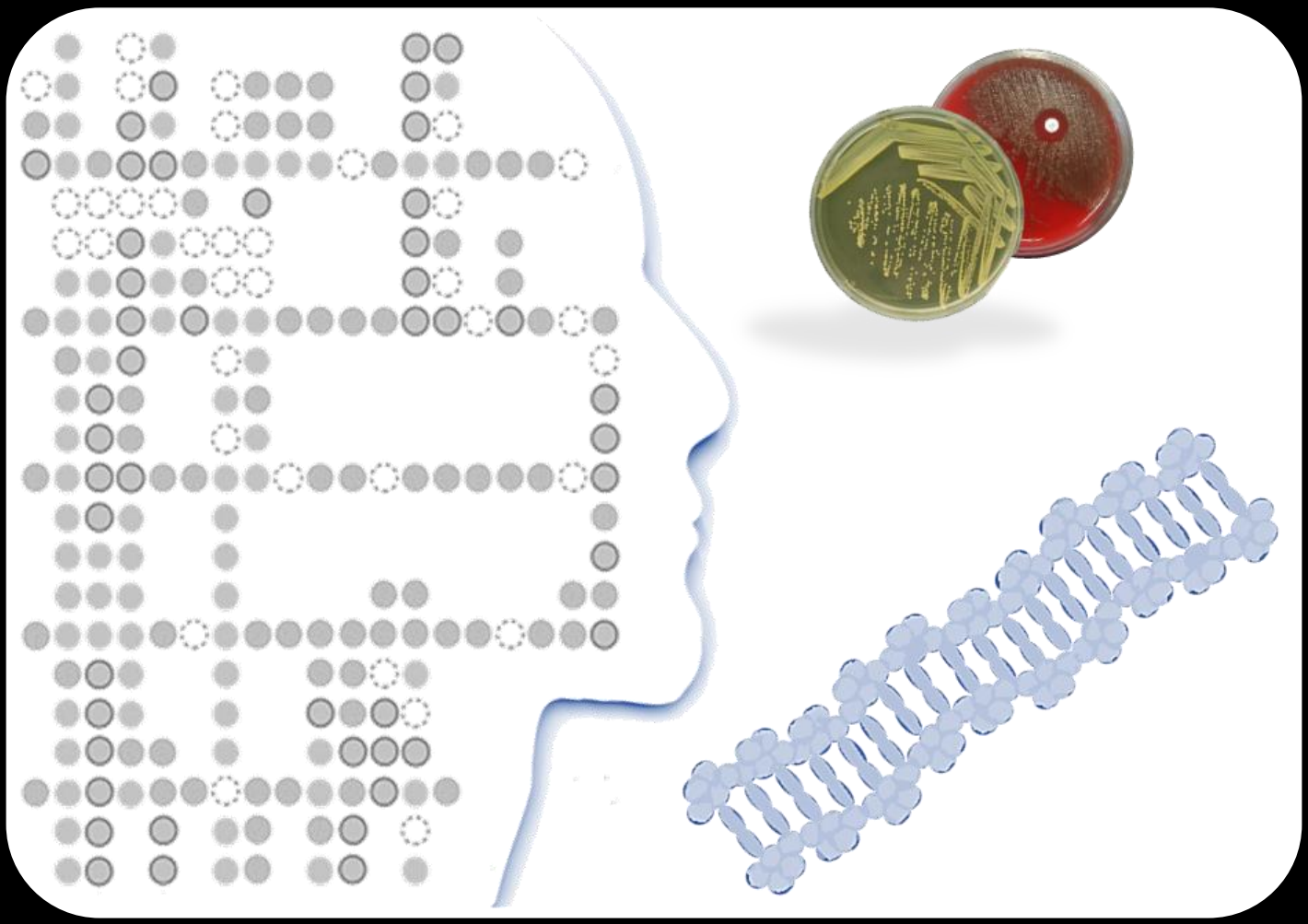
Dynamics of bacterial colonization in the upper respiratory tract of the adult host

Sónia Almeida



Dissertation presented to obtain the Ph.D degree in Biology | Molecular Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
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Abstract

Bacterial infections caused by microorganisms such as, *Streptococcus pneumoniae* and *Staphylococcus aureus* are a main cause of morbidity and mortality worldwide, particularly among young children, the elderly, and the immunocompromised of all ages. However, disease is incidental, and the natural lifestyle of these bacteria is through asymptomatic colonization of the upper respiratory tract.

Based on classical culture-based methods, colonization has been accepted to be high among young children and low among immunocompetent healthy adults. In the last years, cross-sectional studies using highly sensitive molecular methods have challenged this observation. While several studies have addressed bacterial colonization in children, much less information is available for the adult host, and even less from a longitudinal design.

In this thesis we conducted four studies aiming at understanding the upper respiratory tract bacterial colonization prevalence, density and dynamics of the immunocompetent adult host in Portugal.

In the first study we aimed to characterize the *S. pneumoniae* colonization dynamics in healthy adults aged between 25-50 years old. For that, nasopharyngeal, oropharyngeal and saliva samples were collected during a 6-month follow-up, and *S. pneumoniae* was detected using real-time PCR. We showed that the cumulative period-incidence of pneumococcal carriage in the adult host was high, 28.7% (95% CI: 20.3–38.9), when real-time PCR was used. Pneumococcal acquisition rate for the first event was estimated as 16.5 cases per 1000 persons-week (95% CI 11.2-24.2). Living with children ≤18 years old (HR:9.7, 95% CI 2.6-20.5; p<0.001) increased the hazard rate for pneumococcal acquisition. In contrast, antibiotic consumption during the 6-month follow-up (HR:0.1, 95% CI 0.01-0.9; p=0.036) decreased the hazard rate for pneumococcal acquisition. We also showed that pneumococcal clearance rate for the first event was estimated as 95.9 cases per 1000 persons-week (95% CI 62.3-145.0), i.e., the clearance half-life was seven weeks. Smoking (HR:2.7, 1.0-7.8; p=0.041) was shown to be an independent predictor of increased pneumococcal clearance. The results obtained in this study are challenging the paradigm of pneumococcal colonization dynamics among the adult host, as they suggest that pneumococcal carriage is higher than frequently estimated and the duration of carriage can be long, lasting several months.

In the second study we evaluated the prevalence of pneumococcal carriage and serotype distribution in nasopharyngeal and oropharyngeal paired samples from adults aged ≥60

years old living in nursing or in family homes using real-time PCR. Results were compared with those obtained by classical culture-based methods. We demonstrated that the use of real-time PCR, compared to classical culture-based methods, increased significantly the detection of *S. pneumoniae* carriers from 5.7% to 12.7% ($p<0.001$) in the nursing home collection and from 4.3% to 8.0% ($p=0.0026$) in the family home collection. We also demonstrated that the use of molecular methods was particularly valuable to detect pneumococci in samples that are highly polymicrobial and/or in which pneumococci are present at low density. Both conditions can often occur in the adult oropharynx. Overall, the results obtained in this study suggested that pneumococcal carriage in Portuguese elderly is higher, c.a. 10%, than the one previously estimated by classical culture-based methods and unveiled a large pool of serotypes circulating in this population.

In the third study we used the same collection of samples used in the second study to evaluate the prevalence of MRSA carriage in adults aged ≥60 years old. The samples corresponding to the nursing or family home collections were inoculated in a semi-selective enrichment medium and evaluated by qPCR targeting *nuc*, *mecA* and *mecC* genes. These results were compared with those obtained by classical culture-based methods. We showed that by semi-selective enrichment followed by qPCR, 34 (2.8%) of the 1,198 samples were positive for MRSA, compared to 21 (1.8%) by classical culture-based methods. The use of enrichment followed by qPCR improved non-significantly the detection of MRSA carriers from 5.4% to 8.0% ($p=0.12$) in the nursing home collection and from 0.3% to 1.7% ($p=0.13$) in the family collection. The results obtained in this study support that although the use of selective enrichment combined with qPCR improves sensitivity of detection of MRSA carriage in the community, its prevalence in adults over 60 years of age is low and is associated with hospital-associated MRSA clones.

In the fourth study we made use of the nasopharyngeal, oropharyngeal and saliva samples obtained during the 6-month follow-up conducted among healthy adults aged between 25-50 years old described in the first study. We aimed to determine the prevalence of *S. aureus* and MRSA carriage in the community, among immunocompetent healthy adults. The use of traditional culture-based methods revealed that 65.5% of the participants carried *S. aureus* at least once. Carriage rates per sampling site were 20.5% in nasopharynx, 18.3% in oropharynx, and 13.5% in saliva. Simultaneous screening of the three sampling sites increased the detection of *S. aureus*, which overall occurred in 34.4% of the sampling time-points. None of these adults carried MRSA during the six months of the study. Our results suggest that, in Portugal, MRSA

does not seem to circulate among healthy adults without risk factors and therefore this age group does not constitute a reservoir of MRSA in the community.

In conclusion, the studies presented in this thesis improved the knowledge on bacterial colonization prevalence, density and dynamics in the upper respiratory tract of the adult host. Our results point to the usefulness of molecular methods to detect pneumococcal carriage in the adult host (both senior and younger adults) and indicate that carriage is relatively frequent in these age groups. In particular, for healthy immunocompetent adults, our studies point out that duration of pneumococcal carriage can be long, lasting for several months. This work also supports the hypothesis that in Portugal, MRSA circulation in the community, among the healthy adult host, without risk factors, is infrequent, and, when occurring, is associated with MRSA clones typically found in hospitals. This population does not constitute, at the current time, a reservoir of MRSA in the community.

The results obtained in this thesis have allowed a better understanding of *S. pneumoniae* and *S. aureus* colonization dynamics among adults in Portugal. These findings should be useful to improve national strategies aiming to prevent infections caused by these two very important pathobionts.

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Resumo

Streptococcus pneumoniae e *Staphylococcus aureus* são bactérias comensais que colonizam assintomaticamente o tracto respiratório superior. No entanto são também responsáveis por infeções que estão associadas a elevadas taxas de mortalidade e morbilidade, especialmente em crianças, idosos e indivíduos imunocomprometidos.

Estudos com base em métodos clássicos de cultura mostraram que a taxa de colonização por estas bactérias é elevada em crianças até aos seis anos, e rara na idade adulta. Nos últimos anos foram desenvolvidos novos métodos de diagnóstico molecular com maior sensibilidade que sugeriram que a prevalência de colonização em adultos saudáveis é superior à prevista, estando, no entanto, pouco estudada.

No âmbito desta tese foram realizados quatro estudos com o objectivo de determinar a prevalência, densidade e dinâmica de colonização bacteriana no tracto respiratório superior de adultos saudáveis em Portugal.

Com o objectivo de caracterizar a dinâmica de colonização por *S. pneumoniae,* foram seguidos durante seis meses adultos saudáveis entre os 25 e os 50 anos. Por cada participante foram recolhidas amostras mensais e/ou semanais da nasofaringe, orofaringe e saliva. A colonização por *S. pneumoniae* foi identificada por métodos clássicos de cultura e por PCR em tempo real. A detecção de *S. pneumoniae* por PCR em tempo real revelou que a incidência cumulativa de colonização por *S. pneumoniae* foi elevada, 28,7% (95% IC: 20,3–38,9) nos seis meses. Dado o desenho longitudinal deste estudo foi possível determinar, para o primeiro evento de colonização, a taxa de aquisição de *S. pneumoniae* e a taxa com que um indivíduo deixa de estar colonizado por esta bactéria. A taxa de aquisição de *S. pneumoniae* foi de 16,5 casos por 1000 pessoas-semana (95% IC 11,2-24,2). Já a taxa com que um indivíduo deixa de estar colonizado por *S. pneumoniae* foi de 95,9 casos por 1000 pessoas-semana (95% IC 62,3-145,0), o que significa que a mediana da duração de colonização foi de sete semanas. A análise de factores de risco revelou que viver com crianças até aos 18 anos de idade (HR:9,7, 95% IC 2,6-20,5; p<0,001) aumentou o risco de aquisição de *S. pneumoniae*. Contrariamente, o consumo de antibióticos durante os seis meses do estudo (HR:0,1, 95% IC 0,01-0,9; p=0,036) diminuiu o risco de aquisição de *S. pneumoniae*. Verificou-se que ser fumador (HR:2,7, IC 1,0-7,8; p=0,041) estava associado a uma eliminação mais rápida de um evento de colonização por *S. pneumoniae*. Os resultados obtidos neste estudo sugerem que a dinâmica de colonização por *S. pneumoniae* nos adultos é diferente daquela que é frequentemente assumida, mostrando que a colonização por *S. pneumoniae* em adultos saudáveis é

elevada e a duração da colonização pode ser prolongada, estendendo-se por vários meses.

O segundo estudo teve como objectivo estimar a prevalência de colonização por *S. pneumoniae* e a distribuição de serotipos em adultos séniores utilizando PCR em tempo real, e comparar os resultados com os obtidos previamente por métodos clássicos de cultura. Analisámos pares de amostras da nasofaringe e da orofaringe de adultos com mais de 60 anos a viver em lares de idosos ou em habitação própria. Demonstrámos que o uso do PCR em tempo real permitiu estimar uma maior prevalência de colonização por *S. pneumoniae* em comparação com métodos clássicos de cultura: um aumento de 5,7% para 12,7% ($p<0,001$) na coleção de adultos a viver em lares e de 4,3% para 8,0% ($p=0,0026$) na coleção de adultos a viver em habitação própria. Adicionalmente observámos que o uso de métodos moleculares era particularmente útil na análise de amostras em que a diversidade microbiana é elevada ou quando *S. pneumoniae* está presente em baixa densidade como é frequentemente o caso na orofaringe de adultos. Assim, podemos inferir que a prevalência de colonização por *S. pneumoniae* em adultos com mais de 60 anos é mais elevada do que previamente estimada por métodos clássicos de cultura, e ainda que existe um elevado número de serotipos a circular nesta população.

O terceiro estudo teve como objectivo avaliar a prevalência de colonização por *S. aureus* resistente à meticilina (MRSA) utilizando um passo de enriquecimento semi-selectivo seguido de PCR em tempo real para três genes específicos (*nuc*, *mecA* e *mecC*), e comparar os resultados com os obtidos previamente por métodos clássicos de cultura. Para tal, foram utilizadas as coleções de amostras descritas no segundo estudo. De um total de 1198 amostras analisadas, 21 (1,8%) foram positivas por métodos clássicos de cultura, enquanto que 34 (2,8%) amostras foram positivas para MRSA quando testadas por enriquecimento semi-selectivo seguido de PCR em tempo real. Esta estratégia permitiu detectar um maior número de portadores de MRSA: houve um aumento de 5,4% para 8,0% ($p=0,12$) na coleção de adultos a viver em lares e de 0,3% para 1,7% ($p=0,13$) na coleção de adultos a viver em habitação própria. Estas observações suportam a hipótese de que o enriquecimento semi-selectivo seguido de PCR em tempo real aumentam a sensibilidade de detecção de colonização de MRSA. No entanto, a prevalência de MRSA na comunidade, em adultos com mais de 60 anos continua a ser baixa e está associada a clones encontrados frequentemente em ambientes hospitalares.

No quarto estudo utilizámos a mesma colecção de amostras descrita no primeiro estudo com o objectivo de determinar a prevalência de colonização por *S. aureus* e MRSA em adultos saudáveis entre os 25 e os 50 anos. As amostras da nasofaringe, orofaringe e saliva foram analisadas por métodos clássicos de cultura. Demonstrámos que 65,5% dos participantes eram portadores de *S. aureus*, pelo menos uma vez durante os seis meses em que decorreu este estudo. As taxas de colonização por *S. aureus* foram de 20,5% na nasofaringe, 18,3% na orofaringe e 13,5% na saliva. A colheita simultânea de amostras da nasofaringe, orofaringe e da saliva permitiu detectar *S. aureus* em 34,4% dos 526 eventos onde os três nichos ecológicos foram amostrados. Nenhum dos participantes era portador de MRSA. Estes resultados sugerem que, em Portugal, a prevalência de colonização de MRSA em adultos saudáveis, sem factores de risco é extremamente baixa e que esta população não constitui um reservatório de MRSA para a comunidade.

Em conclusão, o trabalho apresentado nesta tese aumenta o conhecimento sobre a prevalência, densidade e dinâmica de colonização no tracto respiratório superior do adulto. Os estudos realizados demonstraram que os métodos moleculares são uma ferramenta útil na detecção de colonização por *S. pneumoniae* em adultos (tanto em idosos como em adultos mais jovens) e que a colonização por *S. pneumoniae* é frequente nesta população. Em particular nos adultos imunocompetentes saudáveis, a duração de colonização por *S. pneumoniae* pode ser prolongada e durar vários meses. Este trabalho sugere que a circulação de MRSA entre adultos saudáveis na comunidade em Portugal não é frequente e que, quando ocorre, está associada a clones de MRSA encontrados frequentemente em ambiente hospitalar. Assim, esta população não parece constituir um reservatório de MRSA na comunidade.

Os resultados obtidos nesta tese permitiram uma melhor compreensão da dinâmica de colonização por *S. pneumoniae* e *S. aureus* nos adultos em Portugal. Estes estudos poderão contribuir para o melhoramento de estratégias nacionais que visem a prevenção de infeções causadas por estas duas bactérias.

Thesis outline

The purpose of the work presented in this thesis was to gain insights into the bacterial colonization prevalence, density and dynamics in the upper respiratory tract of the immunocompetent adult host. This thesis is composed of six chapters.

Chapter I provides a general overview focusing on important aspects of the epidemiology of two important pathobionts that frequently colonize the upper respiratory tract: *Streptococcus pneumoniae* and *Staphylococcus aureus*. In particular, the epidemiology of *S. pneumoniae* and *S. aureus* in the adult host, and the methods for the detection of these pathobionts are among the relevant topics addressed under the scope of this thesis. At the end of **Chapter I** the aims of this thesis are described.

Chapter II and **Chapter III** describe studies addressing the prevalence of *S. pneumoniae* colonization in the adult host.

Chapter II describes the dynamics of *S. pneumoniae* colonization in immunocompetent healthy adults aged between 25-50 years old. Nasopharyngeal, oropharyngeal and saliva samples were collected monthly during six months, and the incidence of pneumococcal colonization was determined by both, classical culture-based methods and by real-time PCR. Pneumococcal acquisition, median duration of carriage and clearance rate were assessed. In addition, risk factors associated with pneumococcal acquisition and clearance were also determined.

Chapter III describes a study conducted to evaluate the prevalence of *S. pneumoniae* colonization in adults aged over 60 years old, using highly sensitive molecular methods. Nasopharyngeal and oropharyngeal paired samples of individuals over 60 years of age living in nursing or family homes were analyzed by real-time PCR. The results were compared with those obtained by classical culture-based methods.

Chapter IV and **Chapter V** describe studies addressing the prevalence of *S. aureus* and MRSA in the adult host.

Chapter IV describes a study conducted to evaluate the prevalence of colonization by MRSA in adults older than 60 years of age, using highly sensitive DNA-based methods. The strategy used was based on the identification of MRSA using a semi-selective enrichment medium followed by real-time PCR in nasopharyngeal and oropharyngeal samples from two collections previously studied: individuals living in either nursing or family homes. The results were compared with those obtained by classical culture-based methods.

Chapter V describes a study addressing the prevalence of *S. aureus* and MRSA in the community, in the immunocompetent adult host. Samples obtained from nasopharynx, oropharynx and saliva of adults aged between 25-50 years old, obtained monthly, during a 6-month longitudinal study, were analyzed by widely accepted classical culture-based methods for the identification of *S. aureus*.

Chapter VI presents general conclusions, where the main findings of the studies conducted in this thesis were approached and highlights several questions that remain unanswered and could be the focus of future investigation.

Chapter III is a reproduction of the following publication and can be read independently: **Almeida, S. T., T. Pedro, A. C. Paulo, H. de Lencastre, and R. Sá-Leão.** 2020. Re-evaluation of *Streptococcus pneumoniae* carriage in Portuguese elderly by qPCR increases carriage estimates and unveils an expanded pool of serotypes. Sci Rep: 10(1): 8373. doi: 10.1038/s41598-020-65399-x.

Chapters II and IV have been submitted for publication and **Chapter V** is nearly ready for submission.

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Chapter I

Introduction

Introduction

The human health has always been a fundamental concern of Humankind. In recent years, the way we look at the human body during health and disease has changed profoundly. It is now amply recognized that humans are more than human cells and are, in fact, hosts of a complex network of microbial cells which are an essential part of ourselves. The human microbiome, often referred to as “our second genome”, is a diverse ecosystem. It consists of a wide variety of microorganism (such as bacteria, viruses, fungi and archaea) that inhabit different parts of the human body and co-evolve with it [1].

The interactions between the microbiome itself and with the host cells impact on human health and disease. The microbiome plays a role in human development, immunity, metabolic functions and nutrition, and its dysbiosis has been associated with several diseases such as diabetes, allergies, asthma, and cancer, among others. The microbiome composition and abundance can vary throughout life and between individuals, and can depend on a combination of several factors, such as the colonization site, host genetics, immune system and physiology. In addition, environmental factors such as diet, antimicrobial agents and lifestyles, can also be responsible for alterations in the composition of microbiota [1-3].

The microbiome of the upper respiratory tract is constituted by several bacterial species and virus, some of which are commensals and others are potentially pathogenic (pathobionts). Among the most common bacterial species are *Streptococcus pneumoniae* (or pneumococcus) and *Staphylococcus aureus*. These species are considered leading causes of morbidity and mortality worldwide [4].

Since it was first identified, *S. pneumoniae* has always generated concern due to its undeniable clinical importance. *S. pneumoniae* is responsible for high rates of mortality and morbidity worldwide, despite the advances in the use of antimicrobial agents and the introduction of pneumococcal multivalent conjugate vaccines [5]. On the other hand, *S. aureus* remains a major cause of nosocomial infections, and methicillin-resistant isolates (MRSA) which are difficult to treat and are often multidrug resistant, are emerging in the community [6]. In addition, there is no vaccine to prevent *S. aureus* infections.

Bacterial colonization of the human airways by these pathobionts, a phenomenon that is frequent and mostly asymptomatic, increases the risk of disease and is a source of transmission between hosts. Colonization studies are therefore of utmost importance [7].

In this thesis we focused on the study of both *S. pneumoniae* and MRSA among Portuguese asymptomatic adults.

The history of *Streptococcus pneumoniae*

S. pneumoniae was first documented in 1875 by Klebs in infected sputum and lung tissue. However, only some years later, in 1881, this pathogen was identified and characterized, independently, by two scientists. Pasteur, in France, injected saliva from a child with rabies into rabbits, while Sternberg, in the United States, injected his own saliva into rabbits. Both observed the same elongated diplococci-shaped bacteria in the blood of the rabbits. Pasteur designated them *Microbe septicemique du salive* and Sternberg designated them *Micrococcus pasteuri*. Two years later, pneumococcus was identified as a major cause of bacterial pneumonia in humans, and since then has also been associated with meningitis, bacteremia, sepsis endocarditis, arthritis, pneumonia, sinusitis and otitis media (reviewed in [8]).

Over the years, several names were attributed to pneumococcus: “pneumoniekokken” in 1883 by Mátray and “pneumokokkus” three years later by Fraenkel. In the same year, it was officially designated by *Diplococcus pneumoniae* by Weichselbaum. But it was not until 1974 that the actual name *Streptococcus pneumoniae* was adopted due to its morphology in chains when grown in liquid medium, and the type of disease caused by this bacterium (reviewed in [8]).

Since its discovery, pneumococcus has played a major role in several scientific advances. During the 1880s, this bacterium was one of the first organisms used for the development of the Gram’s stain that allows the distinction between Gram-positive and Gram-negative bacteria. The brothers Klemperer, in 1891, developed the concept of humoral immunity by injecting heat-killed pneumococci in rabbits. They showed that the rabbits’ serum contained factors that conferred immunity to a new infection with the same strain (reviewed in [9]). Years later, Heidelberg and Avery discovered the ability of polysaccharides to induce antibodies and their use as antigens in vaccines [10, 11]. In line with these scientific advances, Felton and Bailey showed that the bacterial capsule was responsible for the immunity against pneumococcal infections [12]. In 1944, Tillett *et al.* demonstrated the therapeutic efficacy of penicillin against pneumococcal pneumonia [13]. Another landmark of the 20th century in the history of *S. pneumoniae* came from the discovery of the “transforming principle”. In the late 1920s Griffith identified the mechanism of bacterial gene transfer [14], and in 1944, Avery, McLeod and

McCarty, identified, unequivocally, DNA as the genetic material [15]. Two decades later, while studying pneumococcal transformation, Tomasz described the first bacterial quorum-sensing factor [16].

Although *S. pneumoniae* was at the center of the development of antimicrobial therapy and vaccine immunity and was called as “captain of the men of death” by William Osler in 1918, it remains today a major cause of mortality and morbidity worldwide.

Streptococcus pneumoniae, the bacterium

S. pneumoniae (Figure 1) is a Gram-positive lancet-shaped bacterium, which is generally found in pairs, in the form of diplococcus or in short chains, and varies between 0.5 - 1.5 μm in diameter. It is a facultative anaerobic microorganism and when cultured on blood agar it shows α-hemolytic activity. Being a fastidious bacterium, it grows best in culture medium supplemented with blood, at 37°C in CO_2 supplemented atmosphere.

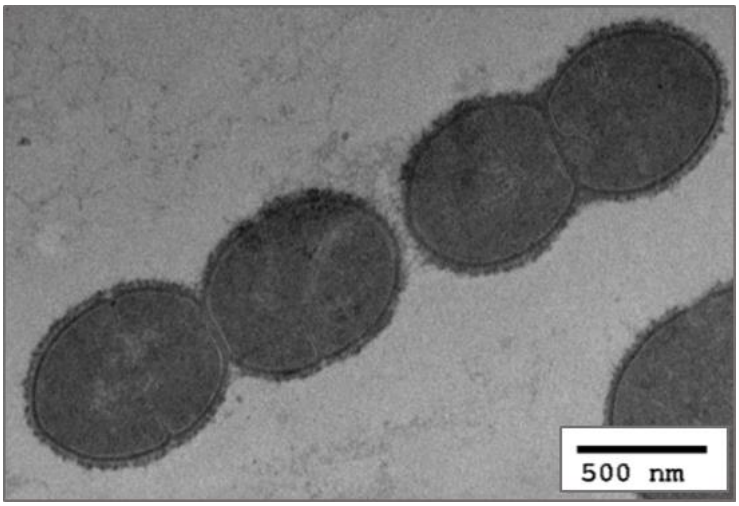

Figure 1. The pneumococcus. Transmission electron microscopy of *S. pneumoniae* cells, using a magnification of 15000X. The form of diplococci can be observed. Figure from the laboratory.

Pneumococcus is usually susceptible to optochin (Figure 2A) and soluble in the presence of bile salts (Figure 2B) [17-19]. These criteria, together with colony morphology on blood agar plates have been routinely used worldwide for pneumococcal presumptive identification [20].

Capsular types (or serotypes, further described below) can be assigned by the Quellung reaction, a method based on an immunological reaction between the polysaccharide capsule and specific antisera [18, 19].

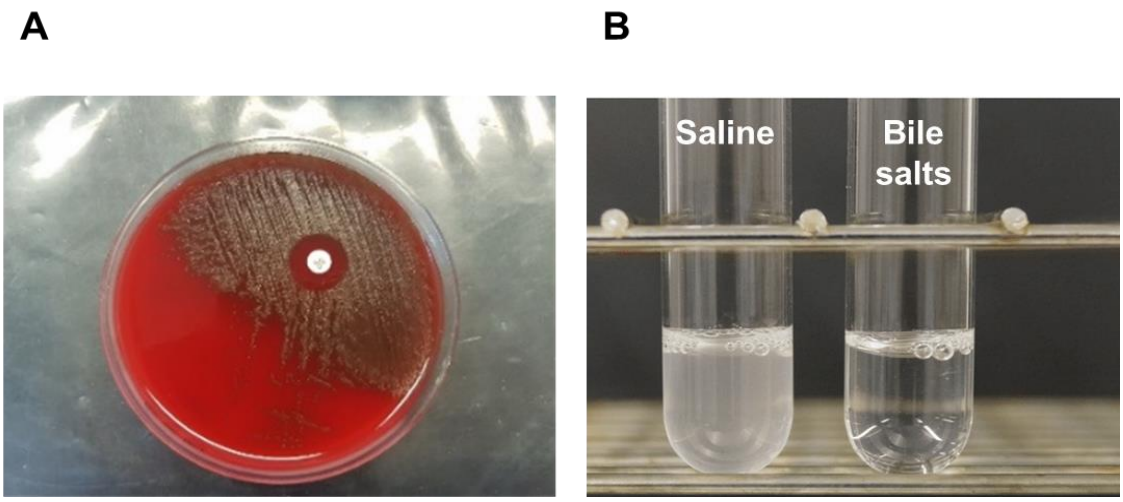

Figure 2. Tests routinely used for the identification of *S. pneumoniae*. (A) Susceptibility to optochin. (B) Solubility in bile salts. Figure 2A from the laboratory. Figure 2B adapted from *https://microbiologynotes.com/bile-solubility-test-principle-procedure-result-interpretation -examples-and-limitation/.*

The pneumococcal polysaccharide capsule

The great majority of pneumococci have a polysaccharide capsule which is considered its main virulence factor. The capsule acts like a shield against the host immune system and plays a major role in both disease and colonization (reviewed in [21]). Although its main function is the protection against opsonophagocytosis, the capsule is also essential to the survival of the bacterium in the lungs and its spread to the bloodstream [22]. Regarding colonization, the capsule is important to avoid entrapment in the mucus, delaying clearance [23].

The pneumococcal capsule is 200 to 400 nm thick and is constituted by an external layer of saccharide repeat units that surrounds the cells [24]. The biochemical structure of the pneumococcal capsules is very diverse and based on these differences more than 95 capsular types have been described up to now [25].

The polysaccharide capsule is covalently attached to the outer surface of the cell wall peptidoglycan, being synthesized by the Wzx/Wzy-dependent pathway in all serotypes, with the exception of serotypes 3 and 37, which are synthesized by the synthase pathway [24]. The genes responsible for the synthesis of the polysaccharide capsule through the Wzx/Wzy-dependent pathway are located at the same chromosomal locus - capsular polysaccharide synthesis (*cps*) locus - between *dexB* and *aliA* (that do not participate in the capsule biosynthesis) [26]. The structure of this conserved locus begins with four conserved genes encoding for loci *cpsA-D* that are involved in the regulation of the capsular biosynthesis [24]. The production of capsular polysaccharide of serotypes 3 and 37 requires a single glycosyltransferase – Cap3B/cps3S and Tts, respectively [26].

The pneumococcal capsule has been considered a key factor in what concerns the invasive disease potential of strains and their capacity to induce prolonged colonization. For example, serotypes 1 and 5 have been associated with a high invasive disease potential and are rarely detected in carriage. In contrast, serotypes 6A, 6B, 19F and 23F, tend to have a low invasive disease potential and have been associated with frequent and prolonged colonization [27-29].

Although the overwhelming majority of pneumococci have a polysaccharide capsule, some strains lack it. These strains are often designated as non-typeable (NT) based on the fact that the Quellung reaction cannot type them. The NT pneumococci are less virulent and are rarely isolated from invasive sources [30, 31]. Nevertheless, they have been associated with outbreaks of conjunctivitis [32] and after the introduction of PCVs, they have become more frequent in colonization [33, 34].

Epidemiology of *Streptococcus pneumoniae*

Pneumococcal colonization

S. pneumoniae colonizes the upper respiratory tract, mainly the human nasopharynx. Colonization is asymptomatic and very frequent in children under 5 years of age [35]. The nasopharynx of children is considered the main reservoir of pneumococci. Virtually every child carries pneumococci during its early years of life [7, 36, 37]. In contrast, colonization prevalence in immunocompetent adults and the elderly has long been considered almost insignificant [38, 39]. Colonization can occur soon after birth [40], being highest around the age of 2-3 years. After that, there is a steady decrease until the age of ten, remaining low during adulthood [35] (Figure 3).

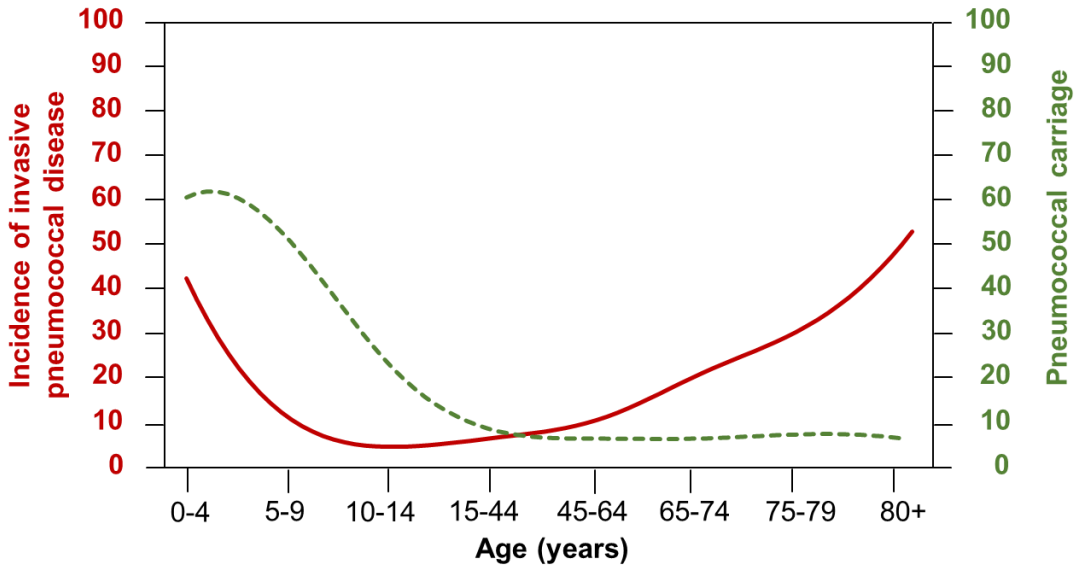

Figure 3. Incidence of invasive pneumococcal disease and pneumococcal carriage. Invasive pneumococcal disease is depicted in red. Pneumococcal carriage detected by classical culture-based methods is depicted in green.

Pneumococcal carriage is not a permanent state, and once in the nasopharynx, pneumococci can be cleared within days to months. Different pneumococcal serotypes can be acquired over time, or even at the same time [28, 41-43]. The duration of carriage depends on several factors such as the colonizing serotype, the age of the host and its immunocompetence, and previous exposure [35, 44-46]. Studies with a longitudinal design have estimated that the median duration of carriage is 60.5 days in children and 31 days in adults [45, 46].

Pneumococcal transmission occurs mainly via close contact between individuals, being young children the most important vehicle of transmission. However, it can also occur by aerosols and by contact with contaminated environmental surfaces [35, 47, 48].

Risk factors for pneumococcal carriage include young age (up to 2-3 years of age), frequent contact with children, crowded environments (day-care centers, nursing homes and prisons, among others), season, socioeconomic conditions, previous respiratory infections, asthma, chronic diseases and cigarette smoking. In addition, some populations, such as native Americans and Australian aborigines can also be more frequently colonized [35, 38, 43, 49, 50].

Pneumococcal colonization among young children has been studied extensively worldwide [33, 51-55]. These studies have shown that the prevalence of carriage and

serotype distribution can vary with the geographic area. For example, a systematic review estimated that in low and lower-middle income countries the prevalence of pneumococcal colonization is very high, especially among young children (up to 93.4% in children aged 2-4 years old) [54]. In contrast, a study conducted between 2009-2016 in Southern Israel among children aged 0-59 months showed pneumococcal carriage rates of c.a. 50% [55]. In Massachusetts, between 2000 and 2014, in children up to 7 years old, carriage rates ranged from 23% in 2004 to 32% in 2014 [52]. In England, between 2015-2016 carriage rates were c.a. 52% in children aged <5 years old [53], and in the Netherlands, between 2012-2013 carriage rates were 59% and 56% in children aged 11 months and 24 months, respectively [51]. All the above-mentioned studies relied on the use of classical culture-based methods in which a nasopharyngeal swab was plated in selective media and presumptive pneumococcal colonies were sub-cultured for species identification. In addition, participants were healthy children or children attending healthcare centers for reasons other than pneumococcal-related diseases.

In Portugal, since 1996 that studies on pneumococcal carriage have been regularly conducted among children 0-6 years old attending day-care centers. Carriage rates have remained stable over the years and are typically between 60%-65% [33, 36, 56-60].

While several studies have addressed bacterial colonization of the upper respiratory tract in children, there is limited information regarding colonization in immunocompetent healthy adults.

Pneumococcal carriage rates among adults can vary depending on several factors such as the geographic area, the age of population studied, the health status and the methodology used to detect pneumococcal colonization, among others [61].

In the beginning of the 20th century, some studies reported pneumococcal carriage rates between 45%-60% in the mouths of healthy adults. These early studies detected mostly pneumococci in samples from throat or saliva that were usually tested using mouse inoculation, a highly sensitive method. In fact, at that time, studies were performed using saliva and less often using nasopharyngeal or oropharyngeal swabs [62-67].

These studies were, overtime, replaced by studies using classical culture-based methods followed by identification of presumptive pneumococcal colonies in nasopharyngeal and/or oropharyngeal swabs. In fact, this became the WHO recommended method for almost 20 years [68]. With this approach the results obtained suggest that colonization prevalence and density in adults is significantly lower than in children (reviewed in [69]). For example, in Israel, in 2001, carriage rates in adults aged

18-40 years old were found to be c.a. 4% [39]. A cross-sectional study among healthy adults living in the Kilifi District, Kenya, in 2004, described carriage rates of c.a. 8% and 3% in individuals aged between 20-29 and 30-49 years, respectively [70]. In the Netherlands, nasopharyngeal and oropharyngeal swabs obtained from parents of 24-month-old children showed a carriage prevalence of c.a. 10% in 2012-2013 [51]. In the United Kingdom, in 2015-2016, two studies targeting different adult populations and using samples collected from different ecological niches showed different carriage rates. Carriage rates from nasopharyngeal samples from adults living with children <5 years old were 2.8%, while carriage rates from nasal washes from healthy adults without contact with children were 6.2% [53, 71].

In Portugal, the prevalence of pneumococcal colonization among the elderly has only been studied using classical culture-based methods. A previous cross-sectional study conducted by our group showed a carriage prevalence of 2.3% [38]. Regarding immunocompetent healthy non-aged adults, no carriage studies had been conducted prior to the work of this thesis.

Other European studies among the healthy senior population using classical culture-based methods have shown carriage rates of up to 5% (reviewed in [69]). In Belgium the pneumococcal colonization rates were c.a. 4% in both community-dwelling elderly and those in nursing homes [72]. In Finland, the carriage rates were similar, c.a. 5% among healthy individuals aged ≥65 years old [73]. In contrast, German adults aged over 65 years old living at home or in nursing homes were not found to be colonized with pneumococci during a cross-sectional study conducted between 2012-2013 [74]. Other countries outside Europe have reported very low carriage rates among the senior population. For example, in Brazil, nasopharyngeal swabs from outpatients aged ≥60 years old showed a pneumococcal carriage prevalence of 2.2% [75]. Among US adults aged ≥65 years old carriage rates were also low, 1.5%, between 2015-2016 [76].

Of note, a cross-sectional study conducted between 2008-2011, in the rural villages of Alaska, where the population lived in highly crowded conditions, showed that 16% of the adults aged 18-49 year old and 11% of those >50 years old were colonized with pneumococci [77].

The few longitudinal studies available that were conducted among healthy adults, showed carriage rates slightly higher than the ones described in studies with a cross-sectional design. Carriage rates of 14% were reported in Alaska in 2008-2012, in adults ≥18 years old [78]. And among Native American communities, pneumococci were

detected in c.a. 11%, 9% and 14% of those aged 17-<40, 40-<65 and ≥65 years old, respectively [79].

Over the years, novel non-culture DNA-based methods have been developed and are now being increasingly used (discussed below in this chapter). In fact, some studies that used real-time PCR (qPCR) to study pneumococcal colonization among adults described carriage rates between 0%-20% among healthy elderly individuals and 20%-40% among immunocompetent healthy adults [80-85]. Furthermore, colonization rates of c.a. 50% (period-prevalence within 7-9 weeks) were described in elderly individuals with influenza-like illness [86].

Pneumococcal disease

Nasopharyngeal colonization is a crucial step for the development of disease. From there, pneumococcus can spread to other body sites such as the middle ear, the lungs, the blood and the brain, causing a wide range of diseases, such as sinusitis, otitis media, pneumonia, bacteremia, meningitis and sepsis.

Risk factors for pneumococcal disease include the extremes of age, affecting disproportionally children under 5 years of age and adults over 65 years old, and individuals with certain underlying medical conditions such as chronic diseases and HIV infection among other immunocompromising conditions [7, 87].

Pneumococcal disease is considered a major public health problem. In fact, in 2017, the World Health Organization (WHO) considered *S. pneumoniae* as one of the “priority” pathogens, due to the high rates of disease and resistance to penicillin. It has been estimated that pneumococci is responsible for more than 11 million episodes of serious pneumococcal disease annually in the world [88] and for over one million deaths annually [89].

Although the global burden of morbidity and mortality associated with pneumococcal infections is very high worldwide, it varies across different geographic areas and with the season of the year [90, 91].

In the USA, pneumococcal disease causes 4 million episodes and 22,000 deaths annually. Pneumococcal pneumonia is utterly relevant in the scenario of pneumococcal disease. Each year, around 160,000 children under 5 years old and over 600,000 adults seek care or are hospitalized with pneumococcal pneumonia [92].

In developing countries pneumococcal pneumonia is one of the major causes of childhood mortality [88]. It is also the most common infectious disease in adults. Adults over 65 years old are the age group with the highest incidence and mortality risk worldwide associated with pneumococcal pneumonia [90].

According to the Annual Epidemiological Report for 2017 from the European Surveillance System, the incidence of invasive pneumococcal disease in Europe was 6.2 cases per 100,000 inhabitants. These rates varied across countries, ranging from 0.2 cases per 100,000 inhabitants in Luxembourg to 15.9 cases per 100,000 inhabitants in Slovenia, respectively. In Portugal the rate was 2.9 cases per 100,000 inhabitants. Invasive pneumococcal disease was especially reported in the elderly and in children under one year of age, where the incidence of invasive disease was 18.9 cases per 100,000 inhabitants and 14.5 cases per 100,000 inhabitants, respectively [93].

Association of serotypes with carriage and disease

The distribution of pneumococcal serotypes depends on several factors such as geographic area, disease manifestation, age, and vaccine coverage [35, 94-96]. In addition, several studies have reported that temporal changes, due to secular trends of specific serotypes, also affect serotype distribution, both in carriage and disease [97-100]. Still, studies have shown that only a small proportion of all serotypes causes most pneumococcal disease worldwide [94, 101].

In the 1990's, serotypes included in the 23-valent polysaccharide vaccine (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F) were associated with more than 80% of pneumococcal disease in adults [102].

Also, before introduction of pneumococcal conjugate vaccines, serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, all targeted by the seven-valent pneumococcal conjugate vaccine (PCV7) were the most prevalent in pediatric invasive disease in the USA [91].

In Portugal, it has been shown that the distribution of pneumococcal serotypes responsible for causing invasive disease remained stable until 2002. Among children <2 years of age, the most prevalent serotypes were 6B, 14, 23F, and 19A. In adults aged ≥60 years old, serotypes 1, 3, 4, 8 and 14 were the most prevalent, accounting for more than half of the isolates [103]. The introduction of pneumococcal conjugate vaccines led to significant changes among the pneumococcal population circulating in Portugal, which will be further discussed in this chapter.

Regarding disease manifestation, serotypes 3, 6A, 6B, 9V, 11A, 14, 19A, 19F and 23F were associated with otitis media, varying with geographic area, and vaccine coverage [104]. In contrast, serotypes 1, 4 and 14 were associated with bacteremia [94]. Serotypes 1 and 3 have also been associated with pneumonia [91].

Several authors have estimated the invasive disease potential of specific serotypes [27, 95, 96, 105]. These studies have shown that serotypes 1, 3, 4, 5, 7F, 9V, 14, 18C and 19A tend to have a high invasive disease potential [27, 95, 96]. In Portugal, the same was observed for those serotypes as well as for serotypes 8, 9N, 9L, 12B and 20 [105].

The same Portuguese study identified serotypes 6A, 6B, 11A, 15B/C, 16F, 19F, 23F, 34, 35F and 37 as being mostly associated with carriage, and having a low propensity to cause invasive disease [105].

Pneumococcal vaccination, the history behind

The first descriptions of vaccines to prevent pneumococcal infections go back to a century ago, in 1911, when Wright and colleagues developed a whole-cell heat-killed pneumococcal vaccine to treat South African gold miners (reviewed in [106]). In the following 30 years, clinical trials to demonstrate the efficacy and the safety of pneumococcal vaccines were performed. However, the validity of these trials was debatable due to flaws in the study design, adverse effects of the vaccines and scarce follow-up of the subjects (reviewed in [107]). By the 1940s, controlled trials of polysaccharide vaccines were made, demonstrating the efficacy of these vaccines in a military population. This led to the licensure of two hexavalent vaccines: one for administration in adults and other for the pediatric population. Almost at the same time, the efficacy of antibiotics to cure pneumococcal pneumonia was reported, and, as a consequence, the interest in pneumococcal vaccines declined and, those commercially available, were withdrawn from the market (reviewed in [106]). It was not until two decades after, that Austrian showed that despite antibiotic treatment, the mortality rate due to pneumococcal invasive disease remained high, c.a. 17%-30%, especially among patients aged over 60 years old [108]. In order to overcome these high mortality rates, Austrian and co-workers conducted clinical trials to develop an effective polyvalent pneumococcal polysaccharide vaccine. These efforts culminated with the licensure of a 14-valent polysaccharide vaccine in the United States in 1977, covering the 14 most common serotypes responsible for pneumococcal disease [109]. In 1983 this vaccine was expanded to protect against the 23 most common serotypes causing invasive

disease in adults (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F). The so called 23-valent pneumococcal polysaccharide vaccine (PPV23, with the commercial name of Pneumovax© 23), is still in the market today, and is recommended for individuals ≥2 and ≤64 years old with certain medical conditions that increase the risk of developing pneumococcal disease, and to all adults ≥65 years old. The major limitation of this vaccine is that it is not consistently effective in children younger than 2 years old, generating a poor immune response [102, 110, 111].

In 2000, the drawbacks of PPV23 were surpassed when the first pneumococcal conjugate vaccine – the 7-valent pneumococcal conjugate vaccine (PCV7, with the commercial name of Prevnar 7®) - reached the market. This vaccine was composed of polysaccharides covalently attached to a protein carrier, CRM197, which induces antibody production and immunological memory. PCV7 was licensed and introduced in the USA National immunization program in 2000. It targeted the most prevalent serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) causing invasive disease in children under 6 years of age in the USA [94, 101, 112]. Since then, two other pneumococcal conjugate vaccines reached the market. In 2009 a ten-valent pneumococcal conjugate vaccine (PCV10, with the commercial name of Synflorix®), targeting PCV7 serotypes plus three additional serotypes – 1, 5 and 7F – conjugated to a carrier protein, either non-typeable *Haemophilus influenzae* protein D, tetanus toxoid or diphtheria toxoid, was licensed. In 2010, a 13-valent pneumococcal conjugate vaccine (PCV13, with the commercial name of Prevnar 13®) replaced PCV7. PCV13 includes PCV10 serotypes plus three additional serotypes – 3, 6A and 19A – conjugated to CRM197 protein.

In Portugal, it was not until August 2015 that a PCV was introduced in the National Immunization Plan (NIP). Still, between 2001 and 2015 PCVs were available through the private market. PCV7 became commercially available in June 2001, PCV10 in April 2009, and PCV13 in January 2010. Usage estimates based on national sales indicated a gradual increase in PCV7 use, reaching 75% in 2008. For PCV13 the coverage estimate was 63% in 2012 (IMS and INE/National Statistic Institute). Regarding the adult population, PPV23 has been available in Portugal since 1996 and its usage has been estimated as approximately 10% between 2003-2007 [113]. In addition, PCV13 was recommended for adults over 50 years of age in 2012. Its usage, however, was low (<10%) between 2012-2015 [114]. In 2015, recommendations from national health authorities suggested sequential vaccination with PCV13 and PPV23 for specific adult risk groups [115].

The beneficial impact of conjugate vaccines on public health is undeniable. However, these vaccines cannot be considered a definitive solution to prevent pneumococcal disease, as they only target a limited number of serotypes. Currently there are several pneumococcal vaccines under investigation not only with higher valency, but also serotype-independent vaccines, which have the potential to elicit anti-pneumococcal protection regardless of capsular type [116].

Vaccination consequences on pneumococcal disease

With the introduction of conjugate vaccines, the pneumococcal disease epidemiology changed deeply worldwide in countries where the vaccine was introduced. Differences in effectiveness of PCVs across countries have been mainly associated with vaccination schemes, vaccine coverage and circulating serotypes [97, 117, 118].

An incredible impact was found early after the introduction of PCV7 in the USA immunization program in 2000. In 2005, the CDC reported a decrease of 77% decline in overall invasive pneumococcal disease incidence and a 98% decline in PCV7 serotypes in children under 5 years of age [118]. In Europe, the impact of PCV7 varied across countries. For example, in countries such as Belgium, France and Spain the incidence of invasive disease caused by PCV7 serotypes decreased by 22%, 52% and 58%, respectively, within two years in Belgium and four years in France and Spain of PCV7 introduction. In these countries PCV7 became available in 2004-2005, 2001-2002 and 2000-2001, respectively, and the proportion of vaccinated children aged <2 years old by two years in Belgium and by four years in France and Spain of PCV7 was 42%, 48% and 33%, respectively, which may explain, in part, the differences observed [97].

The decrease of invasive pneumococcal disease was not only observed among the vaccinated population, but also among those that were not vaccinated, due to a herd immunity effect. In the USA, for example, between 1998 and 2015, a reduction of 50% in invasive disease rates among the adult population was observed [118]. In fact, after about six years of PCV7 use, the rates of invasive disease caused by PCV7 serotypes decreased from 56% to 10% in adults aged between 18-64 years old and from 56% to 9% among adults aged over 64 years old [119]. Also countries such as Canada, Germany, and England and Wales observed a decrease in invasive disease caused by PCV7 serotypes in populations not targeted for PCV immunization (reviewed in [120]).

Although a decline in PCV7 serotypes occurred, an increase in the incidence of invasive pneumococcal disease caused by non-vaccine serotypes (NVT) was observed. In

particular, serotype 19A increased worldwide as an important cause of pneumococcal disease. Its prevalence among invasive disease isolates ranged from 22% in Spanish children under 5 years old, between 2001 and 2005, to 40% in American children of the same age between 2002 and 2005 [121, 122]. Other serotypes – such as serotypes 1, 3, 6A and 7F – were also noted as important causes of replacement disease (reviewed in [123]). This phenomenon was also observed among adults. A study conducted in England and Wales showed that pneumococcal disease caused by non-vaccine types increased by 48% in adults aged over 65 years old [124]. In addition, other study, conducted in Spain, showed that serotypes 1, 3, 7F and 19A accounted for almost 40% of invasive pneumococcal disease cases among this same age group [125].

The introduction of PCV7 had also consequences on antimicrobial resistance levels among pneumococci. There was a decrease in resistant pneumococci causing disease in various countries following PCV7 vaccination. However, almost at the same time, there was an increase of penicillin resistant non-PCV7 serotypes, especially due to serotype 19A [126, 127].

Similar effects as the ones described for PCV7 were observed with the introduction of PCV13. Between 2012 and 2013, in the USA, among children under 5 years old, a decline of 64% and 93% in invasive disease overall cases and in invasive disease due to the six additional serotypes of PCV13, respectively, was observed. Among adults, the overall incidence of invasive disease and invasive disease caused by PCV13-only serotypes declined by 12%-32% and 58%-72%, respectively, depending on age. These observations were mainly attributed to the decrease of invasive disease caused by serotype 19A [128].

In Spain, in the region of Catalonia, the incidence of invasive pneumococcal disease was studied between 2011 (early-PCV13) and 2016 (late-PCV13). The authors observed a decrease in the overall incidence of invasive disease by 31% in children aged <5 years old and by 3% in adults aged between 18-64 years old. The incidence of invasive disease caused by PCV13 serotypes declined in both age groups: by c.a. 36% in children aged <5 years old and by 29% in adults aged between 18-64 years old [129].

The abovementioned studies showed that with the decrease of PCV13 serotypes, non-PCV13 serotypes increased, suggesting serotype replacement, as had previously occurred following PCV7 vaccination [128, 129]. The distribution of the emerging non-PCV13 serotypes varied according to the geographic area. For example, in Europe the most prevalent non-PCV13 serotypes were 10A, 12F, 24F, 22F and 15C, while in North America were serotypes 22F, 33F, 15B, 38 and 35B (reviewed in [130]).

In Portugal, surveillance of invasive pneumococcal disease has been systematically done since 1999 by the Portuguese Group for the Study of Streptococcal Infections. This laboratory-based surveillance system, which includes 30 laboratories of microbiology throughout Portugal, identifies and sends all isolates responsible of invasive pneumococcal disease to a central laboratory for characterization.

Portuguese studies have shown changes for both children and adults, in the distribution of serotypes causing invasive disease, after PCV7 became available. In the early post PCV7 period (2003-2005), despite a moderate PCV7 use (43% in 2004), there was a significant decline in the proportion of invasive infections caused by serotypes 6B, 14 and 23F in children ≤5 years old, and in serotype 4 and 14 in the adult population. The decline in these serotypes was accompanied by an increase in the proportion of invasive infections caused by serotypes 19A (in both groups) and 7F (in adults) [131].

Two studies conducted between 2006 and 2008 among children and adults reinforced the main findings of the previous analysis. In children aged up to 17 years old a decrease in invasive disease caused by PCV7 serotypes, from 56% in 1999-2002 to 17% in 2006-2008, occurred. Besides this decrease, there was an increase in the proportion of serotypes 1, 3, 7F, 14, and 19A that together accounted for c.a. 70% of the pediatric invasive pneumococcal infections [132]. Regarding the adult population, changes were also observed, with a decrease in the proportion of PCV7 serotypes causing invasive disease, from 30% in 1999-2003 to 16% in 2008. The most frequently detected serotypes in this population were the same detected in children that, together, accounted for more than 50% of the adult invasive infections [113].

PCV10 and PCV13 became available in Portugal in mid-2009 and early-2010, respectively. Between 2008 and 2012, the incidence of invasive disease in individuals aged <18 years old decreased by half, from 8.19 cases per 100,000 (in 2008-2009) to 4.52 per 100,000 (in 2011-2012). In this period, serotypes 1, 7F and 19A remained the most prevalent ones, accounting for almost 60% of all invasive isolates [133]. Among Portuguese adults, between 2009-2011, a period of transition from PCV7 to PCV10 and PCV13, the most frequent serotypes, accounting for 40% of all adult invasive disease, were serotypes 3, 7F, 19A, and 14. In parallel, there was a decline in serotypes 1, 5 and 6A, and an increase in serotype 34. Serotypes included in PCV13 declined from 70.2% in 2008 to 53.5% in 2011. Nevertheless, in this period, most of pneumococcal invasive infections were still potentially vaccine preventable [134].

Just before the introduction of PCV13 in the Portuguese National Immunization Program, between 2012 and 2015, the serotype dynamics fluctuated slightly, with serotypes 1, 7F,

14, 19A remaining the most prevalent ones, in addition to serotypes 3, 6B, 10A, 12B, 15B/C and 24F, which in total, accounted for c.a. 69% of all pediatric invasive cases. These results indicated that PCV13 serotypes still accounted for a significant proportion of pediatric invasive infections (c.a. 60%) [135]. In the same period, in adults, serotypes 3, 7F, 8, 14, 19A and 22F were the most prevalent, accounting for 50% of all invasive isolates, being serotype 3 the most prevalent (c.a. 14%). Still, a decrease in the prevalence of invasive disease caused by PCV13 serotypes from 51% in 2012 to 38% in 2014 was observed. This decrease was mainly due to a decline in serotypes 7F and 19A [136].

Vaccination consequences on pneumococcal carriage

Pneumococcal conjugate vaccines impact not only on disease, but on colonization as well. Before the introduction of PCVs, the serotypes most associated to nasopharyngeal carriage were similar worldwide and included serotypes 6B, 9V, 14, 19F, and 23F, which were all included in PCV7 [35]. These serotypes, together with serotypes 6A and 19A (later included in PCV13), were also associated with the majority of antimicrobial resistance in pneumococcus [137].

Several studies conducted worldwide have shown that following PCV7 vaccination, there was a decrease in the nasopharyngeal carriage of PCV7 serotypes. This was accompanied by an increase in the non-PCV7 serotypes, a phenomenon known as serotype replacement [59, 138-140]. In Massachusetts, results from a carriage study among children showed a decrease of vaccine types following introduction of PCV7 and, by 2007, the most common carried serotypes were all non-PCV7 serotypes - 6A, 11A, 15B/C, 19A and 35B- accounting for more than half of the isolates [138].

Across Europe, in countries such as Norway, Italy and the Netherlands, similar results were reported [51, 139, 141]. In Norway, two years after PCV7 became commercially available, it was shown that the overall prevalence of carriage remained unchanged as PCV7 serotypes, previously prevalent, were replaced by non-PCV7 serotypes 9N, 16F, 24F, 35B and 35F serotypes [139]. Bosh *et al*. reported that, in the Netherlands, in the post-PCV7 period (seven years after introduction of PCV7 in the Dutch national immunization program), serotype 19A was the main serotype carried by children and their parents [51]. In addition, in the United Kingdom, a cross-sectional study conducted among children, their parents and older adults showed that, following PCV7 introduction, low carriage rates of PCV7 serotypes among the three groups were observed. PCV7

serotypes accounted for 1.5%, 0.0% and 15.4% of the carried strains by children, their parents and older adults, respectively [142].

The dynamics of nasopharyngeal carriage after the introduction of PCV13 has also been monitored. In France, Cohen *et al.* described the impact of this vaccine on pneumococcal nasopharyngeal carriage of children with acute otitis media shortly after its introduction (2010-2011). A decrease in PCV13 serotypes 19A and 7F, in addition to the non-PCV 6C serotype was observed in PCV13 vaccinated children when compared with children only vaccinated with PCV7 [143]. In the United Kingdom, the introduction of PCV13 led to a decrease in PCV13 serotypes carriage being noted in 2013-2014, in both vaccinated and non-vaccinated individuals [144]. Five years after the introduction of PCV13, carriage of serotypes 19A, 6C and 7F (all included in PCV13) had decreased among vaccinated children. The most prevalent serotypes carried by children were by that time the non-vaccine types 15B/C, 23B, and 11A [145].

In Portugal, since colonization studies in children 0-6 years old attending day-care centers have been performed for more than 20 years, it was possible to establish a robust pre-PCV baseline enabling the study of the impact of pneumococcal conjugate vaccines in carriage and in antimicrobial resistance. These studies showed that, five years after PCV7 commercialization, a significant decline in PCV7 serotypes had occurred as well as serotype replacement with no changes in the overall prevalence of carriage in this age group (c.a. 65%). In fact, vaccine types decreased from 53.1% to 11.2%, and the most prevalent serotypes were the non-PCV7 types 1, 6C, 7F, 15A, 16F, 21, 23A, 29, and NT. Although at the time, it was not statistically significant, an increase in 19A was also observed [59]. More recently, the impact of the private use of PCV7 in 2009-2010 was also studied. Our group observed that serotype replacement was extensive, and with the exception of serotype 19F, all PCV7 serotypes were rarely detected. The most prevalent serotypes in these years were 6C, 15A, 15B/C, 16F, 21, 23B and NT [33].

The impact of the private use of PCV13 in Portuguese children attending day-care centers has also been evaluated. Between 2009 and 2016 cross-sectional studies were carried out in urban and rural areas of Portugal. It was observed that carriage of PCV13 serotypes decreased significantly in both regions. This occurred mostly due to a reduction in the prevalence of serotype 19A. In addition, following introduction of PCV13 in the national immunization program in 2015, serotype replacement continued to occur and serotypes 11D, 15B/C and 23B became the most prevalent in the urban areas, while serotypes 15A, 23A and 35F became the most prevalent in the rural areas [146].

The effects of both PCV7 and PCV13 on co-colonization, defined as simultaneous carriage of more than one pneumococcal strain, were also evaluated in Portuguese children attending day-care centers. Valente and colleagues showed that the prevalence of co-colonization was lower among PCV7 and PCV13 vaccinated children. These studies suggested an unanticipated potential benefit of pneumococcal conjugate vaccines, as a decrease in co-colonization may translate in reduced opportunities for horizontal gene transfer [42, 147].

The challenge of identification of *S. pneumoniae*

S. pneumoniae belongs to the mitis group of streptococci, which also includes other species such as *S. pseudopneumoniae*, *S. mitis*, *S. oralis*, *S. gordoni*, *S. sanguis* and *S. parasanguis*. [148, 149]. All streptococci of the mitis group are part of the commensal flora of the human upper respiratory tract. However, the closest relatives of pneumococcus - *S. oralis*, *S. pseudopneumoniae* and *S. mitis* - have also been identified as cause of infections, especially in immunocompromised individuals [150-152].

In the nasopharynx streptococcus of the mitis group co-habit together and, being naturally competent for genetic transformation, exchange of genetic material can occur. As a consequence of evolutionary relationships and occurrence of horizontal gene transfer, it is often difficult to define and correctly speciate isolates of streptococcus of the mitis group as there are several examples in which the species boundaries seem to have been blurred [153-155].

The significance of correct identification of pneumococcus

Given the high burden of morbidity and mortality that pneumococcus is responsible for, its correct identification is of utmost importance for both, clinical diagnosis and surveillance studies.

Despite the advances in science and the epidemiological relevance of invasive pneumococcal disease, the clinical diagnosis of pneumococcus infections is still mostly dependent on classical culture-based methods with isolation of live pneumococcus from a normally sterile body site, followed by characterization of the strain. The incorrect identification of the pathogen that is responsible for causing an infection has not only an impact in the accuracy of the disease burden, but also in the assessment of the effectiveness of vaccination against pneumococcal disease [92, 156].

Accurate identification of *S. pneumoniae* is also crucial in carriage surveillance studies aiming, for example, to study the impact of pneumococcal conjugate vaccines and to better elucidate the link between carriage and disease. Moreover, misidentification of *S. pneumoniae* can lead to a false increase in the rates of antimicrobial resistance [157, 158].

The correct identification of pneumococcus is only possible with good and adequate typing methods. An optimal typing method should be unambiguous to allow comparisons between laboratories around the world, have high typeability and discriminative power. It should also be easy to perform, quick and cost-effective [159].

Methods for the detection of *S. pneumoniae*, the road so far

Between 1998 and 2001, the World Health Organization united, for the first time a working group to establish a set of standard methods for the study of pneumococcal colonization. Since then, significant advances in typing methods have occurred and, given the importance of carriage studies, the WHO asked, once again, a group of experts to do an update of the consensus standard methods, which was published in 2013 [20, 68].

The current gold standard method approved by WHO for detecting *S. pneumoniae* in carriage surveillance is based on classical culture-based methods. Traditionally, identification of pneumococcus implies the collection of a nasopharyngeal swab and isolation of live pneumococci followed by identification of presumptive colonies. In order to distinguish pneumococcus from other closely related species its phenotypic characteristics are used. Usually, pneumococcal colonies have a central depression or mucoid morphology, are susceptible to optochin and soluble in bile salts. In addition, a capsular type can be frequently assigned to pure cultures using serotype-specific sera [17-19]. This is considered the standard method for pneumococcal serotyping [20]. However, it is an expensive and time-consuming methodology that is prone to some subjectivity when interpreting the results, being necessary some technical expertise.

Most of the epidemiological surveillance studies on pneumococcal carriage have been conducted among children. Recommendations from WHO state that only nasopharynx should be collected in this age group [20].

In contrast, when studying pneumococcal carriage in adults, the updated recommendations from WHO state that both nasopharyngeal and oropharyngeal

samples should be collected [20]. In fact, for adults, the added value of oropharyngeal samples has been suggested [81, 83, 160]. Watt and colleagues, in 2004, conducted a pneumococcal carriage study among the Navajo and White Mountain Apache adult population. They found that although pneumococcal carriage rates and density were higher in nasopharyngeal samples than in oropharyngeal samples, sampling both sites significantly increased the rates of carriage in this population [160]. However, some studies have shown that the microbiota complexity varies in different ecological niches, with the oropharynx having a higher bacterial density and diversity when compared to the nasopharynx [161, 162]. Furthermore, it has been reported that the high polymicrobial nature of oropharyngeal samples impairs the sensitivity of the classical culture-based methods to detect pneumococcus [163]. In addition, the poor sensitivity of classical culture-based methods has also been observed in samples from aged adults where pneumococcal prevalence and density are usually low [164].

It is interesting to note that studies from the early 1900s, in the pre-antibiotic period, used saliva to detect carriage, and described it as being one of the most sensitive sampling methods. At that time, mice susceptible to pneumococcal infection were inoculated with human saliva, and pneumococcus was then isolated from the blood of the infected mice. Based on these studies, it was estimated that between 45%-60% of all adults, including elderly, carried pneumococcus in saliva [62-67]. Since then, the preferential sampling site to detect pneumococcus moved from saliva to the nasopharynx. This was mostly because of the highly polymicrobial nature of saliva that makes it almost impossible the detection of pneumococcus by classical culture-based methods. Nevertheless, already in this century, Trzciński et al. provided evidence for the superiority of saliva samples over nasopharyngeal or oropharyngeal samples for the detection of pneumococcal carriage in children and, especially, in adults [82, 86, 165]. The authors overcame some of the limitations of the classical culture-based methods by using highly sensitive molecular methods for pneumococcal identification. These methods have been validated and are now being increasingly used worldwide [80, 81, 83, 86, 166].

The identification of *S. pneumoniae* by molecular methods usually relies on the identification of ubiquitous genes, essentially virulence factors, that are not present in its less pathogenic close relatives. Today, methods based on *lytA* gene (encoding for the major pneumococcal autolysin) as molecular target, are widely used for identification of pneumococcus.

In the last two decades, several other molecular methods have been described. For example, multilocus sequence typing (MLST), multilocus sequence analysis (MLVA) and

bacterial identification by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) [167-169]. In recent years, whole genome sequencing (WGS) is being increasingly used for the characterization of pneumococci. This methodology is able to provide an accurate identification of the species and also allows identification of several other characteristics such as serotype, gene allelic profiles, virulence determinants and antibiotic resistance profiles [170-172].

Currently, the WHO gold standard method for culture-independent identification of *S. pneumoniae* in carriage samples is a qPCR targeting the *lytA* gene as described by Carvalho *et al.* [20, 173].

PCR-based assays, are also being used for the direct detection of pneumococci from clinical samples, since they offer a quick and sensitive approach when compared to classical-culture based methods, even in pediatric patients who have received antimicrobial therapy [174, 175]. For example, a study conducted in Barcelona, between 2007 and 2009, among children <5 years of age showed that pneumococci were exclusively detected by qPCR in 66.5% of the invasive isolates, in contrast to 33.5% by classical culture-based methods. In addition, serotype 3, which was rarely detected by classical culture-based methods, was frequently detected by qPCR [175]. Recent findings from Portugal also confirmed that the use of molecular methods improved the detection of pneumococci in disease isolates. In that study, 135 pleural fluid samples obtained from pediatric patients with complicated pneumonia, previously considered pneumococcal culture-negative samples, were tested by qPCR. Of these, 68% were identified as pediatric complicated pneumonia caused by *S. pneumoniae*. The most frequent serotype detected was serotype 3 [176].

Improving *S. pneumoniae* molecular identification by real-time PCR

Nowadays, *lytA* qPCR is widely accepted as a molecular target for the identification of pneumococcus [83, 173]. However, *lytA* homologs have been described in some streptococcal species [177]. An additional qPCR targeting *piaB* (encoding for the iron uptake ABC transporter lipoprotein PiaB) has been proposed to be performed in parallel with *lytA* qPCR [83, 165]. Although the use of both assays increases specificity, some limitations should be taken into consideration. *piaB*, which was first described as ubiquitous in pneumococci [178], was later shown to be absent in some non-typeable pneumococci, and more recently, in a serotype 6B pneumococcus [166, 179].

In 2019, Tavares *et al.* described a new qPCR targeting SP2020 gene (a putative transcriptional regulator). The authors compared this identification target with *lytA* and *piaB.* The specificity was 99.5%, 99.5% and 99.8% for *lytA*, *piaB* and SP2020, respectively. They also observed that a combination of *lytA* and SP2020 assays resulted in no misidentifications of pneumococci, suggesting that this combined strategy can be a good method for the identification of pneumococci, not only in pure cultures, but also in polymicrobial samples [166].

For an efficient prevention of pneumococcal disease, the knowledge of *S. pneumoniae* reservoirs is essential, and carriage surveillance studies are a way to achieve that. However this type of studies are almost inexistent in the adult population, since carriage rates detected by classical culture-based methods are almost neglectable (less than 5% among the elderly) [38, 39, 72].

Recent studies have indicated that the use of qPCR, due to its high sensitivity, enables detection of pneumococci in samples where pneumococci is at low density. It has been suggested that this is the case in adult populations, which has led to an underestimation of carriage prevalence [69]. With such approaches, carriage prevalence in this group has been re-evaluated.

A cross-sectional study, conducted among the Navajo Nation and White Mountain Apache Tribal lands in the US, between 2010-2012, evaluated differences between classical culture-based methods and molecular methods to detect pneumococcal carriage. The effects of age and density in the detection of pneumococci in healthy individuals were also evaluated. The authors observed that use of qPCR increased the detection of pneumococcal carriage when compared to classical culture-based methods, from 35% to 60%. This increase was particularly valuable among adults with lower pneumococcal density [180].

In the Netherlands, a pneumococcal surveillance study conducted among healthy adults, parents of 24-month-old children, in 2010-2011, reported a carriage rate of c.a. 40% (opposed to 19% by classical culture-based methods) in nasopharyngeal and oropharyngeal samples [83]. In addition, when nasopharyngeal, oropharyngeal and saliva samples obtained from parents of 24-month-old children and from childless adults were screened, it was observed that qPCR improved the detection of pneumococcal carriage and that carriage rates were higher in parents compared to childless adults (34% *vs.* 7%) [82].

Among healthy elderly, studies from the Netherlands (2007-2008) and Italy (2012) reported a prevalence of pneumococcal carriage of 20% and 18.7%, respectively [81, 84]. Furthermore, a longitudinal study where adults aged ≥65 years old were followed biweekly for one year, showed a cumulative incidence of pneumococcal carriage of 41% by qPCR and 14% by classical culture-based methods. Monthly prevalence ranged from 0% to 17% [80]. Pneumococcal carriage rates among adults aged over 60 years old with influenza-like illness in the Netherlands have been observed to be even higher, reaching c.a. 50% period-prevalence [86].

It is worth mentioning that some concerns regarding the specificity of molecular methods were raised and are not completely clarified. While the use of qPCR has been especially useful in samples from oropharynx and saliva [82, 83, 86], a study by Carvalho *et al.* reported high rates of false positives of pneumococcal strains in the adult oropharynx if only molecular methods were used [181].

Molecular assays have also been developed to determine a pneumococcal serotype. Both conventional PCR and real-time PCR overcome some of the limitations of the Quellung reaction that is considered the gold standard method. qPCR has advantages over conventional PCR: is quicker, more sensitive, and allow detection of lower limits [86, 165, 182-184].

Currently, some qPCR-serotyping assays have been published [182, 185]. And if it is well accepted that these assays have high sensitivity and specificity when applied to pure pneumococcal samples [182, 183, 186], some concerns have been raised regarding its specificity when applied to polymicrobial samples. In fact, some studies reported the existence of false positive signals when analyzing samples with a polymicrobial nature, such as those obtained from the oral cavity [86, 165, 187, 188]. The source of misidentification has been found to be due to the fact that non-pneumococcal mitis group strains can have homologues of pneumococcal capsular genes, which can be a source of false positives. Thus, when testing polymicrobial samples, the reliability of qPCR requires continue validation, careful optimization and the use of rigorous controls to avoid over-detection of pneumococci and misidentification.

Overall, *lytA* qPCR in combination with *piaB* or *SP2020* qPCR, and serotyping-qPCR are promising approaches for pneumococcal epidemiological studies, mostly due to its capability of a low limit of detection, even in low density samples. In addition, this methodology does not require isolation of live organisms, being possible to directly apply it to analyze a sample.

The history of *Staphylococcus aureus*

S. aureus was one of the first bacterial pathogens to be identified in the 19th century. It was first discovered in 1880 by Pasteur and Ogston who observed “cluster forming coccus” bacteria in pus from a human abscess. However, it was Ogston who found that the introduction of pus into human volunteers produced similar abscesses and named the clustered coccus based on their aspect in the microscope. Since the bacteria formed grape-like clusters, Ogston named them as *Staphylococcus* (from the Greek *Staphyle*, “a bunch of grapes”; and *kokkos*, meaning grain or berry) (reviewed in [189]). Four years later, the German physician Rosenbach isolated two species with different pigmentation and provided the first taxonomic description of the *Staphylococcus* genus. He was able to grow a more pathogenic “golden” species, which he named *Staphylococcus aureus* (from Latin *aurum*, meaning gold) and a less pathogenic “white” species that he named *Staphylococcus albus* (from Latin *albus*, meaning white), later renamed as *Staphylococcus epidermidis*. This division persisted for several decades due to the importance of differentiating between pathogenic strains and other commensal species (reviewed in [189]).

Over the years, new *Staphylococcus* species were described. *S. aureus* has been associated with at least two major discoveries in science: the description of the first bacteriophage in 1915 by Twort, [190] and the discovery of penicillin in 1928 by Fleming [191].

Nowadays, nosocomial infections caused by *S. aureus* remain a major problem worldwide. In fact, methicillin-resistant *S. aureus* (MRSA) is included in the WHO global priority list, being the fifth priority pathogen. In Europe, MRSA is one of the most common causes of serious bacterial infections. It has been estimated that MRSA was responsible for 148,727 infections and nearly 8,000 deaths in 2015 [192].

Staphylococcus aureus, the bacterium

Staphylococcus aureus is a Gram-positive cocci bacterium (Figure 4), which is generally found in pairs, in the form of grape-like clusters, tetrads or short chains, and varies between 0.5-1.5 μm in diameter. It is catalase and coagulase positive, facultative anaerobic and, being non-fastidious can be easily cultured on a standard nutrient medium, at the optimal temperature of 37°C in aerobic conditions. This microorganism is one of the hardiest non-spores forming bacteria, being capable of growing on media

containing high salt concentrations and at a wide range of temperatures (from 15ºC to 45ºC). *S. aureus* can be distinguished from other staphylococci because it is able to produce coagulase and to ferment mannitol [189, 193].

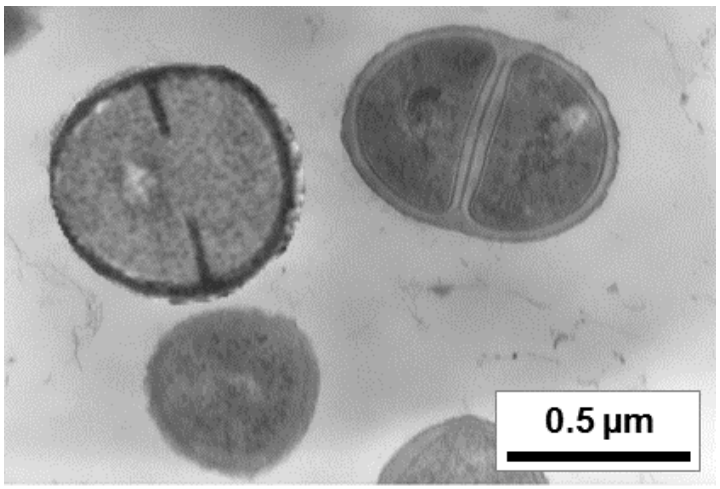

Figure 4. *Staphylococcus aureus*. Transmission electron microscopy of *S. aureus* cells. The form of cocci can be observed. Figure adapted from *www.sciencephoto.com/media/12805/view/tem-of-staphylococcus-aureus-bacteria*.

Epidemiology of *Staphylococcus aureus*

Staphylococcus aureus as a colonizer

S. aureus is a pathobiont that frequently colonizes asymptomatically the skin and mucous membranes of humans and several animals such as pigs, cattle, dogs and cats, among others [194, 195]. In humans, the preferential niche of this versatile bacterium is the anterior nares, although it can also colonize other body sites such as the skin, perineum, pharynx and less frequently the gastrointestinal tract, the vagina and axillae [196].

S. aureus can survive for several months on any type of surface. Transmission occurs mainly via hands when in contact with colonized or infected individuals or contaminated surfaces. Although less frequently, *S. aureus* can also reach the nose directly through the air [196].

Longitudinal studies addressing nasal colonization have shown that 20%-30% of the healthy general population are persistent *S. aureus* nasal carriers, while 30% are

intermittent carriers and approximately 50% are non-carriers. The prevalence of carriage is variable, depending on the characteristics of the populations studied, the quality of sampling, the use of different culture techniques, and the use of different interpretation guidelines (for a detailed review see [197]). The prevalence of carriage is dependent on the age of the host and has been reported to be significantly higher in children when compared to adults. Colonization can occur within days to a few weeks after birth, with carriage rates between 50%-70%. Approximately eight weeks after birth, carriage rates decrease, and have been estimated as being c.a. 20% by one year of age. After that carriages rates increase again, reaching 40% at the age of ten. During adolescence there is a transition from persistent carriage to intermittent or non-carrier states. From there onwards there is a decrease that remains stable during adulthood. Among the elderly carriages rates decline slowly with increased age, being c.a. 15% (reviewed in [196]).

S. aureus carriage rates have also been shown to vary across countries. A cross-sectional study aiming to assess the prevalence of nasal carriage across nine European countries (Austria, Belgium, Croatia, France, Hungary, Spain, Sweden, The Netherlands and the UK) in healthy individuals aged 4 years or older showed that the prevalence of carriage varied between countries ranging from around 12% in Hungary to 30% in Sweden [198].

In Portugal, in a study conducted between 2006 and 2009, nasopharyngeal carriage rates of *S. aureus* were reported to be 17.4% in children aged up to 6 years old attending day-care centers, ranging from 13.2% in 2007 to 21.6% in 2009. Moreover, the authors reported that carriage rates were also variable with age, ranging from 6.3% among children less than two years of age to 27.5% among children with 6 years of age [199]. In the Portuguese adult population, carriage rates of *S. aureus* were 31.6% among young adults, and 20.1% in senior adults according to studies conducted between 1993-2000 and 2010-2012, respectively [200, 201].

High rates of *S. aureus* carriage have been associated with specific groups: patients with diabetes mellitus, individuals undergoing hemodialysis, patients with HIV infections or viral infections of the URT and individuals with skin infections and skin diseases, among others. In addition, hospitalization, frequent contact with healthcare settings or living in nursing homes or other settings in the community where individuals have close contact with each other are also risk factors for being colonized with *S. aureus* (reviewed in [197]). For example, data from American hospitalized patients indicated that carriage rates were 28.6% in 2003-2004 [202], and data from several studies showed that around 25% of the health care workers were colonized with *S. aureus* [203]. In addition, in

Portugal, a longitudinal cohort study conducted among nursing students suggested that this population may be an important reservoir of *S. aureus*. Overall, 83% of the nursing students carried *S. aureus* at least once during the four years of the study (2012-2016) [204].

Staphylococcus aureus as an opportunistic pathogen

S. aureus can behave as a pathogen given the right occasion. For example, when the skin and mucosal barriers are disrupted it can cause a wide range of opportunistic infections, being one of the leading causes of infections worldwide [194, 205]. *S. aureus* infections range from skin and soft tissue infections (such as impetigo, cellulitis, folliculitis, mastitis, superficial and deep skin abscesses, and wound infections) to life threatening diseases (such as pneumonia, bacteremia, osteomyelitis, myocarditis, endocarditis and meningitis). It is also responsible for toxin-mediated disease such as toxic shock syndrome and food poisoning (reviewed in [197]).

S. aureus is one of the most common causes of nosocomial infections worldwide. During a European point-prevalence survey of healthcare-associated infections conducted between 2016 and 2015, it was shown that *S. aureus* was the second most prevalent pathogen responsible for healthcare-associated infections (11.6%) [206]. In addition, estimates from CDC showed that over 300,000 cases of MRSA in hospitalized patients and over 10,000 occurred in the USA in 2017 [205].

Methicillin-resistant *Staphylococcus aureus*

Before the discovery of antibiotics, more than 80% of patients with bacteremia caused by *S. aureus* died [207]. However, in the 1940s the usefulness of penicillin to treat these infections was discovered and its subsequent use led to a significant decline in the mortality rate associated with *S. aureus* infections. However, two years later, antimicrobial resistance was noted. By the 1960s almost all *S. aureus* strains were resistant to penicillin. To overcome this problem, penicillin was replaced by the semi-synthetic antibiotic methicillin. Once again, antimicrobial resistance appeared very quickly (reviewed in [208]). In fact, the first description of MRSA goes back to 1961, in England, soon after the introduction of methicillin into clinical practice [209].

Resistance to methicillin is conferred by the acquisition of the *mecA* gene, which encodes for a low affinity penicillin binding protein, PBP2A, and is located on a mobile genetic

island, the staphylococcal chromosomal cassette *mec* (SCC*mec*) [210]. More recently, an homologue of *mecA*, the *mecC* gene, was described in *S. aureus* strains of both animal and human origin [211, 212].

Hospital-associated methicillin-resistant *S. aureus*

Since its first appearance in the 1960s, MRSA disseminated in several countries becoming a leading cause of infections in health-care settings worldwide. Still, the burden of MRSA is dependent on the geographical area, ranging from less than 5% in Nordic European countries to more than 50% in some countries of South America and Asia (Figure 2) [213, 214].

Surveillance data from the United States indicated an increase in hospital-associated MRSA (HA-MRSA) infections between 1998-2005. In 2005, more than 50% of *S. aureus* clinical isolates were methicillin-resistant [215]. Since then, the incidence of HA-MRSA has been declining. In fact, from 2005 to 2012, the rates of MRSA bloodstream infections decreased by 17% each year. However, from 2013 to 2016 the decline in MRSA bloodstream infections slowed [205].

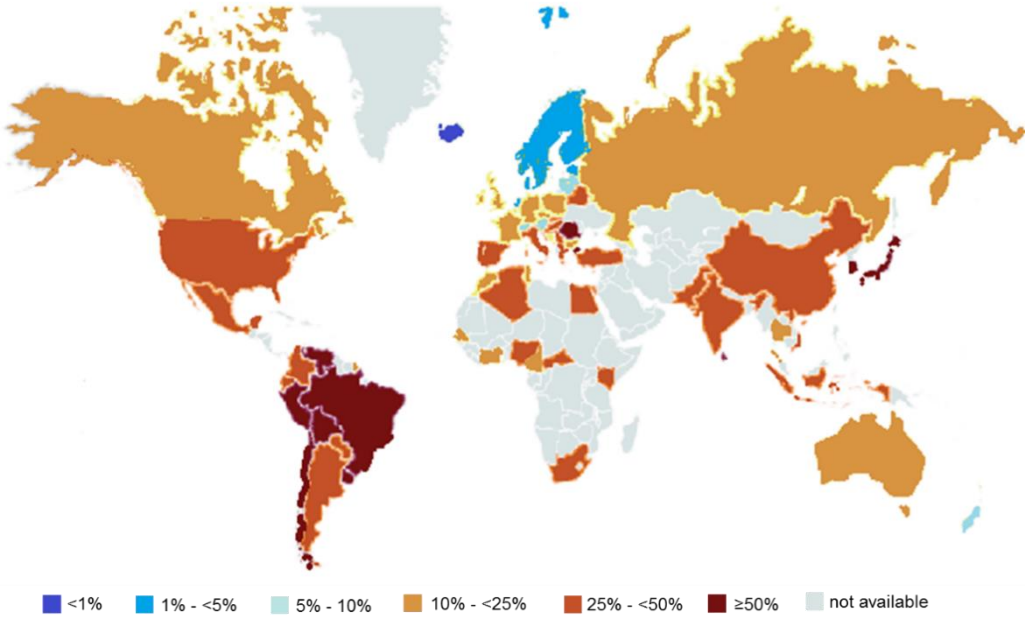

Figure 5. Prevalence of MRSA worldwide. The percentage of *S. aureus* that are resistant to methicillin is shown. Figure adapted from Lee *et al*. [214].

Several epidemiological studies from Asia-Pacific countries have shown high rates of HA-MRSA with a peak in the late 1990s, ranging from 28% in Hong Kong and Indonesia to >70% in South Korea. Nevertheless, since the 2000s there was a decrease in the MRSA prevalence in some countries [216, 217]. For example, in Taiwan the nosocomial bloodstream infections caused by MRSA decreased significantly from around 69% in 2000 to approximately 56% in 2010 [218].

The few data available from Africa suggested a prevalence of HA-MRSA lower than 50% for most of the African regions, with variable prevalence across countries [219]. In Tunisia the MRSA prevalence increased from 16% to 41%, between 2002 and 2007, while in South Africa it decreased from 36% to 24% between 2006 and 2007 [220, 221].

Regarding Europe, the prevalence of HA-MRSA varied considerably across countries, after years of increased prevalence since the early 2000s. Based on the last published report (2019) from the European Antimicrobial Resistance Surveillance Network (EARS-NET) the burden of MRSA infections in Europe is 16.4%, ranging from 0%-2% in Nordic countries such as Iceland, Norway, Sweden, and Finland, to 35%-45% in countries such as Malta, Greece, Portugal, Cyprus and Romania. Between 2015-2018, close to one third of the European countries reported a significantly decline in HA-MRSA rates, including countries with both low and high rates of HA-MRSA. Overall, HA-MRSA rates declined from 19.0% in 2015 to 16.4% in 2018. For example, in Austria and Belgium the HA-MRSA rates decreased from 7.5% and 12.3% in 2015 to 6.4% and 9.1% in 2018, respectively. This decrease was also observed in Romania, the country with the highest rates of HA-MRSA, from 57.2% in 2015 to 43.0% in 2018. Nevertheless, MRSA remains an important pathogen circulating in Europe due to its still high prevalence in several countries [213].

In the specific case of Portugal, since the early 2000s until 2012 the rates of HA-MRSA increased over time, from 31.9% in 2001 to 53.8% in 2012 [222]. Since then it has followed the decreasing trend of Europe, declining from 46.8% in 2015 to 38.1% in 2018. Nevertheless, the burden of MRSA infections in Portugal remains extremely high, being the third European country with the highest rate of HA-MRSA [213].

Nasal carriage of *S. aureus* is considered a risk factor for the development of nosocomial infections, especially during hospitalizations. Colonization with MRSA is even a higher risk factor. In fact, in one study, nasal MRSA carriage increased the risk of developing a nosocomial infection in approximately four-fold [223]. Other common risk factors for HA-MRSA infections include prolonged hospitalization, indwelling catheters, IUC exposure, history of surgery, being on hemodialysis, presence of skin lesions, wounds or ulcers,

co-morbidity conditions, prior antimicrobial therapy, older age, and living in nursing homes [224]. Moreover, HA-MRSA is mostly associated with pneumonia, bloodstream, surgical wound and urinary tract infections [225].

HA-MRSA are usually multidrug resistant and are characterized by carrying large SCC*mec* of types I, II and III [226]. Nowadays, some highly epidemic MRSA clones are successfully disseminated worldwide in hospitals: the New York/Japan clone (ST5-II) and the Pediatric clone (ST5-VI/IV) belonging to clonal complex (CC) 5; EMRSA-15 clone (ST22-IVh) belonging to CC22; EMRSA-16 clone (ST36-II) clone belonging to CC30; and the Berlin clone (ST45-IV) belonging to CC45 [227].

Community-associated methicillin-resistant *S. aureus*

The perception that MRSA infections were strictly confined to nosocomial settings started to change in the early 1990s, when MRSA was described as emerging in the community, among healthy individuals without previous healthcare contact. These strains of MRSA were apparently acquired only in the community and were referred to as community-associated MRSA (CA-MRSA) [6, 228]. The first cases of CA-MRSA were reported among indigenous populations in remote communities in Australia, in the 1980s [229] and then in four healthy children, who died from sepsis and necrotizing pneumonia, in the upper Midwestern region of the United Sates, in 1997-1999 [230]. Since then, reports of CA-MRSA in the general population have increased and CA-MRSA is now considered a public health treat [92].

The most common clinical manifestations of CA-MRSA are skin and soft-tissue infections (SSTIs), such as furuncles, boils, abscesses, and pus formation, accounting for 90% of the infections [231]. CA-MRSA can also be associated to more serious invasive infections such as necrotizing pneumonia, necrotizing fasciitis and bacteremia [232, 233]. The prevalence of SSTIs is different from country to country. For example, in the USA, during one month of sampling in 2004, CA-MRSA were responsible for around 60% of SSTIs [231]. In Portugal, a study conducted among children attending the pediatric emergency in a hospital in Lisbon showed that 7.9% of the SSTIs were caused by MRSA [234].

Specific population groups, especially closed and crowded populations, have a higher risk of developing a CA-MRSA infection, namely, children attending day-care centers [235], athletes [236], prisoners [237], military personnel [238, 239] and intravenous drug users [240].

Nasal and skin colonization of healthy individuals with CA-MRSA can increase the risk of developing infections, which are easily transmitted by skin-to-skin contact or through contaminated fomites [241]. Despite the increasing emergence of CA-MRSA infections worldwide, the few large-scale studies about the prevalence of CA-MRSA colonization in the healthy population seem to suggest that carriages rates remain low in most parts of the world, varying among different populations and geographic area. A study conducted among children attending the emergency ward and ambulatory services of a pediatric hospital in Luanda showed a considerable prevalence of MRSA in the community (12.7%) [242]. In the USA the prevalence of nasal CA-MRSA was only 1.5% between 2003 and 2004, among the healthy population [202]. Even in Japan or Taiwan, where the rates of HA-MRSA are extremely high, low rates of CA-MRSA were reported: 3.7%-4.3% in Japan and 1.9%-11.6% in Taiwan [243-246]. Similarly, several European countries reported low carriage rates. For example, studies from Ireland, Malta, and Greece estimated that the prevalence of CA-MRSA ranged between 0.7%-5.2% among adults aged between 16-60 years old [247-249]. In addition, carriage rates among adults aged over 65 years old living in Germany were similar to the ones described for younger adults, 0.7% [74].

In Portugal, while the epidemiology of HA-MRSA has been regularly studied since the 1990s, less is known about the epidemiology of MRSA in the community, which remains incompletely understood [250, 251]. A study conducted between 1993-2000 among different types of populations: young adults (draftees and university students), high-school students and children attending day-care centers found that the prevalence was very low. Prevalence of MRSA was 0.12%, 0.21%, 0.0% and 0.24% among draftees, university students, high-school students and children, respectively [200]. Years later, two other studies were conducted among children attending day-care centers between 2006-2009, and among adults over 60 years of age between, 2010-2012. Both studies reported low carriage rates: 0.14% among children attending day-care centers and 1.8% in adults over 60 years of age [199, 201]. Other Portuguese studies conducted among individuals without known healthcare-associated risk factors reported rates higher than 20% in the community, however in the these studies, most of the participants were enrolled at healthcare centers and both colonization and infection samples were analyzed [252, 253].

CA-MRSA have distinct phenotypic and genotypic features from HA-MRSA, which include a limited resistance profile, a frequent production of virulence factors, such as Panton-Valentine leukocidin (PVL) and the carriage of the smaller SCC*mec* element types IV and V. In addition, CA-MRSA are associated with five specific epidemic clones:

USA400 (ST1-IV), USA300 (ST8-IV), South West Pacific (ST30-IV), Taiwan (ST59-V) and European clone (ST80-IV) [226, 254]. While, initially CA-MRSA clones were associated to a specific geographic location, in more recent years this barrier has vanished [255, 256]. Currently, an increasing blur between CA-MRSA and HA-MRSA clones has been described in several countries. Some CA-MRSA clones such USA300 and USA400 have been identified as a cause of HA-MRSA [257, 258]. Moreover, the inverse situation has also been observed and several studies have reported the dissemination of traditional HA-MRSA clones, such as New York/Japan and EMRSA-15 into de community [249, 259, 260]. Studies conducted in Portugal also showed that HA-MRSA clones are no longer confined to the hospital and most of the MRSA isolates found in the community were associated with HA-MRSA clones: New York/Japan, Pediatric and EMRSA-15 [201, 252, 253]. Interestingly, studies conducted in Portuguese public buses circulating close to hospitals were often contaminated with EMRSA-15 clone, suggesting a possible transmission route for the dissemination of HA-MRSA outside the healthcare settings [261, 262].

Bacterial identification methods: the demand against *S. aureus*

The significance of correct identification of *S. aureus*

Given that MRSA infections continue to be a source of morbidity and mortality in healthcare settings worldwide and represent additional costs for society (due to prolonged hospitalization and costly medical procedures) its correct identification is essential. Understanding MRSA prevalence and relatedness of strains is crucial to prevent and fight nosocomial infections. Methods that are easy to deploy and reliably differentiate bacterial isolates of the same species are of instrumental use in epidemiological surveillance studies, outbreak situations, and for the study of the population structure, genetics and infection pathogenicity of a given pathogen [159, 263].

Methods for the detection of *S. aureus*

Classical culture-based methods are still considered as the gold standard to identify *S. aureus* or MRSA from clinical samples. However, before the identification of a given strain, the choice of the anatomical site or sites to be sampled is also very important. The screening of MRSA is generally obtained by swabbing the anterior nares, the preferential ecological niche of *S. aureus*. In fact, the sensitivity of the nasal screening

was reported to be more than 80%, which can increase to 95% with the screen of additional sampling sites [264-268].

The traditional detection of MRSA relies on selective culture in liquid and/or solid media. The most common media used as the first screening for *S. aureus* is the selective mannitol-salt agar medium [189]. More recently, chromogenic selective agar media have been used for the rapid identification of MRSA. In contrast with mannitol-salt agar, chromogenic media allow a faster and direct colony identification from the primary culture. Usually, using this type of medium, the need for subculture decreases and MRSA can be detected in 20-24h (reviewed in [264]. In addition, it has been reported that the use of a pre-enrichment step with a selective broth medium increases the sensitivity of these media by 15%-30%, despite the increase in costs and delay in obtaining the results (reviewed in [264]). For example, Böcher and colleagues showed that the use of a semi-selective enrichment broth containing 3.5mg/L cefoxitin, 20mg/L aztreonam and 2.5% NaCl followed by direct plating on chromogenic selective agar improved significantly the detection of MRSA. The use of this semi-selective medium detected 18% more MRSA than direct culture plating alone and 10% more than using a non-selective enrichment broth followed by culture plating [269]. Following the isolation of presumptive *S. aureus* colonies more tests can be performed, including tests for coagulase production, which can be assessed by available commercial kits or the tube coagulase test [270].

Improving *S. aureus* and MRSA identification by real-time PCR

Classical culture-based methods are laborious, time-consuming (3-5 days) as they are often preceded by a selective enrichment step, require isolation of a pure culture and its characterization, and potential false-negative results can occur when samples are obtained after antimicrobial therapy (reviewed in [271]). Currently, to overcome these limitations, in clinical practice, DNA-based methods, such as qPCR, are used for direct detection of the *mecA* gene in combination with specific genes for *S. aureus*. qPCR has several advantages: can yield results in approximately 1-3h because MRSA can be directly identified from nasal swabs, it has a high sensitivity and specificity, and a low contamination risk [272]. A possible challenge for this molecular method may be the detection of MRSA false-positives in samples containing a mixture of MRSA with other methicillin-resistant coagulase negative staphylococci (CoNS).

Several qPCR assays are commercially available and have been used in clinical laboratories and hospitals. Examples of commercial molecular assay for detection of *S.*

aureus and MRSA in clinical specimens include Xpert MRSA, BD GeneOhm MRSA and MRSA Advanced Test, among others [271].

Besides the commercial qPCR assays, other in-house multiplex qPCR have been developed. For example, Boye and colleagues developed a qPCR targeting *nuc* (encoding for a *S. aureus* thermonuclease), *femA* (encoding for proteins which influence the level of methicillin resistance), *mecA* and *mecC* (for the detection of not only *mecA* methicillin-resistant *S. aureus*, but as well as of *mecC* methicillin-resistant *S. aureus*), preceded by the use of a semi-selective enrichment broth [273]. Wang and colleagues developed a multiplex qPCR for the simultaneous detection of MRSA, *S. aureus* and CoNS using specific primers and probes targeting *16S rRNA*, *nuc* and *mecA* genes [274]. In addition, Kilic and colleagues described a triplex qPCR targeting *tuf* (encoding for the elongation factor Tu), *nuc* and *mecA* genes [275].

Typing methods for characterization of *S. aureus*

After obtaining pure cultures of *S. aureus*, characterization of the strain is required. In the past 50 years, several typing methods, both phenotypic and genotypic have been used to characterize *S. aureus*. Phenotypic methods were the earliest method to be used to identify and type bacteria and include biotyping, antimicrobial susceptibility testing, and phage typing, among others. However, only a small set of phenotypic methods are currently used [159, 276].

Antimicrobial susceptibility test is still routinely used in microbiology laboratories and indicates the pattern of resistance or susceptibility of a strain to a panel of antimicrobial agents. For example, methicillin resistance can be screened by cefoxitin susceptibility, using agar disk diffusion methods or broth microdilution in an automated way (reviewed in [159]). This technique is not only easy and fast to perform, but also cheap and readily available. However, it can have poor discriminatory power, and cannot be used as the unique typing method for MRSA (reviewed in [276]).

Several molecular typing methods have been developed to characterize *S. aureus*. Currently only a few are well-accepted and used, such as pulsed-field gel electrophoresis (PFGE), MLST, *spa* typing and SCC*mec* typing [277].

One of the most important scientific advances in diagnostic and surveillance in the past years was the use of Whole Genome Sequencing analysis. The utility of this methodology has already been demonstrated in the study of *S. aureus*, providing

information about antibiotic resistance and population dynamics of MRSA. In addition, WGS is also useful in diagnostic, and in the analysis of MRSA transmission both during nosocomial outbreaks and in community settings (reviewed in [214]). This methodology is able to provide a massive amount of data and allows the extraction of information on antimicrobial resistance, *spa* type, MLST and SCC*mec* type.

Aim of the thesis

Respiratory infections caused by *S. pneumoniae* and *S. aureus* are a main cause of morbidity and mortality worldwide. The incidence of infections is highest at the extremes of age, affecting disproportionally young children and the senior population. Colonization of the upper respiratory tract by *S. pneumoniae* and *S. aureus* is asymptomatic and a prerequisite for infection and transmission.

Despite the wealth information about *S. pneumoniae* carriage in children, limited information is available for the adult host. Based on limited classical culture-based methods, colonization has been accepted to be low among adults. Recent evidence, however, suggests that carriage in this group has been underestimated, and detection can be improved by the use of highly sensitive molecular methods, combined with samples collected from additional body sites (such as oropharynx and saliva). In addition, carriage detection may be also improved by longitudinal studies as they can increase the probability of carriage detection among adults and provide information on the dynamics of colonization.

Given the carriage rates among adults may be higher than initially expected, these individuals, that we have a scarce knowledge about, may represent a potential source for bacterial transmission in the community. Thus, an accurate assessment of the true colonization prevalence, and a better understanding of the patterns of colonization among the adult host will be essential to provide valuable information that will allow to create better prevention strategies for respiratory infections.

In this thesis we proposed to study the colonization patterns of two important pathobionts – *S. pneumoniae* and MRSA – both circulating in the community, among two groups of adults: immunocompetent healthy adults aged between 25-50 years old, and among senior adults aged over 60 years old. To access colonization dynamics in these two groups of adults we resorted to longitudinal and cross-sectional designs and made use of highly sensitive molecular methods that are currently available.

Specifically, the aims of this thesis were: (i) to determine the prevalence, density, dynamics and risk factors for colonization with *S. pneumoniae* in immunocompetent healthy adults aged between 25-50 years old, exploring differences between smokers and non-smokers; (ii) to evaluate carriage prevalence of *S. pneumoniae* in healthy adults over 60 years of age using real-time PCR; (iii) to evaluate carriage prevalence of MRSA in healthy adults over 60 years of age using real-time PCR; and (iv) to determine carriage prevalence of *S. aureus* and MRSA in immunocompetent healthy adults aged between 25-50 years old.

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Chapter II

Dynamics of pneumococcal carriage in adults: a new look at an old paradigm

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Contributions:

The study was conceived by RSL. Medical consultations were done by FF. Experiments were done by STA. Statistical analyses were done by ACP. Data interpretation was done by STA, ACP and RSL. RSL and HdL contributed with reagents and materials. The manuscript was written by STA, ACP and RSL and critically revised by all authors. All authors read and approved the final version of the manuscript.

Summary

Streptococcus pneumoniae (pneumococcus) is a leading cause of morbidity and mortality worldwide. Colonization of the upper respiratory tract is asymptomatic and a prerequisite for infection and transmission. Limited information on pneumococcal colonization among adults is available. We aimed to study pneumococcal carriage dynamics in healthy adults using approaches aiming to increase sensitivity of detection of carriage events.

A cohort of eighty-seven adults, aged 25-50 years old, was followed for six months in Portugal. Nasopharyngeal, oropharyngeal and saliva samples were obtained monthly and pneumococcal carriers were also sampled weekly. Carriage was investigated by real-time PCR (targeting *lytA* and *piaB*) and by culture. Positive samples were serotyped.

Approximately 70% of the participants were non-carriers, 20% were intermittent carriers and 10% were persistent carriers (>4 months). Median duration of carriage was seven weeks. Pneumococcal acquisition and clearance rates were 16.5 (95% CI 11.2-24.2) cases and 95.9 cases (95% CI 62.3-145.0) per 1000 persons-week, respectively. Living with children ≤18 years increased pneumococcal acquisition (HR:9.7, 95% CI 2.6-20.5; p<0.001). Smoking (HR:2.7, 1.0-7.8; p=0.041) was marginally associated with increased pneumococcal clearance. Serotypes carried by adults were broadly the same as those associated with disease in Portugal and with carriage in children suggesting an epidemiological link between them.

The pneumococcal carrier state in healthy adults is more dynamic than generally assumed: acquisition is frequent, and duration of carriage is higher than previously estimated with evidence of some persistent carriers. These findings are important when designing strategies to prevent pneumococcal disease in adults.

Introduction

Streptococcus pneumoniae (or pneumococcus) is a major cause of infectious disease worldwide, such as otitis media, pneumonia, bacteremia and meningitis. The ecological niche of the pneumococcus is the upper respiratory tract of humans and colonization is mostly asymptomatic [1]. Colonization is a pre-requisite for disease and transmission and therefore is a key target in strategies aiming to prevent disease.

Several studies have investigated the pneumococcal carrier state in children, in whom colonization is very frequent often exceeding 50% at a given time [2, 3]. By contrast, there is only limited information available on colonization among adults. Cross-sectional data based on culture-based methods estimated a low (1-10%) colonization prevalence among adults in high-income countries [2, 4]. Recent studies using real-time PCR (qPCR) indicate that pneumococcal colonization among adults is higher with estimates of 20-40% among Dutch parents of 24-month-old children [5, 6].

The dynamics of the adult carrier state have remained poorly studied. A few longitudinal studies have been conducted, mostly in families or in populations living in crowded conditions [7-10].

A previous cross-sectional study conducted by our group among elderly in Portugal identified cigarette smoking as the strongest risk factor for pneumococcal colonization [11].

Given the current gaps in knowledge and the observation that smokers may be at higher risk of pneumococcal carriage, we aimed to investigate pneumococcal carriage dynamics in healthy adults aged between 25-50 years taking into account the smoking status. We combined intensive sampling of multiple sites (nasopharynx, oropharynx and saliva at multiple time points) and sensitive detection of pneumococci (real-time PCR and culture-based methods) coupled with capsular type assignment to estimate acquisition and clearance rates and associated risk factors. The results unveiled a mostly unknown dynamic carrier state that challenges the current paradigm of pneumococcal carriage.

Materials and methods

Study design and population. This was a 6-month longitudinal study. Immunocompetent healthy adults aged 25-50 years old were enrolled between February 2015 and June 2016. Three samples were obtained monthly from each participant: nasopharyngeal and oropharyngeal swabs, and saliva. Samples were processed within eight hours as described below. Individuals testing positive for pneumococci were sampled weekly in the niche that tested positive and in saliva. This was maintained until two consecutive negative samples were obtained.

Two groups were defined: smokers and non-smokers. Smokers were defined as individuals smoking at least 10 cigarettes per day for the past five years. Non-smokers

were defined as individuals who had never smoked or only occasionally and, in any case, had not smoked in the past five years. Exclusion criteria included chronic obstructive pulmonary disease (COPD), diabetes, hepatic or renal disease, HIV infection, daily inhalation therapy, immunosuppression therapy, admission to a hospital within the last three months and regular contact with sick persons or patients.

An initial medical consultation was performed. Thoracic and pulmonary function, and the maximum expiratory flow were evaluated and a questionnaire regarding demographic information, smoking habits and brief medical history was obtained. All patients presented normal expiratory flow rates. A second questionnaire regarding recent medical history was repeatedly applied at each sampling occasion throughout the study.

The study was approved by the ethical committee of the Instituto de Higiene e Medicina Tropical of Universidade Nova de Lisboa and was registered at National Commission of Data Protection (ref. 3803/2014). Signed informed consent was obtained from all participants; samples and questionnaires were processed anonymously.

Sampling. Sampling was performed by a nurse following WHO recommendations [12]. A flexible swab with a flocked nylon fiber tip (ESwab 482CE, Copan) was used trans-nasally to reach the nasopharynx. A rigid swab with a flocked nylon fiber tip (ESwab 480CE, Copan) was used trans-orally to reach the oropharynx. Swabs were placed in STGG medium [12]. Saliva was collected by spitting into a tube from which 1ml was transferred to a tube containing 500µl of sterile 50% glycerol. Samples were kept on wet ice and were processed within eight hours.

Detection of *S. pneumoniae*. Samples (100µl for nasopharyngeal samples or 50 µl for oropharyngeal and saliva samples) were plated onto gentamycin blood agar (GBA) and incubated overnight at 37ºC in anaerobiose jars. Suspected pneumococcal colonies were isolated and identified as described [12].

In parallel, all visible bacterial growth was collected from each GBA plate and total DNA was extracted using the MagNA Pure Compact Nucleic Acid Isolation Kit (Roche Diagnostics GmbH).

S. pneumoniae was identified by real-time PCR using specific primers and probes for *lytA* [13] and *piaB* [5]. Samples were considered positive for pneumococci when cycle threshold (Ct) values for both genes were <40.

Capsular typing. Samples positive for pneumococci were pooled by five as described [6] and were typed by uniplex qPCR, using primers and probes targeting 41

serotypes/serogroups (Table S1) [14, 15]. For reactions yielding Ct<40, each sample of the pool was tested individually for the serotype that originated the signal. Samples were considered positive if Ct<40. One hundred pneumococcal negative samples randomly selected were pooled by 10 and also tested. If a Ct<40 was obtained for a given serotype/serogroup, this was considered non-reliable and similar results were excluded from all samples.

Pneumococcal positive samples were also individually screened by conventional multiplex PCR for sixteen additional serotypes/serogroups (Supplementary Table 1, [16, 17] and www.cdc.gov).

For pure pneumococcal cultures, serotypes were assigned using conventional multiplex PCR and/or the Quellung reaction using commercially available pneumococcal antisera (Statens Serum Institute, Copenhagen, Demmark) [12].

Definitions and statistical analysis. Sample size was calculated to detect a 0.2 difference between pneumococcal carriage of smokers vs non-smokers assuming a cumulative incidence of 0.3 and 80% power [18].

Categorical variables were compared using the Fisher's exact test. Continuous variables were compared using a Student's t-test. Kruskal-Wallis test was used to compare serotypes composition between the niches. Concordance was estimated using the Rand coefficient and the jackknife pseudo-values method to calculate the corresponding 95% CI [19].

Pneumococcal acquisition during the follow-up was defined as detection of a serotype following two consecutive negative swabs. Acquisition was assumed to have occurred during the time interval between the second negative swab and the first positive swab. Incidence of pneumococcal acquisition was calculated as events per 1000 persons-weeks. For the first time-point, in which pneumococcal carriage was detected, acquisition was assumed to have occurred in the previous week. This assumption was needed in order to retain as much information as possible regarding the carrier state.

Clearance was assumed to have occurred during the time interval between the last positive swab and the first of two consecutive negative swabs. Carriage duration was defined as the period between acquisition and clearance of a given serotype.

Events were right censored if no serotype acquisition or clearance was observed until the end of follow-up or if participants were lost to follow-up.

The Anderson-Gill regression model, which is an extension of the Cox model, was used to model repeated events and allow for censored follow-up times and correlations between successive observations of the same individual. The model was used to estimate hazard ratios (HR). Variables with $p<0.10$ in the univariable analysis were included in a multivariable model and backwards selection was used to identify the strongest associations ($p<0.05$). The final model was the one with the lowest likelihood cross-validation criterion.

Analysis of the regression models was done using R package frailtypack [20]. Exploratory and statistical analyses were performed using R version 3.6.2 [21].

Results

Characteristics of the population. Thirty-eight smokers and 49 non-smokers were enrolled. Table S2 summarizes the socio-demographic characteristics of the two groups. The only significant difference was the uptake of PCV13, which was higher among non-smokers (0.0% *vs.* 12.2%, p=0.032).

Comparison of methods and sampling sites to detect *S. pneumoniae* carriage. A total of 1,718 samples were obtained: 552 nasopharyngeal samples, 568 oropharyngeal samples and 598 saliva samples. Of note, there were 526 sampling occasions when simultaneous sampling of the three sites occurred enabling direct comparison between samples (Table 1 and Figure 1).

For all sampling sites, the use of qPCR was better than classical culture-based methods in detecting *S. pneumoniae*. This difference was statistically significant for oropharyngeal and saliva samples but not for nasopharyngeal samples (Table 1). Of note, all positive samples detected by culture were also detected by qPCR.

Detection of carriage by qPCR yielded comparable results between nasopharyngeal and oropharyngeal samples (14.8% vs 18.4%, p=0.136), and was significantly lower in saliva (10.5%, p=0.041 and p<0.001, respectively) (Table 1).

Concordance between qPCR results was highest (0.86, 95% CI 0.82-0.90) between nasopharyngeal and oropharyngeal samples (Figure 1).

qPCR results (based on distribution of Ct values for *lytA* and *piaB*) suggested that the density of pneumococcal colonization was highest in nasopharyngeal samples and lowest in saliva samples (Figure 2).

Table 1. Comparison of pneumococcal carriage detection obtained by culture methods and real-time PCR in nasopharyngeal, oropharyngeal and saliva samples obtained in parallel in 526 sampling occasions.

Sampling site	No. of samples	Samples positive for pneumococci		p-value
		Culture % (n)	qPCR %(n)	
Nasopharynx	526	12.9% (68)	14.8% (78)	0.422
Oropharynx	526	2.3% (12)	18.4% (97)	<0.001
Saliva	526	0.0% (0)	10.5% (55)	<0.0001

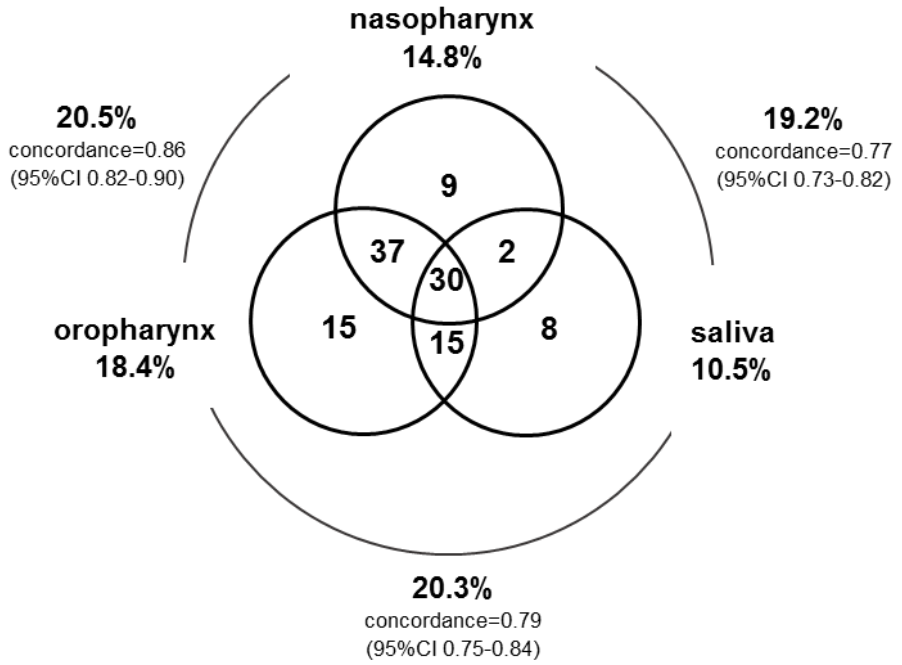

Figure 1. Comparison of pneumococcal carriage detection in nasopharyngeal, oropharyngeal and saliva samples obtained in parallel in 526 sampling occasions. There were 410 sampling events in which the three types of samples were negative for pneumococci. Numbers in Venn's diagram indicate the number of positive samples for pneumococci depending on the sampling site. The proportions of positive samples (out of 526) are indicated as well. Concordance between sampling sites was calculated using the Rand coefficient.

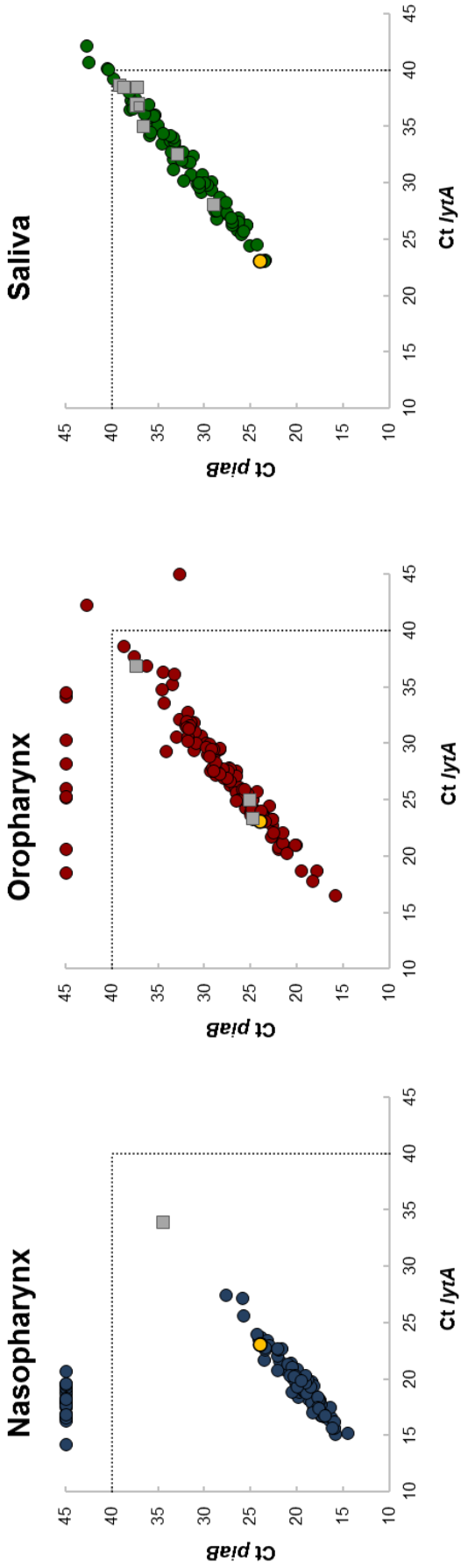

Figure 2. Detection of *S. pneumoniae* by real-time PCR in adults aged between 25-50 years old in nasopharyngeal, oropharyngeal and saliva samples, using primers and probes for two pneumococcal genes: *lytA* and *piaB*. Each circle represents an individual sample and its position corresponds to the Ct values obtained for each gene. Blue circles, nasopharyngeal samples, red circles, oropharyngeal samples, green circles, saliva samples; yellow circles, positive control (*S. pneumoniae* TIGR4); grey squares, positive samples in which the serotype was not determined (one sample in nasopharynx; three samples in oropharynx; and eight samples in saliva). Dashed lines indicate the threshold limit defined to discriminate between positive and negative samples.

Overall, when all the 1,718 samples were taken into account, the use of qPCR led to the detection of pneumococci in 289 samples (98 of 552 nasopharyngeal, 120 of 568 oropharyngeal and 71 of 598 saliva samples), while the use of culture-based methods led to the detection of pneumococci in 94 samples (82 and 12 in nasopharyngeal and oropharyngeal samples, respectively, Table 2).

Detection of *S. pneumoniae* serotypes. By qPCR serotyping, non-specific results for serotypes 4 and 5 were observed: 25 samples tested positive for serotype 4 and 18 tested positive for serotype 5 – all these samples differed in more than 2 Ct from the values obtained for *lytA* and *piaB* genes [22]; by conventional PCR these samples tested negative for these serotypes; qPCR serotyping of 100 randomly selected *S. pneumoniae* negative samples yielded false positive results for serotypes 4 (n=10) and 5 (n=12). For these reasons, results for serotype 4 and 5 were considered non-reliable (Table 2).

A serotype/serogroup was assigned to 95.5% (277 of 289) of the qPCR pneumococcal positive samples and to all 94 isolated strains (Table 2).

There was 100% concordance in serotype assignment between isolated strains and the corresponding qPCR pneumococcal positive samples.

Seventeen capsular types were found plus non-capsulated strains. Simultaneous carriage of two serotypes was detected in 18 qPCR pneumococcal positive samples from three individuals (A44, A66 and A74 in Figure 3).

We found no evidence for niche specificity among the serotypes detected (Kruskal-Wallis, p=0.147).

Dynamics of pneumococcal carriage. For 69 (79%) of the 87 participants, all planned swabs were obtained. Missing samples were due to unavailability of the participants to provide sample on the scheduled occasion for professional or personal reasons such as travelling and holidays. The mean number of samples per participant was 7±4, [IQR 5-6.5], ranging from a minimum of three samples (five adults) to 22 samples (one adult) (Figure 3, Figure S1).

A total of 164 pneumococcal carriage events were detected (Figure 3) in 25 participants, i.e., the cumulative period-incidence of pneumococcal carriage was 28.7% [95% CI 20.3–38.9]. Among these, 17 (68.0% [95% CI 48.4–82.8]) had more than one carriage event (Figure 3).

Table 2. Serotypes/serogroups detected among nasopharyngeal, oropharyngeal and saliva samples positive for pneumococci.

Serotypes/ serogroups	Pneumococcal positive samples by qPCR (n)		Pneumococcal positive samples by classical culture-based methods (n)
	Serotyping by qPCR[a]	Serotyping by cPCR[b]	Serotyping by cPCR or Quellung
3	3	-[c]	3
4	NR[d]	0	0
5	NR	0	0
6A/6B/6C/6D	14	-	5 (6C)
7A/F	22	-	0
7B/7C/40	-	2	0
8	28	-	5
9N/L	3	-	1 (9L)
10A	13	-	6
11A/D	27	-	2
15B/C	-	23	3
19A	39	-	23
22A/F	2	-	0
23A	21	-	7
23B	-	2	1
33A/33F/37	33	-	17 (37)
34	-	2	1
35B	-	1	0
35F/47F	-	31	12 (35F)
NT	-	29	8
Subtotal	205	90	
Not assigned[e]	12		0
Total	307[f]		94

[a]Uniplex qPCR reactions targeting 41 serotypes/serogroups as described in Materials and Methods; [b]multiplex conventional PCR targeting 15 serotypes/serogroups plus non-typeables as described in Materials and Methods; [c]not tested; [d]non-reliable assay as detailed in Results section; [e]serotype could not be assigned either due to low sample density or lack of adequate specific primers; [f]for 18 samples, the simultaneous presence of two serotypes was observed: serotypes 7A/F and 15B/C (n=14), 7B/C and 11A/D (n=2), and 11A/D and 23A (n=2).

Most (20 of 25) carriers were colonized with a single serotype; four were sequentially colonized with two serotypes (A13, A15, A44, A53) and one was sequentially colonized with three serotypes (A20). In three adults (A44, A66 and A74) simultaneous colonization with two serotypes was detected. Reacquisition of the same serotype appeared to have occurred in 24% of the carriers (A13, A24, A44, A53, A66 and A74, Figure 3).

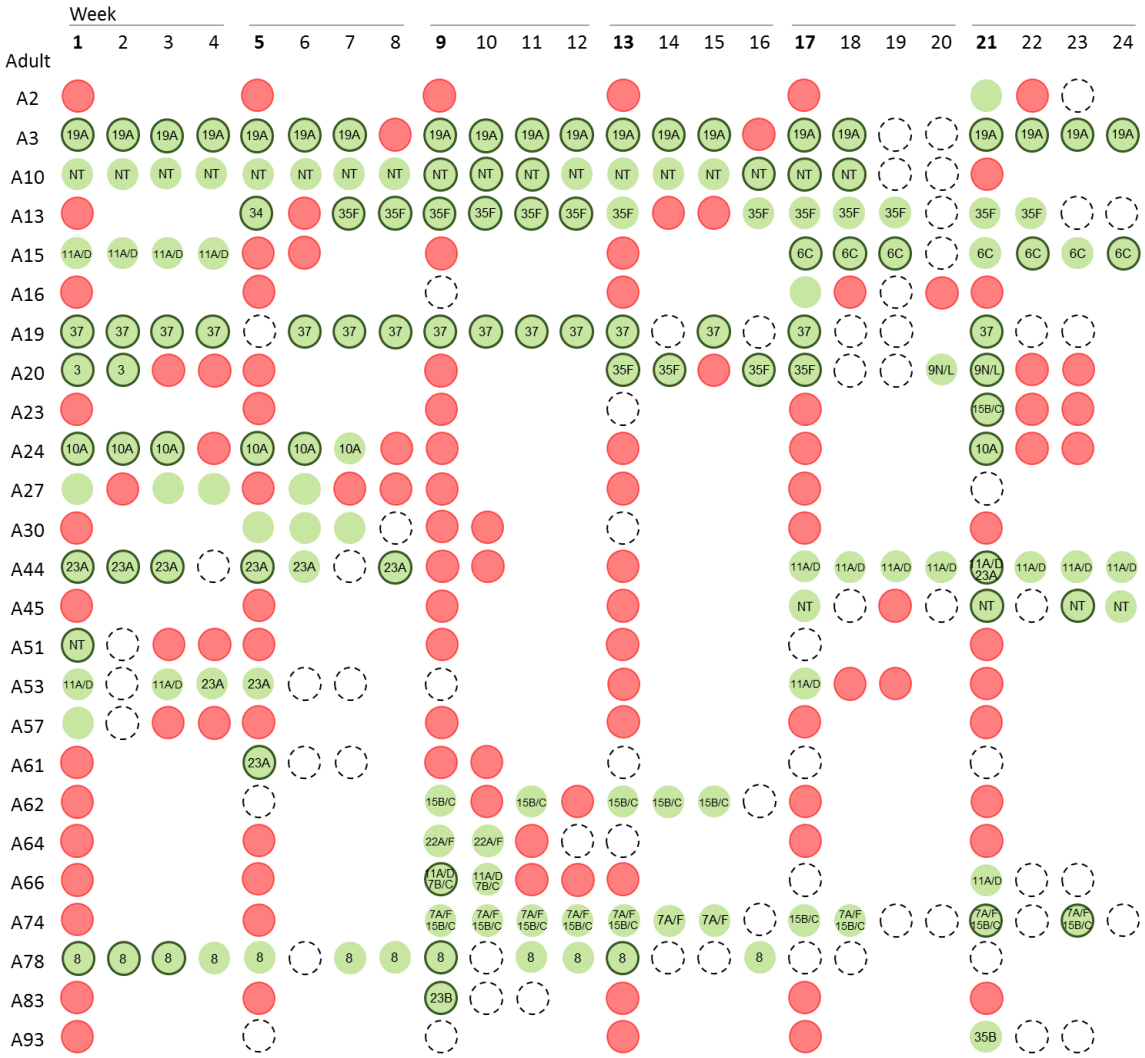

Figure 3. Representation of the carriage dynamics of the 25 participants that were colonized at least once with pneumococci during the six-month follow-up. Green circles, pneumococcal positive samples; solid dark green line delimiting green circle, pneumococcal culture was isolated; red circles, samples negative for pneumococci; dotted circles, sampling at that time point did not occur although planned per protocol; numbers in circles indicate the serotype.

Among the carriers, 12 individuals were smokers (or 31.6% of the 38 smokers) and 13 were non-smokers (or 26.5% of the 49 non-smokers). No significant differences in serotype distribution between these two groups were observed (Mann-Whitney test, p-value=0.1192).

Potential vaccine coverage based on the 164 carriage events by PCV13, PCV20 and PPV23 was 19.5%, 47.6% and 48.8%, respectively.

Thirty-nine acquisition events were detected in 25 of the 87 individuals (28.7%; 95% CI 20.3–38.9); and twenty-six clearance events were detected in 18 of the 25 carriers (72.0%; 95% CI 52.4–85.7). Duration of carriage of a given serotype was variable: in eight individuals pneumococcus was only detected once (32.0%; 95% CI 17.2-51.6), in ten individuals it ranged from 2-<8 weeks (40%; 95%CI 23.4-59.3) and in seven individuals it was over two months (28.0%; 95% CI 14.3-47.6). Notably, in individuals A3, A10, A13, A19, A74, A78 carriage of the same serotype for four months or more was noted (Figure 3).

Pneumococcal acquisition rate for the first event was estimated as 16.5 cases per 1000 persons-week [95% CI 11.2-24.2], i.e., the risk of being colonized with pneumococci, at least once, during a period of one year was 57.5%.

Pneumococcal clearance rate for the first event was estimated as 95.9 cases per 1000 persons-week [95% CI 62.3-145.0], i.e., the clearance half-life (i.e. the time needed for half of the carriers to become clear of pneumococci) was seven weeks and the cumulative risk of clearance during one year was 99.3%.

Risk factors for pneumococcal acquisition and clearance. In the univariable analysis of risk factors for pneumococcal acquisition, living with children ≤18 years old and seasonal flu vaccination were independently associated with increased pneumococcal acquisition. Consumption of antibiotics during the 6-month follow-up was associated with decreased pneumococcal acquisition. Other variables, including smoking, were not significant (Table 3). In the multivariable analysis, living with children ≤18 years old increased significantly the hazard rate for pneumococcal acquisition (HR:9.7, 95% CI 2.6-20.5; p<0.001). Consumption of antibiotics during the 6-month follow-up decreased significantly the hazard rate for pneumococcal acquisition (HR:0.1, 95% CI 0.01-0.9; p=0.036) (Table 3).

Table 3. Risk factors associated with pneumococcal acquisition.

Variable	Crude rate per 1000 persons-week (95% CI)	Univariable analysis		Multivariable analysis	
		HR (95% CI)	p value	HR (95% CI)	p value
Age class (years)			0.153		
<35 years old	15.2 (8.5-27.0)	1			
≥35 years old	31.7 (22.1 - 45.5)	2.2 (0.7-6.1)			
Gender			0.833		
female	26.4 (14.3-39.9)	1			
male	22.2 (14.1-34.8)	0.9 (0.3-2.8)			
Body mass index (kg/m^2)			0.836		
normal/underweight[a]	25.5 (17.1-37.6)	1			
overweight	24.1 (14.6-39.4)	0.9 (0.3-2.6)			
Household size			0.194		
≤2	17.8 (11.1-28.4)	1			
>2	33.6 (22.3-50.0)	1.9 (0.7-5.3)			
Living with adults ≥65 years			0.392		
no	25.2 (18.4-34.4)	1			
yes	10.0 (0.6-54.6)	0.3 (0.03-4.2)			
Living with children (≤18 years)			**<0.001**		**<0.001**
no	4.1 (1.4-11.9)	1		**1**	
yes	41.3 (30.0-56.6)	11.4 (2.8-25.3)		**9.7 (2.6-20.5)**	
Smoker			0.585		
no	20.2 (12.8-31.7)	1			
yes	29.4 (19.3-44.6)	1.3 (0.5-3.8)			
No. of years as smoker					
zero	20.2 (12.8-31.7)	1			
≤15	18.1 (8.3-38.9)	0.9 (0.2-3.4)	0.818		
>15	39.4 (24.0-63.9)	1.7 (0.6-5.3)	0.357		
No. of cigarettes per day					
zero	20.2 (12.8-31.7)	1			
≤15	27.0 (15.1-47.7)	0.9 (0.3-3.5)	0.983		
>15	32.6 (17.8-59.1)	1.9 (0.5-7.1)	0.365		
Smoke exposure			0.877		
no	25.4 (16.5-38.9)	1			
yes	23.3 (14.9-36.0)	0.9 (0.3-2.6)			
Chronic diseases[b]			0.918		
no	23.4 (16.2-33.6)	1			
yes	26.8 (15.0-47.4)	0.9 (0.3-3.3)			

Table 3. *(cont.)*

Variable		Crude rate per 1000 persons-week (95% CI)	Univariable analysis		Multivariable analysis	
			HR (95% CI)	p value	HR (95% CI)	p value
Long term medication				0.616		
	no	22.7 (15.8-32.7)	1			
	yes	29.3 (16.4-51.6)	1.2 (0.6-2.4)			
Seasonal flu vaccination				**0.053**		0.553
	no	21.8 (15.6-30.5)	1		1	
	yes	63.8 (29.6-132.3)	2.4 (1.0-5.9)		1.6 (0.4-6.7)	
Vaccination with PCV13				0.347		
	no	22.6 (16.2-31.4)	1			
	yes	50.0 (21.5-111.7)	2.6 (0.4-18.1)			
Antibiotic consumption within the 6 months preceding enrollment				0.812		
	no	22.2 (15.5-31.7)	1			
	yes	33.3 (18.2-60.3)	1.2 (0.3-4.1)			
Disease within the 6 months preceding enrollment				0.885		
	no	23.7 (17.0-32.9)	1			
	yes	29.2 (12.5-66.6)	0.8 (0.1-4.4)			
Antibiotic consumption during the 6-month follow-up				**0.021**		**0.036**
	no	29.4 (21.5-40.1)	1		**1**	
	yes	3.2 (0.2-17.8)	0.08 (0.01-0.7)		**0.1 (0.01-0.9**)	

[a]Underweight/normal weight classes were grouped together as there were no pneumococcal carriers classified as underweight; [b]when only respiratory chronic diseases (asthma, sinusitis and allergic rhinitis) were considered there was still no association with carriage (p=0.745). Not shown: the only adult vaccinated with PPV23 did not carry pneumococci; hospitalization within the 6 months preceding enrollment occurred twice, both individuals did not carry pneumococci.

In the univariable analysis of risk factors for pneumococcal clearance, a household size >2, living with adults ≥65 years old and smoking (regardless of the number of years as smoker or the number of cigarettes smoked per day) were independently associated with increased pneumococcal clearance (Table 4). In the multivariable analysis, only smoking (HR:2.7, 1.0-7.8; p=0.041), remained an independent predictor for increased pneumococcal clearance.

Table 4. Risk factors associated with pneumococcal clearance rate.

Variable	Crude rate per 1000 persons-week (95% CI)	Univariable analysis		Multivariable analysis	
		HR (95% CI)	p value	HR (95% CI)	p value
Age class (years)			0.456		
<35 years old	96.4 (49.6-178.8)	1			
≥35 years old	130.0 (84.1-196.8)	1.5 (0.5-4.5)			
Gender			0.119		
female	90.1 (51.5-155.4)	1			
male	150.0 (93.1-232.8)	2.0 (0.6-6.6)			
Body mass index (kg/m^2)			0.104		
normal/underweight[a]	98.7 (60.7-156.4)	1			
overweight	159.4 (91.4-263.3)	1.9 (0.6-6.1)			
Household size			**0.034**		0.062
≤2	75.0 (39.9-136.4)	1		1	
>2	168.3 (107.8-253.1)	4.4 (1.1-17.5)		3.4 (0.9-11.7)	
Living with adults ≥65 years			**0.078**		ns[b]
no	114.1 (78.5-163.3)	1			
yes	500.0 (25.6-974.3)	9.8 (0.8-123.7)			
Living with children (≤18 years)			0.186		
no	33.3 (1.7-166.7)	1			
yes	130.0 (90.2-186.1)	4.5 (0.5-40.9)			
Smoker			**0.006**		**0.041**
no	80.3 (45.4-138.0)	1		**1**	
yes	178.5 (111.3-273.9)	2.9 (1.0-8.7)		**2.7 (1.0-7.8)**	
No. of years as smoker					
zero	80.3 (45.4-138.0)	1			
≤15	161.2 (70.9-326.2)	3.1 (0.7-14.5)	0.157		ns
>15	188.7 (105.8-313.6)	2.8 (0.9-9.2)	**0.078**		ns
No. of cigarettes per day					
zero	80.3 (45.4-138.1)	1			
≤15	189.2 (94.8-342.0)	3.3 (0.8-12.7)	**0.085**		ns
>15	179.5 (89.8-326.7)	2.7 (0.8-9.3)	0.131		ns
Smoke exposure			0.217		ns
no	93.0 (54.0-155.6)	1			
yes	152.2 (92.8-239.4)	2.0 (0.7-6.4)			
Chronic diseases			0.345		
no	109.8 (71.4-165.2)	1			
yes	145.8 (72.5-271.7)	1.7 (0.5-5.9)			

Table 4. *(cont.)*

Variable		Crude rate per 1000 persons-week (95% CI)	Univariable analysis		Multivariable analysis	
			HR (95% CI)	p value	HR (95% CI)	p value
Long term medication				0.157		
	no	100.0 (64.1-152.5)	1			
	yes	195.1 (102.3-340.1)	2.1 (0.7-6.1)			
Seasonal flu vaccination				0.325		
	no	118.9 (79.8-173.5)	1			
	yes	111.1 (44.1-253.1)	0.9 (0.2-3.7)			
Vaccination with PCV13				0.812		
	no	115.2 (77.3-168.2)	1			
	yes	100.0 (34.5- 256.2)	0.8 (0.2-4.2)			
Antibiotic consumption within the 6 months preceding enrollment				0.900		
	no	115.2 (74.9-172.8)	1			
	yes	125.0(61.8-236.2))	1.1 (0.3-3.7)			
Disease within the 6 months preceding enrollment				0.139		
	no	106.7 (71.5-156.4)	1			
	yes	266.7 (108.9-519.5)	2.7 (0.7-10.5)			
Antibiotic consumption during the 6-month follow-up				0.749		
	no	117.4 (80.8-167.5)	1			
	yes	125.0 (6.4-470.8)	0.7 (0.1-8.9)			

[a]Underweight/normal weight classes were grouped together as there were no pneumococcal carriers classified as underweight; [b]this variable was not selected after backwards selection in the multivariate model. Not shown: the only adult vaccinated with PPV23 did not carry pneumococci; hospitalization within the 6 months preceding enrollment occurred twice, both individuals did not carry pneumococci.

Discussion

As far as we are aware this is the first study that did intensive consecutive sampling of healthy adults combining three sampling sites and highly sensitive molecular methods to study adult pneumococcal carriage. We obtained nasopharyngeal, oropharyngeal and saliva samples at weekly or monthly intervals and systematically investigated the presence of pneumococci by qPCR (targeting both *lytA* and *piaB*) and by conventional culture-based methods. Positive samples were serotyped. Risk factors for pneumococcal acquisition and clearance were investigated.

The parallel sampling of nasopharynx, oropharynx and saliva also enabled the investigation of the adequacy of each sampling site to detect pneumococcal carriage in adults, a subject of current debate [6, 23, 24]. When using qPCR, the oropharyngeal sample was superior to the nasopharyngeal sample, although this difference was not significant. Saliva samples were the ones with the lowest detection rate. These observations support current recommendations of combining oropharyngeal and nasopharyngeal samples and the use of qPCR to study pneumococcal carriage in adults [5, 12]. We highlight that the nasopharyngeal sample has the advantage of frequently enabling the isolation of pure cultures of pneumococci allowing their further characterization.

In contrast with recent studies we did not find saliva samples to be superior for the detection of pneumococci [6, 25]. The reasons for these discrepancies are currently unknown and may have resulted from differences in sampling technique, intrinsic differences of the human populations studied (such as diet, vaccination status and genetic differences) or others. As saliva sampling is easy and minimally invasive, future studies aiming to clarify these discrepancies should be carried out.

We observed that colonization among healthy adults was relatively frequent: the pneumococcal acquisition rate for the first event was 16.5 cases per 1000 persons-week, i.e., the risk of being colonized, at least once, during a period of one year was 57.5%. This is comparable to previous estimates for adults: 10 and 28 episodes per 1000 person-week in adults of families in Bangladesh or the UK [7, 26], respectively; and 35 cases per 1000 person-week among mothers in Thailand [8]. It is also in line with recent findings on pneumococcal detection in healthy adults, suggesting that carriage prevalence varied between 20-40% [5, 6].

In addition, two-thirds of the carriers experienced more than one carriage event suggesting a dynamic state of acquisition, carriage and clearance. Reacquisition of a serotype was detected in approximately one-quarter of the carriers. Whether these were true re-acquisitions or, alternatively, resulted from lack of ability to detect pneumococci in some occasions is unknown.

Pneumococcal clearance rate was estimated as 95.9 cases per 1000 persons-week resulting in a cumulative risk of clearance of 99.3% in one year. The median duration of carriage was seven weeks.

The clearance rate observed is shorter than previous estimates: in adults in Bangladeshi families the clearance rate ranged from 259-343 cases per 1000 person-week depending

on the serotype [7]. In line with this observation, the median duration of carriage observed in our study is higher than the one reported in other studies: 19 days in adults of families in the UK [26], four weeks in mothers in the Myanmar camp [8]; 2-4 weeks in adults from Gambia [10]. Of note, these studies used nasopharyngeal swabs and culture-based approaches to identify pneumococcal carriers and thus the sensitivity of the methods applied was likely lower than the one used in the current study. The sampling frequency also varied across studies.

Overall, approximately 70% of the participants were non-carriers, 20% were intermittent carriers and 10% were persistent carriers (with some examples of a serotype being carried for over six months).

In this study living with children increased the risk of pneumococcal acquisition by 10-fold. This is in line with current evidence that children are major reservoirs and transmitters of pneumococci in the community [27, 28]. Recent antibiotic consumption, by contrast, and as expected, decreased the risk of being colonized with pneumococci [29-31].

Smoking was associated with marginally significant shorter carriage duration. While cigarette smoking is a strong risk factor for invasive pneumococcal disease, its association with increased risk of carriage has not been consistent across studies [11, 32-34]. As smoking seems to impair antimicrobial defenses a decrease in pneumococcal clearance would be plausible [35]. On the other hand, this impairment may facilitate colonization by novel microorganisms, which may compete with previous colonizers and increase its clearance [35].

Of interest, the serotypes carried by adults in this study were often the same serotypes that are main causes of invasive and non-invasive pneumococcal disease in Portugal [36-39] and that are carried by children [40], suggesting an epidemiological link between them (Table S3).

As the number of vaccinated individuals was low (8.0%) we were unable to draw conclusions regarding the potential effect of PPV23 or PCV13 in the dynamics of carriage.

Our study has some limitations. The analysis of the clearance rate was based on a small sample size that did not allow distinction between clearance by the immune system vs by serotype displacement. These two processes can impact differently the clearance rate. Secondly, the study was designed to observe carriage episodes of shorter duration than the ones found. Hence, the six-month follow-up limited the observation of clearance

in prolonged carriers. Our study has also some strengths. The sampling of multiple sites, systematic use of qPCR targeting two probes, and frequent (weekly) sampling of carriers, are factors that likely increased the chance of detecting carriage events.

In conclusion, this study showed that pneumococcal acquisition in healthy adults is frequent, the duration of carriage is higher than previous estimates and some individuals appear to be persistent carriers; living with children increases significantly the likelihood of pneumococcal acquisition and the serotypes carried by adults are the same that colonize children and cause disease in adults suggesting an epidemiological link between them. These findings are important when designing strategies to prevent pneumococcal disease in adults.

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Supplementary material

Table S1: Pneumococcal serotypes/serogroups screened in the study by PCR-based methods.

Serotypes/ Serogroups	Method	Reference
1, 2, 3, 4, 5, 6A/B/C/D, 7A/F, 9A/V, 11A/D, 12A/B/F/44/46, 14, 15A/F, 16F, 18A/B/C/F, 19A, 19F, 22A/F, 23A, 23F, 33A/F/37	uniplex qPCR	[14]
8, 10A/B, 38	uniplex qPCR	[15]
7B/C/40, 9N/L, 15B/C, 17F, 21, 23B, 24, 34, 35B, 35F/47F	multiplex conventional PCR	[16] and www.cdc.gov
NT	multiplex conventional PCR	[17]

Table S2: Socio-demographic characteristics of the participants by smoker status.

Variable		Smokers (n=38)	Non-smokers (n=49)	p-value
Mean age (years)		38.1 ± 7.8	36.7 ± 5.9	0.370
Age class (years)				1.000
	<35 years old	36.8% (14)	34.7% (17)	
	≥35 years old	63.2% (24)	65.3% (32)	
Gender				1.000
	female	52.6% (20)	46.9% (23)	
	male	47.4% (18)	53.1% (26)	
Body mass index (kg/m^2)[a]				0.901
	normal weight	63.2% (24)	59.2% (29)	
	underweight	2.6% (1)	2.0% (1)	
	overweight	34.2% (13)	38.8% (19)	
Household size				0.922
	≤2	60.5% (23)	57.1% (28)	
	>2	39.5% (15)	42.9% (21)	
Living with adults ≥65 years		10.5% (4)	4.1% (2)	0.453
Living with children (≤ 18 years)		55.3% (21)	59.2% (29)	0.882
No. of years as smoker				NA
	≤15	44.7% (17)	NA	
	>15	55.3% (21)	NA	
No. of cigarettes per day				NA
	≤15	57.9% (22)	NA	
	>15	42.1% (16)	NA	
Smoke exposure[b]		76.3% (29)	32.6 (16)	**<0.001**
Chronic diseases[c]		31.6% (12)	22.4% (11)	0.463
Long term medication[d]		23.7% (9)	22.4% (11)	1.000
Seasonal flu vaccination		2.6% (1)	10.2% (5)	0.225
Pneumococcal vaccination with PCV13		0.0% (0)	12.2% (6)	**0.032**
Pneumococcal vaccination with PPV23		0.0% (0)	2.0% (1)	1.000
Antibiotic consumption within the 6 months preceding enrollment		23.7% (9)	16.3% (8)	0.425
Hospitalization within the 6 months preceding enrollment		0.0% (0)	4.1% (2)	0.502
Disease within the 6 months preceding enrollment[e]		10.5% (4)	8.2% (4)	0.725
Antibiotic consumption at least once during the 6-month follow-up		28.9% (11)	20.4% (10)	0.450

[a]Body mass index calculated as weight/height^2 and classified according to WHO as underweight if BMI<18.5, normal weight if 18.5≤BMI≤24.9, and overweight if BMI≥25; [b]at home (n=8), at the working place (n=20), by a partner who smoke (n=23), independently of being a smoker; [c]sinusitis (n=10), asthma (n=3), allergic rhinitis (n=2), heart diseases (n=2), bronchiectasis, hypertension, hypothyroidism, obesity, neurological diseases and psoriasis (n=1 each); [d]oral contraceptives (n=12), α-blockers for hypertension (n=4), antihistamines (n=1) and medication for hypothyroidism (n=1), venous insufficiency (n=1), asthma (n=1) and psychiatric disorders (n=1); [e]respiratory infections (n=4), gynecologic disorders, cutaneous infection, urinary tract infection and blunt trauma (n=1 each).

Figure S1: Representation of the 62 out of 87 participants that were never colonized with pneumococci during the six months of the study. Samples of participants that were non-carriers were always collected monthly, at weeks one, five, nine, 13, 17 and 21. Red circles, negative samples; dotted circles, samples that were not possible to obtain at that time point, although planned by protocol.

Table S3. Serotypes detected in this study and their occurrence in disease and children carriage in Portugal

Serotype/serogroup detected in this study-carriage in adults (2015-2016)	Epidemiological relevance				
	Adult non-invasive pneumococcal pneumonia (2012-2015)[a]	Adult invasive pneumococcal disease (2012-2014)[b]	Pneumococcal carriage among children (2015-2016)[c]	Pediatric complicated pneumonia (2010-2015)[d]	Children invasive pneumococcal disease (2012-2015)[e]
3	one of the most prevalent	one of the most prevalent	found	one of the most prevalent	one of the most prevalent
6A/6B/6C/6D	one of the most prevalent (6C)	found (6A/B/C)	found (6A/B/C)	found	one of the most prevalent (6B)
7A/F	found (7F)	one of the most prevalent (7F)	found	found	one of the most prevalent (7F)
7B/7C/40	found (7C)	found (7C)	found (7C)	-	found
8	found	one of the most prevalent	found	found	found
9N/L	one of the most prevalent (9N)	found (9N)	found (9L)	-	found
10A	found	found	found	found	one of the most prevalent
11A/D	one of the most prevalent (11A)	found (11A)	one of the most prevalent (11D)	-	found (11A)
15B/C	found	found	one of the most prevalent	-	one of the most prevalent
19A	one of the most prevalent	one of the most prevalent	found	one of the most prevalent	one of the most prevalent
22A/F	found (22F)	one of the most prevalent (22F)	found	-	found (22F)
23A	one of the most prevalent	found	one of the most prevalent	-	found
23B	one of the most prevalent	found	one of the most prevalent	-	found
33A/33F/37	found (37)	-	found (37)	found	found
34	found	found	found	-	-
35B	found	found	found	-	-
35F/47F	found (35F)	found (35F)	one of the most prevalent (35F)	-	-
NT	one of the most prevalent	found	found	-	found

[a]Horácio et al., 2018. Serotypes 3, 6C, 9N, 11A, 23A, 19A, 23B, and NT accounted for 46.1% of the isolates [39].
[b]Horácio et al., 2016. Serotypes 3, 7F, 8, 19A, and 22F accounted for 43.7% of the isolates [36].
[c]Félix, et al., 2018. Serotypes 11D, 15B/C, and 23B were the most prevalent in an urban area; 15A, 23A, and 35F were the most prevalent in a rural area [40].
[d]Silva-Costa et al., 2018. Serotypes 3 and 19A accounted for 43.1% of the isolates [37].
[e]Silva-Costa et al., 2019. Serotypes 3, 6B, 7F, 10A, 15B/C, and 19A accounted for 40.5% of the isolates [38].

Chapter III

Re-evaluation of *Streptococcus pneumoniae* carriage in Portuguese elderly by qPCR increases carriage estimates and unveils an expanded pool of serotypes

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Contributions:

The study was conceived by RSL. Experiments were done by STA and TP. Statistical analyses was done by ACP. Data interpretation was done by STA, ACP and RSL. RSL and HdL contributed with reagents and materials. The manuscript was written by STA and RSL and critically revised by all authors. All authors read and approved the final version of the manuscript.

Summary

Streptococcus pneumoniae (pneumococcus) is a leading cause of infections worldwide. Disease is preceded by asymptomatic colonization of the upper respiratory tract. Classical culture-based methods suggest that colonization in the elderly is <5%. Recently, use of qPCR has challenged these observations.

We estimated pneumococcal carriage prevalence and serotypes among Portuguese elderly using qPCR and compared results with those obtained by classical culture-based methods.

Nasopharyngeal and oropharyngeal paired samples (599 each) of individuals over 60 years living in nursing (n=299) or family (n=300) homes were screened for the presence of pneumococci by qPCR targeting *lytA* and *piaB.* Positive samples were molecular serotyped.

Use of qPCR improved detection of pneumococci in oropharyngeal samples compared to classical culture-based methods: from 0.7% to 10.4% ($p<0.001$) in the nursing home collection, and from 0.3% to 5.0% ($p<0.001$) in the family home collection. No significant differences were observed between both methods in nasopharyngeal samples (5.4% *vs.* 5.4% in the nursing homes; and 4.3% *vs.* 4.7% in the family homes). Twenty-one serotypes/serogroups were detected by qPCR compared to 14 by classical culture-based methods.

In conclusion, use of qPCR suggests that pneumococcal carriage in Portuguese elderly is approximately 10%, and unveiled a large pool of serotypes. These results are important to understand progression to disease and impact of pneumococcal vaccines in the elderly.

Introduction

Streptococcus pneumoniae (or pneumococcus) is a leading cause of infectious diseases worldwide, such as otitis media, pneumonia, bacteremia and meningitis. The incidence of pneumococcal infections is highest at the extremes of age affecting disproportionally young children and the elderly [1-3]. This latter group is increasingly important in our society as the world population is aging, resulting in an increasing demand for strategies to maintain quality-adjusted life years in advanced ages [2].

To prevent pneumococcal disease three vaccines are currently available and several are under investigation [4]. These vaccines target 10, 13, or 23 serotypes of the over 95 described to date [5]. The 13-valent pneumococcal conjugate vaccine (PCV13) is approved for all age groups and the 23-valent pneumococcal polysaccharide vaccine (PPV23) is approved for individuals over 2 years of age. Several countries have issued recommendations for vaccination of adults over 65 years of age with one or both of these vaccines [1, 6, 7].

In Portugal, PCVs for children were commercially available through the private market until mid-2015 (PCV7 became available in June 2001, PCV10 in April 2009, and PCV13 in January 2010). In August 2015, PCV13 was introduced in the National Immunization Plan for all children born after January 2015 [8]. PPV23 and PCV13 are commercially available for adults but its usage has been low (<10% by 2015) [9, 10].

Pneumococcal disease is always preceded by colonization of the upper respiratory tract, a phenomenon that is mostly asymptomatic [11]. Several studies have described high pneumococcal colonization rates (frequently over 60%) in young children [12, 13]. By contrast, studies conducted in elderly populations have suggested, until recently, that pneumococcal colonization occurred at a very low prevalence (1%-5%) [14-16]. These studies relied on the use of classical culture-based approaches in which swabs of the nasopharynx and/or oropharynx were plated in selective media and pneumococcal presumptive colonies were sub-cultured for species identification [17]. This strategy, recommended by the WHO, has a good specificity but a low sensitivity [17, 18].

Recently, new strategies to detect pneumococcal carriage based on real-time PCR (qPCR) have been proposed, validated, and are now being increasingly used [19-23]. The use of qPCR enables high sensitivity in samples where pneumococci is at low density. With such approaches, carriage prevalence in the elderly has been re-evaluated with studies suggesting it may range between 0%-20% in healthy individuals and reach approximately 50% period-prevalence (within 7-9 weeks) in individuals with influenza-like illness [20-22, 24].

In Portugal, the prevalence of pneumococcal carriage among the elderly has been studied by classical culture-based methods only. A previous study conducted by our group estimated a carriage prevalence of 2.3% [15].

The aim of this study was to re-evaluate the prevalence of nasopharyngeal and oropharyngeal colonization by *S. pneumoniae* in adults over 60 years of age using qPCR and compare the results with those obtained by classical culture-based approaches. We

further characterized positive samples by serotyping and compared serotype distribution and diversity.

Materials and methods

Study participants and samples. This study was nested on a study previously described [15]. Briefly, pneumococcal carriage prevalence and associated risk factors were studied among 3,361 adults older than 60 years of age, between April 2010 and December of 2012. For each participant, one nasopharyngeal and one oropharyngeal sample were obtained. Pneumococci were isolated and identified by routine procedures based on classical culture-based methods [25, 26], and serotyped by conventional multiplex PCR and/or by the Quellung reaction [27, 28]. Briefly, in the initial study, swabs were plated on gentamycin blood agar and incubated overnight at 37°C in anaerobiose jars. On the following day, suspected pneumococcal colonies were isolated and identified by routine culture methods. All the remaining total bacterial growth was collected and frozen at -80°C.

In the current study, two subsets of samples of the initial study were re-examined. One set corresponded to 600 paired samples (300 nasopharyngeal and 300 oropharyngeal obtained from 300 individuals) randomly selected from a pool previously obtained from 3,062 individuals living in family homes. The second set corresponded to 598 samples (299 nasopharyngeal and 299 oropharyngeal) obtained from all individuals living in nursing homes at the time of the study [15].

Identification and serotyping of *S. pneumoniae* by molecular methods. Total bacterial growth was thawed on ice, vortexed for 20 seconds and 200µl were transferred into a sample tube with 200µl of a lysis buffer (MagNA Pure Compact Nucleic Acid Isolation Kit, Roche Diagnostics GmbH), and incubated for 20 min at 37ºC. Total DNA was then extracted using the MagNA Pure Compact instrument (Roche Diagnostics GmbH) as recommended by the manufacturer.

Pneumococcal carriage was evaluated by real-time PCR, targeting two pneumococcal genes: *lytA* (major pneumococcal autolysin) and *piaB* (iron uptake ABC transporter lipoprotein PiaB) [19, 29]. Reactions for both genes were performed in a final volume of 25µl, containing 1x FastStart TaqMan Probe Master (Roche), 150nM of each primer, 75nM of probe and 2.5µl of total DNA. DNA amplification was performed in the CFX96 Real-Time System Amplification (Bio-Rad) using the following conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Samples were

considered positive for pneumococci when both genes had cycle threshold (Ct) values below 40.

S. pneumoniae capsular types were accessed in positive samples by uniplex qPCR following a pooling strategy as previously described [30]. Pools of five samples containing 2µl of each sample were tested. Each pool was used as template for qPCR in a total volume of 25µl. If a Ct<40 was obtained in a given pool of samples, these were tested individually for the serotype that originated the signal. In this latter case, 2.5µl of DNA was used per reaction. A panel of 23 sets of primers and probes was used targeting serotypes or serogroups: 1, 2, 3, 4, 5, 6A/6B/6C/6D, 7A/F, 9A/V, 11A/D, 12A/12B/12F/44/46, 14, 15A/F, 16F, 18A/18B/18C/18F, 19A, 19F, 22A/F, 23A, 23F, 33A/33F/37 [31], 8, 10A/B and 38 [32]. Samples were considered positive for a given serotype when Ct values were below 40. Following previous studies that indicated that non-pneumococcal streptococci in the oropharynx can confound molecular serotyping assays, we disregarded positive results obtained by qPCR for serotypes 4 and 5 [20, 33, 34]. In addition, conventional multiplex PCR was used to detect serotypes 7B/7C/40, 9N/L, 15B/C, 17F, 20, 21, 23B, 24, 31, 34, 35B, 35F/47F and non-typeables using primers previously described [27, 35].

Statistical Analysis. The geometric mean was used to summarize the distribution of Ct values from *lytA* and *piaB* of nasopharyngeal and oropharyngeal samples. To look for associations between Ct values from nursing home *vs.* family home and nasopharynx *vs.* oropharynx an adjusted generalized linear model (GLM) using a Gaussian distribution and a log link function was used.

The McNemar's test was used to compare culture and real-time PCR methods based on paired individuals. The Chi-square test was used to compare prevalence of *S. pneumoniae* between nasopharyngeal and oropharyngeal samples and between nursing home and family home by real-time PCR or classical culture-based methods. A p-value of <0.05 was considered statistically significant for all the tests used. The Gini-Simpson index of diversity (GSID) was used to calculate serotype diversity. All analyses were performed using R version 3.2.3 [36].

Ethics statement. This study was conducted in accordance with the European Statements for Good Clinical Practice and the declaration of Helsinki of the World Health Medical Association. In addition, it was registered and approved at health care centers of Oeiras and Montemor-o-Novo that report to Administração Regional de Saúde (ARS, “Regional Health Administration”) of Lisboa e Vale do Tejo, and Alentejo, respectively, from the Ministry of Health. Informed written consent was obtained from all participants.

All samples and questionnaires were attributed a numeric code and were processed anonymously.

Results

Pneumococcal carriage. We evaluated pneumococcal carriage by real-time PCR targeting *lytA* and *piaB* genes in nasopharyngeal and oropharyngeal samples from 599 adults older than 60 years of age living in nursing homes (n=299) and family homes (n=300). Socio-demographic characteristics of the population were previously described [15] and are summarized in Supplementary Table S1. In general, individuals living in a nursing home, were older, had less years of formal education, and were less active than those living in family homes. Results were compared to those obtained by classical culture-based methods.

Ct values for *lytA* and *piaB* genes were not significantly different between samples from adults living in nursing homes compared to those living in family homes (adjusted GLM, p=0.065 for *lytA*; p=0.048 for *piaB*). However, in both groups, the geometric Ct mean was significantly lower in positive nasopharyngeal samples than in positive oropharyngeal samples (p<0.001 for both *lytA* and *piaB*), suggesting higher quantity of pneumococci in the harvests of cultures originating from nasopharyngeal samples compared to those originating from oropharyngeal samples (Figure 1 and Supplementary Table S2).

The use of real-time PCR, when compared with classical culture-based methods, increased significantly the detection of pneumococcal carriage in oropharyngeal samples from 0.7% to 10.4% (p<0.001) in the nursing home collection, and from 0.3% to 5.0% (p<0.001) in the family home collection. By contrast, no significant differences were observed in carriage detection in nasopharyngeal samples when both methods were compared: 5.4% *vs.* 5.4% in the nursing home collection; and 4.3% *vs.* 4.7% (p=1.0) in the family home collection (Table 1 and Figure 2).

Overall, in adults living in nursing homes*, S. pneumoniae* was more frequently detected in oropharyngeal samples than in nasopharyngeal samples: 10.4% *vs.* 5.4% (p=0.0093), respectively; in adults living in family homes no significant differences were observed between the two sampling sites: 4.7% *vs.* 5.0% (p=1.0) (Table 1). When results of the two sampling sites were combined, the use of real-time PCR, compared to classical culture-based methods, increased significantly the detection of *S. pneumoniae* carriers

from 5.7% to 12.7% (p<0.001) in the nursing home collection and from 4.3% to 8.0% (p=0.0026) in the family home collection (Table 1).

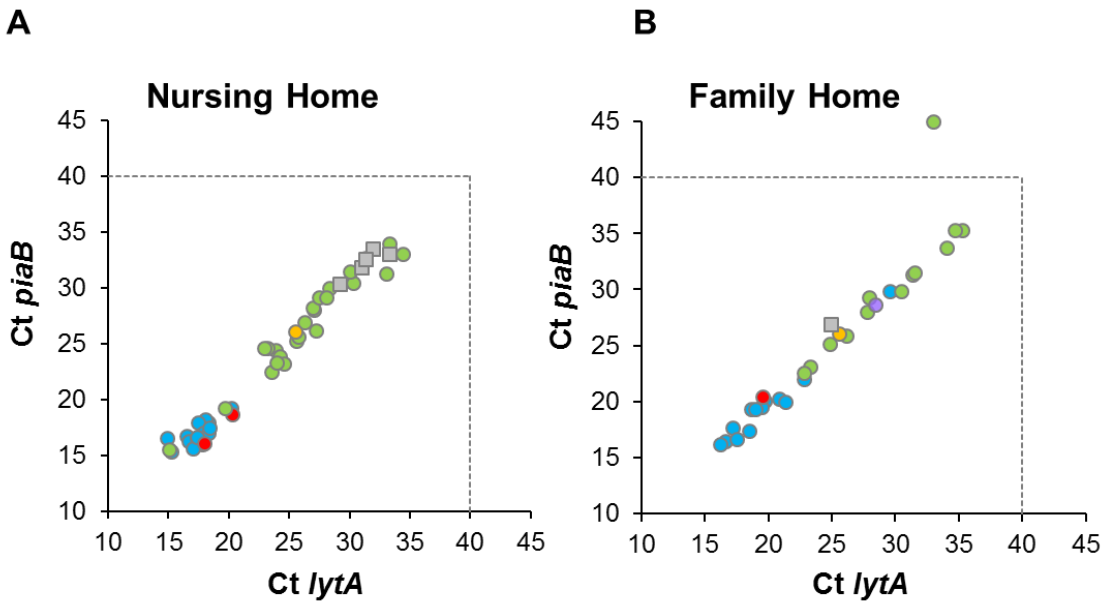

Figure 1. Detection of *S. pneumoniae* by real-time PCR in adults over 60 years of age living in nursing homes (A) and family homes (B). Blue circles, nasopharyngeal samples positive by culture; purple circles, nasopharyngeal samples negative by culture; red circles, oropharyngeal samples positive by culture; green circles, oropharyngeal samples negative by culture; yellow circles, positive control (*S. pneumoniae* TIGR4); grey squares, positive oropharyngeal samples in which the serotype was not determined. Dashed lines indicate the Ct value above which results were considered to be negative.

Molecular serotyping. Molecular serotyping allowed assignment of a serotype/serogroup in 70 (40 from oropharynx and 30 from nasopharynx) of the 76 qPCR positive samples. A single capsular type was detected in 67 qPCR pneumococcal positive samples and two serotypes were detected in three samples: one individual was co-colonized with serotypes 19A and 22A/F which were detected both in the nasopharynx and the oropharynx; other individual was co-colonized with serotypes 3 and 23A in the oropharynx. The six samples for which a serotype could not be assigned were all from the oropharynx (Figure 1). Failure of assigning a capsular type to these samples suggests that they may have not been targeted by the primer set tested.

Table 1. Detection of *S. pneumoniae* carriers according to sampling site and methodology used.

Collection	Participants n	Sampling Site n (%)								Carriers n (%)			
		Oropharynx (OP)				Nasopharynx (NP)							
		Culture positive	qPCR positive	Culture and/or qPCR positive	p-value	Culture positive	qPCR positive	Culture and/or qPCR positive	p-value	Culture positive (NP and/or OP)	qPCR positive (NP and/or OP)	Total	p-value
Nursing home	299	2 (0.7%)	31 (10.4%)	31 (10.4%)	**<0.001**	16 (5.4%)	16 (5.4%)	16 (5.4%)	NA	17 (5.7%)	38 (12.7%)	38 (12.7%)	**<0.001**
Family home	300	1 (0.3%)	15 (5.0%)	15 (5.0%)	**<0.001**	13 (4.3%)	14 (4.7%)	14 (4.7%)	1.0	13 (4.3%)	24 (8.0%)	24 (8.0%)	**0.003**

p-values determined using McNemar's Chi-squared test for paired individuals.

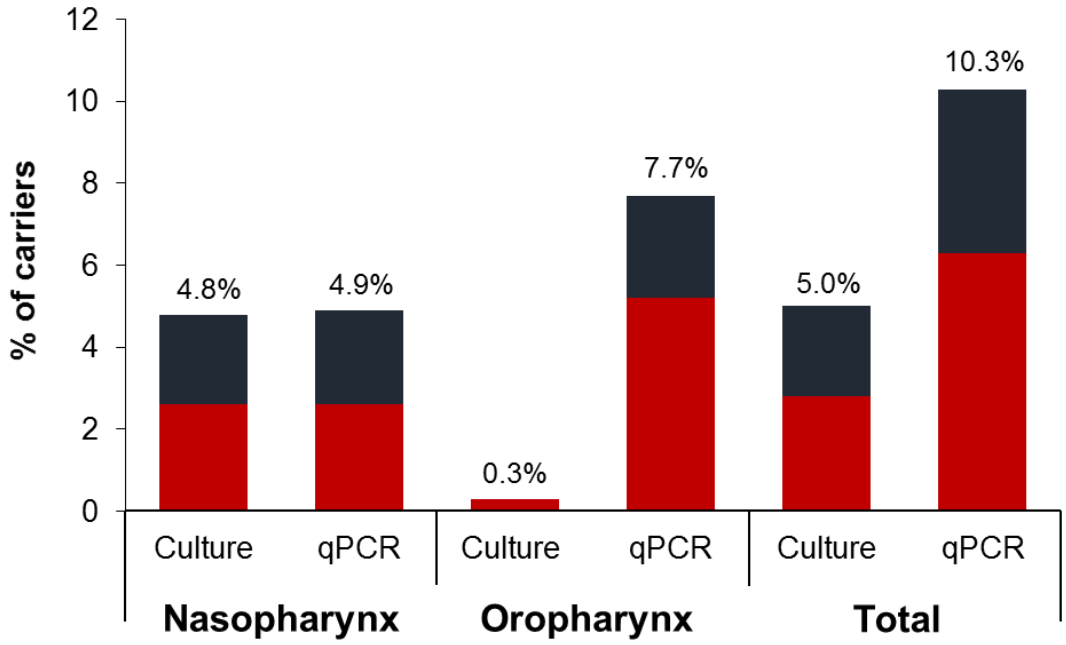

Figure 2. Proportion of pneumococcal carriers detected by classical culture-based methods or qPCR. Red bars, adults over 60 years of age living in nursing homes; blue bars, adults over 60 years of age living in family homes.

In total, twenty-one different serotypes/serogroups were detected among 76 samples that were positive for pneumococci by qPCR compared to 14 serotypes present in the 32 samples that were positive for pneumococci by classical culture-based methods. Serotype diversity (GSID=0.897, 95% CI: 0.896 – 0.898) was slightly higher among samples detected by qPCR compared to those by classical culture-based methods (GSID=0.879, 95% CI: 0.877 – 0.881). Serotypes/serogroups 15A/F, 24 and 38, which are not included in any pneumococcal multi-valent vaccine, and serotypes/serogroups 7A/F, 10A, 12A/B/F and 20 potentially targeted by PPV23 (7F is also targeted by PCV13), were only detected in qPCR positive samples (Table 2).

Combined nasopharyngeal and oropharyngeal sampling did not yield significantly more different serotypes than each sampling site alone (14 serotypes/serogroups detected in nasopharyngeal samples and 17 detected in the oropharyngeal samples, p=0.166 and p=0.564, respectively).

The most frequent serotypes/serogroups were 19A (n=17), 23A (n=9), 35F (n=7), 6C/D (n=6), 22A/F (n=5) and 11A/D (n=4), which, together, accounted for 68.6% of all serotyped samples (Table 2). Serotypes 7B/C (n=1), 9L (n=1), 23B (n=2) and 31 (n=1) were only found in nasopharyngeal samples, while serotypes 7A/F (n=1), 10A (n=1),

12A/B/F (n=1), 15A/F (n=1), 20 (n=2), 24 (n=3) and 38 (n=1) were only found in oropharyngeal samples (Table 2).

Table 2. *S. pneumoniae* serotypes/serogroups detected in samples obtained from adults over 60 years old.

Serotype/ serogroup	Vaccine[1]	Number of samples with serotype/serogroup			
		Nursing homes		Family home	
		Classical culture-based approaches[2]	qPCR[3]	Classical culture-based approaches[2]	qPCR[3]
3	PCV13/PPV23	-	-	2	3
5	PCV13/PPV23	-	1[4]	-	-
6A	PCV13/PPV23	-	-	2	2
6C/D	-	-	2	2 (6C)	4
7A/F	PCV13/PPV23	-	-	-	1
7B/C	-	-	-	1	1
9L	-	-	-	1	1
10A	PPV23	-	-	-	1
11A/D	PPV23	-	3	1 (11A)	1
12A/B/F	PPV23	-	-	-	1
15A/F	-	-	-	-	1
16F	-	-	-	1	2
17F	PPV23	1	3	-	-
19A	PCV13/PPV23	7	16	-	1
20	PPV23	-	2	-	-
22A/F	PPV23	2 (22F)	4	-	1
23A	-	4	6	2	3
23B	-	1	1	1	1
24	-	-	1	-	2
31	-	-	-	1	1
35F	-	3	6	1	1
38	-	-	-	-	1
ND	-	-	5	-	1

[1]Indicates whether the serotype/serogroup is potentially targeted by the 13-valent pneumococcal conjugate vaccine (PCV13: 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) or by the 23-valent polysaccharide vaccine (PPV23: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F).

[2]Detection of pneumococci by identification and characterization of presumptive colonies grown on gentamicin blood agar, as described in the Methods section.

[3]Detection of pneumococci using a qPCR targeting *lytA* and *piaB* genes, as described in the Methods section.

[4]Given that this specific assay is known to yield false positive results, it was not taken into consideration.

ND, not determined.

Overall, among the 62 carriers (38 living in nursing homes and 24 living in family homes) a maximum of 25.8% and 46.8% had serotypes potentially covered by PCV13 and PPV23, respectively.

Of note, within nursing homes, there was evidence of cross-transmission with a common serotype being frequently isolated from multiple individuals (Figure 3). This contributed to a lower serotype diversity in the nursing home collection (GSID=0.778, 95% CI: 0.775 – 0.782) when compared to the family home collection (GSID=0.919, 95% CI: 0.918 – 0.920).

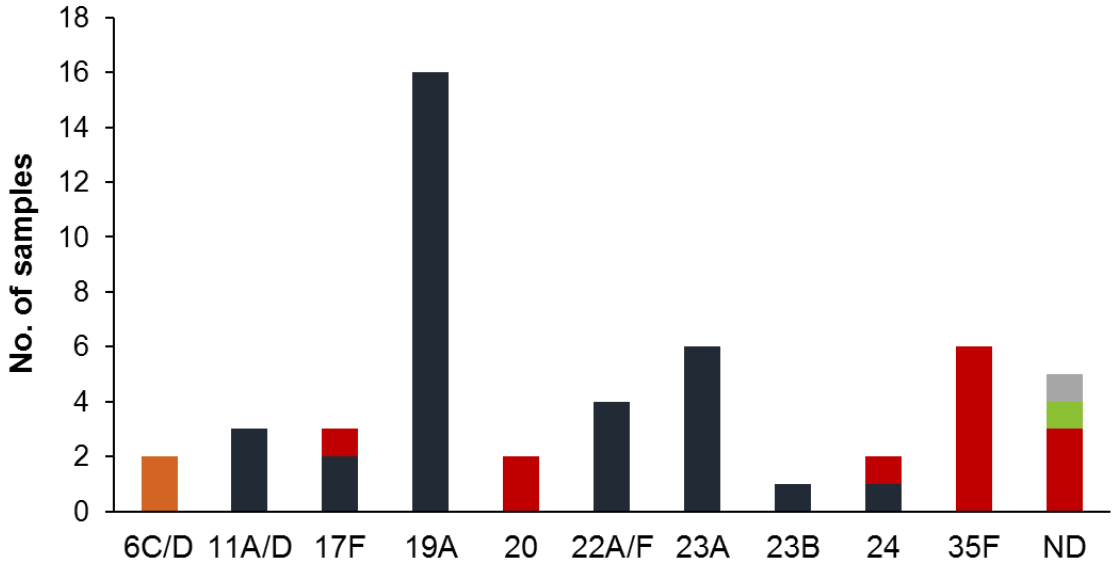

Figure 3. Serotype distribution of pneumococcal isolates carried by adults over 60 years of age living in nursing homes. Different colors indicate different nursing homes.

Discussion

In this study qPCR targeting *lytA* and *piaB* was used to investigate the prevalence of nasopharyngeal and oropharyngeal pneumococcal carriage in two collections of samples obtained from adults over 60 years of age living in either nursing homes or family homes. Positive samples were further characterized by molecular serotyping. These collections were previously characterized by classical culture-based methods as part of a much larger pneumococcal carriage study [15].

In our previous study, as well as in studies from countries such as Belgium, Finland and Israel – which also used conventional culture followed by identification of presumptive

colonies of *S. pneumoniae* – pneumococcal carriage rates were estimated to be in the range of 3%-5% among the elderly [14-16, 37].

In this study, use of qPCR increased the detection of pneumococci in oropharyngeal samples by approximately 15 times in both the nursing home and family home collections. By contrast, there was no significant added value in using qPCR to detect pneumococci in nasopharyngeal samples. The use of qPCR is known to be particularly valuable to detect pneumococci in samples that are highly polymicrobial and/or in which pneumococci are present at low density. In oropharyngeal samples both conditions tend to be common [19, 20, 22]. The nasopharynx, by contrast, is known to be less polymicrobial and pneumococci, when present, tend to thrive [15, 33, 38-40].

When all results were compared, pneumococci were more frequently detected in oropharyngeal samples than in nasopharyngeal samples (7.7% *vs.* 5.0%, McNemar's, $p<0.05$) and there were also twice more individuals positive for pneumococci exclusively in oropharyngeal samples (n=32) compared to individuals positive exclusively in nasopharyngeal samples (n=16). These observations suggest that pneumococci, in the elderly, tend to preferentially colonize the oropharynx.

They also support the recommendation that to detect pneumococci in senior adults it is important to combine both nasopharyngeal and oropharyngeal samples and that qPCR (targeting more than one validated gene, such as *lytA* and *piaB*) should be used when analyzing oropharyngeal samples [19, 20, 22]. In our study, half of the pneumococcal carriers were only detected in these conditions.

Overall, we estimated that approximately 10% of the individuals were pneumococcal carriers independently of whether they lived in a nursing home or in a family home. These estimates are lower than those described in studies conducted in Italy or the Netherlands, which were closer to 20% [22, 24]. These differences are likely to be due to seasonality: the latter studies were carried out in winter or winter/spring, while ours took place throughout the year. In fact, a variation between 0%-17% in pneumococcal carriage prevalence among adults over 65 years of age was noted in the US longitudinal study of Branche et al. [21].

While we did not observe differences in density of colonization between individuals living in nursing homes or family homes, we observed (unsurprisingly) lower serotype diversity and evidence for cross-transmission within nursing homes. These are settings where individuals are often confined and where transmission of infectious agents is often facilitated [41].

Molecular serotyping of qPCR pneumococcal positive samples enabled assignment of a serotype/serogroup to most (92%) samples. Furthermore, the number of different serotypes that were detected increased by 50% (21 in qPCR samples *vs.* 14 by classical culture-based processed samples) expanding considerably the pool of serotypes detected in the population. This included non-vaccine serotypes and vaccine serotypes that, otherwise, would remain undetected and that may be important to monitor intervention strategies such as the use of pneumococcal vaccines.

In conclusion, carriage of pneumococci in senior adults is significantly higher than the one estimated by classical culture-based methods alone. Our current estimates suggest that, in Portugal, it is approximately 10%. The high number of serotypes circulating in this population and the transmission observed within nursing homes warrant additional studies aimed to assess its implications in disease. As the number and proportion of aged individuals increases in societies worldwide, accurate estimates of pneumococcal carriage prevalence and circulating serotypes in this group are crucial to better understand colonization dynamics, serotype disease potential, and impact of pneumococcal vaccines in this age group.

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Supplementary material

Table S1. Socio-demographic characteristics of the population.

Variable		Participants[1] (total=3361)	Nursing home collection[2] (n=299)	Family home collection[2] (n=300)
Living area				
	urban	1945 (57.9%)	27 (9.3%)	101 (33.7%)
	rural	1416 (42.1%)	272 (90.7%)	199 (66.3%)
Mean age (yrs)		74.5 ± 8.2	82.5 ± 6.9	73.8 ± 7.7
Gender				
	female	1935 (57.6%)	194 (64.9%)	170 (56.7%)
	male	1426 (42.4%)	105 (35.1%)	130 (43.3%)
Years of school education				
	0	315 (9.4%)	72 (24.1%)	33 (11.0%)
	1-4	2799 (83.3%)	226 (75.6%)	254 (84.7%)
	≥ 5	246 (7.3%)	1 (0.3%)	13 (4.3%)
Retirees				
	retired	3015 (89.7%)	299 (100%)	266 (88.7%)
	active	346 (10.3%)	0 (0.0%)	34 (11.3%)
Housing				
	family home	3062 (91.1%)	0 (0.0%)	300 (100%)
	retirement home	299 (8.9%)	299 (9.3%)	0 (0.0%)
Weekly contact with children ≤ 6 yrs				
	yes	650 (19.3%)	1 (0.3%)	46 (15.3%)
	no	2711 (80.7%)	298 (99.7%)	254 (84.7%)
Recreational activities				
	at least one activity	1119 (33.3%)	9 (3.0%)	131 (43.7%)
	club	339 (10.1%)	4 (1.3%)	30 (10.0%)
	day center	652 (19.4%)	6 (2.0%)	94 (31.3%)
	senior university	51 (1.5%)	0 (0.0%)	1 (0.3%)
	other	99 (2.9%)	0 (0.0%)	10 (3.3%)
Smoker				
	yes	126 (3.7%)	5 (1.7%)	12 (4.0%)
	no	3235 (96.3%)	294 (98.3%)	288 (96.0%)
Vaccination with PPV23				
	yes	122 (3.6%)	8 (2.7%)	11 (3.7%)
	no	3239 (96.4%)	291 (97.3%)	289 (96.3%)

[1]Data described in Almeida *et al*. [15].

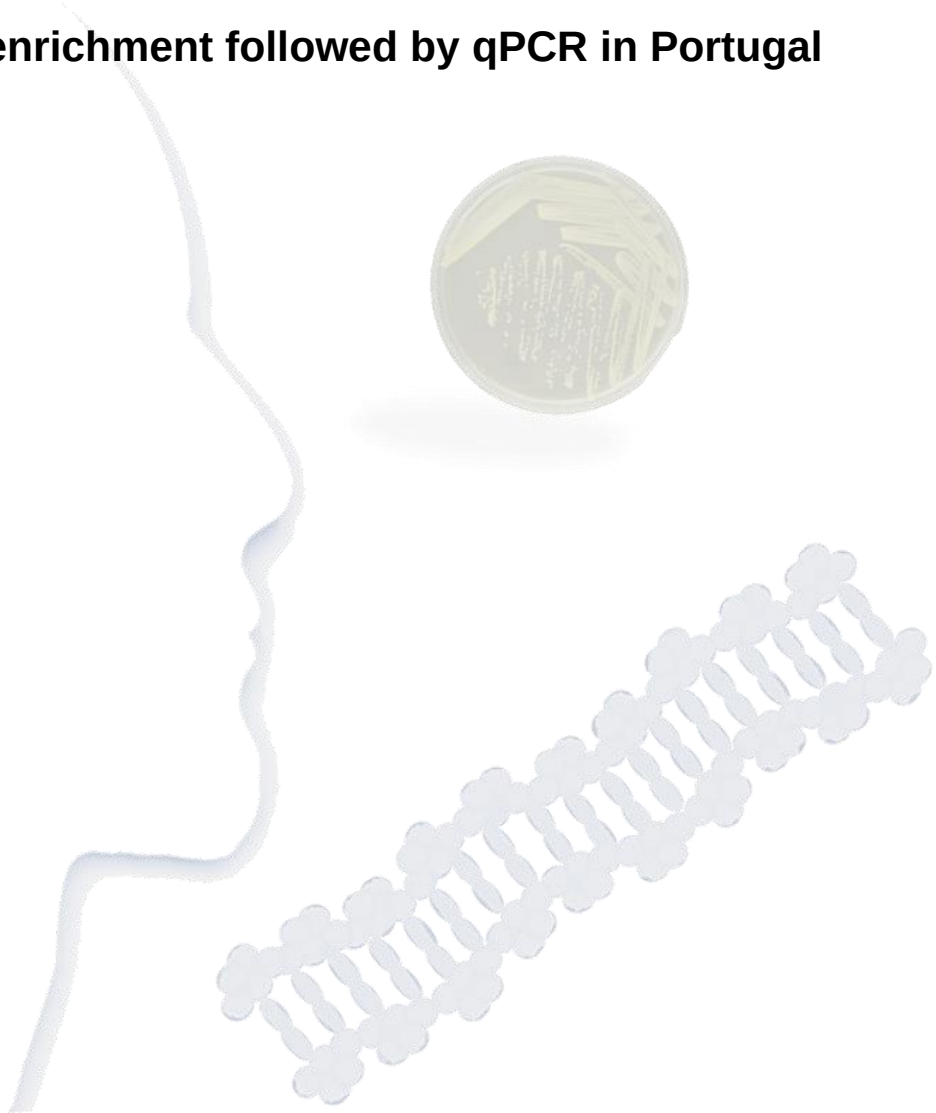
[2]Data relative to samples analyzed in this study.

Table S2. Geometric mean of the distribution of Ct values for *lytA* and *piaB* genes of oropharyngeal and nasopharyngeal samples.

	Geometric mean ± Geometric Sd	
	lytA	*piaB*
Oropharyngeal samples	26.79 ± 1.19	26.90 ± 1.29
Nasopharyngeal samples	19.11 ± 1.17	18.52 ± 1.19

Chapter IV

Evaluation of methicillin-resistant *Staphylococcus aureus* carriage in senior adults by selective enrichment followed by qPCR in Portugal



Submitted:

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Contributions:

The study was conceived by RSL. Experiments were done by STA. Statistical analyses were done by ACP. Data interpretation was done by STA and RSL. RSL and HdL contributed with reagents and materials. The manuscript was written by STA and RSL and critically revised by all authors. All authors read and approved the final version of the manuscript.

Summary

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals in Portugal is worrisome and among the highest in Europe. Surprisingly, MRSA prevalence in the community was described as very low (<2%) based on studies that used classical culture-based methods.

We investigated whether the apparent limited spread of MRSA in the community in Portugal might result from low sensitivity of classical culture-based methods. Nasopharyngeal and oropharyngeal paired samples obtained from senior adults living in nursing (n=299) or family homes (n=300) previously characterized by classical culture-based methods were re-analyzed. Samples were inoculated in a semi-selective enrichment medium and evaluated by qPCR targeting *nuc*, *mecA* and *mecC* genes.

By semi-selective enrichment medium followed by qPCR, 34 of the 1,198 (2.8%) samples were MRSA positive compared to 21 (1.8%) by classical culture-based methods. The use of a semi-selective enrichment medium followed by qPCR improved non-significantly detection of MRSA carriers from 5.4% to 8.0% (p=0.12) in the nursing home collection, and from 0.3% to 1.7% (p=0.13) in the family home collection. MRSA isolates belonged to three HA-MRSA clones widely disseminated in Portuguese hospitals.

We conclude that although the use of a semi-selective enrichment medium followed by qPCR improves sensitivity of detection of MRSA in community carriage samples, it does not change the overall scenario previously described. In Portugal, MRSA circulation in the community among senior adults is low.

Introduction

Staphylococcus aureus is an opportunistic Gram-positive bacterium that colonizes asymptomatically the human anterior nares. It has been estimated that 20%-40% of the adult population is colonized with *S. aureus* [1]. It is also a main cause of a wide range of infections including skin and soft tissue infections, pneumonia, endocarditis and bacteremia affecting primarily debilitated individuals such as those with diabetes, undergoing hemodialysis or with HIV [2].

Methicillin-resistant *S. aureus* (MRSA), in particular, is a leading cause of morbidity and mortality worldwide and has been associated for more than 40 years with nosocomial

settings only (hospital-associated, HA-MRSA). In the late 1990's, however, MRSA emerged in the community affecting healthy individuals without typical risk factors for MRSA carriage (community-associated MRSA, CA-MRSA) [3-6]. During the past decade, the prevalence of CA-MRSA increased very rapidly worldwide, varying from country to country [6, 7]. In parallel, an increasing blur between CA-MRSA and HA-MRSA clones has been described in several countries. For example, the EMRSA-15 clone traditionally associated with hospital infections has been found circulating in the community in England, Italy, and Portugal [8-10]. Furthermore, some CA-MRSA clones such as USA300 and USA400 have also been associated with nosocomial infections [11, 12]. In Greece, a country with high rates of both HA-MRSA and CA-MRSA, the presence of the CA-MRSA European clone ST80-IV was identified not only in the community (c.a. 90%) but also in hospitals (c.a. 11%) [13].

In Portugal the burden of MRSA infections is among the highest in Europe. Still, in recent years the rates of HA-MRSA (isolated from blood or CSF) have been declining: from c.a. 50% in 2015 to c.a. 40% in 2018 [6]. This declining trend parallels what has been observed in most European countries although the reasons underlying it are poorly understood, as these declines have not always been directly correlated with improved infection control practices and/or antibiotic stewardship.

The molecular epidemiology of nosocomial MRSA has been extensively studied in Portugal since the early 1990s. At that time the Iberian clone (ST247-IA) was dominant in several Portuguese hospitals, having been replaced by the Brazilian clone (ST239-IIIA) in the mid 1990's [14, 15]. In 2001, the EMRSA-15 (ST22-IVh) clone emerged in Portugal and soon became the dominant clone in nearly all hospitals, a situation that has persisted for several years. Since 2010, in parallel with EMRSA-15, the New York/Japan related clone (ST105-II) and the Pediatric and its related clones (ST5-IVc and ST125-IVc, respectively) were also found to be frequent in Portuguese hospitals, representing together c.a. 20% of the MRSA isolates [14-17].

In Portugal, the available studies suggest that the prevalence of CA-MRSA is low [18-20], a somewhat surprising observation given its high prevalence in hospitals [6]. Previous studies conducted in the community among children attending day-care centers, young healthy adults (draftees and university students) and adults aged over 60 years old reported a prevalence of MRSA asymptomatic colonization lower than 2% [18-20]. Two other studies showed MRSA rates higher than 20% in the community. However, differently from the studies mentioned above, where only colonization samples were obtained, these latter studies included not only colonization samples, but also infection

samples. One study included individuals attending health care centers due to skin and soft tissue infections [16]. The other included individuals attending health care centers and hospital inpatients from which MRSA was isolated within the first 48 hours upon admission [10].

The gold standard for routine detection of MRSA from clinical specimens are culture-based approaches. These methodologies are laborious and time-consuming as they are often preceded by a selective enrichment step, require isolation of a pure culture and its characterization [21]. In recent years, in-house and commercial PCR-based methods have been developed [22, 23]. These strategies enable the reliable identification of MRSA within hours and have a high sensitivity, which is important in low density samples [24].

In carriage studies, culture-based approaches are also the most frequent ones for the identification of MRSA [24]. The few studies available that have used qPCR (qPCR)-based methods to investigate MRSA carriage, in patients admitted to an intensive care unit and individuals attending health care organizations, have shown that these methods yield comparable results to those obtained by using culture-based approaches [23, 25].

We hypothesized that the low prevalence of MRSA observed among individuals in the community in Portugal, might be due to an underestimation of carriage due to the use of low sensitivity classical culture-based approaches.

In this study we re-visited two collections of carriage samples previously characterized by classical culture-based approaches obtained from adults over 60 years of age living in either nursing homes or family homes in Portugal. We combined the use of a semi-selective enrichment step followed by qPCR to re-evaluate the prevalence of MRSA carriers in this age group.

Material and methods

Study population and samples. This study was nested on a larger one conducted previously [18]. Briefly, the prevalence of *S. aureus* and MRSA carriage, associated risk factors and the properties of circulating MRSA lineages were studied in a Portuguese population of 3,361 adults of over 60 years of age, between April 2010 and December 2012. Nasopharyngeal and oropharyngeal samples (3,361 samples each) were obtained for each participant, as previously described [26]. *S. aureus* and MRSA isolates were identified by classical culture-based methods: nasopharyngeal and oropharyngeal

swabs were plated onto mannitol salt agar (Difco, Detroit, MI) and incubated in aerobic conditions, overnight at 37°C; the yellow colonies were streaked on tryptic soy agar (TSA) (Difco) and, on the following day, presumptive *S. aureus* colonies were tested for the production of coagulase using Staphaurex test (Remel, Lenexa, KS). Oxacillin and cefoxitin susceptibility was tested for all *S. aureus* isolates, by agar disk diffusion, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [27]. Screening for the *mecA* gene was performed by PCR in order to identify MRSA [28]. Genotyping of MRSA was carried out by SCC*mec* typing, *spa* typing and MLST following previously described protocols [19, 28]. Nasopharyngeal and oropharyngeal swabs were frozen in skim milk, tryptone, glucose and glycerin (STGG) and stored at -80°C.

In the current study, two collections of samples from the earlier initial study were re-examined. One collection corresponded to 600 paired samples (300 nasopharyngeal and 300 oropharyngeal) obtained from 300 individuals randomly selected from a pool of 3,062 individuals living in their own family home. The second collection corresponded to 598 paired samples (299 nasopharyngeal and 299 oropharyngeal) obtained from all individuals (n=299) living in nursing homes at the time of the initial study [18].

Identification of MRSA by molecular methods. To screen for MRSA, an enrichment step was used: 200µl of the frozen stock of each (nasopharyngeal and oropharyngeal) sample were inoculated into 2mL of a semi-selective enrichment broth - tryptic soy broth (Difco), containing 2.5% of NaCl (Merck), 3.5mg/L cefoxitin and 20mg/L aztreonam (Sigma-Aldrich) [29] - and were incubated overnight at 37°C in ambient air with agitation. Total DNA was extracted from turbid cultures (indicative of bacterial growth) using the MagNA Pure Compact system (Roche Diagnostics GmbH) according to a modified protocol: 200µl of the enriched culture were centrifuged for 1 min at 13,000 rpm. The supernatant was discarded, the pellet was resuspended in 250µl of PBS, and 200µl were transferred into a tube containing 200µl of a lysis buffer (Roche) and 10µl of lysostaphin 10mg/mL (AMBI products LLC). Tubes were incubated for 20 min at 37°C, and the subsequent isolation steps were performed in the MagNA instrument.

DNA samples were tested by qPCR targeting *mecA* (confers resistance to methicillin), *mecC* (homologue of *mecA*, confers resistance to methicillin) and *nuc* (encodes for a *S. aureus* thermonuclease) genes. The sequences of primers and probe for *mecA* were described before [30]; for *mecC* and *nuc* they were kindly provided by Prof. Henrik Westh from the University of Copenhagen. For all samples two qPCRs were performed. The first qPCR targeted the *nuc* gene and was performed in a final volume of 25µl, containing 1x FastStart TaqMan Probe Master (Roche), 250nM of each primer, 125nM of probe and

2.5µl of total DNA. The second qPCR targeted *mecA* and *mecC* genes and was also performed in a final volume of 25µl, containing the same concentration of primers and probes as described for the *nuc* gene. DNA was amplified using the CFX96 QSystem Amplification (Bio-Rad) using the following cycling conditions: 95°C for 10 min followed by 45 cycles of 95°C for 10 sec, 55°C (for *nuc*) or 60°C (for *mecA* and *mecC*) for 25 sec, and a final step of 40°C for 30 sec. Samples were considered positive for MRSA when *nuc* and *mecA* or *mecC* genes had cycle threshold (Ct) values below 40. Strains COL, LGA251, ATCC25923 and ATCC14900 were used as controls.

Characterization of MRSA isolates. Samples positive for MRSA by qPCR were re-examined in order to try to isolate a pure culture of MRSA. 200µl of each MRSA-positive previously enriched culture were plated on a chromogenic selective medium CHROMagar MRSA (ChromAgar, Paris, France) and incubated overnight at 37ºC with ambient atmosphere. Strains COL (MRSA) and ATCC14990 (*Staphylococcus epidermidis*) were used as positive and negative controls, respectively. For each sample up to 50 presumptive MRSA colonies were picked and isolated in TSA plates. The presence of *mecA* was confirmed by PCR, using the same primers mentioned above. From each sample, one positive MRSA colony was further cultivated and characterized by SCC*mec* typing, *spa* typing and MLST, as previously described [19, 28]. DNA sequencing reactions were performed at STAB VIDA (Caparica, Portugal).

Statistical Analysis. The Ct values of *nuc* and *mecA* genes from nasopharyngeal and oropharyngeal samples were summarized by their geometric mean and corresponding standard deviation. Statistical associations between the Ct values from nursing homes *vs.* family home and nasopharynx *vs.* oropharynx were obtained by using a generalized linear model (GLM) with a Gaussian distribution and a log link function.

Culture and qPCR approaches were compared by the McNemar's test based on paired individuals. To compare the prevalence of MRSA between nasopharyngeal and oropharyngeal samples and between nursing home and family home by qPCR or classical culture-based methods the Chi-squared test was used. A p-value of <0.05 was considered statistically significant for all tests used. All analyses were performed using R version 3.2.3 [31].

Ethics statement. The initial study was performed in line with the European Statements for Good Clinical Practice and the principles of the Declaration of Helsinki of the World Health Medical Association. The study was registered and approved at health care centers of Oeiras and Montemor-o-Novo that report to Administração Regional de Saúde (ARS, “Regional Health Administration”) of Lisboa e Vale do Tejo, and Alentejo,

respectively, from the Ministry of Health. Informed written consent was obtained from all participants. A numeric code was attributed to all samples and questionnaires all of which were processed anonymously. The current study – a retrospective re-analysis of samples – did not require ethical approval.

Results

Detection of MRSA carriage by semi-selective enrichment followed by qPCR. We used semi-selective enrichment followed by qPCR to re-evaluate MRSA carriage in samples obtained from 599 adults aged over 60 years old, divided in two collections: participants living in nursing homes (n=299) and participants living in family homes (n=300). These samples are a subset of 6,722 samples (3,361 nasopharyngeal samples and 3,361 oropharyngeal samples) obtained in a study previously described [18]. Overall, in the current study we analyzed 1,198 paired samples (599 nasopharyngeal samples and 599 oropharyngeal samples) and compared the results with those previously obtained by classical culture-based approaches for the same samples [18]. The socio-demographic characteristics of the population were described before [18] and are summarized in Supplementary Table S1.

By qPCR, 34 of the 1,198 (2.8%) samples were positive for *nuc* and *mecA* genes suggestive of the presence of MRSA. There were two samples that were only positive for *mecA* indicating the presence of coagulase negative methicillin-resistant staphylococci (Figure 1). All samples were negative for *mecC* (Ct values ≥40). Of note, 10 samples had Ct values for *nuc* that were lower in more than two Cts than those obtained for *mecA* suggesting those samples contained a mixture of MSSA and MRSA strains (Figure 1). Similarly, there were three samples in which the opposite occurred suggesting that these contained a mixture of MRSA and coagulase negative methicillin-resistant staphylococci (Figure 1).

Concordance between oropharyngeal and nasopharyngeal swabs for detection of MRSA by qPCR. Among the 34 MRSA positive samples detected by qPCR, 20 were nasopharyngeal samples and 14 were oropharyngeal samples. Of these, only twelve were concordant (paired) samples obtained from six individuals. Compared to sampling the oropharynx only, the addition of nasopharyngeal sampling improved significantly the overall detection of MRSA carriage (18/599, 3.0% *vs.* 34/599, 5.7%; p=0.03). In contrast, compared to sampling the nasopharynx only, the addition of oropharyngeal sampling did

not improve significantly the detection of MRSA carriage (22/599, 3.7% *vs.* 34/599, 5.7%; p=0.13).

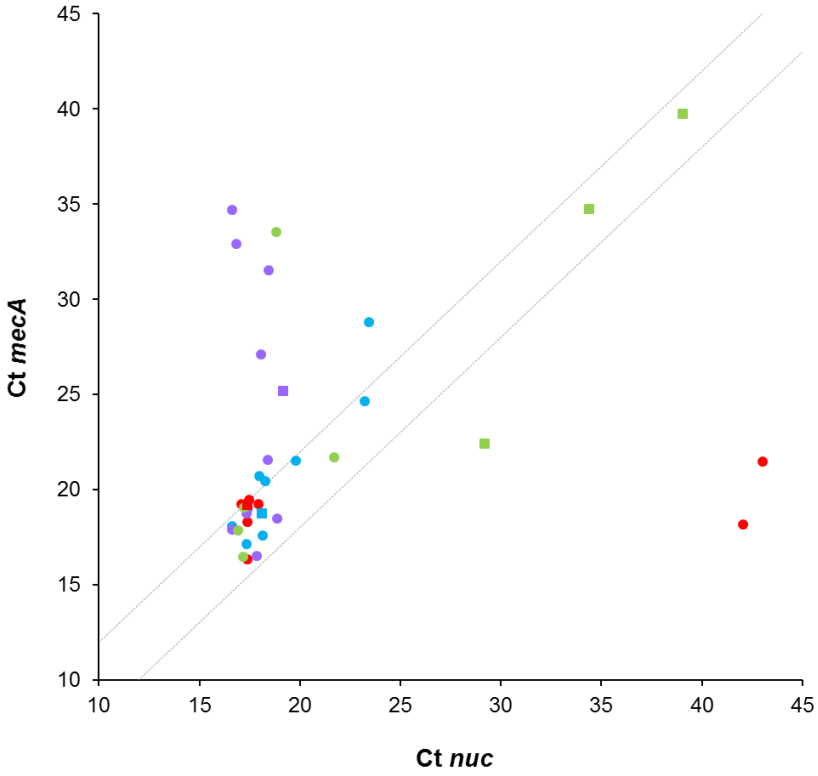

Figure 1. Detection of MRSA by real-time PCR in adults over 60 years of age living in nursing homes (circles) and family homes (squares). Each dot represents a sample and its position corresponds to the Ct values for *nuc* and *mecA* genes. Blue, nasopharyngeal samples that were positive by culture; purple, nasopharyngeal samples that were negative by culture; red, oropharyngeal samples that were positive by culture; green, oropharyngeal samples that were negative by culture. Diagonal dashed lines indicate the threshold of 2 Ct values difference between *nuc* and *mecA* genes. Samples with Ct values above 40 were considered to be negative. Ct values for *mecC* gene are not shown, since all samples were negative for this gene, with Ct values ≥40.

Comparison of semi-selective enrichment followed by qPCR with classical culture-based approaches for detection of MRSA nasopharyngeal and oropharyngeal carriage. The use of semi-selective enrichment followed by qPCR, when compared with classical culture-based methods, improved detection of MRSA carriage in oropharyngeal and nasopharyngeal samples in both the nursing home and

family home collections (Table 1 and Figure 2). This increase was, however, only statistically significant for the nasopharyngeal samples from the nursing home collection: by culture, MRSA was detected in 10 (3.3%) samples, while by qPCR, MRSA was detected in 18 (6.0%) samples (p=0.04) (Table 1 and Figure 2).

MRSA carriage in nursing homes and family homes. In both groups of individuals, we observed that the geometric Ct mean was not significantly different between positive nasopharyngeal samples and positive oropharyngeal samples (p=0.092 for *nuc*; p=0.965 for *mecA*) suggesting that both anatomical sites were colonized at comparable MRSA densities.

Nevertheless, Ct values for *nuc* gene were significantly lower in samples from adults living in nursing homes than in samples from adults living in the family home (adjusted GLM, p<0.001) indicating higher density of *S. aureus* colonization in the former case. On the other hand, Ct values for the *mecA* gene were not significantly different between samples from both collections (adjusted GLM, p=0.069) (Figure 1 and Supplementary Table S2) suggesting that colonization by MRSA occurred at a comparable density.

When results of the two sampling sites were combined, the use of qPCR, compared to classical culture-based methods, improved detection of MRSA carriers from 5.4% to 8.0% in the nursing home collection and from 0.3% to 1.7% in the family home collection (Table 1). However, probably due to the low number of positive results, these increases were not statistically significant. In fact, when results from the two collections were analyzed together, the use of qPCR improved significantly the detection of MRSA carriers compared to classical culture-based methods (from 17 (2.8%) to 29 (4.8%), p=0.03).

Characteristics of MRSA isolates. Overall, 40 samples were positive for MRSA by culture and/or by qPCR (18 from oropharyngeal samples and 22 from nasopharyngeal samples; Table 1 and Supplementary table S3). From these, pure MRSA cultures were obtained from 38 samples and were further characterized. All isolates were found to be related to three HA-MRSA epidemic clones: 19 (50.0%) isolates were related to the New York/Japan clone, 17 (44.7%) belonged to the Pediatric clone, and two (5.3%) belonged to the EMRSA-15 clone (Table 2). The six MRSA carriers yielding both a positive nasopharyngeal and oropharyngeal sample carried the same strain in both samples.

Table 1. Detection of MRSA carriers according to sampling site and methodology used.

Collection	Participants n	Sampling Site n (%)								Carriers n (%)			
		Oropharynx (OP)				Nasopharynx (NP)							
		Culture positive	qPCR positive	Culture and/or qPCR positive	p-value	Culture positive	qPCR positive	Culture and/or qPCR positive	p-value	Culture positive (NP and/or OP)	qPCR positive (NP and/or OP)	Total	p-value
Nursing home	299	9 (3.0%)	10 (3.3%)	14 (4.7%)	1.0	10 (3.3%)	18 (6.0%)	20 (6.7%)	**0.04**	16 (5.4%)	24 (8.0%)	29 (9.7%)	0.12
Family home	300	1 (0.3%)	4 (1.3%)	4 (1.3%)	0.25	1 (0.3%)	2 (0.7%)	2 (0.7%)	1.0	1 (0.3%)	5 (1.7%)	5 (1.7%)	0.13

p-values determined using McNemar's Chi-squared test for paired individuals.

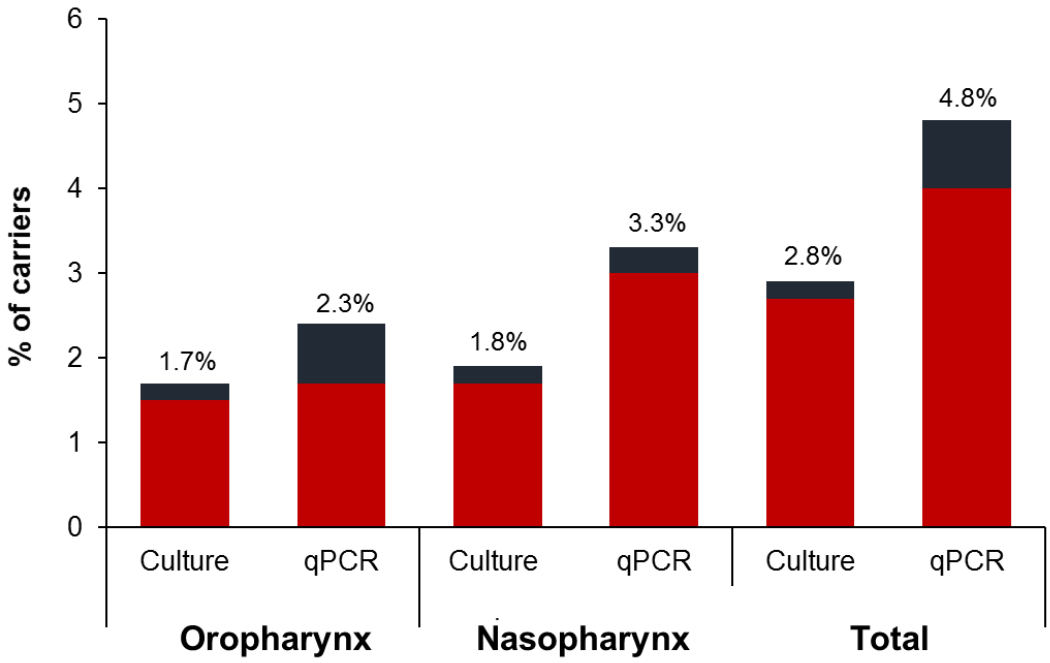

Figure 2. Proportion of MRSA carriers (adults over 60 years old) detected by classical culture-based methods and real-time PCR. Red bars, carriers living in nursing homes; blue bars, carriers living in family homes.

Table 2. Characteristics of the 38 MRSA isolates carried by adults aged over 60 years old.

Genotype			Related clones	Collection	No. of isolates detected by	
MLST (CC)	SCC*mec* type	spa types			qPCR	Culture
ST105 (CC5)	II	t002	New York/Japan	Nursing home, Family home	19[1]	19[1]
ST5 (CC5)	IVc	t002, t535	Pediatric	Nursing home	17	17
ST22 (CC22)	IVh	t2357	EMRSA-15	Nursing home	2	2

[1]15 isolates from the nursing home collection and 4 isolates from the family home collection.

CC, clonal complex.

Discussion

In Portuguese hospitals, MRSA prevalence is among the highest in Europe [6]. By contrast, previous studies suggested that its prevalence in the community is very low and is associated with well-defined risk groups [18-20]. We used qPCR, preceded by an enrichment step to investigate MRSA carriage in nasopharyngeal and oropharyngeal paired samples obtained from adults older than 60 years of age living in nursing homes or in family homes. We compared results with those obtained using classical culture-based methods.

We observed that the use of enrichment followed by qPCR increased detection of MRSA in the nasopharynx and oropharynx both among individuals living in family homes and in nursing homes. Compared to previous estimations based on classical culture-based methods, the current strategy increased detection by an average of 1.5-fold of MRSA carriers in the nursing home collection and by an average of 6-fold of MRSA carriers in the family home collection. Still, MRSA carriage prevalence was relatively low in both groups: 8.0% among individuals living in nursing homes, and 1.7% among individuals living in family homes. These findings are similar to the ones described in studies from Brazil, Malta and USA that estimated a prevalence of MRSA around 2%, 5% and 8%, respectively, among elderly adults living in the community [32-34].

We also observed that the combined use of nasopharyngeal and oropharyngeal swabs increased detection of MRSA as concordance between sampling sites was not high. Nevertheless, the nasopharyngeal swab alone enabled significantly higher detection of MRSA carriers than the oropharyngeal swab in the nursing home collection (in the family home collection the numbers were too low to draw conclusions). As density of colonization was comparable in both sites, the results suggest that the nasopharynx may be a preferred colonization niche compared to the oropharynx.

Genotyping of MRSA isolates identified three HA-MRSA clones widely disseminated in Portuguese hospitals with no evidence for specific CA-MRSA clones in circulation.

Our study has some limitations. First, due to the relatively low prevalence of MRSA, the study may have been underpowered to detect significant differences between methodologies or sampling sites. Secondly, samples from the anterior nares were not obtained despite the fact that this is considered to be the preferential human niche [1]. This was due to the fact that the study was nested in another one aimed to detect *Streptococcus pneumoniae*. Still, it is known that *S. aureus* colonizes multiple human body sites (skin, perineum, pharynx and axillae, among others) and is a frequent

colonizer of the nasopharynx and the oropharynx [1, 18, 19]. The qPCR used in this study did not identify six MRSA samples that were previously identified by culture-based methods despite repeated attempts. The reasons for this are unclear and may have been due to low density or sample heterogeneity.

One strength of our study was the use of paired samples for all individuals. We also combined an enrichment step with qPCR increasing the detection of MRSA compared to culture alone. In addition, we were able to isolate 38 MRSA strains from the 40 qPCR positive samples allowing culture confirmation of results and genotyping of the strains.

In conclusion, although the use of selective enrichment combined with qPCR improves sensitivity of detection of MRSA in community carriage samples, it does not change the overall scenario previously described in Portugal: MRSA circulation in the elderly is low and associated with HA-MRSA clones.

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Supplementary material

Table S1. Socio-demographic characteristics of the population.

Variable		Participants[1] (total=3361)	Nursing home collection[2] (n=299)	Family home collection[2] (n=300)
Living area				
	urban	1945 (57.9%)	27 (9.3%)	101 (33.7%)
	rural	1416 (42.1%)	272 (90.7%)	199 (66.3%)
Mean age (yrs)		74.5 ± 8.2	82.5 ± 6.9	73.8 ± 7.7
Gender				
	female	1935 (57.6%)	194 (64.9%)	170 (56.7%)
	male	1426 (42.4%)	105 (35.1%)	130 (43.3%)
Years of school education				
	0	315 (9.4%)	72 (24.1%)	33 (11.0%)
	1-4	2799 (83.3%)	226 (75.6%)	254 (84.7%)
	≥ 5	246 (7.3%)	1 (0.3%)	13 (4.3%)
Retirees				
	retired	3015 (89.7%)	299 (100%)	266 (88.7%)
	active	346 (10.3%)	0 (0.0%)	34 (11.3%)
Housing				
	family home	3062 (91.1%)	0 (0.0%)	300 (100%)
	retirement home	299 (8.9%)	299 (9.3%)	0 (0.0%)
Weekly contact with children ≤ 6 yrs				
	yes	650 (19.3%)	1 (0.3%)	46 (15.3%)
	no	2711 (80.7%)	298 (99.7%)	254 (84.7%)
Recreational activities				
	at least one activity	1119 (33.3%)	9 (3.0%)	131 (43.7%)
	club	339 (10.1%)	4 (1.3%)	30 (10.0%)
	day center	652 (19.4%)	6 (2.0%)	94 (31.3%)
	senior university	51 (1.5%)	0 (0.0%)	1 (0.3%)
	other	99 (2.9%)	0 (0.0%)	10 (3.3%)
Smoker				
	yes	126 (3.7%)	5 (1.7%)	12 (4.0%)
	no	3235 (96.3%)	294 (98.3%)	288 (96.0%)
Vaccination with PPV23				
	yes	122 (3.6%)	8 (2.7%)	11 (3.7%)
	no	3239 (96.4%)	291 (97.3%)	289 (96.3%)

[1]Data described in Almeida *et al*. [18].

[2]Data relative to samples analyzed in this study.

Table S2. Geometric mean of the distribution of Ct values for *nuc* and *mecA* genes of samples from the nursing home collection and the family home collection.

	Geometric mean ± Geometric Sd	
	nuc	*mecA*
Nursing home collection	18.15 ± 1.10	21.14 ± 1.26
Family home collection	24.86 ± 1.42	25.59 ± 1.36

Table S3. Detection of MRSA by classical culture-based methods and by selective enrichment followed by qPCR.

No. of MRSA isolates (n=40)	Classical culture-based[1]	Selective enrichment followed by qPCR[2]
15	positive	positive
19	negative	positive
6	positive	negative
Total (positive)	**21**	**34**

Chapter V

Prevalence of methicillin-resistant *Staphylococcus aureus* colonization among immunocompetent healthy adults: insights from a longitudinal study

In preparation:

Almeida, S. T., A. C. Paulo, J. Babo, J. Borralho, C. Figueiredo, B. Gonçalves, J. Lança, M. Louro, H. Morais, J. Queiroz, H. de Lencastre, and R. Sá-Leão. Prevalence of methicillin-resistant *Staphylococcus aureus* colonization among immunocompetent healthy adults: insights from a longitudinal study.

Contributions:

The study was conceived by RSL. The experimental work was performed under the supervision of STA by undergraduate students JB, JB, CF, BG, JL, ML, HM and JQ as part of the Curricular Unit “Estudo Orientado em Biologia Molecular e Genética”, degree in Biology from Faculdade de Ciências da Universidade de Lisboa. The statistical analysis was performed by ACP. RSL and HdL contributed with reagents and materials. Data interpretation was done by STA and RSL. The manuscript was written by STA and RSL and critically revised by all authors. All authors read and approved the final version of the manuscript.

Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) has long been known as a major cause of hospital-acquired (HA-MRSA) infections worldwide. For the past twenty years, an increasing number of studies have described its emergence in the community as well. In Portugal, a country with a high-prevalence of HA-MRSA, there are only limited data available on the epidemiology of MRSA in the community.

We studied the prevalence of *S. aureus* and MRSA colonization among healthy adults in Portugal. Between February 2015 and December 2016, a longitudinal study was conducted in which 87 adults aged 25-50 years old were followed for six months. For each participant nasopharyngeal, oropharyngeal and saliva samples were obtained monthly and, in some cases, weekly. A total of 1,578 samples (n=526 for each sampling site) were examined for the presence of *S. aureus* and MRSA by classical culture-based methods.

Fifty-seven adults (65.5%) carried *S. aureus* at least once during the six months period of the study: 19.5% were persistent *S. aureus* carriers and 46.0% were intermittent carriers. Carriage rates per sampling site were 20.5% in nasopharynx, 18.3% in oropharynx, and 13.5% in saliva. Simultaneous screening of the three sampling sites increased detection of *S. aureus*, which overall occurred in 34.4% of the 526 sampling time-points. No MRSA were isolated.

In conclusion, this study adds novel information about the MRSA scenario in the Portuguese community. Our results indicate that, in Portugal, MRSA does not seem to circulate among healthy adults without risk factors and therefore this age group does not constitute, at the current time, a reservoir of MRSA in the community.

Introduction

Staphylococcus aureus is a common colonizer of the human anterior nares. About 20%-40% of the general population is colonized with this bacterium. It is also an important pathogen that is responsible for both health-care and community infections, such as skin and soft tissue infections, pneumonia, endocarditis and bacteremia [1, 2].

Methicillin-resistant *S. aureus* (MRSA), in particular, is responsible for high rates of nosocomial infections worldwide and in the last two decades, has also emerged and spread in the community (community-associated MRSA, CA-MRSA) worldwide [3-7].

In recent years, although the rates of hospital-associated MRSA (HA-MRSA) have decreased in most European countries, including Portugal, this pathogen continues to be a serious cause of bacterial infections. In Portugal, the prevalence of HA-MRSA, among all *S. aureus* obtained from blood and cerebrospinal fluid, is the third highest in Europe having been estimated as 38.1% in 2018 [6].

In Portugal, national surveillance studies have been conducted for almost 30 years in order to follow the prevalence of HA-MRSA over time. In the early 1990s the dominant clone in the Portuguese hospitals was the Iberian clone (ST247-IA) that was replaced by the Brazilian clone (ST239-IIIA) in 1995. In 2001, the EMRSA-15 clone emerged in the country and soon became the dominant clone in most hospitals. Today, it still remains one of the most prevalent clones [8, 9].

Despite the emergence of CA-MRSA infections worldwide, studies among healthy populations suggest that carriage rates remain low in most parts of the world. Cross-sectional analysis among USA adults aged between 20-49 years old and Queensland adults aged between 18- >59 showed a prevalence of CA-MRSA nasal colonization of 0.8% and 0.7%, respectively [10, 11]. In Europe, studies from Ireland, Malta, and Greece estimated that the prevalence of CA-MRSA ranged between 0.7%-5.2% among adults aged between 16-60 years old [12-14]. In addition, a longitudinal study conducted among the German general population (aged between 7-97 years old) also showed very low (0.7%) rates of MRSA [15]. Furthermore, colonization rates among senior adults aged ≥65 years old living in Germany and Brazil were similar to the ones described for younger adults, 0.7% and 3.7%, respectively [16, 17].

In Portugal, although several studies have been conducted in the nosocomial setting, less is known about the epidemiology of MRSA in the community. Previous screenings of MRSA among young adults, such as draftees (aged 17-22 years old), non-medical university students (aged 21-24 years old) and high-school students (aged 13-16 years old), and among the elderly reported a very low prevalence of MRSA carriage, 0.7% and 1.8%, respectively [18, 19]. In addition, a carriage study conducted in children up to 6 years old attending day-care centers, also showed a very low (0.2%) prevalence of MRSA in the nasopharynx [20].

Regular surveillance studies are needed to monitor and prevent dissemination of potential pathogens – such as MRSA – and adapt strategies to prevent infections. To our best knowledge, in Portugal, MRSA colonization studies among immunocompetent healthy adults have not been performed before, and it is unknown whether this age group may constitute a reservoir of MRSA in the community.

The aim of this study was to evaluate the prevalence of asymptomatic colonization of *S. aureus* and MRSA in the community among immunocompetent healthy adults aged between 25-50 years old, living in Portugal.

Material and Methods

In order to conduct this work, we took advantage of a longitudinal study that our group conducted previously, among adults aged between 25-50 years old. The details of the study design have been described in Chapter II. Briefly, between February 2015 and December 2016, 87 adults aged 25-50 years old, living in the Lisbon area, were followed for 6 months. For each participant nasopharyngeal, oropharyngeal and saliva samples were obtained monthly. In some cases, individuals were sampled weekly. For the purpose of this study, we focused on sampling time-points in which the three types of samples were obtained. Overall, 1,578 samples (526 nasopharyngeal samples, 526 oropharyngeal samples and 526 saliva samples) were analyzed.

Nasopharyngeal samples were collected using a flexible swab with a flocked nylon fiber tip (reference 482CE from Copan) and oropharyngeal samples were collected with a rigid swab with a flocked nylon fiber tip (reference 480CE from Copan) as described in Chapter II. Saliva was collected by spitting into a tube. 50µl of each sample were plated onto mannitol salt agar (Difco, Detroit, MI) and incubated in aerobic conditions, overnight at 37ºC. On the following day, one mannitol-positive colony was streaked onto tryptic soy agar (Difco) and incubated overnight at 37°C. All presumptive *S. aureus* cultures were tested for coagulase production using the latex agglutination test Staphaurex (Remel, Lenexa, KS).

Samples considered to be *S. aureus* positive were tested for cefoxitin susceptibility using agar disk diffusion, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [21]. *S. aureus* isolates displaying an inhibition zone against cefoxitin ≤21 mm were considered to be putative MRSA.

Persistent *S. aureus* carriers were defined as individuals with at least three consecutive positive monthly samples. Intermittent carriers were defined as individuals carrying *S. aureus* with less than three consecutive monthly positive samples. Non-carriers were defined as individuals from which *S. aureus* was never recovered.

To compare the prevalence of *S. aureus* between nasopharyngeal, oropharyngeal and saliva samples the McNemar's test was used. A p-value of <0.05 was considered statistically significant. All statistical analysis was performed using R version 3.6.2 [22].

Results

A total of 87 adults between the ages of 25-50 years old, living in the Lisbon region, participated in this study. The characteristics of the population are described in Table 1. Briefly, the mean age of the participants was 37.1 ± 6.4 years and 49.4% were female. More than half of the participants (57.5%) lived with children under 18 years old, while few participants (6.9%) lived with adults aged ≥65 years old. A total of 43.7% of the participants were smokers and 51.7% were exposed to smoke. Hospitalization within the six months preceding enrollment was low with 2.3% of the participants reporting previous hospitalization (Table 1).

During the six months of the study, from the estimated 522 (87x6) monthly sample time-points, 455 (87.2%) were obtained. In addition, there were 145 weekly sampling time-points that occurred given the original study design, aiming to closely monitor the dynamics of carriage of *Streptococcus pneumoniae*. Overall, there were a total of 600 sampling time-points. Of these, in 526 the three types of samples (nasopharynx, oropharynx and saliva) were obtained, yielding 1,578 samples (526 each), all of which were screened for the presence of *S. aureus* and MRSA.

Prevalence of *S. aureus* by sampling site is summarized in Table 2. Among the 1,578 samples screened, 275 samples (17.4%) were positive for *S. aureus*. There were no significant differences in the detection of *S. aureus* when comparing the nasopharynx with the oropharynx: 20.5% *vs.* 18.3% (p=0.306), respectively. In contrast, *S. aureus* was more frequently detected in nasopharyngeal samples or oropharyngeal samples than in saliva samples: 20.5% *vs.* 13.5% (p<0.001) and 18.3% *vs.* 13.5% (p=0.01), respectively. Of note, 17.2% (n=15) of the participants carried *S. aureus* in the three sampling sites simultaneously, at least once.

Table 1. Socio-demographic characteristics of the participants.

Variable		Participants (total=87)
Mean age (years)		37.1 ± 6.4
	<40 years old	40.2% (52)
	≥40 years old	59.8% (35)
Gender		
	female	49.4% (43)
	male	50.6% (44)
Body mass index (kg/m^2)[a]		
	normal weight	60.9% (53)
	underweight	2.3% (2)
	overweight	36.8% (32)
Household size		
	≤2	58.6% (51)
	>2	41.4% (36)
Living with adults ≥65 years		6.9% (6)
Living with children (≤ 18 years)		57.5% (50)
Smoker		43.7% (38)
No. of years as smoker		
	≤15	19.5% (17)
	>15	24.1% (21)
No. of cigarettes per day		
	≤15	25.3% (22)
	>15	29.9% (26)
Smoke exposure[b]		51.7% (45)
Chronic diseases[c]		26.4% (23)
Long term medication[d]		23.0% (20)
Seasonal flu vaccination		6.9% (6)
Pneumococcal vaccination		8.0% (7)
Pneumococcal vaccination with PCV13		6.9% (6)
Pneumococcal vaccination with PPV23		1.1% (1)
At enrollment		
Antibiotic consumption within the 6 months preceding enrollment		19.5% (17)
Hospitalization within the 6 months preceding enrollment		2.3% (2)
Disease within the 6 months preceding enrollment[e]		9.2% (8)
Antibiotic consumption at least once during the 6-month follow-up		24.1% (21)

[a]Body mass index calculated as weight/height^2 and classified according to WHO as underweight if BMI<18.5, normal weight if 18.5≤BMI≤24.9, and overweight if BMI≥25; [b]at home (n=8), at the working place (n=20), by a partner who smoke (n=23), independently of being a smoker; [c]sinusitis (n=10), asthma (n=3), allergic rhinitis (n=2), heart diseases (n=2), bronchiectasis, hypertension, hypothyroidism, obesity, neurological diseases and psoriasis (n=1 each); [d]oral contraceptives (n=12), α-blockers for hypertension (n=4), antihistamines (n=1) and medication for hypothyroidism (n=1), venous insufficiency (n=1), asthma (n=1) and psychiatric disorders (n=1); [e]respiratory infections (n=4), gynecologic disorders, cutaneous infection, urinary tract infection and blunt trauma (n=1 each).

Table 2. Detection of *S. aureus* according to the sampling site.

Sampling site	No. of isolates (%)
Nasopharynx (n=526)	108 (20.5%)
Oropharynx (n=526)	96 (18.3%)
Saliva (n=526)	71 (13.5%)

Simultaneous sampling of the three sites led to detection of *S. aureus* carriage events in 34.4% of the 526 sampling time-points. Sampling both nasopharynx and oropharynx allowed detection of virtually all positive samples (30.4%) (Figure 1).

Overall, 65.5% (n=57/87) of the participants carried *S. aureus* at least once during the six-month follow-up: 19.5% (n=17) were persistent *S. aureus* carriers and 46.0% (n=40) were intermittent carriers (Figure 2). There were 30 (34.5%) adults for whom *S. aureus* was never detected suggesting they were persistent non-carriers (Figure S1).

Cefoxitin susceptibility test was performed for the 275 *S. aureus* isolates; all were susceptible displaying halos ranging from 23 to 38 mm. More than half (66.5%) of the *S. aureus* isolates displayed halos between ≥28 - ≤32 mm. No MRSA were detected.

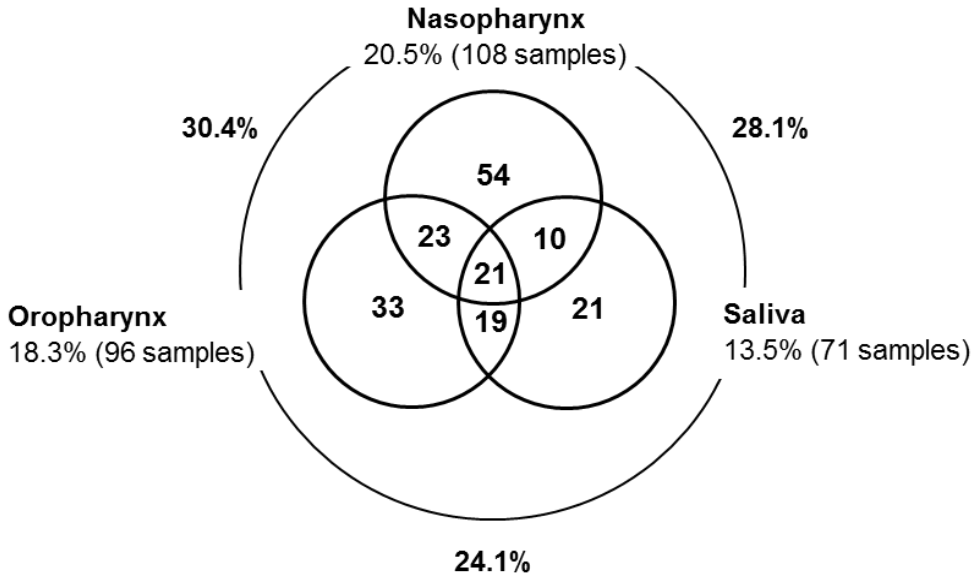

Figure 1. Positive sites for *S. aureus* among the 526 sampling time-points in which three sampling sites (nasopharynx, oropharynx, and saliva) were screened. There were 345 sampling time-points in which the three sampling sites were negative for *S. aureus*. Each circle represents the indicated sampling site. The numbers inside circles indicate the number of positive samples for *S. aureus*. Overlapping areas indicate the number of positive samples in which simultaneously detection of *S. aureus* in more than one sampling site occurred. Percentages of positive samples are indicated, as well as concordance between sampling sites.

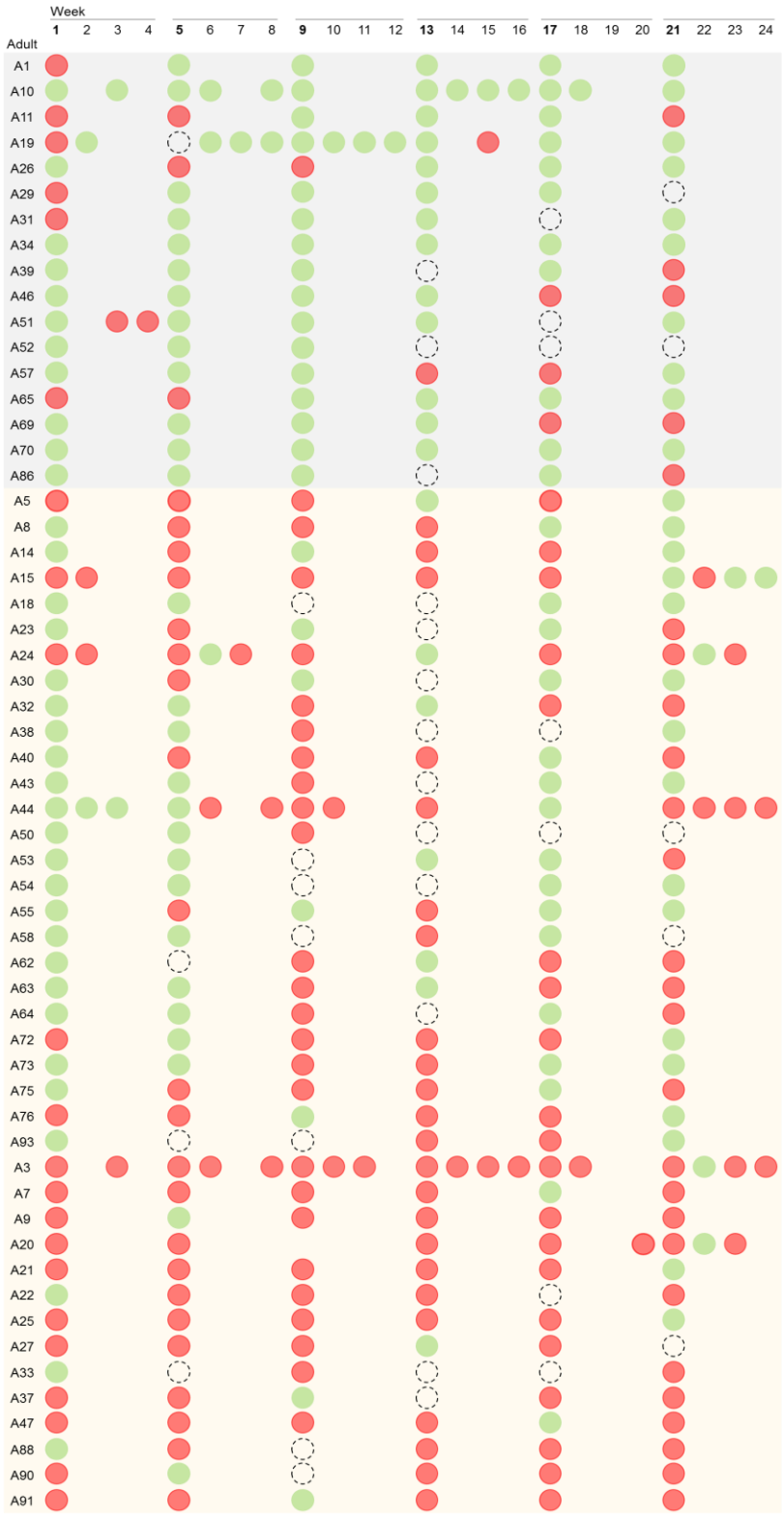

Figure 2. *S. aureus* carriage dynamics of the 57 participants that were colonized at least once during the six months of the study. Red circles represent negative samples; green circles represent positive samples for *S. aureus*; dotted circles represent expected samples (as per protocol) that were not obtained. The grey light area indicates persistent carriers; the orange light area indicates intermittent carriers.

Discussion

We evaluated the prevalence of *S. aureus* and MRSA colonization among immunocompetent healthy Portuguese adults aged between 25-50 years old. We observed that c.a. two thirds (65.5%) of the individuals carried *S. aureus* at least once during the six months: 20% were persistent carriers and 46% were intermittent carriers. Our results, although not directly comparable, are in line with recent studies from other countries conducted among the general population. In a study conducted in Mexico, throat swabs were collected annually during six years from individuals aged 17-66 years old. The authors observed that 85.5% of the population carried *S. aureus* [23]. In a study conducted in Germany, nasal swabs were collected thrice in intervals of 6-8 months from individuals 7-97 years old. The proportion of *S. aureus* carriers was 40.9% [15, 24]. Collectively, these and other studies (for a detailed review see [25]) indicate that a significant proportion of the general population is regularly colonized with *S. aureus*.

Although we screened a substantial number of samples from three sampling sites (526 samples for each), we did not find any MRSA carrier.

In Portugal, over the years, the study of MRSA in the community has spanned different groups of the population (summarized in Table 3 and Figure 3). In Portuguese children, previous findings reported very low rates of MRSA carriage, c.a. 0.2% [18, 20]. In the 1990's a study of adolescents and young adults, namely, high-school students aged 13-16 years old, nonmedical university students aged 21-24 years old, and draftees aged 17-22 years old, also reported very low MRSA colonization, <1% [18]. Two other studies, focusing on adults over 60 years of age estimated MRSA carriage rates as <2% among individuals living in their family homes and 5%-8% among individuals living in nursing homes [19]. Taken together, these results suggest that, although Portugal has a high prevalence of nosocomial MRSA, the prevalence of MRSA in the community is low among the healthy population without known risk factors.

Our study has several limitations. First, we did not obtain samples from the anterior nares, which have been traditionally considered the preferential site of *S. aureus* colonization. However, we did obtain samples from different sites, in line with current recommendations to increase the detection of *S. aureus* and MRSA carriage [25-29]. Our study supports this recommendation as the combined use of three sampling sites led to the detection of *S. aureus* in 34.4% of the sampling time-points, while the use of a single sampling site would have detected *S. aureus* in a maximum of 20.5% of the

samples (Figure 1). Secondly, we did not use an enrichment step, which some have found to increase detection of *S. aureus* and MRSA [12, 26]. However, this limitation was, in part, overcome by the collection of multiple samples from the same individual, which resulted in the detection of *S. aureus* in several individuals at several time-points, as described above and in line with findings from other studies. A third potential limitation of our study was the fact that typically a single colony with the characteristic properties of *S. aureus* was isolated and studied to evaluate whether the sample contained MRSA (evaluated through susceptibility to cefoxitin). In samples containing more than one strain of *S. aureus* this strategy would likely lead to the isolation of only the dominant strain. It is not impossible that MRSA present at a lower density might have been missed. Still, we consider this unlikely to have occurred with a frequency high enough to potentially change our main conclusions. Indeed, we have recently carried out a study among elderly adults where selective enrichment of samples followed by real-time PCR (qPCR) were used to increase the capacity to detect MRSA, and we found evidence that MRSA were rare and tended to be present as dominant population (Chapter IV).

In conclusion, this study adds novel information about the MRSA scenario in the Portuguese community. Our results support that, in Portugal, MRSA does not seem to circulate among healthy adults without risk factors and therefore this age group does not constitute, at the current time, a reservoir of MRSA in the community.

Table 3: Summary of studies on MRSA prevalence performed among different populations of different ages groups since 1993.

Study period	Study design	Population	No. of participants	Sampling site	No. of samples	Method	*S. aureus* carriage %	MRSA carriage %	Reference
1993-2000	cross-sectional	draftees; non-medical university students; high-school students; children attending day care centers	3525	anterior nares (nasopharynx for children)	3525	selective culture-based methods	28.4%	0.7%	[18]
2006, 2007, 2009	cross-sectional	children attending day care centers	2100	nasopharynx	2100	selective culture-based methods	17.4%	0.14%	[20]
2010-2012	cross-sectional	adults ≥ 60 years	3361	nasopharynx and oropharynx	6722	selective culture-based methods	20.1%	1.8%	[19]
2010-2012	cross-sectional	adults ≥ 60 years living in either nursing homes or family homes	599	nasopharynx and oropharynx	1198	selective enrichment and qPCR	ND[a]	8.0% (nursing home); 1.7% (family home)	chapter IV
2015-2016	longitudinal	adults 25-50 years	87	nasopharynx, oropharynx and saliva	1578	selective culture-based methods	65.5%[b]	0.0%	this study

[a]nd, not determined; [b]cumulative carriage over six months.

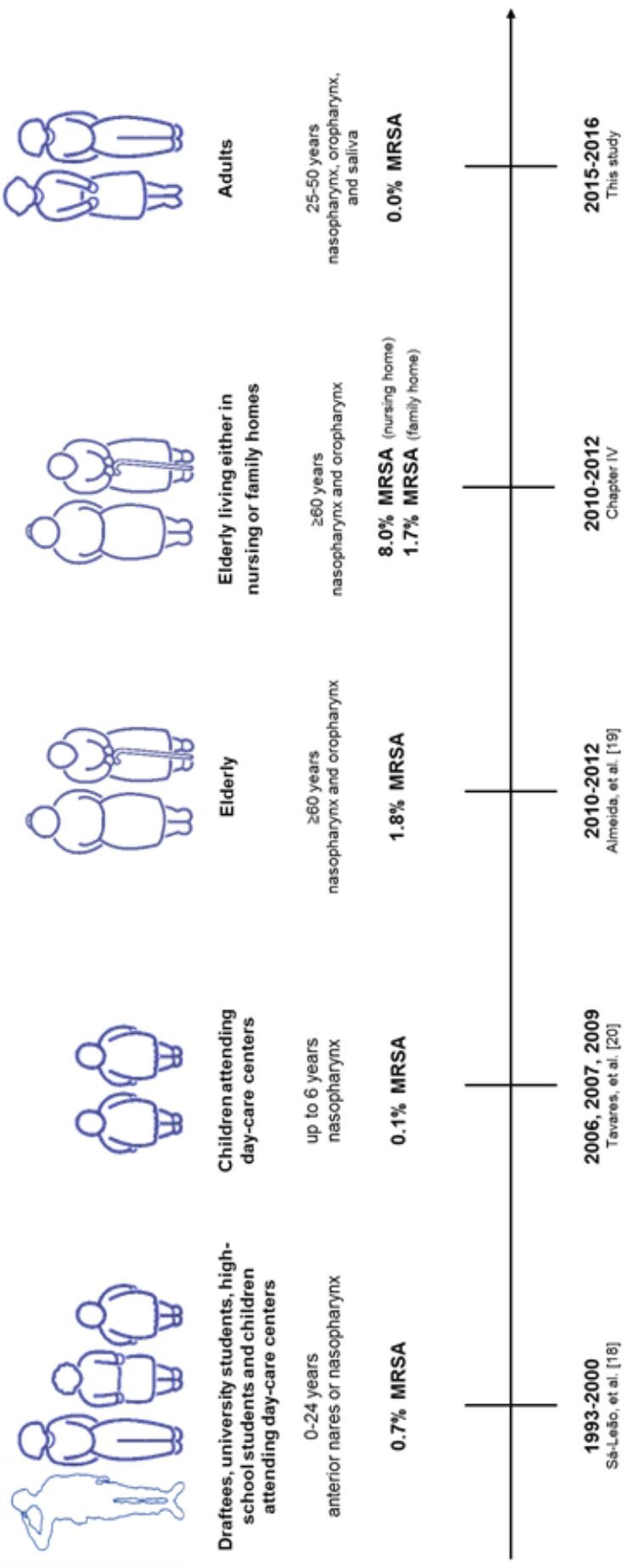

Figure 3. MRSA prevalence in the community in Portugal. Summary of studies performed among different populations of different age groups since 1993. For each study the age groups, sampling site and MRSA prevalence are indicated. See also Table 3.

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Supplementary material

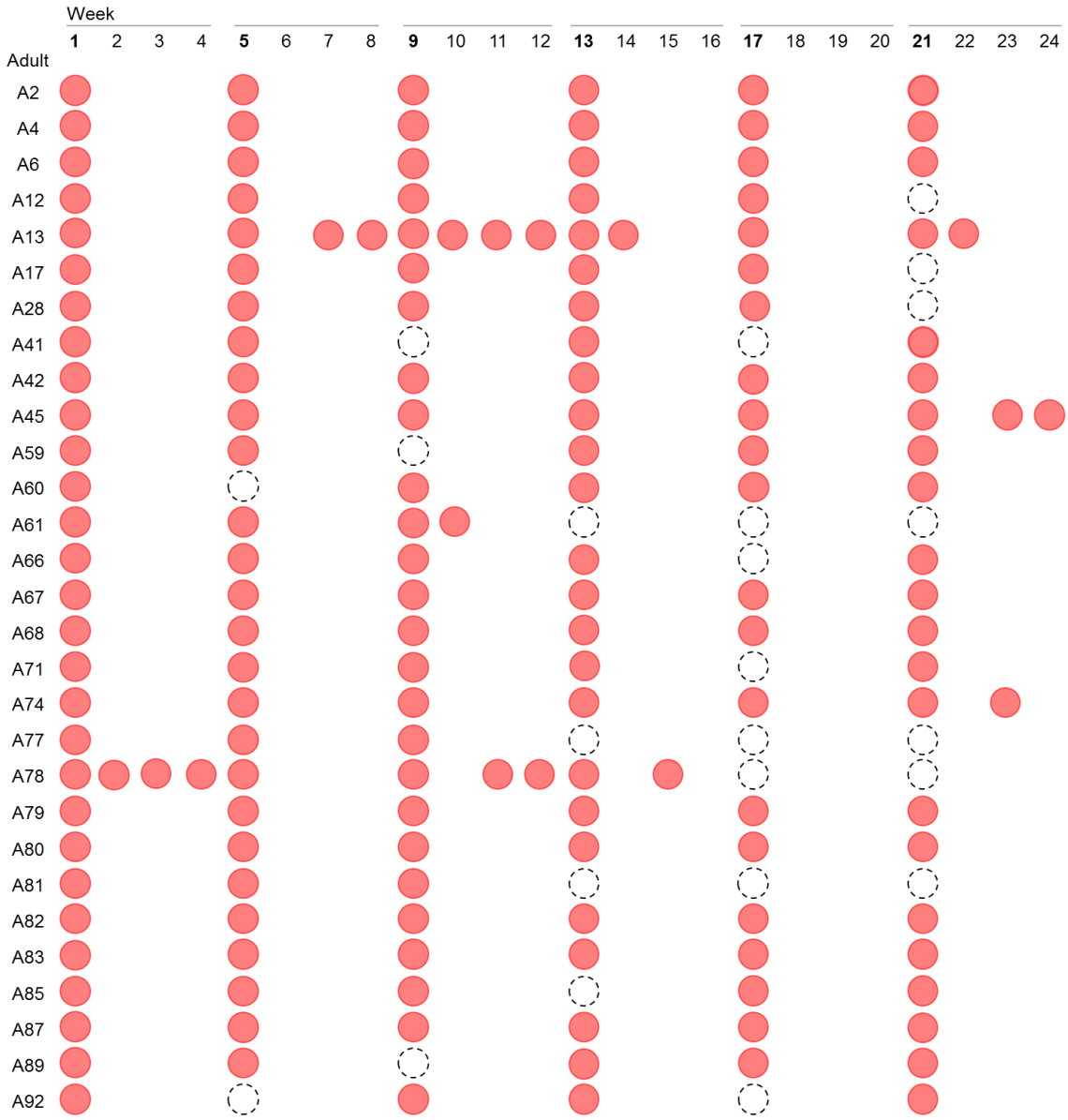
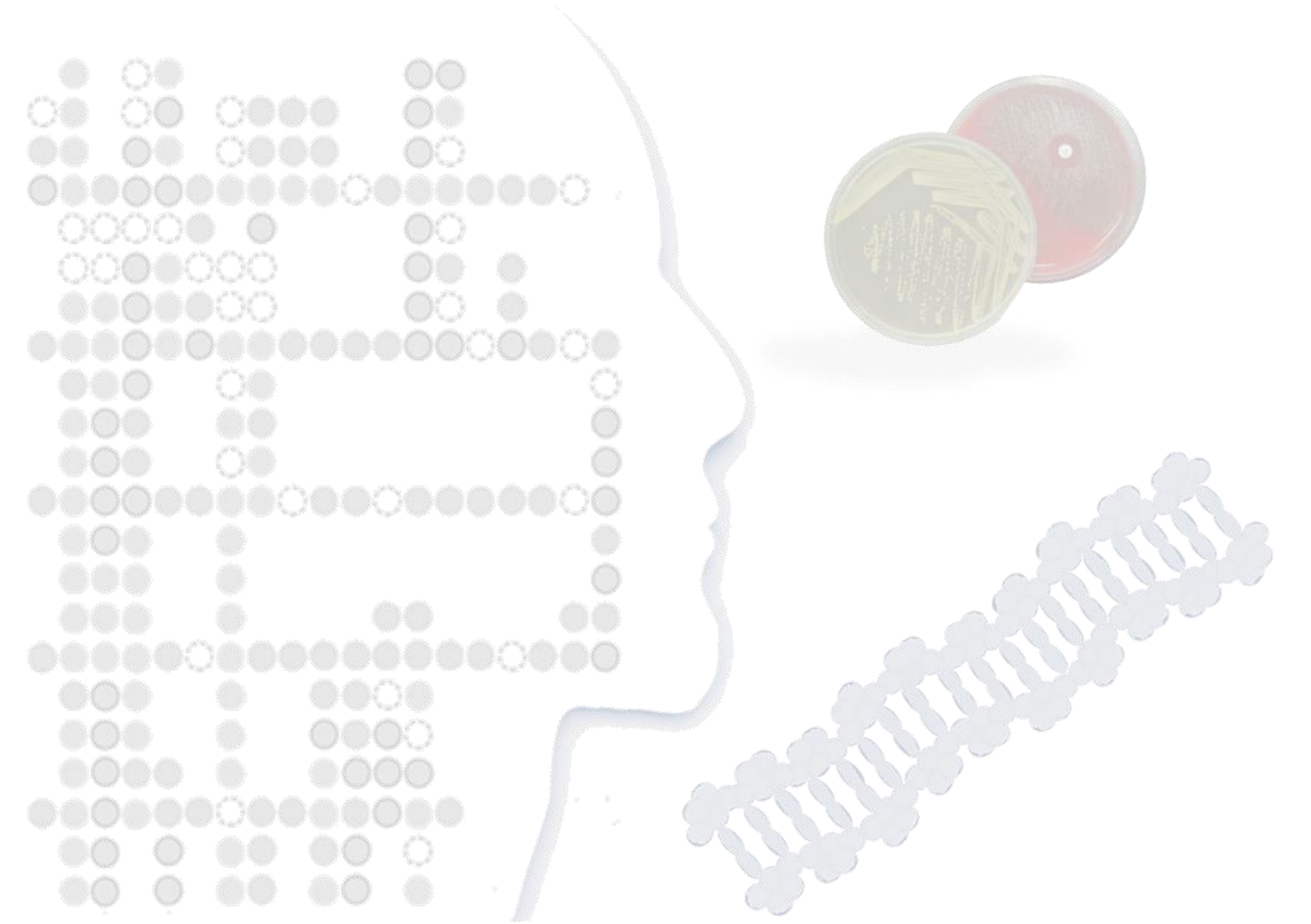


Figure S1. Representation of the 30 out of 87 participants that were never colonized with *S. aureus* during the six months of the study (non-carriers). Red circles represent negative samples; dotted circles represent expected samples (as per protocol) that were not obtained.

Chapter VI

Concluding remarks



Concluding remarks

The human upper respiratory tract is frequently colonized asymptomatically by *S. pneumoniae* and *S. aureus* both of which are important causes of morbidity and mortality worldwide [1].

Colonization is the first step for the infection process and children are frequently colonized with these pathobionts and are regarded as the main reservoir for bacterial transmission to others in the community [2].

Several studies have been conducted worldwide on the subject of pneumococcal colonization, and the focus of most of them were young children, given the high carriage prevalence of over 50% in this age group [3-5]. In fact, the study of pneumococcal carriage in healthy individuals goes back to more than a century ago.

Early studies on pneumococcal colonization among healthy adults reported carriage rates between 45%-60% in oral samples (saliva, throat swabs or throat washes) inoculated in mice susceptible to pneumococcal infection (reviewed in [6]). Since then, nasopharynx became the preferential sampling site to detect pneumococcal carriage. This was mostly because of the highly polymicrobial nature of the oral samples, which makes detection of pneumococcus by WHO recommended classical culture-based methods, almost impossible. Thus, the use of nasopharyngeal samples became a worldwide accepted method to detect pneumococcal carriage, and the early findings were mostly forgotten.

In contemporary studies, based on pneumococcal detection using classical culture-based methods the prevalence of colonization and density in the adult host is significantly lower than in young children, being routinely estimated as less than 5% [7-9]. With these low carriage rates, surveillance studies in adults have been neglected and therefore there is limited information regarding colonization in adults and the elderly.

Over the years, with advances in technology, novel non-culture DNA-based methods have emerged. Its development took a long time, as it was essential to have ubiquitous pneumococcal targets, not present in other close relatives in order to prevent the existence of non-specific or misleading results. In addition, the fieldwork on colonization studies in the community, among the adult host, is difficult to implement, and enrollment of a large number of participants to have meaningful results may be challenging.

Recent surveillance studies have shown that in children both methods (culture and DNA-based) often give comparable results for pneumococcal carriage rates [10, 11] while in adults, the use of highly sensitive non-culture methods is expected to increase colonization rates. In fact, studies using highly sensitive molecular methods to test samples obtained from nasopharynx, oropharynx and saliva showed that adult pneumococcal carriage is more frequent than estimated previously [12-15]. However, still today, these molecular methods are not yet widely used for pneumococcal detection.

In the early 2000s, the introduction of multivalent conjugate vaccines into clinical practice led to a massive shift in the colonizing pneumococcal flora [16-18]. Almost at the same time, MRSA infections were no longer confined to nosocomial settings and started to emerge among individuals with no health-care associated risk factors [19].

The prevalence of MRSA in hospitals in Portugal is worrisome and among the highest in Europe [20]. National surveillance studies have been conducted for almost 30 years in order to follow the prevalence of hospital-associated MRSA (HA-MRSA) over time [21, 22]. In contrast, less is known about the epidemiology of MRSA in the community. Previous screenings among adults showed that MRSA circulation in the community was low, being less than 2% [23, 24].

In MRSA carriage studies the gold standard are culture-based approaches. The few studies available that have used qPCR-based methods to investigate MRSA carriage have shown that these methods yield comparable results to those obtained by using classical culture-based methods [25, 26]. It is unclear if the low prevalence of MRSA observed among individuals in the community in Portugal, might be due to an underestimation of carriage due to the use of low sensitivity classical culture-based approaches, and whether the adult population may constitute a reservoir of MRSA in the community.

The work developed under the scope of this thesis enabled us to gain further insights into bacterial colonization prevalence, density and dynamics in the upper respiratory tract of the healthy adult host. Moreover, by combining epidemiological and molecular approaches, the usefulness of molecular methods, such as real-time PCR for *S. pneumoniae* and *S. aureus* carriage detection were evaluated.

In **Chapters II** and **III** we studied *S. pneumoniae* colonization in the healthy adult host: adults aged between 25-50 years old and adults aged ≥60 years old.

In the study described in **Chapter II** a 6-month longitudinal study was conducted among immunocompetent healthy adults aged between 25-50 years old. Here, we combined intensive sampling of multiple sites (nasopharynx, oropharynx and saliva) and sensitive detection of pneumococci (real-time PCR and classical culture-based methods) to study the dynamics of pneumococcal carriage. We demonstrated that pneumococcal colonization can be frequent, when highly sensitive molecular methods were used, as we estimated pneumococcal acquisition as 16.5 cases per 1000 persons-week. This rate was comparable to previous estimates [12, 13, 27-29]. We have also observed that the median duration of carriage was seven weeks. These results challenge the paradigm of pneumococcal colonization dynamics among adults, since our observations suggest that pneumococcal acquisition is frequent, and the duration of carriage is higher than previous estimated, often lasting for several months, with some individuals appearing to be persistently colonized with pneumococci. Previous studies reported lower median duration of carriage than our estimate. For example, a 10-month longitudinal study, conducted in England, between 2001-2002, among children ≤5 years old and their families showed a median duration of carriage of 19 days for older family members (>5 years old) [28]. In addition, studies from non-Western countries reported a median carriage duration of four weeks in Myanmar camp and 2-4 weeks in adults from Gambia [29, 30]. Together, this study suggest that the adult carrier state is more dynamic than generally assumed.

Following the results presented in Chapter II, in **Chapter III** we evaluated the prevalence of pneumococcal colonization in senior adults using qPCR and compared the results with those obtained previously by classical culture-based approaches [7]. We further characterized pneumococcal positive samples by serotyping and compared serotype distribution and diversity. In this study we used nasopharyngeal and oropharyngeal paired samples of individuals over 60 years old living in nursing or family homes. Here, we were able to show that pneumococcal colonization in this age group is significantly higher (c.a.10%) than the one estimated by classical culture-based methods alone. The use of qPCR was particularly relevant to detect pneumococci when present at low density, as it is often the case for oropharyngeal samples. Our study supports the recommendation that to detect pneumococci in senior adults it is important to combine both nasopharyngeal and oropharyngeal samples and that qPCR should be used when analyzing oropharyngeal samples [13, 15, 31]. In addition, the use of molecular serotyping of qPCR pneumococcal positive samples enabled the detection of an expanded pool of serotypes detected in this population, that otherwise, would remain undetected. These results may have important

implications since that, as the number and proportion of aged individuals increases in societies worldwide, accurate estimates of pneumococcal carriage prevalence and circulating serotypes are crucial to understand progression to disease and impact of pneumococcal vaccines in this age group.

In **Chapters IV** and **V,** we explored the prevalence of MRSA in the adult host, using the same collection of samples studied in Chapters II and III.

In **Chapter IV** we used a real-time PCR targeting *nuc*, *mecA* and *mecC* genes, preceded by a semi-selective enrichment step to evaluate the carriage of MRSA in adults ≥60 years old. Here we analyzed the same collection of samples described in Chapter III. This study showed that although the use of a semi-selective enrichment step combined with qPCR increased sensitivity of MRSA carriage detection, it did not change the overall scenario previously described in Portugal: MRSA circulation in the community among senior adults remains low. Our carriage estimates are similar to the ones described in studies from Brazil, Malta and the USA that estimated a prevalence ranging between 2%-8% among senior adults living in the community [32-34]. We were also able to isolate pure MRSA cultures in almost all samples, which allowed an extension of previous findings indicating that colonizing MRSA strains were associated to clones typically described as HA-MRSA. This scenario seems to be relatively common not only in Portugal [24, 35, 36], but also in other European countries [37-39].

Still in the context of evaluating the prevalence of MRSA in the Portuguese community, in **Chapter V,** a 6-month follow-up was conducted among immunocompetent healthy adults aged between 25-50 years old. Using classical culture-based methods we showed that 65.5% of the participants carried *S. aureus* at least once, and none of the participants carried MRSA. Our data indicates that in Portugal, despite the high prevalence of MRSA in hospitals, its prevalence in the community is low among healthy adults without known risk factors. This low prevalence is in line with past studies conducted among healthy Portuguese populations that showed a carriage rate lower than 2% [23, 24, 40].

Altogether, the work developed under the scope of this thesis contributed with important knowledge on the epidemiology of two remarkable pathobionts, *S. pneumoniae* and *S. aureus*, and about the molecular-based identification methods. Nevertheless, several questions related to the work developed in this thesis are still unanswered and could be the basis of future investigation.

First, the work developed in this thesis highlighted important considerations for further surveillance studies on *S. pneumoniae* colonization, not only in children, but also in the adult host, since our results suggest that pneumococcal carriage in this latter age group is higher than previously estimated by classical culture-based methods only. An inaccurate understanding of pneumococcal distribution in the population may limit our understanding of the impact of vaccination strategies.

In Portugal, several studies aiming to study the impact of pneumococcal vaccines in invasive disease among adults have been conducted. These studies have shown a decrease in PCV7 serotypes in adult invasive disease after the introduction of PCV7, followed by an increase of non-PCV7 serotypes 1, 7F and 19A [41]. Nevertheless, PCV7-serotypes persisted as a cause of invasive disease in adults, accounting for c.a. 15% of the cases [41], and as a cause of c.a. 20% of the cases of invasive pneumococcal disease in children [42]. Following the introduction of PCV13, there was an additional decrease in PCV7 serotypes. The PCV13-only serotypes, particularly serotypes 7F and 19A, declined among cases of pneumococcal invasive disease occurring in children [43]. The same declines were observed in adult invasive disease suggesting a herd immunity effect. Concomitantly, an increase in non-PCV13 serotypes, namely in serotypes 8, 15A, 20 and 22F was observed. Still, PCV13 serotypes remain responsible for a significant proportion (38%) of invasive disease in adults [44]. In children, the most important non-vaccine serotypes causing invasive disease in the PCV13 era were 15B/C, 10A, 12B and 24F [45]. Taken together, these results suggest that, in Portugal, vaccination of children only may not be enough to achieve an effective protection among adults against invasive pneumococcal disease. These studies, together with those presented in this thesis, point out important considerations for future studies on pneumococcal reservoirs in adults in order to understand if they may represent a source of pneumococci independent from the reservoir in children, and thus act as a potential vector for transmission. Moreover, if some adults are reservoirs of pneumococci, it will be important to gain insights on how transmission between adult to adult or adult to children occurs.

Recently, a cross-sectional study performed by Wyllie and colleagues, among parents of 24-month-old children and childless adults showed that pneumococci were virtually absent in this latter group, in contrast to parents, suggesting that children are the true reservoir of the circulating pneumococcal strains [12]. Our study described in Chapter II also found that living with children increased the likelihood of being a pneumococcal carrier. Still, whether these adults may act as hubs of dissemination to other adults and even to their own children is not known and could be addressed in future studies.

A second question that remains unanswered is the usefulness of saliva for pneumococcal carriage detection. In contrast to our findings that suggest that sampling saliva of the adult host does not increase significantly the detection of *S. pneumoniae,* studies conducted among Dutch aged adults (≥60 years old) and parents of 24-month-old children argue the superiority of saliva samples over nasopharyngeal and oropharyngeal samples, and thus propose that saliva should be considered for future carriage studies [12, 15]. Indeed, studies from the early 1900s reported saliva as the most sensitive sampling site to detect pneumococcal colonization in adults. Based on these studies, it was estimated that between 45%-60% of all adults carried pneumococcus in saliva (reviewed in [6]). Differences in the methodology used to collect saliva samples, vaccination, or even intrinsic differences of the population, such as diet, genetic differences, among others, may underlie the differences found between our study and those conducted in the Netherlands. To address these questions, the first key aspect that should be taken into consideration should be the implementation of a standard detailed protocol that would include all steps on how to collect and to process saliva samples. This protocol should then be disseminated within a consortium of groups to promote saliva testing by researchers in different settings and countries. A study among adult population of different countries aiming to understand the value of saliva to study pneumococcal colonization should be conducted. If proven that the use of saliva increases the detection of pneumococcal carriage, its use may be advantageous as it is likely that it will be easier to enroll volunteers in community studies. Saliva sampling is easy and minimally invasive when compared to nasopharyngeal or oropharyngeal sampling. Nevertheless, it is also important to keep in mind that sampling only saliva may have disadvantages, as it is nearly impossible to isolate pure pneumococcal cultures, which are important for detailed characterization of a given strain. In addition, an increase of non-specific or misleading results may occur because of the highly polymicrobial nature of saliva.

Several studies that addressed the colonizing bacterial pathogens that inhabits the human upper respiratory tract (including those described in this thesis) have been mainly focused on elucidating the biology aspects of a single species. However, colonization of the upper respiratory tract is not exclusive of one bacterial species. In fact, colonization is affected by several factors, including not only interactions with the host, but also, interactions with other bacterial species inhabiting the same niche. For example, epidemiological studies have shown an antagonistic association between carriage of *S. pneumoniae* and *S. aureus*, with carriers of pneumococcus being less prone to carry *S. aureus* (reviewed in [46]).

With the introduction of species-specific vaccines, the competition for the niche may become more pronounced and may disturb the stability of the ecosystem in unanticipated ways, which may promote either health or disease. Thus, it is essential to understand the upper respiratory tract microbial interactions. Future studies might address how the composition of the upper respiratory tract microbiome affect pneumococcal colonization and vice-versa. Microbiota studies have demonstrated high variability in the composition of the microbial community across individuals, which may be affected by factors such as age and seasonality, among others [47, 48]. The nasopharynx of young children is commonly colonized by members of phyla Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes, being the latter two less abundant. During puberty there is a shift that is maintained in healthy adults ≤65 years old and phyla Firmicutes and Actinobacteria, in particular the genera *Corynebacterium and Propionibacterium,* become the most predominant ones (reviewed in [49]). It has also been established that the presence of the commensals *Corynebacterium* spp. and *Dolosigranulum* spp. are associated to a healthy microbiota and its stability (reviewed in [46]). In addition, a cross-sectional 16S rRNA-based microbiome study has shown an association between pneumococcal colonization and specific bacterial genera. Given the cross-sectional design, it was not possible to study the causal relationships between the upper respiratory tract microbiota and pneumococcal carriage [50]. Longitudinal data on these interactions in healthy adults are scarce. Cremers and colleagues employed an experimental human pneumococcal carriage model to study associations between pneumococcal carriage and the nasopharyngeal microbiota, suggesting that pneumococcal acquisition may promote a more diverse microbiome [51]. Understanding microbiota composition and structure is also very important to determine the risk of developing invasive disease. In fact, a recent study showed that children with invasive pneumococcal disease had a higher bacterial diversity and richness when compared to healthy children. Furthermore, the authors found a different microbiota profile associated either with invasive disease or asymptomatic colonization. The microbiota of children with invasive disease was mainly associated with the presence of *Streptococcus*, while the microbiota of healthy children was mainly associated with the presence of *Dolosigranulum* [52].

Overall, these studies suggest that interactions in the context of a polymicrobial environment cause alterations in the upper respiratory tract microbiome and should not be disregarded. Thus, taking advantage of our longitudinal collection of adult samples, a future study could aim to investigate the dynamics of the upper respiratory tract microbiome in the presence and absence of naturally acquisition of *S. pneumoniae*.

With the use of molecular methods, surveillance studies have suffered enormous changes. In addition, in recent years, with the increasing use of whole genome sequencing (WGS) these changes became even more pronounced. The use of WGS has enabled a higher resolution and a deeper detail about genes of interest that may play a role in antimicrobial susceptibility, virulence, or adaptation to the host [53-55]. Recently, by combining epidemiological modelling of longitudinal data with WGS data, it has been demonstrated the contribution of *S. pneumoniae* genome in carriage duration. The authors estimated that sequence variation in the pneumococcal genome explained most (63%) of the variability observed in carriage duration, while age of the host and previous carriage accounted for less than 5% of the variability [56]. Following these observations, it would be very interesting to characterize our longitudinal collection by WGS to investigate the within-host genetic changes occurring during pneumococcal colonization of adult hosts. This genomic investigation might increase our understanding, not only of the bacterial factors leading to successful colonization, but also the role of colonization in disease transmission and the spread of genetic traits in the pneumococcal population.

Overall, the studies presented in this thesis give a significant contribution to the scarce knowledge on bacterial colonization prevalence, density and dynamics in the upper respiratory tract of the adult host. We showed that, in Portugal, pneumococcal carriage is frequent among the adult host (both senior and younger adults). In particular, for healthy immunocompetent adults, our studies point out that duration of pneumococcal carriage can be long, lasting for several months. This thesis has also confirmed that MRSA circulating in the community in Portugal among adults is low and, when occurring, is associated with MRSA clones typically found in hospitals. As a final point, we provide evidence supporting the value of highly sensitive molecular methods for carriage detection in the adult host, particularly in the detection of pneumococcal carriage and in expanding considerably the pool of serotypes detected in this population.

The results obtained in this thesis have allowed a better understanding of *S. pneumoniae* and *S. aureus* colonization dynamics among adults in Portugal. These findings should be useful to improve national strategies aiming to prevent infections caused by these two very important pathobionts.

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