12]. Beside the Nethmap data also other surveillance programs on antibiotic resistance only report data from clinical samples (https://www.ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc). However, surveillance of antibiotic resistance in commensal microbiota is an indicator for the situation in the general population and need to be monitored. Especially given the fact that carriership is the first step to IPD. Therefore, such data also should contribute to support the empiric choice and the development of antibiotic prescription guidelines.

CHOICE OF THE GOLD STANDARD FOR PNEUMOCOCCAL IDENTIFICATION:

Correct identification of *S. pneumoniae* remains a challenge due to the lack of a reliable gold standard. In order to redress this issue, a single signature sequence-based method was chosen to identify the isolates in our study. This method is based on the detection of a single signature on the position 203 of the 16SrRNA gene[20]. In *S. pneumoniae,* this position is a cytosine (C) whereas in other mitis group streptococci this is an adenosine (A), **Chapters 2** and **3**). This method has shown similar accuracy as the Multilocus Sequence Analysis (MLSA), and was implemented with success for the identification of other bacterial species that were difficult to differentiate such as *Burkholderia* species, *Streptococcus dysgalactiae, Haemophilus* species and *Borrelia* species[20].

For the identification of *S. pneumoniae* conventionally phenotypic characteristics of the bacteria are used. In the last decades, other methods based on the genetic characteristics were implemented. These methods ranged between gene amplification of virulence factors, analysis of nucleotide sequences of 16S rRNA and genomic subtractive hybridization and proteomic based methods (MALDI-TOF MS)[21-24]. In **chapter 2**, the sensitivity and specificity of the performance of several phenotypic, molecular and proteomic based methods were compared. No method could accurately discriminate the different species of the viridans group streptococci. The sensitivity of the phenotypic methods ranged between 81.7% (for optochin susceptibility) and 97.9% (for bile solubility), and the specificity between 64.0% and 96.8% respectively. Although bile solubility was the most accurate method, the method is time consuming and difficult to assess making this method not feasible in the routine di-

agnostic laboratories. Also, an algorithm based on the phenotypic methods i.e. morphology, optochin susceptibility and bile solubility had low performances (sensitivity and specificity of 96.3% and 61.7%, respectively).

In the last years, in an increasing number of reports, *S. pseudopneumoniae* has been associated to lower respiratory tract infections[25,26] and high antibiotic resistance prevalence to penicillin, macrolides, tetracycline and co-trimoxazole[26-28]. Other studies report that this bacterium can be isolated from invasive sites but also, unlike ono pneumococcal mitis group members, from non-invasive sites[29]. Misidentification of this bacterium may lead to an erroneous treatment during infection with *S. pseudopneumoniae*[30]. It has been reported that misidentification of streptococci may cause of overestimation of resistance among *S. pneumoniae*[8]. The emergence of *S. pseudopneumoniae* as potential pathogen accentuates the need for correct identification and determination of susceptibility patterns is therefore essential.

MALDI TOF MS is a useful method to identify a wide variety of mainly Gram-negative bacteria, is cost effective and rapid. However, this method could not discriminate *S. pneumoniae* from the closely related species using the standard identification database (**chapter 3**). Development of Spectral analysis resulted in an accurate pneumococcal identification and differentiation between this species and other related streptococci with a sensitivity and specificity of 99.4% and 98.8%, respectively. These results were obtained with colonization isolates. The usefulness for the identification of clinical isolates requires further investigation. If similar results are obtained, implementation of this method in routine diagnostics will improve the identification of pneumococci both in pneumococcal carriage and IPD.

PNEUMOCOCCAL CARRIAGE

Different challenges are met when interpreting the results of *S. pneumoniae* carriage described in the literature. In large cross-sectional studies, feasibility to enrol sufficient participants remains a challenge. In **chapter 4**, nasal swabs from 32.161 patients visiting general practitioners in nine European countries (Austria, Belgium, Croatia, Hungary, France, the Netherlands, Spain, Sweden and the UK) were analysed. The overall prevalence of pneumococcal carriage was 2.9%. A wide range in carriage rate was found between the participating countries after correction for age, gender and general practitioner. The highest carriage rate was found in France (4.4%) and the lowest in Austria and the UK (1.2%). Carriage rate was higher in children of 4-9 years of age (2.7%) than in older participants (older children and adults) (1.9%). Vaccination, including the national vaccination strategy, the vaccine type, whether all children >2 years were vaccinated, or only clinical risk groups (risk based), whether the implementation of a vaccine was followed by a catch-up pneumococcal vaccination and the number of doses given may influence pneumococcal

colonization. Of 937 pneumococcal strains cultured from the 32.161 outpatients, 18.1% were PCV10 types and 15.9% were PCV7 types. Vaccinated persons were significantly less often carriers of PCV10 serotypes (9.6%) compared to non-vaccinated individuals (19.0%). Being vaccinated was also associated with an increase in of pneumococcal colonization with non-vaccine serotypes due to sero-replacement.

In a study including more than 15000 adults from Alaska (US) before and after introduction of PCV7, Hammitt et al., reported a significant post-PCV7 decrease of pneumococcal carriage. Also, in Norway Steens et al., compared carriage prevalence in more than 1000 children from day-care centers between 2006, 2008 and 2013 (pre- and post PCV13). A decrease of pneumococcal carriage in 2013 (post-PCV13) was observed compared to previous years. In contrast to these studies, our study showed an increase in prevalence of non-vaccine serotypes in all age groups (OR=0.70) after vaccination[31,32]. Our results are supported by a Swedish study including both invasive (n=2336, collected in pre-PCV7 until post PCV13 period) and carriage samples (n=2880, pre-and post- PCV13), from children and adult participants. The researchers concluded that PCV introduction resulted in an almost complete replacement of vaccine types to non-vaccine serotypes in healthy children, without affecting carriage rates substantially [33]. Differences in results between the above-mentioned studies might be due to the age of the population tested, and the time between vaccination and testing. Shortly after vaccination, the immediate immune response may play a role in the decrease in vaccine serotypes. Over time the protective effect will decrease and vaccine and non-vaccine serotypes (the so-called sero-replacement) will (re-) emerge. Vaccination results not only in emergence of new non-vaccine serotypes but in these isolates (both as carriage and as clinical isolates) are more resistant to antimicrobial agents[34,35].

SEROTYPES AND VACCINATION:

To date more than 93 pneumococcal serotypes are known, most of which are not included in the pneumococcal vaccines. The serotypes vary in their ability to cause infection and in antibiotic resistance. As described in **Chapter 5**, serotypes 11A, 19A, 3, 6C, 23A and 23B were the most frequent serotypes among nasal carriers, and were either PPV23 vaccine serotypes or non-PCV vaccine types at the time of this study. The high prevalence of these serotypes especially serotype 11A is in part due to sero-replacement. Our results are in concordance with the sero-replacement as described by McElligott et al.,[36]. Serotype 23F was the most frequently isolated one among *S. pneumoniae* carriers in Croatia which was next to Spain, without PCVs in the National Immunization Programme. In Sweden which introduced PCV7 and PCV10 in 2009, and PCV13 in 2010 in the immunization program, serotypes 23F and 11A were the most prevalent serotypes. Serotype 6C was highly frequent in the Netherlands and Spain. Serotypes 3, 15A, 11A and 10A have the highest prevalence

in Austria, Belgium, France and UK, respectively and in Hungary it were serotypes 23A and 15 B. Differences in vaccine effect per geographical region may be related to variation in host background, the microbiota of the upper respiratory tract[37,38], differences in immunisation programme such as vaccination schedule, age of vaccination[39], prior pathogen exposure[40] as well as crowding, day care centrers attendance, age distribution, antibiotic use, and climatic conditions[41].

ANTIBIOTIC RESISTANCE:

Information on antibiotic use and prevalence of resistance are essential to make a rational empiric antibiotic choice and to make antibiotic prescription guidelines for the treatment of (*S. pneumoniae*) infections. As the majority of human antibiotic use is prescribed by general practitioners, optimal use of antibiotics needs to be based on actual resistance data of the target population in order to control the antibiotic resistance problem. Data regarding antibiotic resistance are mostly collected from clinical settings e.g. Nethmap (https://swab.nl/nl/nethmap). This thesis focussed on antibiotic resistance in carriage isolates.

Numerous reports have shown a worldwide increase of resistance of *S. pneumoniae* to both penicillin and erythromycin in the past years[42-46]. The variation in antimicrobial resistance including the emergence of MDR (Multidrug resistance i.e. resistant to three or more different classes of antibiotics)[47] depends in part on the use of specific antimicrobial agents[13,14].

In the APRES study, a wide variation in antibiotic resistance of *S. pneumoniae* was found among the participating countries **(chapters 4-5).** Resistance to cefaclor was most commonly averaging 52.3%, ranging between 30.6% in the UK to 77.3% in Belgium. Tetracycline showed the highest intercountry variability being lowest in Sweden (1.9%) and highest in Spain (29.9%). The resistance to the most frequently used antibiotics in outpatients, i.e. penicillin, macrolides and tetracycline ranged between 3.9% (Sweden) and 31.7% (Spain) for penicillin, 1.9% (Sweden) and 29.3% (Spain) for clarithromycin, and 1.9% (Sweden) and 29.9% (Spain) for tetracycline. MDR was observed in 33.9% of the pneumococcal isolates, ranging from 3.8% in Sweden to 50.4% in Spain[44]. No resistance was found for moxifloxacin and ciprofloxacin.

In 2010/2011 the APRES countries, Belgium, France, Spain and Croatia had a high penicillin consumption[48] and also high penicillin resistance rates in *S. pneumoniae*. The Netherlands, with a low penicillin consumption, had one of the lowest resistance rates. Sweden, despite a higher rate of penicillin consumption, forms an exception to the correlation between antibiotic consumption and resistance with a low penicillin resistance rate[48]. This might be explained by the use in Sweden of small spectrum penicillin's (60% of the used penicillin's)[49]. Use of broad-spectrum antibiotic selected for resistance not

only in the causative bacteria but also in the other pathogenic and commensal bacteria exposed to the antibiotic. Antibiotic resistance in commensal bacteria forms a reservoir for resistance genes which can be transferred to pathogenic bacteria[50]. Next to antibiotic use also the national vaccination policy influences the antibiotic resistance prevalence. Vaccination has led to increased carriership of more resistant non-vaccine serotypes at the expense of susceptible vaccine serotypes, thereby resulting in an increase in the prevalence of antimicrobial resistance[34,35]. In the APRES study serotype 15A a non-PCV-13 vaccine serotype representing 2.6% of the pneumococcal isolates, was one of the serotypes with the highest prevalence of resistance to penicillin, ceftazidime, clarithromycin, clindamycin and tetracycline. Serotype 19A, a PCV13 vaccine serotype, a frequent causative agent of invasive pneumococcal diseases[51] was, one of the most frequent carriage isolates (5.5%) and resistant to the antimicrobial agents tested i.e. ceftazidime 67.3%, clarithromycin 53.8%, clindamycin 50.0%, penicillin 67.3%, tetracycline 55.8% and trimethoprim-sulfamethoxazole 30.8%, 61.5% of all 19A serotype isolates were multidrug resistant.

A recent post PCV 13 study of clinical samples from children of 6 to 36 months of age, showed an increase in resistance to penicillin, third generation cephalosporin's and fluoroquinolones of non-PCV-13 serotypes 11A, 15A, 35B and 35F compared to the pre-PCV13 era[52]. In the APRES study, serotype 11A was the most frequently isolated pneumococcal vaccine serotype with low resistance to the antibiotics tested. On the other hand, the non-vaccine serotype 35B had a high resistance rate to penicillin and ceftazidime (38.2% for both antibiotics).

The variation in prevalence of resistance between the participating countries can be explained at least in part by differences in the use of antibiotics[13]. Other explanations are clonal spread of resistant microorganisms[53] and antimicrobial cross-resistance between members of antimicrobial agents' classes[54]. The antibiotic resistance data obtained in nasal carriers of *S. pneumoniae* in **Chapter 4** and **5** represent the prevalence of antibiotic resistance in the community. Since carriage is the first step in the development of an infection, these data should be taken into account when making antibiotic treatment guidelines

INTERACTION BETWEEN *S. PNEUMONIAE* AND *S. AUREUS* IN THE NASAL CAVITY

Imbalanced microbiome in the upper respiratory tract is the first step toward pneumococcal infections in the upper and lower respiratory tract[55]. In the upper respiratory tract *S. pneumoniae* interacts with the host and with other co-existing bacteria, such as *S. aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis*[56]. Interactions between these species are in some cases beneficial and sometimes not. A positive correlation, i.e. synergy has been demonstrated between carriage of *S. pneumoniae* and growth of *H. influenzae* and

M. catarrhalis[56]. A negative correlation or antagonism was observed between carriage of piliated *S. pneumoniae* and *S. aureus*[2,57]. This may be explained by the presence of pili, which induce a host immune response resulting in growth inhibition of colonizing. *S. aureus* in the respiratory tract [58]. In addition, the adherence of piliated pneumococci to the respiratory tract is better than that of *S. aureus* and piliated pneumococci inhibit staphylococcal colonization by production of hydrogen peroxidase[59].

Two pneumococcal serotypes, serotype 3 and 10A, are part of the PPV-23 vaccine and were frequently related with co-colonization with *S. aureus*. In this study (**Chapter 6**), in 31.7% and 42.4% in children younger than 10 years, respectively. PPV-23 is used in risk populations and not recommended as vaccine for young children. PPV-23 pneumococci are mostly non-pilliated serotypes [60] and not able to enhance immune reactions inducing an inflammatory reaction and elimination of *S. aureus*[61]. Nasal co-colonization with *S. aureus* may be due to the presence of non- piliated pneumococcal serotypes.

Several studies have described the effect of pneumococcal vaccination on *S. aureus* colonization[57-59,62]. In children younger than one year, pneumococcal conjugate vaccination resulted in an increase of *S. aureus* carriage and infections[62,63]. This may be due to the elimination of *S. pneumoniae* serotypes resulting in more ''space" for the *S. aureus* [62,63]. More recent studies in children younger than two years, have shown that the effect on *S. aureus* after pneumococcal conjugate vaccination was only temporary[64,65]. In this study, overall, no significant difference was observed in *S. aureus* carriage between vaccinated and not vaccinated individuals. However, higher *S. aureus* carriage was noticed in age groups 11-15 years and 31-40 years. These results might be explained by the fact that in addition to filling the gap left by *S. pneumoniae* shortly after vaccination in young children, *S. aureus* is able to co-colonize the upper respiratory tract next to re-emerged non-vaccine *S. pneumoniae* serotypes during years after vaccination[63]. This is due to the absence of competition by pneumococcal pili and production of hydrogen peroxide. The 11-15 years age group forms then a reservoir to transmit *S. aureus* to the exposed individuals, especially their parents who remain colonized for a long time[63]. This phenomenon was not reported in vaccinated older adults which might be explained by the lower density pneumococcal colonization in this age group[66].

SUMMARY AND IMPLICATIONS FOR FUTURE RESEARCH:

Invasive pneumococcal diseases (IPD) such as severe respiratory tract infections and blood stream infections result in high morbidity and mortality. Attempts to reduce morbidity and mortality focus on vaccination and antibiotic therapy. Despite the availability of conjugate and polysaccharides vaccines and antibiotics, IPD is still a leading cause of severe diseases worldwide.

This thesis focussed on carriage of *S. pneumoniae* as carriership is the first step in the development of disease. The effect of vaccination in nine European countries on the prevalence of carriage isolates in different age groups are described as well as the serotypes of these isolates and the antibiotic resistance in relation to the serotypes. A great variation in prevalence of circulating serotypes was observed depending on the vaccination policy, (i.e. the time of introduction of the vaccination, type of vaccine used, the target population and the presence of a catch-up campaign). Several serotypes such as serotype 7, 14 and 19A were related to antibiotic resistance and multidrug resistance. Surveillance of *S. pneumoniae* carriage isolates to determine the prevalence, the serotypes and the antibiotic resistance of the isolates is needed to contribute to optimal vaccine composition and antibiotic therapy of IPD. Change in prevalence of circulating non-vaccine serotypes (due to sero-replacement) might result in adaptation of the serotypes of the vaccine used. Information on the antibiotic resistance of the circulating serotypes is needed to make a rational empiric choice for the treatment of IPD and to set up an antibiotic stewardship program.

The first step to study the effect of vaccination on the circulating serotypes is a correct identification of the presumed *S. pneumoniae*. The adaptation of the current MALDI TOF MS method as developed in this thesis improved the identification of carriage isolates significantly and will be tested for clinical isolates. Vaccination will not only result in the elimination of vaccine serotypes and replacement by non-vaccine serotypes but will also influence the co-colonization of other species such as *S. aureus*. Surveillance of serotypes and antibiotic resistance in both clinical and carriage isolates in different age groups in relation to vaccination policy is strongly recommended to update and or adapt the composition of the vaccine and to optimize the empiric antibiotic choice of IPD.

RECOMMENDATIONS FOR FUTURE RESEARCH

Many challenges remain when studying *S. pneumoniae* regarding identification, carriage, antibiotic resistance and vaccination. Some of these are:

- To improve identification methods of *S. pneumoniae* and other mitis group members in order to avoid erroneous treatment or antibiotic resistance over- or underestimation.
- To widely use whole genome sequence in order to better understand the epidemiology of *S. pneumoniae* and the related species especially clinically relevant species as *S. pseudopneumoniae*.
- To test whether the MALDI TOF MS method as developed in this thesis for carriage isolates is also applicable for clinical isolates in routine microbiological laboratories.
- To perform surveillances of clinical isolates but also of carriership of pneumococci, in all age groups to keep an up to date database on the prevalence and antibiotic resistance of circulating vaccine and non-vaccine serotypes in relation to the vaccination policy. Such a national database will provide /information concerning:
  - The influence of vaccination policy on the distribution of serotypes of carriage and clinical isolates
  - The serotype adaptation of vaccines
  - The serotypes having a high tendency to cause IPD
  - The optimal treatment of IPD as part of an antibiotic stewardship program
- To study effect of viral infections (e.g. respiratory viruses) on the microbiome of the upper respiratory tract and the evolution of colonization to infection.
- To improve pneumococcal vaccine by developing a whole cell vaccine. This might be the solution to the replacement of pneumococcal serotypes after implementation of new vaccines. Awaiting the realization of such a vaccine, surveillance of (non) vaccine circulating serotypes, including the virulence and antibiotic resistance in relation to the current vaccines remains necessary for optimal vaccine composition.

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8 Nederlandse samenvatting

Streptococcus pneumoniae (pneumokok) behoort tot de commensale bacteriën van de bovenste luchtwegen. De pneumokok komt het meest voor bij kinderen onder 5 jaar (tot 60%) en ouderen (+60 jaar oud). In het merendeel van de personen leidt aanwezigheid van deze bacterie in de bovenste luchtwegen niet tot klachten. Men spreekt dan van asymptomatisch dragerschap. Echter, bij daling van de weerstand van de patiënt kan dragerschap leiden tot een infectie. Deze infecties kunnen relatief mild zijn zoals voorhoofdholte en/of oorontsteking maar pneumokokken kunnen ook ernstige invasieve infecties veroorzaken zoals hersenvliesontsteking, longontsteking of bloedstroominfecties. Verspreiding van pneumokokken vindt vooral plaats via contact met besmette persoon, via aanhoesten of via besmette oppervlakken en voorwerpen. In 2000, werden wereldwijd 14 miljoen ernstig invasieve pneumokokkeninfecties (IPD) gerapporteerd1. Jaarlijks overlijden ongeveer 1,6 miljoen patiënten aan deze infecties, waaronder een miljoen kinderen jonger dan 5 jaar.

In 2017 heeft de Wereldgezondheidsorganisatie (WHO) *S. pneumoniae* toegevoegd aan de lijst van twaalf belangrijke pathogenen die de grootste bedreiging voor de gezondheid van de mens vormen. De lijst is opgesteld om onderzoek en ontwikkeling van nieuwe antibiotica tegen deze micro-organismen te bevorderen als onderdeel van de inspanningen van de WHO om de wereldwijde resistentie tegen antibiotica tegen te gaan.

In deze thesis beschrijf ik het onderzoek dat is uitgevoerd om meer te begrijpen van het effect van vaccinatie op dragerschap en infectie door pneumokokken.

DEEL I. PNEUMOKOKKENIDENTIFICATIE:

S. pneumoniae maakt deel uit van een grote familie van streptokokken, de *S. mitis* groep waar ook *S. oralis* en *S. mitis* toe behoren. De verwantschap tussen de verschillende soorten streptokokken is groot, 99% van de DNA-volgorde komen met elkaar overeen. Correcte identificatie van *S. pneumoniae* is belangrijk voor inzicht in het vóórkomen en antibiotica resistentie van deze bacterie. *S. oralis* en *S. mitis* zijn in vergelijking met *S. pneumoniae* minder gevoelig voor penicilline en andere antibiotica en zijn vaak ongevoelig voor meerdere antibiotica tegelijk.

De identificatie van *S. pneumoniae* is tot nu toe vooral gebaseerd op fenotypische kenmerken van de bacterie. In de afgelopen jaren zijn andere methoden ontwikkeld gebaseerd op genetische eigenschappen van de bacterie zoals vermeerdering van genen die coderen voor virulentiefactoren, analyse van nucleotidensequenties van 16S rRNA en op proteoom gebaseerde methoden zoals de MALDI-TOF MS.

In Hoofdstuk 2 worden de resultaten beschreven van een vergelijkend onderzoek naar de toepasbaarheid (sensitiviteit en specificiteit) van de verschillende fenotypische en genotypische methoden voor de identificatie van *S. pneumoniae*. De fenotypische testen omvatten groei van de bacteriekolonie, optochine gevoeligheid en gal oplosbaarheid. De

genotypische test betreft de polymerase kettingreactie (PCR). De te testen bacteriën waren afkomstig uit de neus van 1371 patiënten uit huisartsenpraktijken in 9 Europese landen. Geen van de onderzochte methodes afzonderlijk was bruikbaar met een voldoend hoge sensitiviteit en specificiteit om onderscheid te maken tussen de *S. oralis, S. mitis* en *S. pneumoniae*. De meest gebruikte methode in routine laboratoria, de optochine gevoeligheid had een sensitiviteit en specificiteit van 81.7%, respectievelijk 64.0%.

Een proteomische test, MALDI TOF MS, werd toegepast voor de identificatie van 496 *S. pneumoniae* en 83 non- *S. pneumoniae* in **Hoofdstuk 3**. MALDI TOF MS, kan veel verschillende bacterie soorten (met name Gram negatieven) snel en correct te identificeren. Echter de methode was niet goed genoeg om met de standaard database onderscheid te maken tussen *S. pneumoniae* en zijn nauw verwante "soortgenoten". De sensitiviteit en specificiteit van deze methode waren 99.8%%, respectievelijk 44.6%. Aanpassing van de routine methode door gebruik te maken van spectrale analyse resulteerde in een correcte identificatie van pneumokokken en de andere streptokokken met een sensitiviteit van 99.4% en specificiteit van 98.8%. Deze resultaten zijn verkregen met "dragerschap isolaten" en deze methode lijkt dus zeer bruikbaar voor studies als in dit proefschrift uitgevoerd. De bruikbaarheid bij bacterie afkomstig van infecties vereist verder onderzoek. Als vergelijkbare resultaten worden verkregen zal het toepassen van deze methode in de routine diagnostiek de identificatie van pneumokokken verbeteren.

DEEL II. PNEUMOKOKKEN DRAGERSCHAP EN ANTIBIOTICA RESISTENTIE:

Pneumokokken dragerschap

Aandachtspunten bij de vergelijking van de verschillende studies naar de prevalentie van pneumokokken dragerschap, zijn de leeftijd van de onderzoekspopulatie en de wijze van afname van het patiënten materiaal. De meeste onderzoeken zijn uitgevoerd bij kinderen en maar beperkt bij volwassenen. Voor het afnemen van patentenmateriaal beveelt de WHO een nasopharyngeal uitstrijk (keelwat achter in de keel) aan. Echter voor een onderzoek met een groot aantal deelnemers, zoals beschreven in **Hoofdstuk 4**, is het afnemen van een dergelijke uitstrijk tijdrovend, belastend voor de personen en moeilijk uitvoerbaar. Bovendien is uit verschillende onderzoeken gebleken dat de resultaten verkregen met neusuitstrijken zoals in onze studie gebruikt niet significant verschilden met nasopharyngeale uitstrijken.

Hoofdstuk 4 beschrijft de prevalentie van pneumokokken dragerschap in 9 Europese landen (Oostenrijk, België, Kroatië, Frankrijk, Hongarije, Nederland, Spanje, Zweden en de Verenigde Koninkrijk) bij personen ouder dan 4 jaar, alleen in de UK was de leeftijdsgrens > 18 jaar vanwege ethische bezwaren. Gemiddeld werd bij 2.9%, Van de onderzochte personen de bacterie aangetroffen. Het voorkomen van de bacterie was afhankelijk van de leeftijd van

de onderzochte personen. Ook was er een grote variatie tussen de verschillende landen. Bij kinderen tussen 4-9 jaar in Spanje werd de bacterie het meest aangetroffen (24.0%) en het minst in België (12.8%). Bij deelnemers (ouder dan 10 jaar) waren de percentages het hoogst in Frankrijk (2.8%) en het laagste in het Verenigd Koninkrijk (2.1%). Het verschillende percentage zijn deels te verklaren door verschillen in het nationale vaccinatieprogramma zoals het gebruikte vaccin, het aantal toegediende doses, de doelgroep (alle kinderen of alleen de risicogroepen inclusief volwassene), en latere vaccinatie van personen buiten de beoogde leeftijdsgroep. Er was geen verschil in pneumokokken dragerschap tussen gevaccineerde (3.5%) en niet-gevaccineerde personen (2.9%).

Serotypes en vaccinaties

Zowel de hoge ziektelast van invasieve pneumokokken infecties en de toenemende antibiotica resistentie onderstrepen het belang van effectieve preventie. Vaccinatie is de hoeksteen van preventie van pneumokokken dragerschap en (invasieve) pneumokokken infecties. Er zijn twee verschillende soorten pneumokokkenvaccins op de markt: polysacharidenvaccins (PPV) en conjugaat vaccins (PCV). Polysacharidevaccin (PPV-23) beschermt tegen 23 verschillende pneumokokken serotypes. Dit vaccin bevat delen van het polysacharidekapsel die dienen als antigeen. Deze zorgen voor het opwekken van een T-cel onafhankelijke immuunrespons Omdat het immuunsysteem bij kinderen nog niet voldoende ontwikkeld is, wordt met dit vaccin bij hen geen immunologisch geheugen opgebouwd en wordt dit vaccin niet gegeven aan kinderen.

In conjugaat vaccins, worden de polysacharide antigenen gekoppeld aan een dragereiwit waardoor een T-cel afhankelijk immuunrespons wordt opgewekt. Tevens wordt na de vaccinatie een immunologisch geheugen opgebouwd. Momenteel zijn drie conjugaat vaccins beschikbaar PCV-7, PCV-10 en PCV-13 die bestaan uit 7 (4, 6B, 9V, 14, 18C, 19F en 23F), 10 (PCV7 serotypes plus serotypes 4, 5 en 7F) respectievelijk 13 serotypes (PCV10 serotypes plus serotypes 6, 6A en 19A).

Het gebruik van PCVs bij kinderen vermindert het dragerschap en het vóórkomen van ernstige pneumokokken infecties bij zowel kinderen als niet-gevaccineerde volwassenen door de opgebouwde "herd-immunity". Vaccinatie heeft echter als keerzijde dat door de afname van de vaccin serotypes, de "vrijgekomen plaatsen" ingevuld worden door niet-vaccintypes. Dit fenomeen wordt "Sero-replacement" genoemd. Monitoring van nieuwe opkomende pneumokokken-serotypes is belangrijk om inzicht te krijgen in serotypes die in de bevolking voorkomen, Deze informatie wordt gebruikt om indien nodig de samenstelling van de huidige vaccins aan te passen. Tot nu toe zijn meer dan 93 pneumokokken-serotypes bekend die verschillen in ziekmakend vermogen en in antibioticaresistentie. In **hoofdstuk 5** is de verdeling van serotypes en de relatie met antibioticaresistentie in pneumokokkendragers beschreven in de APRES-studie populatie. Serotypes 11A, 23A, 19A, 3, 6C, en 23B waren de meest voorkomende serotypes bij 6.4%, 6.2%, 5.5%, 5.3%, 4.7% respectievelijk

4.2% van de geteste populatie. Ten tijde van het onderzoek kwamen deze serotypes niet voor in de gebruikte PCV-vaccins. De meest voorkomende PCV7, PCV10 en PCV13 vaccin serotypes waren 19F (3.9%). 7F (1.8%) en 19A (5.5%). Resistentie tegen penicilline en ceftazidim was gerelateerd aan serotype 14 dat voorkomt in een PPV-23 en PCV7-vaccin (81.3 % voor beide antibiotica).

Antibiotica resistentie

Antibioticaresistentie is wereldwijd een toenemend probleem. Infecties veroorzaakt door antibioticaresistente *S. pneumoniae* zijn moeilijker te behandelen in vergelijking tot infecties veroorzaakt door streptokokken die gevoelig zijn voor antibiotica[2,3]. Ook de ziekteduur is langer en een eventuele ziekenhuisopname en de kans op overlijden is hoger. De meeste gegevens over het voorkomen van antibioticaresistentie zijn afkomstig van patiënten uit ziekenhuizen. Verschillende studies hebben een toenemende resistentie tegen zowel penicilline als erythromycine beschreven[4-8]. Zo steeg in de periode tussen 2011 en 2018 de penicilline resistentie van ziekenhuis isolaten in Frankrijk van 23.8% in 2011 naar 29.1% in 2018 en in Nederland van 1.1% in 2011 naar 3.2% in 2018[9]. Echter in andere landen zien we een daling in prevalentie van penicilline resistentie: in België van 0.8% in 2011 naar 0.1% in 2018 en in Roemenië van 61.1% in 2011 naar 40.0% in 2018[9] Het merendeel (~90%) van de antibiotica wordt door huisartsen voorgeschreven. Voor het opstellen van therapierichtlijnen voor de huisartsen is inzicht in de antibiotica resistentie van isolaten van patiënten uit de huisartsenpraktijk essentieel.

In het APRES-onderzoek werden neusuitstrijken van 32.000 patiënten uit huisartsenpraktijken uit negen Europese landen onderzocht op het vóórkomen en de antibiotica resistentie van *S. pneumoniae* **(Hoofdstuk 4)**. Er was een grote variatie in voorkomen van antibioticaresistente pneumokokken tussen de deelnemende landen. Resistentie voor cefaclor kwam het meeste voor (52,3%), variërend van 30,6% in het Verenigde Koningrijk tot 77,3% in België. Resistentie tegen tetracycline was het laagst in Zweden (1,9%) en het hoogst in Spanje (29,9%). De resistentie tegen penicilline was gemiddeld 19.4% en varieerde tussen 4.7% in Nederland en 31.7% in Spanje. Alle isolaten waren gevoelig voor moxifloxacine en ciprofloxacine.

DEEL III. INTERACTIE TUSSEN *S. PNEUMONIAE* EN *S. AUREUS*.

In de bovenste luchtwegen zijn verschillende bacterie soorten aanwezig waaronder *S. pneumoniae* en *S. aureus*. **Hoofdstuk 6** beschrijft de interactie tussen deze bacteriën en de vaccinatie status van de studie populatie. Er werd geen verband gevonden tussen de aanwezigheid van beide bacterien en vaccinatiestatus van de deelnemers, en ook niet met de serotypes van de pneumokokken in het gebruikte vaccin.

Twee pneumokokken serotypes, serotype 3 en 10A, kwamen vaker voor bij co-kolonisatie, in vergelijking met kolonisatie met pneumokokken alleen**.** Serotype 3 is opgenomen in PCV-13 (niet in gebruik in de tijd van deze studie). Beide serotypes 3 en 10A maken deel uit van het PPV-23-vaccin, in gebruik tijdens dit onderzoek in zeven van de negen participerende landen (Oostenrijk, België, Frankrijk, Nederland, Spanje, Zweden en Verenigd Koninkrijk).

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9

PhD portfolio

Publications

About the author

Dankwoord

PORTFOLIO

Name PhD student: Rachid Youcef Yahiaoui
PhD period: 2011-2020
Promotor: Pr.dr. Annelies Verbon
Co-Promoter: Dr. Ellen Stobberingh

Seminars and workshops:

Infectieziekten bij ouderen. Maastricht, Netherlands	2011
Results of a 4-year EC-funded project (APRES) and applications to antibiotic stewardship initiatives in Europe - Antibiotics and Resistance in Primary Care, the European Experience: What are the links between antibiotic prescriptions, treatment guidelines and anti-microbial resistance patterns in primary care in Europe? Amsterdam, Netherland	2014

Posters and oral presentations:

Scientific Spring Meeting NVMM 2014. Papendal, Netherlands. Commensal *Streptococcus pneumoniae* in Europe: prevalence and antibiotic resistance (poster)	2014
ECCMID 2014. Barcelona, Spain. Prevalence and resistance of commensal *Streptococcus pneumoniae* in nine European countries (poster)	2014
Scientific Spring Meeting NVMM. Papendal, Netherland. Commensal *Streptococcus pneumoniae* in Europe: capsular serotypes distribution (poster)	2016
ECCMID 2016. Amsterdam, Nederland Distribution of commensal Streptococcus pneumoniae capsular types in nine European countries (e-poster)	2016
European meeting on molecular diagnostics. Noordwijk, Netherlands Differentiation between *Streptococcus pneumoniae* and other viridians group streptococci by MALDI-TOF MS (poster)	2017
Scientific Spring Meeting NVMM 2018. Papendal, Netherlands Co-colonization between *Streptococcus pneumoniae* and *Staphylococcus aureus affects antibiotic resistance (poster)*	2018
ECCMID 2018. Madrid, Spain Effect of *Streptococcus pneumoniae* and *Staphylococcus aureus* co-colonization on antibiotic resistance (e-poster)	2018

Teaching

Co-supervised Bsc thesis "Identification of *S. pneumoniae* in clinical samples"	2017

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This thesis

1. **R.Y. Yahiaoui**, C.D.J. den Heijer, P. Wolffs, C.A. Bruggeman, E.E. Stobberingh. Evaluation of phenotypic and molecular methods for identification of *Streptococcus pneumoniae*. Future Microbiol. 2016;11(1):43-50. doi: 10.2217/fmb.15.124.
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5. **R.Y. Yahiaoui**, Casper D.J. den Heijer, Ellen E. Stobberingh. Effect of *Streptococcus pneumoniae* and *Staphylococcus aureus* co-colonization on antibiotic resistance. Submitted

Other:

1. E. van Elzakker, **R. Yahiaoui**, C. Visser, P. Oostvogel, A. Muller, Y.-R. Ho, J.-J. Wu, A. van Belkum. Epidemiology of, and prenatal molecular distinction between invasive and colonizing group B streptococci in The Netherlands and Taiwan. Eur J Clin Microbiol Infect Dis. 2009 Aug; 28(8): 921–928. https://link.springer.com/article/10.1007/s10096-009-0726-4.
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OVER DE AUTEUR

Rachid Yahiaoui werd geboren op 16 april 1974 in Alkmaar. Op drie jarige leeftijd is hij verhuisd naar Tunesië waar hij zijn basis, middelbare, en een deel van zijn universitaire opleiding heeft gevolgd. In 1998, behaalde hij de medische biologie analist diploma van de "l'École Supérieure des Sciences et Techniques de la Santé, université El Manar, Tunesië". Na een stop vervolgde hij zijn studie in Frankrijk waar hij in 2010 het masterdiploma in microbiologie heeft behaald. In 2011, krijgt hij de gelegenheid om als externe promovendus een promotieonderzoek op *Streptococcus pneumoniae* dragerschap in het kader van het project '' The appropriateness of prescribing antimicrobial agents in primary health care in Europe with respect to antimicrobial resistance" te beginnen in het Academische Ziekenhuis, Maastricht onder begeleiding van dr. Ellen Stobberingh en Pr.dr. Cathrien Bruggeman. Na het emeritaat van Pr.dr. Bruggenman, heeft Pr.dr. Annelies Verbon de stok overgenomen.

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