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The asymptomatic carriage of four potentially pathogen respiratory tract bacteria in children attending communities

PhD thesis

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Abbreviations

AIDS: acquired immune deficiency syndrome

AOM: acute otitis media

ATCC: American Type Culture Collection

BLNAR: β-lactamase-non-producing ampicillin resistance

BLPAR: β-lactamase positive ampicillin resistance

BLPACR: β-lactamase positive amoxicillin/clavulanic acid resistance

CA-MRSA: community-acquired methicillin resistant *S. aureus*

CAP: community acquired pneumonia

CDC: Centers for Disease Control and Prevention

COPD: Chronic obstructive pulmonary disease

DCC: day-care center

ECDC: European Centre for Disease prevention and Control

EUCAST: European Committee on Antimicrobial Susceptibility Testing

EU/EEA: European Union/European Economic Area

Hap: *Haemophilus* adhesion protein

HA-MRSA: hospital-associated methicillin resistant *S. aureus*

Hia: *H. influenzae* adhesion

Hib: *H. influenzae* serotye b

H. influenzae: Haemophilus influenzae

HIV: human immunodeficiency virus

HMW1/HMW2: high molecular weight proteins

ICAM1: intercellular adhesion molecule 1

IgA: immunoglobulin A

IgD: immunoglobulin D

IgG: immunoglobulin G

IPD: invasive pneumococcal disease

LA-MRSA: livestock-associated methicillin resistant *S. aureus*

LOS: lipooligosaccharide

M. catarrhalis: Moraxella catarrhalis

MHC II: major histocompatibility complex class II

MIC: minimum inhibitory concentration

MID/Hag: *M. catarrhalis* immunoglobulin D-binding protein/hemagglutinin

MLST: multilocus sequence typing

MRSA: methicillin resistant *S. aureus*

MSSA: methicillin-sensitive *S. aureus*

NAD: nicotinamide adenine dinucleotide

NT: non-typeable

NTHi: non-typeable *Haemophilus influenzae*

NVT: non-vaccine type

OM: otitis media

ON: overnight

PCho: phosphorylcholine

PCR: polymerase chain reaction

PBP: penicillin binding protein

PCV7: 7-valent pneumococcal conjugate vaccine

PCV13: 13-valent pneumococcal conjugate vaccine

PFGE: Pulsed-field gel electrophoresis

PPV23: 23-valent pneumococcal polysaccharide vaccine

PVL: Panton-Valentine Leukocidin

S. aureus: Staphylococcus aureus

SCCmec: staphylococcal cassette chromosome *mec*

S. pneumoniae: Streptococcus pneumoniae

SSSS: staphylococcal scalded skin syndrome

TEA: Tris-acetate EDTA

TMP/SMX: trimethoprim/sulfamethoxazole

TSS: Toxic shock syndrome

USA: United States of America

Usp: Ubiquitous surface protein

VT: vaccine type

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1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*), *Staphylococcus aureus* (*S. aureus*), *Haemophilus influenzae* (*H. influenzae*) and *Moraxella catarrhalis* (*M. catarrhalis*) are common pathogens found in the respiratory tract. Except for *S. aureus*, they are obligate human pathogens (1-4). Preschool children carry these pathogens often asymptomatically, but occasionally, when their immune system is compromised by a simultaneous viral infection or other enhancing factor, even severe diseases can develop. Disease is most common in young children (< 5 years) and in elderly (> 65 years) who are often contacting with grandchildren (5, 6). The following detailed introduction summarizes the most important characteristics of the four investigated species.

1.1. *S. pneumoniae*

1.1.1. *S. pneumoniae* in general

S. pneumoniae is a Gram-positive, catalase negative coccus, sized 0.8-1.5µm, displayed as diplococci (arranged in pairs) or short chains under the microscope. It has α-hemolytic activity, which means that on blood agar plate a greenish discoloration can be seen around the colonies due to partial lysis of red blood cells (Figure 1). *S. pneumoniae* belongs to the mitis group of streptococci and other mitis group streptococci (*S. mitis*, *S. oralis*, and *S. pseudopneumoniae*) are associated with human diseases, therefore it is crucial to identify them accurately for the proper treatment (3, 7-10). The routine culture-based identification of *S. pneumoniae* involves bile solubility and optochin susceptibility testing (11). However, exceptions were also reported (12, 13).

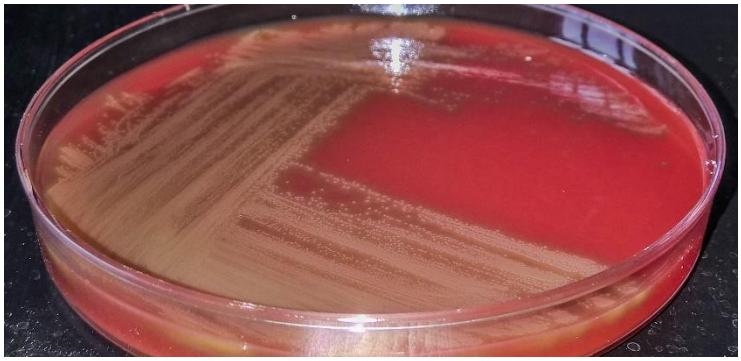

Figure 1. Culture of *S. pneumoniae* on blood agar (Photo by E. Kovács)

Nowadays, a lot of molecular methods can be involved in *S. pneumoniae* identification, like PCR of species-specific target genes (pneumolysin, autolysin, pneumococcal surface antigen A) (14-16), multilocus sequence typing (MLST) or whole genome sequencing (WGS) (17, 18).

1.1.2. Virulence factors

S. pneumoniae, like many other bacterial species, produces toxins that are harmful to its host, has several surface proteins and physical structures, which play a vital role in its pathogenesis (19). Some selected, important virulence factors are listed in Table 1.

The most important virulence factor of *S. pneumoniae* is its polysaccharide capsule, which protects the microbe from phagocytosis. The capsule is strongly antigentic, therefore the antibodies produced by the immune system against it can neutralize the pneumococcus. Unfortunately, more than 95 serotypes are distinguished currently, which challenges vaccine improvement (20).

First, Fred Neufeld described a process to differentiate serotypes with the help of type-specific antisera in 1902. Basically, the capsule will appear to swell due to binding of antibodies which cause a surface tension. This phenomenon can be visualized under a microscope. The method is also called as Quellung reaction (21, 22).

Capsule switching ability further increases its virulence. Mutations in the capsule polysaccharide synthesis genes (*cps*) promote serotype switching. This phenomenon can be observed often under antibiotic or vaccine pressure (23).

Table 1. Selected virulence factors of *S. pneumoniae*, their location, and function (24).

Virulence factor	Location on *S. pneumoniae*	Function
Polysaccharide capsule	Layer of polysaccharides on cell wall	• Allows the bacteria to escape the nasal mucus • Inhibits phagocytosis by innate immune cells • Escapes neutrophil net traps • Inhibits complement and recognition by immunoglobulins • Allows adherence and colonization of the nasopharynx
Pneumolysin	Cytoplasmic toxin	• Binds to membranes with cholesterol • Forms pores which cause cell lysis • Induces inflammation • Drives host-to-host transmission

		• Can activate complement and modulate chemokine and cytokine production
Autolysin (lytic amidase)	Intracellular enzyme produced by Gram-positive bacteria	• Cell lysis • Break down peptidoglycan • Exposes hosts cell to pneumolysin and teichoic acid • Enhances bacterial colonization
Pneumococcal surface protein A	Bound to the cell wall via phosphorylcholine (PCho) moiety	• Protects against complement system of the host • Enhances colonization by adhering to epithelial cell membranes • Decreases the deposition of the complement
Pneumococcal surface protein C, also known as choline-binding protein A (CbpA)	Bound to the cell wall via PCho moiety	• Protects against the complement system of the host • Binds to receptors such as the human polymeric immunoglobulin A (IgA) • during colonization and invasion the nasopharynx • Cell adhesion and colonization of nasopharynx
Pneumococcal surface adhesin A (PsaA)	Surface of the cell wall	• Transports magnesium and zinc into the cytoplasm of the bacteria • Enhances invasion of epithelial cells during nasopharynx colonization
Other choline-binding proteins: LytB, LytC, CbpC, CbpG	Bound to the cell wall via PCho moiety	• Promote bacterial colonization of the nasopharynx • Modify proteins on cell surfaces and allows for binding to host cell receptors • Important for host cell recognition
Non-classical surface proteins	Surface of the cell wall	• Act as adhesins • Promote immune system evasion by inhibiting complement • Controls inflammation and affects cytokine production
Pili	Cell surface	• Promotes adherence and colonization of the epithelial cells within • the nasopharynx • Inhibits phagocytosis by immune cells
Bacteriocin	Produced and secreted by the bacterium	• Inhibits the growth of competing bacterial cells
Neuraminidase	Cell wall bound	• Degrades mucus • Promotes growth and survival • Enhances cell adherence
Biofilm formation capability		• Helps to reduce bacterial recognition by the host immune system • Reduces the impact of antimicrobial agents on bacteria
IgA protease	Secreted by the bacteria into the extracellular environment	• Breaks down IgA during mucosal infections
Lipoteichoic acid	Membrane bound	• Causes inflammation

1.1.3. Diseases caused by *S. pneumoniae*

Pneumococci can cause both life threatening invasive and severe or milder mucosal infections. *S. pneumoniae* causes more than 50% of bacterial meningitis in the USA (25, 26) and it is predominantly seen in young children. Bacteremia refers to bacterial invasion of the blood. By colonizing the middle ear, it can cause otitis media (OM). According to CDC data, approximately 60% of young children would have at least one episode, but at least 40% of children have recurrent forms, thus acute otitis media (AOM) is the number one reason for antibiotic prescription in childhood (26). *S. pneumoniae* can be responsible also for sinusitis and pneumonia. Pneumococcus is the leading etiological agent of community acquired pneumonia (CAP) in the USA and worldwide (26). In Europe, an average of 6.4 invasive pneumococcal disease (IPD) cases per 100000 population occurred in 2018 (27).

1.1.4. Antibiotic resistance

The first penicillin-resistant strains were recorded in the 1970s (28). A significant proportion of *S. pneumoniae* is showing intermediate level resistance to penicillin (0.06 mg/L < MIC ≤ 2 mg/L) but some strains can fall into the resistant category (MIC >2 mg/L). Penicillin resistance is related to structurally modified penicillin-binding proteins of *S pneumoniae.* The modified proteins allow peptidoglycan synthesis despite the presence of penicillin. However, treatment can still be successful with higher dose penicillin and cephalosporins. *S. pneumoniae* can also acquire resistance to erythromycin, tetracycline, fluoroquinolones, trimethoprim/sulfamethoxazole (TMP/SMX). Vancomycin can be added to the therapy if necessary (29).

1.1.5. Vaccination

B cell immunity is important against encapsulated bacteria. B cell responses are T cell dependent mainly, but responses to polysaccharides are T- independent, therefore memory B cells are not developed. As a result, polysaccharide vaccines are poorly immunogenic under two years of age. With conjugation methods, where the polysaccharide antigen is linked with a protein carrier, T cells are involved into the immune response thus polysaccharide-specific memory B cells are generated even in young children (30, 31).

Complement and spleen also has a crucial role in protection against encapsulated bacteria. Spleen facilitates phagocytosis and produces opsonins and components of the complement pathway. Asplenic or hyposplenic individuals (e.g., post-splenectomy, sickle cell disease) are therefore at much higher risk of a serious infection. Therefore, vaccination is highly recommended for individuals with impaired splenic function as well (32).

The currently marketed 23-valent pneumococcal polysaccharide vaccine (PPV23) has been available since 1983. It contains purified polysaccharides from 23 of the currently known more than 95 serotypes (Table 2) (33).

Due to non-immunogenic effect of PPV23 under two years of age, pneumococcal conjugated vaccines (PCVs) were developed (Table 2). First, PCV7 (4, 6B, 9V, 14, 18C, 19F, 23F) was implemented in 2000 in the USA, after conducting a milestone clinical trial in which 89% efficacy against the seven vaccine serotypes was shown (34). The chosen seven serotypes were associated with the majority of invasive diseases in the USA that time and were typically antibiotic resistant (35, 36).

Routine use of PCV7 resulted in a sharp decrease in PCV7 serotypes among invasive diseases, but on the other hand, an increased incidence of non-PCV7 types, especially 19A was observed (37-39). This vaccination-induced selective pressure lead to the need to widen the spectrum of PCVs. Higher valent PCV formulations (PCV10 and PCV13) were intended to target the residual burden of invasive pneumococcal disease, non-invasive infections (pneumonia and otitis media) and infections caused by non-PCV7 serotypes. These higher valency PCV vaccines were first introduced in Germany within Europe (PCV10 in April 2009 and PCV13 in December 2009) and subsequently in other countries (40).

In Hungary, PCV7 became available in 2005 and in 2008 vaccination became freely available for children <2 years. In 2009, vaccination was officially recommended; it was replaced by PCV13 in 2010, which was then made mandatory in July 2014 in a 2 + 1 scheme (41, 42). From 2009 onwards, the vaccination rate has quickly raised to >80% and now is close to 100%, according to the latest available data (43, 44).

Nonetheless, the dynamic serotype rearrangement of pneumococci did not stop, therefore further extension of the included serotypes is continuously required. New 15-valent and 20-valent conjugated vaccines are in the pipeline, with the following additional planned serotypes: 8, 10A, 11A, 12F, 15B, 22F, 33F (45, 46).

The burden of invasive pneumococcal disease among older children and adults who did not receive PCV has also fallen markedly in the context of routine pediatric vaccination programs. This herd-protection effect is the result of reduced transmission and carriage of vaccine type pneumococci in the community (38, 47).

Table 2. Pneumococcal serotypes included in the licensed pneumococcal vaccines (33, 40)

Vaccine	Manufacturer	Serotypes	Type of vaccine
PPV23 (Pneumovax 23)®	MSD	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F	polysaccharide
PCV7 (Prevenar) ®	Pfizer	4, 6B, 9V, 14, 18C,19F, 23F	conjugated
PCV10 (Synflorix) ®	GSK	4, 6B, 9V, 14, 18C,19F, 23F, **1, 5, 7F**	conjugated
PCV13 (Prevenar 13) ®	Pfizer	4, 6B, 9V, 14, 18C,19F, 23F, **1, 3, 5, 6A, 7F, 19A**	conjugated

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1.2. *S. aureus*

1.2.1. *S. aureus* in general

S. aureus is a Gram-positive coccus of 1 μm in size, forming grape-like clusters under the microscope. They are catalase and coagulase positive, β-hemolytic (i.e., completely hemolyze red blood cells on blood agar plate), and produce golden-pigment (Figure 2) (2).

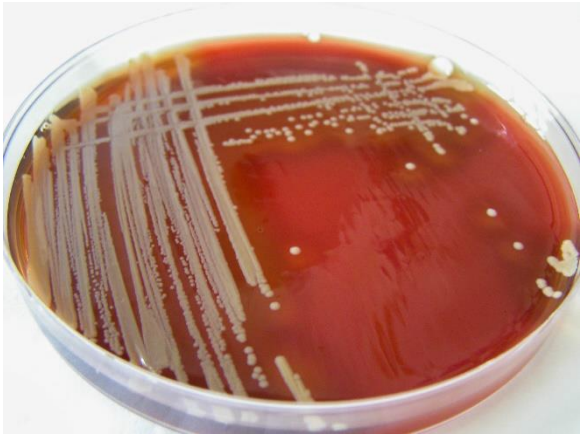

Figure 2. Culture of *S. aureus* on blood agar (Photo by E. Kovács)

1.2.2. Virulence factors

Like many other bacteria that can cause invasive diseases, *S. aureus* also produces a polysaccharide capsule which inhibits phagocytosis. Capsule production by *S. aureus* was first described in 1931 by Gilbert (48).

Catalase activity facilitates intracellular survival by breaking down hydrogen peroxide, a host defense mechanism (49).

Coagulase has two types in *S. aureus*. One of them is the bound coagulase or clumping factor. Another type is the so called free coagulase. They catalyse the fibrinogen→fibrin conversion, protecting it from phagocytosis (49).

Several enzymes help the bacterium spreading: hyaluronidase disrupts proteoglycans in connective tissue; proteases destroy proteins; staphylokinase lyses formed fibrin clots; lipase degrades fats and oils. Lipase enzyme facilitates *S. aureus* colonization of sebaceous glands (49).

Protein A is expressed on the surface of the bacteria. It has sites that bind the Fc receptors of immunoglobulin G (IgG) antibodies. This may protect *S. aureus* from opsonisation and phagocytosis (50).

There are four types of hemolysins: alpha, beta, gamma and delta with cytolytic activity. Hemolysin-α and leukotoxin AB play a synergistic role in promoting macrophage dysfunction and eliciting cell death when *S. aureus* organizes as a biofilm (51).

Hemolysin-β is highly hemolytic towards erythrocytes in sheep, but not in rabbits. The difference in susceptibility for erythrocytes may be due to the different sphingomyelin contents of these cells since the toxin is also known as sphingomyelinase (52, 53). So far, four bi-component leukotoxins were described that are structurally similar to hemolysin-α: Panton-Valentine Leukocidin (PVL), γ-hemolysin, leukotoxin ED and leukotoxin AB/GH. These are pore-forming toxins with different cell specificity, targeting leukocytes, neutrophils, monocytes, dendritic cells and red blood cells (54). PVL is present in a small percentage (approximately 5%) in clinical *S. aureus* strains, but it is strongly associated with community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains (approximately 85%), particularly those causing pneumonia, and skin and soft tissue infections (54).

Exfoliative toxins (type A, B, C and D), also known as epidermolytic toxins, are extremely specific serine proteases which cause the staphylococcal scalded skin syndrome (SSSS). Exfoliative toxins are "molecular scissors" associated with cleavage of keratinocytes junctions and cell-cell adhesions in the epidermis of the host which can induce skin peeling and blister formation (55).

There are more than 23 staphylococcal superantigen toxins described, particularly the toxic shock syndrome toxin (TSST-1) and the staphylococcal enterotoxins (SEA to SEE, SEG to SEJ, SEL to SEQ and SER to SET), and 11 staphylococcal superantigen-like toxins (SEIK to SEIQ, SEIU to SEIX) (56-58). Superantigens are able to bind to major histocompatibility complex class II (MHC II) molecules on antigen presenting cells. This induces a massive T cell response and cytokine release, thereby causing generalized shock-like symptoms (e.g., high fever, rash, desquamation, vomiting, diarrhea, hypotension, and frequently can result in multiple organ failure) (59).

1.2.3. Diseases caused by *S. aureus*

Diseases can be separated into two groups. The first group comprises of exotoxin caused diseases, like gastroenteritis (food poisoning), toxic shock syndrome and SSSS.

Gastroenteritis is manifested clinically as emesis with or without diarrhea. This condition is resulted by ingestion of one or more heat-stable staphylococcal enterotoxins on food. Fever and hypotension is rarely observed in cases of staphylococcal food poisoning.

Gastroenteritis caused by staphylococcal enterotoxins is self-limited and typically resolves within 24 to 48 h of onset (60).

Toxic shock syndrome (TSS) is an acute and potentially fatal illness that is characterized by a high fever, diffuse erythematous rash, desquamation of the skin 1 to 2 weeks after onset (if not fatal before this time), hypotension and multisystem involvement. In the early 1980s, an epidemic of TSS occurred among young women in the United States. Nearly all of these cases were associated with menstruation, the use of tampons and vaginal or cervical colonization (61, 62). There are also important nonmenstrual subsets of TSS, including influenza-associated cases in which *S. aureus* superinfects tracheal lesions caused by the virus, as well as postsurgical TSS (63). Also in acquired immune deficiency syndrome (AIDS) patients, TSS was observed manifested as a recalcitrant, erythematous, desquamating disorder (64).

SSSS is a syndrome characterized by skin exfoliation, but its early manifestations include fever, skin hypersensitivity, and erythema, followed by superficial fluid-filled blister formation and skin separation. If it affects only a restricted area it is called as bullous impetigo (65).

The second group is caused by the direct organ invasion by *S. aureus*: pneumonia, meningitis, osteomyelitis, acute bacterial endocarditis, septic arthritis, skin infections, bacteremia, sepsis, urinary tract infection.

In the industrialized world, the incidence of *S. aureus* bacteremia ranges from 10 to 30 per 100,000 person per year (66). In the United States, the incidence of endocarditis was calculated to increase from 11.4 per 100,000 person-years in 1999 to 16.6 per 100,000 person-years in 2006 with the increase of *S. aureus* driven endocarditis. *S. aureus* endocarditis was also associated with increased mortality compared to other causative pathogens (67-69).

Impetigo is the most common bacterial skin infection of children (70). While *S. aureus* cellulitis most commonly involves the lower extremities, it may also involve other regions, including the upper extremities, abdominal wall, and face (71). There is a strong epidemiological association between PVL and abscesses and furuncles (72). A carbuncle

is a contiguous collection of two or more furuncles. It affects the surrounding skin and deep underlying subcutaneous tissue of the infected hair follicles (73).

In Taiwan, a review of 53 patients with necrotizing fasciitis revealed that 38% of the infections were caused by *S. aureus*, 60% of which were caused by methicillin-resistant *S. aureus* (MRSA) (74).

Pyomyositis is more typical in tropical countries. In temperate climates, it occurs primarily in children and in young adults and it was also reported in association with human immunodeficiency virus (HIV) (75, 76).

Surgical site infections are also common among *S. aureus* infections. *S. aureus* is the most common cause of postoperative mediastinitis (77).

Osteomyelitis is an infection of bone resulting in its inflammatory destruction, bone necrosis, and new bone formation. In Denmark, *S. aureus* caused 55% of vertebral osteomyelitis cases and the incidence of *S. aureus* vertebral osteomyelitis increased from 1.6 to 2.5 per 100,000 person-years (78).

Septic arthritis is quite rare in Western Europe but there is a higher incidence in socioeconomically disadvantaged populations (e.g., 29.1 per 100,000 person-years in aboriginal Australians) (79).

S. aureus is particularly adept at prosthetic device infections due to its biofilm formation ability (80).

Pneumonia, caused by *S. aureus*, was first described during the influenza pandemic in 1918. Thereafter, it remained an infrequent but well-documented cause of CAP (81, 82). Recently, new clinical strains causing severe necrotizing pneumonia have emerged (83). *S. aureus* pneumonia or empyema may occur as a result of hematogenous spread from an infected cardiac valve or via local extension from another infected source (84).

S. aureus is an uncommon cause of bacterial meningitis. It can be derived from a hematogenous spread of an *S. aureus* infection or a neurosurgical intervention (85, 86).

S. aureus urinary tract infections are more common in patients with an indwelling urinary catheter (87).

1.2.4. Antibiotic resistance

Penicillin resistance in *S. aureus* can occur in two different ways. One is producing β-lactamase (penicillinase) enzyme which disrupts the β-lactam ring of the penicillin molecule, thereby inactivating β-lactam antibiotics (88, 89). Methicillin, oxacillin etc. are however penicillinase resistant penicillins. The other way is to have a novel, modified penicillin binding protein (PBP). PBP is also called transpeptidase and is responsible for cell wall formation due to its enzymatic activity however, penicillin inhibits this activity (90). The novel PBP, called PBP2a (encoded by *mecA* gene) is resistant to penicillinase-resistant penicillins and all β-lactam antibiotics (91, 92).

The first MRSA strain was reported in 1961 (93), two years after methicillin was introduced into clinical practice. The spread of MRSA may occur at least by two different mechanisms: spread of existing resistant clones and acquisition of staphylococcal cassette chromosome *mec (SCCmec)* by a methicillin-sensitive *S. aureus* (MSSA) strain. Sequencing of the region containing *mecA* revealed a distinct mobile genetic element (*SCCmec)*, that is present in MRSA but absent in MSSA (94). In 2011, a new *mecA* gene homologue, $mecA_{LGA251}$, was found in isolates from both humans and animals. The International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements renamed it to *mecC (95). MecC* occurs predominantly in a single lineage of MRSA in Europe with a very low prevalence (96).

Originally, MRSA were limited to hospitals (hospital-associated MRSA (HA-MRSA)). These strains are resistant to multiple antibiotics, usually have large *SCCmec* elements and have sacrified virulence for high levels of resistance to β- lactams (97, 98). In the past two decades, CA-MRSA have emerged. These strains carry a small *SCCmec* cassette, are not resistant to multiple antibiotics and have enhanced virulence (99, 100). CA-MRSA typically express lower levels of resistance to β-lactams (98). Since the mid‑2000s, it has also been associated with livestock exposure (livestock-associated MRSA (LA‑MRSA)) (101).

MRSA strains are usually multidrug resistant and vancomycin is one of the few antibiotics useful to treat MRSA infections. However, vancomycin resistance was already reported (102).

1.2.5. Vaccination

Staphylococcal toxins are promising vaccine targets. Vaccines should induce antibody response to prevent bacterial adherence and allow the neutralization of the produced toxins. However, vaccine development against *S. aureus* is an extraordinary challenge as there is a large number of toxins and the expression of the toxins vary significantly with the bacterial genotype. Hence, a broad-spectrum anti-toxin vaccine would be needed (103).

1.3. *H. influenzae*

1.3.1. *H. influenza*e in general

H. influenzae is a nonmotile Gram-negative coccobacillus which requires blood (hemin),- and nicotinamide adenine dinucleotide (NAD)-containing medium for growth (Figure 3). Chocolate agar, where red blood cells are lysed by heat and therefore hemin and NAD (X and V factors) are released, is appropriate for culturing. It is catalase positive, sized approximately 0.3 µm (104).

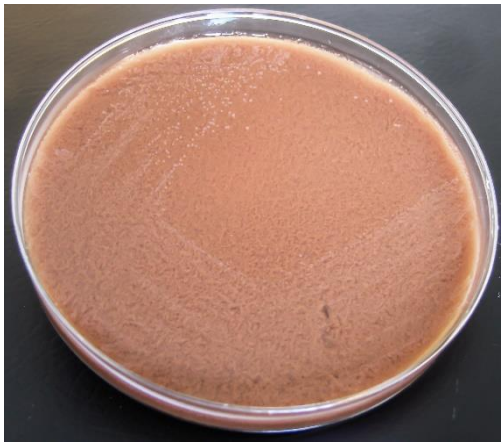

Figure 3. Culture of *H. influenzae* on chocolate agar plate (Photo by E. Kovács)

1.3.2. Virulence factors

Polysaccharide capsule is one of the most important virulence factors of *H. influenzae* and based on it we classify them into six serotypes (a-f). Of these, serotype b is mostly associated with invasive diseases, such as meningitis. Capsule prevents complement-mediated killing and opsonization thus it protects the bacteria from phagocytosis. Unlike capsulated strains, the lack of a polysaccharide capsule on non-typable strains (non-typable *H. influenzae*, NTHi) means the absence of ability to block some binding or deposition of host innate immune factors (105, 106).

In NTHi, adhesion is achieved by two main groups of adhesion proteins - high molecular weight proteins (HMW1/HMW2) and *H. influenzae* adhesin (Hia). The level of expression of HMW1/HMW2 is associated with the development of OM in children and

bronchitis in patients with chronic obstructive pulmonary disease (COPD). Hia analogue in encapsulated *H. influenzae* serotypes is Hsf adhesin (107).

Haemophilus adhesion protein (Hap) mediates binding to epithelial cells, and is able to do this through binding to laminin, fibronectin, and collagen IV (108, 109). Hap is noted for acting in concert with protein E in order to bind laminin (110). The ABC transporter protein F also bind to host cells via laminin (111). Hap and protein E has a multitasking ability as they also play role in entry into human bronchial respiratory epithelial cells (112, 113).

Protein D is a surface-exposed-associated glycerophosphodiester phosphodiesterase that is crucial for survival in the host, where it is able to bind to and facilitate entry into mononuclear cells (114).

The pilus of NTHi, specifically the pilus tip, PilA, was recently shown to bind intercellular adhesion molecule 1 (ICAM1) on the surface of epithelial cells. Pili occur on NTHi as well as on the typeable strains, where the pili are able to bind mucin (115, 116).

Outer membrane proteins are six to eight in number. Some of them such as P2 and P6 are being intensively studied as antigens that may be included in vaccine preparations against nontypable strains. They contribute to the adhesion and invasion of host tissues. (117). Other outer membrane proteins, like porin P5 are able to bind human factor H, which enables it to avoid C3 complement deposition. P4 is able to use its vitronectin-binding ability to resist serum killing (118, 119).

IgA1 protease inactivates human immunoglobulin A1 and facilitates the colonization of mucosae. The presence of a second IgA protease has been detected, more common in isolates from patients with COPD (120).

Like all Gram-negative microorganisms, *H. influenzae* has a lipopolysaccharide layer in its cell wall, but it is with a shorter polysaccharide chain hence it is termed lipooligosaccharide (LOS). *H. influenzae* expresses host carbohydrate structures within the oligosaccharide portion of the LOS, including phosphorylcholine, N-acetyl-lactosamine, paragloboside, and sialoparagloboside. The expression of these structures is a means of molecular mimicry for the bacterium, and may allow for the evasion of the

immune response or for the utilization of host receptors (121-124). The LOS is able to mediate entry into host cells via the platelet-activating factor receptor, causing remodeling of the cytoskeleton (125). Phosphorylcholine modulates the outer membrane and protects *H. influenzae* against IgG and consequently the bactericidal effects by complement activation (126).

The NTHi strains can bind plasma proteins and then manipulate the host through bacterial surface– LOS interactions. Hence, this slows down and/or inhibits the host complement cascade preventing immune factor recognition (127-129).

H. influenzae is able to form a protective biofilm that possibly enables it to cause either subsequent infections at later periods or disease recurrence/relapse (4, 130).

1.3.3. Diseases caused by *H. influenzae*

COPD and AOM are two important pathological conditions caused by *H. influenzae* in addition to sinusitis and conjunctivitis, that can also arise from colonization with this bacterium. For the very young and elderly, pneumonia and bacteremia are possible diseases caused by *H. influenzae* (131, 132). In 2016, 3 379 confirmed cases of invasive *H. influenzae* disease were reported in the EU/EEA, this means 0.7 cases per 100 000 population. Meningitis occurred in 13% of the known clinical presentations and 1% was presented with both septicaemia and meningitis. Across serotypes, meningitis was the most common presentation for *H. influenzae* serotye b (Hib) and d (133). Epiglottitis is classically seen in older children between 2 and 7 years of age. In cases of suspected epiglottitis, direct laryngoscopy with placement of an endotracheal tube in a controlled environment is indicated, as sudden deterioration can occur (134). NTHi can be responsible of around 60% of AOM cases when taking into account recurrent episodes and seasonal variations. In 13-50% of COPD exacerbations, *H. influenzae* can be detected (135, 136). *H. influenzae* is regularly involved in acute exacerbations of cystic fibrosis (137).

1.3.4. Antibiotic resistance

Meningitis, acute epiglottitis and bacterial sepsis are rapidly fatal without antibiotic therapy. Ampicillin was used until resistance emerged. Currently, third generation cephalosporins are chosen in case of serious infections.

The most common antibiotic resistance mechanism is the plasmid-mediated production of β-lactamase of types TEM-1 and ROB-1, the affected strains are the so called β-lactamase positive ampicillin-resistant strains (BLPAR) (138). The use of oral cephalosporins and amoxicillin-clavulanate has contributed to the spread of β-lactam resistance due to modification of penicillin-binding proteins (PBP3) resulting in β-lactamase-non-producing ampicillin resistance (BLNAR) (139). Some strains of *H. influenzae* can produce β-lactamase, while showing resistance to amoxicillin/ clavulanic acid, and are called β-lactamase positive amoxicillin/clavulanic acid-resistant (BLPACR) (140).

Resistance to TMP/SMX is quite common in *H. influenzae* and is due to increased production of dihydrofolate reductase with reduced affinity for trimethoprim (141).

1.3.5. Vaccination

Conjugated Hib vaccine is composed of the type b capsule and diphtheria toxoid. Similarly to the above seen pneumococcal conjugate vaccine, addition of protein toxin was necessary to activate T lymphocytes, this way T-cell dependent immune response is induced also in children under two years of age. Hib conjugated vaccine has been available since the late 1980s (142). In Hungary, Hib vaccination is mandatory in a 3+1 scheme since 1999 (143).

The first vaccine to protect against Hib diseases was introduced in the United States in 1985, it was a pure polysaccharide vaccine; an improved, conjugated vaccine was licensed two years later. The conjugate vaccines differ by protein carrier, polysaccharide size, and method of chemical conjugation. The conjugate could be diphtheria toxoid, (diphtheria CRM197 protein), or meningococcal protein (144, 145).

The efficacy of one of the conjugated vaccines (with meningococcal protein) for infants was demonstrated in a randomized, placebo-controlled double-blind trial performed among 3486 Navajo infants, half of whom were vaccinated at 2 and 4 months of age and the vaccine was licensed for use for infants beginning at 2 months of age in December 1990 (146). Later on, the vaccine with diphtheria CRM197 protein conjugate was licensed to give the first dose in the age of 2 months (147, 148).

The incidence of Hib invasive disease among children aged 4 years or younger has declined by 98% in the USA by 1994-1995 (149).

There was a placebo-controlled randomized clinical trial with oral NTHi vaccination in 40-85 years COPD patients in 2011. The enteric-coated tablet contained a well defined formalin inactivated NTHi strain (HI-164). In the entire study population, there was no significant difference between placebo and active groups regarding NTHi colonisation. In patients aged <65 years, there was a 54% reduction in rate of exacerbations at the first end-point and 65% at the second end-point among active treatment patients. Furthermore, hospital admission rate decreased by 57% and hospitalized patients spent 60% less time in hospital. In the placebo group (patients <65 years) exacerbations occurred significantly higher. On the other hand, its safety is questionable as cardiac disorders occurred significantly higher in the vaccinated group. The results suggested that age influences the response to this vaccine and immune senescence is a more likely cause of apparent vaccine failure in the 65+ age group rather than different colonization rates in younger or older people (150).

1.4. *M. catarrhalis*

1.4.1. *M. catarrhalis* in general

M. catarrhalis is a Gram-negative diplococcus with flattened abutting sides. Colonies on blood agar are nonhemolytic, round, opaque, convex, and snow white (Figure 4). The colony remains intact when pushed across the surface of the agar. It is oxidase, DNase positive, capable to reduce nitrate and nitrite as well as can hydrolyse trybutirin (151, 152).

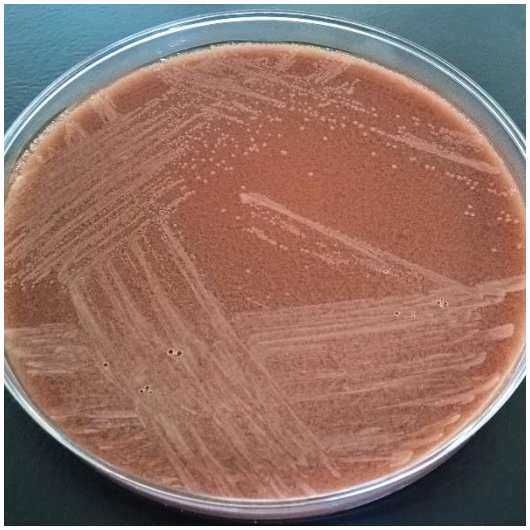

Figure 4. Culture of *M. catarrhalis* on chocolate agar plate (Photo by E. Kovács)

1.4.2. Virulence factors

Ubiquitous surface protein A1 (UspA1) is an outer membrane protein that is involved in adherence to epithelial cells, the extracellular matrix and biofilm formation (153-156).

The uspA2 locus can contain one of three mutually exclusive alleles known as uspA2, uspA2H and uspA2V, each encoding a different outer-membrane protein. Usp2A is the most prevalent with 72-77% of isolates followed by UspA2H with 15-21% and UspA2V with around 10%. They are all involved in serum resistance and adherence to the extracellular matrix, moreover UspA2H has a role in biofilm formation (153-155, 157-162).

M. catarrhalis immunoglobulin D-binding protein/ hemagglutinin (MID/Hag) is located in the outer membrane and mediates haemagglutination and non-immune binding of IgD by *M. catarrhalis*, while it also functions as an adhesin for cells derived from the human lung, middle ear and ciliated bronchial epithelium (163-165).

Catarrhalis outer membrane protein B (CopB) is involved in iron acquisition and elicits a systemic humoral immune response (166, 167).

CD and E porins are both outer membrane proteins (OmpCD and OmpE) and are involved in nutrient acquisition as well as adherence (168, 169).

M. catarrhalis LOS is a surface-exposed glycolipid found in the outer membrane and is involved in adherence to and invasion of epithelial cells, and serum resistance (170, 171). There are three major serotypes of LOS (A, B and C) and they represent 95% of isolates (172). However, this antigen is not the best option as a vaccine candidate because the predominant antibody response to the LOS is toward the serospecific epitopes (173).

1.4.3. Diseases caused by *M. catarrhalis*

M. catarrhalis is the third most common pathogen associated with OM worldwide behind *S. pneumoniae* and NTHi and the second associated with exacerbations of COPD following NTHi, ranking equally with *S. pneumoniae* (174, 175). *M. catarrhalis* is a predominant pathogen in subacute or chronic sinusitis in children with respiratory allergy (176). Occasionally, *M. catarrhalis* can cause lower respiratory infections (laryngitis, tracheitis, bronchitis, pneumonia). Tracheobronchitis is a typical *M. catarrhalis* infection

with cough and purulent sputum. Pneumonia caused by *M. catarrhalis* tends to be a relatively mild disease. Its X-ray image shows mostly lower-lobe infiltrates (177, 178)

Ocular infections, like conjunctivitis and keratitis can be attributed to this microorganism as well (179, 180). It has a role as a nosocomial pathogen, furthermore it can cause bacteremia and endocarditis (181, 182). Meningitis is rarely caused by *M. catarrhalis* and it occurs mostly among newborns (183).

1.4.4. Antibiotic resistance

These bacteria produce β-lactamase and are thus resistant to penicillin. The first β-lactamase positive strain was isolated in 1976 (184). Walker et al. followed antibiotic resistance patterns of *M. catarrhalis* in a single hospital over a ten-year period (1984-1994). During this time, the rate of β-lactamase producing strains increased from 30 to 96% (185). Beside the high prevalence of β-lactamase production, *M. catarrhalis* remained universally sensitive to most other antibiotics (186).

β-lactamase is encoded by two different alleles, bro1 and bro2. The bro alleles differ by one amino acid, which has unknown significance, although bro2 has a 21 base pair deletion in its promoter region that results in decreased expression compared to bro1 and may account for differences in MIC values. MIC is usually higher for BRO-1 isolates than for BRO-2 (187, 188).

1.4.5. Vaccination

There are currently no vaccines licensed that specifically target *M. catarrhalis*, however there are numerous vaccine candidates.

UspA2 was already applied in an NTHi-*M. catarrhalis* common vaccine clinical trial between 2015 and 2017. Three conserved proteins were selected from NTHi, a free recombinant protein D (PD) and a recombinant fusion protein combining protein E and Pilin A (PE-PilA) and UspA2 from *M. catarrhalis*. The multi-component vaccine showed acceptable safety, reactogenicity and immunogenicity in the study population who immunologically represented well the COPD patients: older adults with a smoking history (189).

One possible solution for a future vaccine would be to have a mixture of different antigens that covers as broad range of circulating strains as possible. Another option may be to

identify and target key virulence factors involved in colonization or disease. In either case, further investigations are required for a better understanding of the link between virulence determinants and disease. The development of a *M. catarrhalis* vaccine could have far-reaching implications, reducing both infections by *M. catarrhalis* and co-infections synergized by *M. catarrhalis* (190).

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2. Objectives

- The objective of this study was to survey the nasal carriage rates of four important respiratory pathogens (*S. pneumoniae, S. aureus, M. catarrhalis* and *H. influenzae*) in three different age groups of children attending communities, in 2015-2016. This was the first time in Hungary to conduct a survey about the asymptomatic carriage of *M. catarrhalis* and *H. influenzae*. Besides their individual carriage rates, we could also examine the co-carriage of the four species.
- We tried to specify certain risk factors for their nasal carriage as well as determine their antibiotic resistance.
- Serotype distribution was also investigated where applicable. The aim of this study was to follow the effects of the pneumococcal conjugate vaccine on pneumococcal serotype replacement, as well as on the carriage prevalence of the other three species. Hungary is a good model for vaccine efficacy monitoring as it is a country with a very strict vaccination policy, unlike some European countries which have a significant level of vaccine sceptics.
- Genetic relatedness was determined for all *H. influenzae* and *S. aureus* isolates and some selected serotypes of *S. pneumoniae* to gain an insight into their clonal spread. In case of *S. pneumoniae,* four specific serotype 19F isolates were more closely analysed by multilocus sequence typing (MLST), as this type was represented in a surprisingly high proportion despite that it was already included in PCV7 it is due to its elevated capability to cause invasive diseases.
- Clinical pneumococcal isolates were obtained from the same time period (2015-2016) to compare serotype distribution and antibiotic resistance pattern of clinical and carried isolates.
- Epidemiological surveys are valuable tools in predicting changes regarding dominant serotypes, genotypes in the near future, estimating their prevalence among invasive disease causing types. They can also provide crucial information facilitating prevention and control of diseases, such as vaccine development.

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3. Materials and methods

3.1. Study population

3.1.1. Asymptomatic carriage screening

In total, 580 asymptomatic children belonging to three different age groups were tested between March 2015 and May 2016. In total, the genders were absolutely equally represented with 288 males and 288 females.

Children attending nurseries (1-3 years old)

In three Hungarian cities, altogether 336 children were screened: Five nurseries were visited in Budapest (n=200), two nurseries in Székesfehérvár (n=50) and three in Pápa (n= 86). The gender rates were almost equal here: 159 boys and 173 girls. We did not have any data about four children.

The three cities were chosen according the following criteria: Budapest is the capital city of Hungary with around 1.760.000 people based on data from 2015, Székesfehérvár is a chief county town (~98.000 people) and Pápa (~31.000 people) is a small town (191). Besides their population size, they are located in three different counties: Pest, Fejér and Veszprém. Furthermore, Pápa is my birth place.

Children attending day-care centers (DCCs, 3-6 years old)

Ten DCCs agreed to participate in the survey, nine from Pápa (n=135) and one from Budapest (n=51). This resulted 186 participating subjects. Regarding gender, 97 of them were boys and 89 were girls.

Children attending primary school (6-13 years old)

Samples were taken from 58 children (32 boys and 26 girls) in Pápa.

3.1.2. Clinical pneumococcal isolates

As a second part of the study, we obtained 146 clinical pneumococcal isolates from the Institute of Laboratory Medicine, Semmelweis University, Budapest in the same time frame. The samples derived from patients with manifest mucosal infections (sinusitis maxillaris acuta, bronchitis, pneumonia, conjunctivitis, COPD, AOM). The sample types were different: nasal swab n=81, tracheal/bronchial exudate / bronchoalveolar lavage /

sputum n=41, ear n=9, conjunctiva n=9, pleura/urine/abscess n=1 each, haemoculture n=3. The patients' age varied between 0 and 89 years. A more precise age distribution is shown on Figure 5. The vast majority of patients were either under 4 years (n = 49) or over 50 years (n = 56). The average age of all patients was 31.9 years. We received pure cultures on blood agar plates after routine laboratory identification, with a designated laboratory number, hence the patients' personal data was not available for us.

To make them comparable with the carried pneumococcal isolates, we divided them into two age groups: 0-<7 years (27 males, 36 females) and ≥7 years (45 males, 38 females).

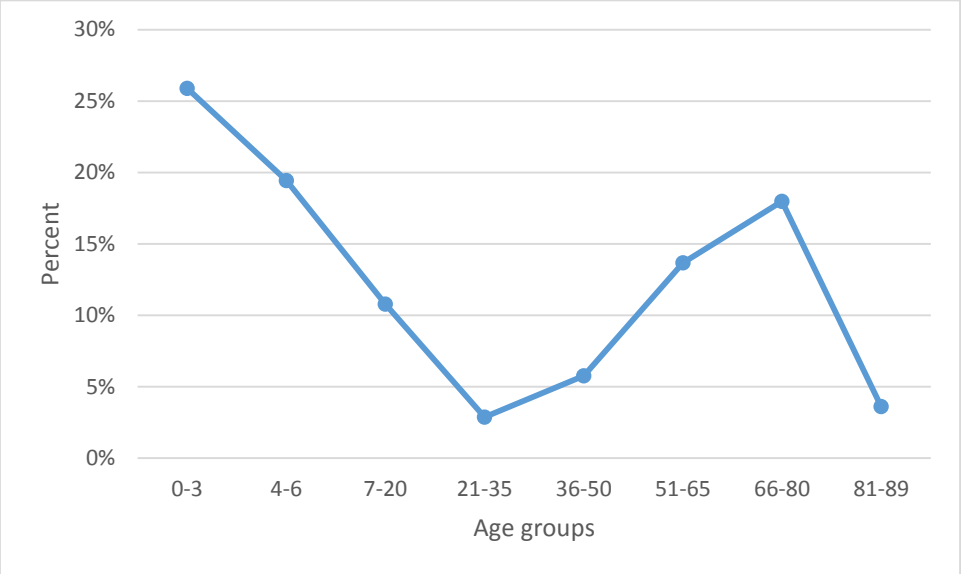

Figure 5. Age distribution of the patients

3.2. Specimen collection from carriers

During screening, nasal samples were taken from both nostrils with sterile cotton swabs and inserted into active charcoal containing Amies transport media (Transwab, Medical Wire & Equipment, Corsham, UK). The swabs were transported to the microbiology laboratory within 24 hours. A questionnaire was filled in by the participants anonymously with questions related to risk factors, such as gender, previous antibiotic use, exposure to active or passive smoking, number of siblings, pneumococcal vaccination status. The number of ethical permit for this study is TUKEB 4-4/2009, issued by the Regional and Institutional Committee of Science and Research Ethics of Semmelweis University. Only those children were enrolled in the study, whose parents provided written informed consent on the child's behalf.

3.3. Phenotypical identification

The collected samples were inoculated onto Columbia blood agar plates (for *S. pneumoniae* and *S. aureus*) and onto vancomycin containing chocolate agar plates for the selective cultivation of *H. influenzae* and *M. catarrhalis* (Thermoscientific, Waltham, USA). Samples were incubated overnight at 37 °C in 5% CO_2 atmosphere. Colonies showing typical phenotypes were chosen to produce pure cultures. These species-specific phenotypes were the following: In case of pneumococcus, the mucoid or flat colonies which were collapsed in the middle and showed α-haemolysis on blood agar were further tested for optochin sensitivity (5µg discs, Mast Group Ltd., Bootle, UK). *S. aureus* was suspected if the colony had a β-haemolytic zone and had a positive catalase and clump test (Pastorex Staph-Plus Kit, Bio-Rad, Marnes-la-Coquette, France). On the chocolate agar plates supplemented with X- and V-factors, the smooth, round, colorless or greyish colonies were identified as *H. influenzae,* subsequently confirmed with a positive catalase and oxidase test. *M. catarrhalis* often grew as a pure culture on the chocolate agar plates. The typical snow white colonies with irregular edge were able to show the "hockey puck sign" (i.e., sliding along the surface without hindrance if taken by an inoculation loop) and were oxidase positive. The phenotypically confirmed isolates were frozen and stored at -80˚C on cryobeads (Cryobank, Mast Group Ltd., Bootle, UK) until further testing.

3.4. Genotypical identification

Species specific gene targets were used to identify each species (Table 3). *LytA* encodes the autolysin which is a pneumococcus-specific virulence factor involved in autolysis (15). Exceptionally, *lytA* positivity can lead to false results because it can be detected in some closely related streptococcal species and in some cases, pneumococci can harbor a non-pneumococcal homologue of the *lytA* gene (192). *NucA* polymerase chain reaction (PCR) was applied to detect the thermostable nuclease of *S. aureus*. In an in-house duplex PCR, *mecA* primer pair was concomitantly added to the *nucA* PCR mix in order to screen for methicillin resistant isolates (193). *OmpP2* (major outer membrane porin protein P2) is present in both capsulated and non-capsulated *H. influenzae* isolates which makes it available for identification (194). For the confirmation of *M. catarrhalis*, a specific *16S rRNA* sequence was amplified as described by Hendolin at al (195). The primer pairs and amplification conditions are listed in Tables 3 and 4.

Table 3. Primers and amplified fragment length size of the identification PCR, bp: base pair; for: forward; rev: reverse

	Primer		Sequence (5'→3')	Amplicon size (bp)	Reference
S. pneumoniae	*lytA*	for	CAACCGTACAGAATGAAGCGG	318	(15)
		rev	TTATTCGTGCAATACTCGTGCG		
S. aureus	*nucA*	for	ATGGACGTGGCTTAGCGTAT	193	(193)
		rev	TGACCTGAATCAGCGTTGTC		
	mecA	for	CCCAATTTGTCTGCCAGTTT	538	(193)
		rev	ATCTTGGGGTGGTTACAACG		
H. influenzae	*ompP2*	for	ATAACAACGAAGGGACTAACG	1043	(194)
		rev	ACCTACACCCACTGATTTTTC		
M. catarrhalis	*16S rRNA*	for	CCCATAAGCCCTGACGTTAC	235	(195)
		rev	CTACGCATTTCACCGCTACAC		

Table 4. PCR cycling conditions of the identification PCR

lytA			*nucA, mecA*		
Repeat	Temperature	Time	Repeat	Temperature	Time
1x	94°C	3 min	1x	94°C	3 min
30x	94°C	1 min	30x	94°C	1 min
	54°C	1 min		54°C	1 min
	72°C	30 sec		72°C	30 sec
1x	72°C	10 min	1x	72°C	10 min
ompP2			*16S rRNA*		
Repeat	Temperature	Time	Repeat	Temperature	Time
1x	94°C	4 min	1x	63°C	3 min
30x	94°C	1 min	38x	94°C	30 sec
	55°C	1 min		66°C	45 sec
	72°C	2 min		72°C	1 min
1x	72°C	8 min	1x	72°C	5 min

3.4.1. Polymerase chain reaction (PCR)

PCR was applied for multiple purposes, like species identification, serotyping if applicable as well as detection of antibiotic resistance genes.

DNA template extraction

From the pure culture of the isolates, a loopful (5 µl) was boiled in 200 µl sterile nuclease free distilled water for 15 minutes (99°C in the PCR machine). The suspension was vortexed briefly and centrifuged for 2 minutes at 7000 rpm (Hermle Z 160M centrifuge). Two µl of the supernatant was used in the PCR reaction as DNA template.

PCR mix

Table 5 details the components of the mix. DNA template was added to the aliquoted 23 µl mix last.

Table 5. Components of the PCR mix

Ingredients	Volume for 1 reaction
DreamTaq Green PCR Master Mix (Thermo Scientific, Waltham, USA)	12.5 µl
primer for*	0.5 µl
primer rev*	0.5 µl
Water, nuclease-free	9 µl
DNA template	2.5 µl
Total	25 µl

* Concentration of primers was 50 pmol/µl in every case

In case of multiplex PCR, further 0.5 µl of both the other forward and reverse primers were added, so an equivalent volume of distilled water was subtracted from the total reaction volume of 25 µl.

PCR cycle

The specific cycling conditions were listed at every single reaction in Table 4. We used a GeneAmp PCR System 9700 (PE Applied Biosystem).

Agarose gel electrophoresis and analysis

1.5% agarose gel was prepared with 1x TEA buffer (for solutions see the end of this chapter). 10 µl of each sample was aliquoted into the gel and run alongside with the GeneRuler 100 bp DNA Ladder (Thermoscientific, Waltham, USA) mixed with 6X DNA Loading Dye as a molecular size marker in every case. The electrophoresis was performed under 100 V for 36 min. With Eco Safe Nucleic Acid Staining Solution (Pacific Image Electronics, Torrance, Canada) the gels were stained and a photo was taken by digital camera (Figure 6).

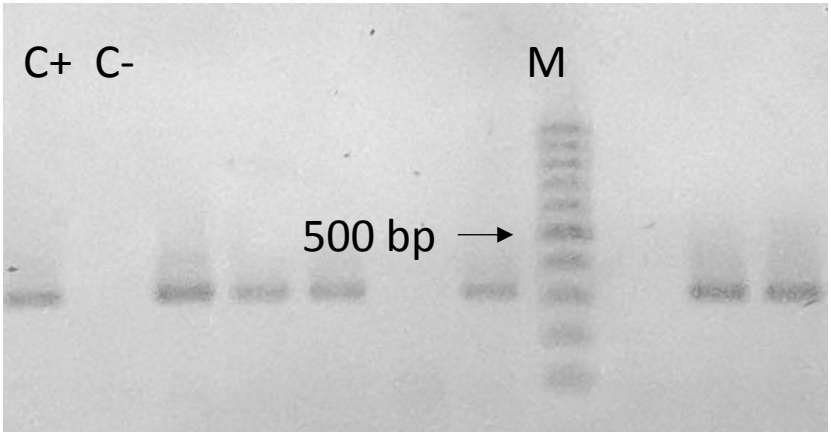

Figure 6. A representative gel image of *lytA* PCR (Photo by E. Kovács)

C+ positive control, C- negative control, M marker

3.5. Serotyping

3.5.1. S. pneumoniae

First latex agglutination was used to determine the serotype or the serogroup. The Pneumotest Latex Kit (Statens Serum Institut, Copenhagen, Denmark) consists of 14 pooled antisera (A-I and P-T). According the manufacturer's instruction, the chessboard system should be applied starting with A-I pools and if there is a positive reaction it defines the next one from the P-T list (Figure 7). The agglutination reaction must be evaluated within 5-10 seconds, otherwise it can be a result of false positivity. PCR was used to confirm the serotypes (e.g. agglutination was positive for B and R, see Figure 7), and if only serogroup could be determined (e.g. H and S pools gave positive result) PCR helped to specify the serotype (Table 6). Serotyping primers for the PCR reaction were described by the CDC and others (196-198).

Pool	P	Q	R	S	T	Non-vaccine groups/types
A	**1**	**18** (18F, 18A, 18B, **18C**)	**4**	**5**	**2**	
B	**19** (19F, **19A**, 19B, 19C)	**6** (6A, **6B**, 6C)	**3**	**8**		
C	**7** (7F, 7A, 7B, 7C)				**20**	24 (24F, 24A, 24B) 31, 40
D			**9** (9A, 9L, 9N, 9V)		**11** (11F, **11A**, 11B, 11C, 11D)	16 (16F, 16A) 36, 37
E			**12** (**12F**, 12A, 12B)	**10** (10F, **10A**, 10B, 10C)	**33** (**33F**, 33A, 33B, 33C, 33D)	21, 39
F				**17** (**17F**, 17A)	**22** (**22F**, 22A)	27 32 (32F, 32A) 41 (41F, 41A)
G						29, 34 35 (35F, 35A, 35B, 35C) 42 47 (47F, 47A)
H	**14**	**23** (**23F**, 23A, 23B)		**15** (15F, 15A, **15B**, 15C)		13 28 (28F, 28A)
I						25 (25F, 25A) 38, 43, 44, 45, 46, 48

Figure 7. The chessboard system of the Pneumotest Latex Kit

Table 6. Serotype specific primer pairs and amplicon size for pneumococcus serotyping PCR

Serotypes and primers		Sequence (5'→3')	Amplicon size (bp)	Ref.
1	for	GGAGACTACTAAATTGTAATACTAACACAGCG	99	(196)
	rev	CAAGGATGAATAAAGTAAACATATAATCTC		
3	for	TTGTTTTTTGTCTTTATTCTTATTCGTTGG	818	(196)
	rev	TTGTTTTTTGTCTTTATTCTTATTCGTTGG		
4	for	CTGTTACTTGTTCTGGACTCTCGTTAATTGG	430	(196)
	rev	GCCCACTCCTGTTAAAATCCTACCCGCATTG		
5	for	GAGACGTCTTTGGGGCATAA	366	(197)
	rev	GCGGAAACGATGAGAAGAAG		
6 A/B/C/D	for	CGACGTAACAAAGAACTAGGTGCTGAAAC	220	(196)
	rev	AAGTATATAACCACGCTGTAAAACTCTGAC		
7C/B/40	for	CTATCTCAGTCATCTATTGTTAAAGTTTACGACGGGA	260	(198)
	rev	GAACATAGATGTTGAGACATCTTTTGTAATTTC		
7F	for	TGACTGCAAGTGTTTCAATGG	528	(197)
	rev	CGTTTCCAAAAATTCCTCCA		
9V	for	AGAGGAGTTCAATCGCCAGA	242	(197)
	rev	ATCGGTTCCCCAAGATTTTC		
10A	for	GGTGTAGATTTACCATTAGTGTCGGCAGAC	628	(198)
	rev	GAATTTCTTCTTTAAGATTCGGATATTTCTC		
10F/10C /33C	for	GGAGTTTATCGGTAGTGCTCATTTTAGCA	248	(198)
	rev	CTAACAAATTCGCAACACGAGGCAACA		
12/44/46	for	GCAACAAACGGCGTGAAAGTAGTTG	376	(198)
	rev	CAAGATGAATATCACTACCAATAACAAAAC		
11A/F/D	for	CGAAATATCGCCATTCATCA	379	(197)
	rev	TCAACAGCAACTGTGCCACT		
13	for	ACGACTTGGAAGTGCTGCTT	308	(197)

	rev	CCAAAAACAAAATCGCTGGA		
14	for	GTCTGTTTATTCTATATACAAAGAGGCTCC	268	(196)
	rev	GCATTGCTACAATCGCTATACTAGATATGC		
15A/F	for	CATTTGCACCCTGACTTCAC	409	(197)
	rev	GTCCCGCAAACTCTGTCCTA		
15B/C	for	TGTTCAAAGAGGCGCTAATG	493	(197)
	rev	TGTTCTGATTCCTGCTCCAA		
16F	for	GAATTTTTCAGGCGTGGGTGTTAAAAG	717	(198)
	rev	CAGCATATAGCACCGCTAAGCAAATA		
18C	for	GCCGTGGGAAGCTTATTTTT	285	(197)
	rev	CCTGCCTAAAGGCAACAATG		
19A	for	GTTAGTCCTGTTTTAGATTTATTTGGTGATGT	478	(196)
	rev	GAGCAGTCAATAAGATGAGACGATAGTTAG		
19F	for	CACCTAATTTTAATACTGAGGTTAAGATTGC	408	(196)
	rev	CATAGGCTATCAGAATTTTAATAATATCTTGC		
20	for	ATCAGGAATACGCCAATCAA	195	(197)
	rev	ATCGGTAATGCAAAGCCAAC		
23A	for	GATTTGGAGCGGATCGATTA	823	(198)
	rev	AATGGGTAATGGAGGGGAGT		
23B	for	CCACAATTAGCGCTATATTCATTCAATCG	199	(198)
	rev	GTCCACGCTGAATAAAATGAAGCTCCG		
23F	for	GTAACAGTTGCTGTAGAGGGAATTGGCTTTTC	384	(196)
	rev	CACAACACCTAACACACGATGGCTATATGATTC		
24A/B/F	for	GCTCCCTGCTATTGTAATCTTTAAAGAG	99	(198)
	rev	GTGTCTTTTATTGACTTTATCATAGGTCGG		
25F/25A /38	for	CGTTCTTTTATCTCACTGTATAGTATCTTTATG	574	(198)
	rev	ATGTTTGAATTAAAGCTAACGTAACAATCC		
33F	for	TCCCCAACGGTTTATGTGTT	171	(197)
	rev	CAATGCAAGGCTCAATACCA		
35B	for	GATAAGTCTGTTGTGGAGACTTAAAAAGAATG	677	(198)
	rev	CTTTCCAGATAATTACAGGTATTCCTGAAGCAAG		
35F/47F	for	GAACATAGTCGCTATTGTATTTTATTTAAAGCAA	517	(198)
	rev	GACTAGGAGCATTATTCCTAGAGCGAGTAAACC		
42/35A/ 35C	for	TCCCTTTTTCAGACGTAGCC	492	(197)
	rev	CAAGAAATTGATCCGCTTGGT		
9N/L	for	GAACTGAATAAGTCAGATTTAATCAGC	516	(198)
	rev	ACCAAGATCTGACGGGCTAATCAAT		
21	for	CTATGGTTATTTCAACTCAATCGTCACC	192	(198)
	rev	GGCAAACTCAGACATAGTATAGCATAG		
31	for	GGAAGTTTTCAAGGATATGATAGTGGTGGTGC	701	(198)
	rev	CCGAATAATATATTCAATATATTCCTACTC		
33F/33A /37	for	GAAGGCAATCAATGTGATTGTGTCGCG	338	(198)
	rev	CTTCAAAATGAAGATTATAGTACCCTTCTAC		
34	for	GCTTTTGTAAGAGGAGATTATTTTCACCCAAC	408	(198)
	rev	CAATCCGACTAAGTCTTCAGTAAAAAACTTTAC		
39	for	TCATTGTATTAACCCTATGCTTTATTGGTG	98	(198)
	rev	GAGTATCTCCATTGTATTGAAATCTACCAA		

for: forward primer, rev: reverse primer bp: base pair

Difficult strains were sent either to the German National Reference Centre for Streptococci (GNRCS), Aachen (to Dr. Mark van der Linden), or the National Public

Health Center, Budapest (to Dr. Tamás Tirczka), where the gold standard capsule swelling method was applied for exact serotype determination.

3.5.2. H. influenzae

For the capsular typing of *H. influenzae*, the capsule producing capability was demonstrated first by PCR. Isolates which carried the capsular gene *bexA* were further tested to distinguish serotypes (a-f) also by PCR as reported by Falla et al (105). Table 7 and 8 show the used primer pairs and PCR cycling conditions.

Table 7. Primer pairs used to determine capsule production capability (*bexA*) and to distinguish the six serotypes

Primer		Sequence (5'→3')	Amplicon size (bp)
bexA	for	CGTTTATATGATGTTGATCCTGAA	343
	rev	TGTCCATATCTTCAAAATGGTG	
serotype a	for	CTACTCATTGCAGCATTTGC	250
	rev	GAATATGACCTGATCTTCTG	
serotype b	for	GCGAAAGTGAACTCTTATCTCTC	482
	rev	GCTTACGCTTCTATCTCGGTGAT	
serotype c	for	TCTGTGTAGATGATGGTTCA	250
	rev	CAGAGGCAAGCTATTAGTGA	
serotype d	for	TGATGACCGATACAACCTGT	146
	rev	TCCACTCTTCAAACCATTCT	
serotype e	for	GGTAACGAATGTAGTGGTAG	1350
	rev	GCTTTACTGTATAAGTCTAG	
serotype f	for	GCTACTATCAAGTCCAAATC	450
	rev	CGCAATTATGGAAGAAAGCT	

for: forward primer, rev: reverse primer bp: base pair

Table 8. Amplifying conditions of *bexA* PCR and the serotype PCR of *H. influenzae*

Repeat	Temperature	Time
1x	94°C	3 min
25x	94°C	1 min
	60°C	1 min
	72°C	1 min
1x	72°C	10 min

3.5.3. M. catarrhalis

Serotypes A, B and C were differentiated based on the method reported by Edwards et al (199). For B and C we have designed new primer pairs because the original primers did not work properly, the PCR reaction did not result any products. Besides, the expected amplicon sizes were shortened this way (3.3 kbp→1488 bp and 4.3 kbp→2485 bp) and a shorter fragment is amplified with a higher efficiency (Table 9). Due to a homologous DNA sequence in both serotypes, the same forward and reverse primer could be applied to identify them in the same reaction. The only difference could be noted in the length of the PCR products. Table 10 contains the detailed PCR settings.

Table 9. Serotyping primers of *M. catarrhalis* and the expected amplicon size

Primer		Sequence (5'→3')	Amplicon size (bp)	Reference
serotype A	for	ATCCTGCTCCAACTGACTTTC	1932	(199)
	rev	CATCAAAAACCCCCCTACC		
serotype B	for	ACTGCCTGTGGCTTTATGCT	1488	own design
	rev	TCGAAGACGCACTTTAGCTG		
serotype C	for	ACTGCCTGTGGCTTTATGCT	2485	own design
	rev	TCGAAGACGCACTTTAGCTG		

for: forward primer, rev: reverse primer bp: base pair

Table 10. PCR cycling conditions of *M. catarrhalis* serotyping

Repeat	Temperature	Time
1x	94°C	3 min
25x	94°C	1 min
	53.1°C	1 min
	72°C	4 min
1x	72°C	10 min

3.6. Antibiotic susceptibility testing

Basically, agar dilution method was used to determine the MIC of all isolates. The following antibiotics were tested, where appropriate: penicillin, ampicillin, amoxicillin-clavulanic acid, oxacillin, cefotaxime, imipenem, tetracycline, erythromycin, clindamycin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, vancomycin, mupirocin and TMP/SMX (Bio-Rad). First, a 0.5 McFarland bacterial suspension was prepared with sterile physiological saline from pure bacterial culture using a VITEK Densichek machine (Biomérieux, Marcy l'Etoile, France). Then 10 µl of each bacterial

suspension was inoculated onto Mueller-Hinton agar plates (for *S. aureus*) or Mueller-Hinton agar plates (Thermoscientific, Waltham, USA) supplemented with 5% horse blood and 20mg/L NAD (for the other three species) using an A400 Multipoint Inoculator (AQS Manufacturing Ltd., Southwater, UK). The plates contained antibiotics in an ascending order. After ON incubation at 37°C, MIC values were determined visually as per EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines (Figure 8), results were interpreted based on the EUCAST breakpoints (200). Control strains of ATCC (American Type Culture Collection) 49619 (for *S. pneumoniae*), ATCC 29213 (for *S. aureus*), ATCC 49766 (for *H. influenzae* and *M. catarrhalis*) were tested as well, and antibiotic free growth controls were also applied.

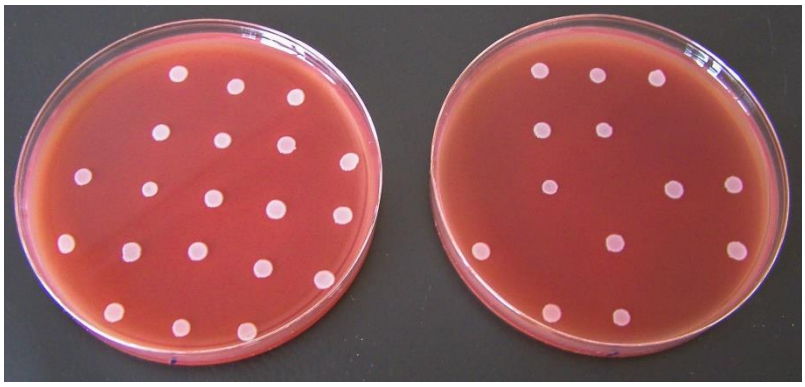

Figure 8. Agar dilution method. Plates contain 0.06 mg/L and 0.125 mg/L cefotaxime and each spot represent a different *M. catarrhalis* isolate. Some strains did not grow under higher antibiotic concentration (Photo by E. Kovács)

3.6.1. Testing macrolide resistance mechanisms in *S. pneumoniae*

Macrolide resistant isolates were checked for four resistance genes (*ermA, ermB, ermTR, mefE/A*) by PCR (Table 11). The same cycling parameters were applied for all four reactions (Table 12). *Mef* primer pair was designed to be able to amplify both *mef(E)* and *mef(A)* due to common nucleotide sequences. *Mef(E)* and *mef(A)* were distinguished by restriction digestion with *Bam*HI enzyme (ThermoScientific, Waltham, Massachusetts, USA) as described by Oster et al.: there is no restriction site in *mef(E)* gene and two fragments (281 bp and 65 bp) are generated in case of *mef(A)*. The reaction composition can be found in Table 13 resulting a total volume of 30 µl. The mix was then incubated at 37°C for 30 minutes and gel electrophoresis was performed in a 1.5% agarose gel (Sigma-Aldrich, USA).

Table 11. Primer pairs of *erm/mef* PCR and the amplified fragment length

Primer		Sequence (5'→3')	Amplicon size (bp)	Reference
ermA	for	TCTAAAAAGCATGTAAAAGAA	645	(201)
	rev	CTTCGATAGTTTATTAATATTAGT		
ermB	for	GAAAAAGTACTCAACCAAATA	639	(201)
	rev	AGTAACGGTACTTAAATTGTTTAC		
ermTR	for	ACAGAAAAACCCGAAAAATACG	679	(202)
	rev	TGGGATAATTTATCAAGATCAG		
mef	for	AGTATCATTAATCACTAGTGC	346	(201)
	rev	TTCTTCTGGTACTAAAAGTGG		

for: forward primer, rev: reverse primer bp: base pair

Table 12. The cycling conditions of the *erm/mef* PCR

Repeat	Temperature	Time
1x	93°C	3 min
35x	93°C	1 min
	52 °C	1 min
	72°C	1 min
1x	72°C	5 min

Table 13. The digestion mix composition to distinguish *mef(E)* and *mef(A)*

Mix	Volume
PCR product FastDigest *Bam*HI enzyme FastDigest Buffer Nuclease free distilled water	10 µl 1 µl 2 µl 17 µl
Total	30 µl

3.6.2. Testing for cefoxitin and mupirocin resistance in *S. aureus*

As stated in the EUCAST guidelines, cefoxitin disc diffusion is more reliable to predict methicillin resistance. The *S. aureus* isolates with an oxacillin MIC ≥0.25 mg/L were also screened with 30 µg cefoxitin discs (Bio-Rad). As described before, a 0.5 McFarland suspension was spread evenly on the surface of Mueller-Hinton agar plates then a disc was placed on it. After ON incubation at 37°C, inhibitory zone diameter was read.

Mupirocin susceptibility of *S. aureus* isolates was tested by gradient strips (E-test) (Liofilchem, Roseto degli Abruzzi, Italy).

Mupirocin resistant *S. aureus* isolates were further investigated by PCR to distinguish low-level and high-level resistance (Table 14). For detecting low-level resistance, we amplified the *ileS* gene which is coding isoleucyl-tRNA synthetase and can be found in every *S. aureus* isolates but the mupirocin resistant isolates contain point mutations (203). The *ileS* PCR products were purified by the QIAquick PCR purification kit (Qiagen, Germany) and sent for sequencing to BIOMI Ltd., Gödöllő, Hungary. The sequences were compared to that of a mupirocin sensitive reference strain, ATCC 25923 (GenBank accession no. CP009361.1) using BLAST. To confirm high-level resistance, we looked for the presence of the plasmid-encoded *mupA* gene (204). We used the originally described cycling condition for both PCR reaction (Table 15 and 16).

Table 14. Primer pairs used to distinguish low and high level mupirocin resistance in *S. aureus*

Primer		Sequence (5'→3')	Amplicon size (bp)	Reference
ileS	for	AAAGAGAAGCGAAAGACTTACTACCAG	790	(203)
	rev	AAGATTGGTGCTAACAACTTCGTCATA		own design
mupA	for	TATATTATGCGATGGAAGGTTGG	458	(204)
	rev	AATAAAATCAGCTGGAAAGTGTTG		

for: forward primer, rev: reverse primer bp: base pair

Table 15. *ileS* PCR settings

Repeat	Temperature	Time
1x	94°C	3 min
30x	94°C	1 min
	52°C	1 min
	72°C	1 min
1x	72°C	10 min

Table 16. *mupA* PCR settings

Repeat	Temperature	Time
1x	95°C	3 min
35x	95°C	30 s
	57°C	60 s
	72°C	30 s
1x	72°C	10 min

3.6.3. Testing for beta-lactamase production in *H. influenzae*

Tests based on a chromogenic cephalosporin like nitrocefin can be used to detect the beta-lactamase production. Therefore, ampicillin resistant *H. influenzae* isolates were tested using nitrocefin discs (Sigma-Aldrich, St. Louis, USA). Nitrocefin has a beta-lactam ring which rapidly turns from yellow to red due to hydrolysis by beta-lactamase. The process is to smear one or two colonies onto the disc on a microscope slide and wait for the colour change. It usually occurs within five minutes, but in some rare cases it can take up to 60 minutes.

3.7. Pulsed-field gel electrophoresis (PFGE)

PFGE was used to get information about the clonal relatedness of *S. pneumoniae*, *S. aureus* and *H. influenzae* isolates. We followed the protocols described by Szabó et al., Hall et al. and Saito et al., respectively (205-207).

3.7.1. Chromosomal DNA preparation

S. pneumoniae and *S. aureus* were grown on 5% blood containing Columbia agar plates whereas *H. influenzae* on chocolate agar plates. Then a cell suspension was made in 200 µl cell suspension buffer (*S. pneumoniae*, *H. influenzae*) or in 200 µl EC lysis buffer (*S. aureus*). This suspension was mixed with 200 µl 2% chromosomal-grade low-melting agarose (Bio-Rad), poured into plug molds and solidified at 4°C for 15-20 minutes. Chromosomal DNA was purified in two lysis steps (Table 17). *S. pneumoniae and H. influenzae* plugs were incubated in the appropriate lysis-1 buffer at 37°C for 3 hours and subsequently ON at 54°C in lysis-2 buffer. *S. aureus* plugs were first incubated at 37°C for 1 h in lysis-1 buffer and then at 54°C for 3 h in lysis-2 buffer.

Table 17. Composition of Lysis-1 and Lysis-2 buffer in case of *S. pneumoniae, S. aureus* and *H. influenzae*

	Lysis-1 buffer for 1 plug		Lysis-2 buffer for 1 plug	
	Ingredient (conc.)	Volume	Ingredient (conc.)	Volume
S. pneumoniae	EC lysis buffer	3 ml	ES lysis buffer	3 ml
	Lysozyme (20 µg/ml)	150 µl	Proteinase K (20 mg/ml)	20 µl
	mutanolysine	2 µl		
	RNase A (25 µg/ml)	20 µl		

S. aureus	EC lysis buffer	1.5 ml	ESTN buffer	1.5 ml
	Lysostaphin (1 mg/ml)	16.7 µl	Proteinase K (20 mg/ml)	10 µl
	Lysozyme (20 mg/ml)	20 µl		
H. influenzae	EC lysis buffer	1 ml	ESTN buffer	1 ml
	Lysozyme (20 mg/ml)	20 µl	Proteinase K (20 mg/ml)	10 µl

The plugs were washed three times in 1 ml TE buffer at room temperature for 30 min each. At the fourth time, the plugs were cut into half and the digestion started immediately or they were stored at 4°C in TE buffer until further usage.

3.7.2. Digestion

Half of the plugs were digested with 15 U FastDigest *Sma*I enzyme (Thermo Fisher Scientific, Waltham, USA) for 30 min at 25°C (Table 18).

Table 18. Composition of the restriction digestion solution for PFGE

Restriction mix	Volume for 1/2 plug
Water, nuclease-free	133.5 µl
10X FastDigest buffer or 10X FastDigest Green buffer	15 µl
SmaI enzyme	1.5 µl
Total	**150 µl**

3.7.3. Gel electrophoresis

The digested plugs were washed with distilled water and were placed in the pockets of a 1% pulsed-field certified agarose gel (Bio-Rad, USA) prepared in 0.5x TBE buffer. To separate DNA fragments by their size, CHEF-DR® II apparatus (Bio-Rad, USA) was used. The temperature was kept constantly at 14°C, the pulse times were increased in two blocks, see details in Table 19.

Table 19. Settings of PFGE gel electrophoresis

Parameter	Block-1	Block-2
Initial time:	5 s	15 s
Final time:	15 s	60 s
Running time:	10 h	11 h
Voltage:	6 V/cm (~ 200 V)	6 V/cm (~ 200 V)

CHEF DNA Size Standard Lambda Ladder (Bio-rad, Budapest, Hungary) was used in three lanes of every gel as a molecular weight control. The marker required a special pretreatment before use, according the manufacturer's instructions. Briefly, they were cut to the required size to fit the sample wells using a razor blade. Then three pieces were placed separately into Eppendorf tubes covered with 200 µl of 0.5x TBE buffer and incubated at 45°C for 10 minutes to get ready to use.

3.7.4. Gel analysis

The gels were stained with GelRed (Biotium, USA) and a photograph was taken by a digital camera. The analysis of the PFGE profiles (normalisation, gel comparison and dendrogram creation) was performed with the BioNumerics software version 2.5 (Applied Maths, Sint-Martens-Latem, Belgium) applying unweighted pair group method using arithmetic averages (UPGMA) and the different bands similarity coefficient, with a band position tolerance of 2.0%. During interpretation, the Tenover's criteria (208) and the suggested designation by van Belkum et al. (209) were applied.

3.8. Multi-locus sequence typing (MLST)

Based on the PFGE dendrogram of *S. pneumoniae* isolates, four of them were chosen to perform MLST. According to the instructions of the MLST website (210), internal fragments of seven housekeeping genes were amplified by PCR except for *recP*, where the *recP* reverse primer was modified by us (Table 20). The products were purified by the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sent for sequencing to the BIOMI Ltd., Gödöllő, Hungary. The allele sequences were compared to the MLST database and the sequence types were identified. The sequenced seven house-keeping genes were: AroE (shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucose kinase), *recP* (transketolase), *spi* (signal peptidase I), *xpt* (xanthine phosphoribosyltransferase), *ddl* (D-alanine-D-alanine ligase).

Table 20. *S. pneumoniae* MLST primer pairs and the expected amplicon size

Primer		Sequence (5'→3')	Amplicon size (bp)
aroE	for	GCCTTTGAGGCGACAGC	479
	rev	TGCAGTTCA(G/A)AAACAT(A/T)TTCTAA	
gdh	for	ATGGACAAACCAGC(G/A/T/C)AG(C/T)TT	659
	rev	GCTTGAGGTCCCAT(G/A)CT(G/A/T/C)CC	
gki	for	GGCATTGGAATGGGATCACC	626
	rev	TCTCCCGCAGCTGACAC	
recP	for	GCCAACTCAGGTCATCCAGG	572
	rev	TGCCAACCGTAGGCATTGTAAC	
spi	for	TTATTCCTCCTGATTCTGTC	560
	rev	GTGATTGGCCAGAAGCGGAA	
xpt	for	TTATTAGAAGAGCGCATCCT	572
	rev	AGATCTGCCTCCTTAAATAC	
ddl	for	TGC(C/T)CAAGTTCCTTATGTGG	514
	rev	CACTGGGT(G/A)AAACC(A/T)GGCAT	

for: forward primer, rev: reverse primer, bp: base pair

3.9. Statistical analysis

The Fisher's exact test of independence was employed to determine statistical significance due to the small numbers. Applying a 95% confidence interval, p value < 0.05 was considered significant.

3.10. Solutions

All chemicals were ordered from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). They were dissolved in distilled water and the pH was adjusted where necessary. The solutions (Table 21) were finally sterilised by autoclave. TE, TEA and TBE buffers were prepared from commercial powder mixtures (Biocenter, Szeged, Hungary).

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Table 21. Ingredients of different solutions used in PCR or PFGE reactions

Solution	Ingredients
1x TEA buffer	20 mM EDTA, 40 mM TRIS, 57.1 ml/L glacial acetic acid
EC lysis buffer	1 M NaCl, 100 mM EDTA, 6 mM TRIS, 0.5% Brij58, 0.2% deoxycholate, 0.5% N-lauroyl sarcosine; pH=7.6
ESTN buffer	20 mM NaCl, 100 mM EDTA, 10 mM TRIS, 1% N-lauroyl sarcosine; pH=8.0
TE buffer	1 mM EDTA, 10 mM TRIS; pH=7.5
0.5x TBE buffer	1.25 mM EDTA, 45 mM TRIS, 45 mM boric acid

DOI:10.14753/SE.2022.2609

4. Results

4.1. Carriage rate

Usually, we had a mixed bacterium flora on the blood agar plates, while the chocolate agar plates were sometimes empty and sometimes a pure (*H. influenzae* or *M. catarrhalis*) culture was detectable. Out of the 580 screened children, 442 (76.2%) carried at least one of the four bacterial species. There was a clear age related bacterial prevalence: *M. catarrhalis, S. pneumoniae* and *H. influenzae* carriage decreased with age, meanwhile *S. aureus* prevalence showed an inverse tendency as shown on Figure 9. *S. aureus* carriage was 11.3% (n=38) in nurseries, 30.1% in DCCs (n = 56) and it peaked with 50.0% (n = 29) in primary school. A more specific age for highest carriage ratio could not be determined, as it was constantly ≥50% in the 9-13 years old children. The other three bacteria were more prevalent among younger children. The carriage rates in the three different age groups were 48.8% (n = 164), 21.5% (n = 40) and 6.9% (n = 4) for *S. pneumoniae*; 60.1% (n = 202), 37.6% (n = 70) and 15.5% (n = 9) for *M. catarrhalis*; 34.2% (n = 115), 19.9% (n = 37) and 0.0% (n = 0) for *H. influenzae*, respectively. Further refining the age, both *M. catarrhalis* and *S. pneumoniae* reached the peak at 1-2 years, whereas the highest rate of *H. influenzae* was observed among three years old children (37.3%, n=55). From that point, *H. influenzae* rate decreased until 0.0% by the age of seven years and it remained absent in older age groups.

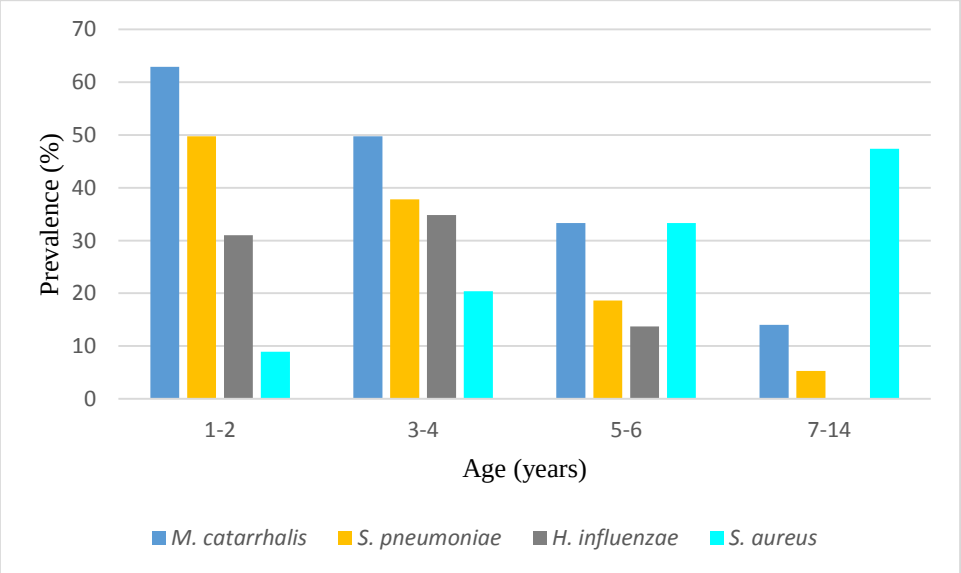

Figure 9. Prevalence of the four bacterial species

We have determined the single and multiple colonization patterns among the three institute types as summarized in Table 21. Multiple carriage occurred more frequently without *S. aureus* which was most obvious in case of triple and double carriage (Figure 10a and 10b). During statistical analysis, we found a significant negative association between *S. aureus* and *S. pneumoniae* or *M. catarrhalis*, and it was nearly significant with *H. influenzae*. On the other hand, positive association existed between *S. pneumoniae-H. influenzae*, *S. pneumoniae-M. catarrhalis* and *H. influenzae-M. catarrhalis* (Table 22). Neither positive nor negative correlation was found between VT pneumococci and the other three species.

Table 21. Nasal colonization patterns of the four respiratory pathogens among different age groups

	Nurseries (1-3y) n=336		DCCs (3-6y) n=186		Primary school (6-13y) n=58	
Non-carriers	54	(16.1%)	60	(32.3%)	24	(41.4%)
Carriers	282	(83.9%)	126	(67.7%)	34	(58.6%)
Carriers of one pathogen	109	(32.4%)	71	(38.2%)	28	(48.3%)
S. aureus	10	(3.0%)	30	(16.1%)	24	(41.4%)
S. pneumoniae	26	(7.7%)	9	(4.8%)	1	(1.7%)
M. catarrhalis	54	(16.1%)	28	(15.1%)	3	(5.2%)
H. influenzae	19	(5.7%)	4	(2.2%)	0	(0.0%)
Carriers of two pathogens	111	(33.0%)	38	(20.4%)	4	(6.9%)
S. pneumoniae + *S. aureus*	2	(0.6%)	3	(1.6%)	0	(0.0%)
S. pneumoniae + *M. catarrhalis*	61	(18.2%)	8	(4.3%)	1	(1.7%)
S. pneumoniae + *H. influenzae*	17	(5.1%)	5	(2.7%)	0	(0.0%)
M. catarrhalis + *H. influenzae*	20	(6.0%)	9	(4.8%)	0	(0.0%)
S. aureus + *M. catarrhalis*	8	(2.4%)	10	(5.4%)	3	(5.2%)
S. aureus + *H. influenzae*	3	(0.9%)	3	(1.6%)	0	(0.0%)
Carriers of three pathogens	60	(17.9%)	12	(6.5%)	2	(3.4%)
M. catarrhalis + *H. influenza* + *S. pneumoniae*	47	(14.0%)	7	(3.8%)	0	(0.0%)
S. pneumoniae + *S. aureus* + *M. catarrhalis*	6	(1.8%)	1	(0.5%)	2	(3.4%)

S. pneumoniae + *S. aureus* + *H. influenzae*	3	(0.9%)	2	(1.1%)	0	(0.0%)
S. aureus + *M. catarrhalis* + *H. influenzae*	4	(1.2%)	2	(1.1%)	0	(0.0%)
Carriers of four pathogens	2	(0.6)	5	(2.7%)	0	(0.0%)

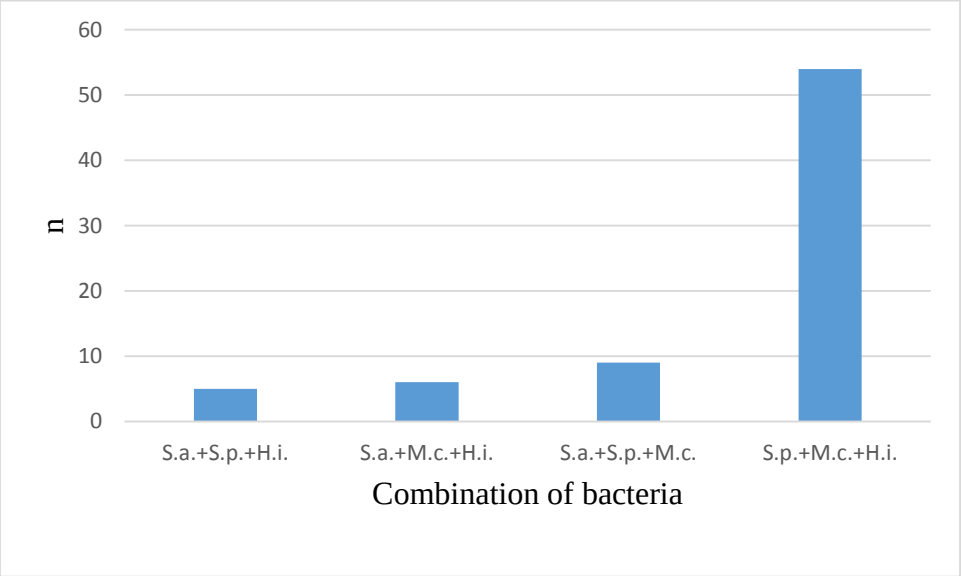

Figure 10a. Triple carriage prevalence

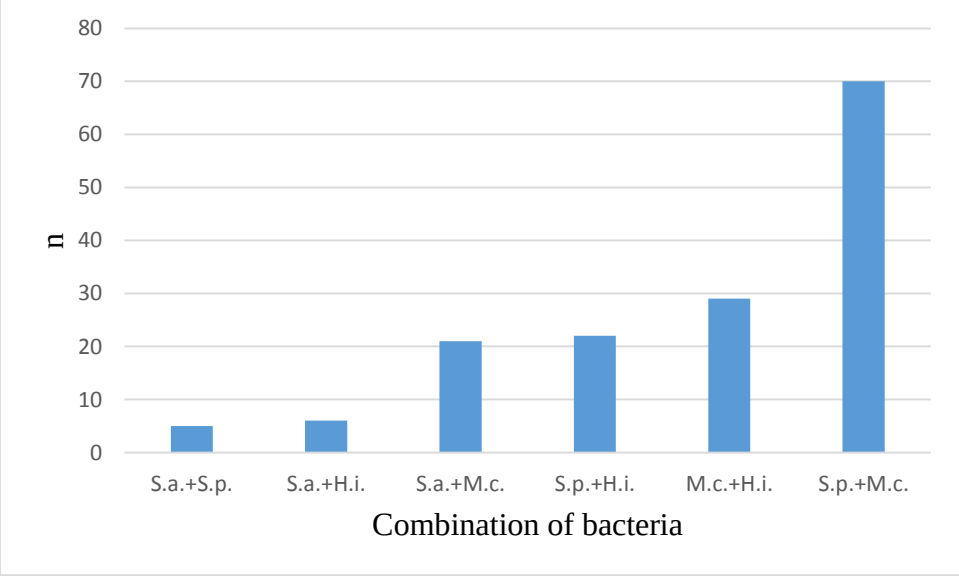

Figure 10b. Double carriage prevalence

Table 22. Co-carriage association between the four species

	S. aureus		p-value	*H. influenzae*		p-value	*M. catarrhalis*		p-value
	Carriers n (%)	Non-carriers n (%)		Carriers n (%)	Non-carriers n (%)		Carriers n (%)	Non-carriers n (%)	
S. pneumoniae carriers n=208	26 (12.5)	182 (87.5)	**1.252×10^{-4} (S)**	88 (42.3)	120 (57.7)	**4.300×10^{-11} (S)**	140 (67.3)	68 (32.7)	**1.077×10^{-11} (S)**
S. pneumoniae non-carriers n=372	97 (26.1)	275 (73.9)		64 (17.2)	308 (82.8)		141 (37.9)	231 (62.1)	
VT *S. pneumoniae* carriers n=13	2 (15.4)	11 (84.6)	0.745 (NS)	6 (46.2)	7 (53.8)	0.772 (NS)	9 (69.2)	4 (30.8)	0.879 (NS)
NVT *S. pneumoniae* carriers n=195	24 (12.3)	171 (87.7)		82 (42.1)	113 (57.9)		131 (67.2)	64 (32.8)	
S. aureus carriers n=123	-	-	-	24 (19.5)	99 (80.5)	0.057 (NS)	43 (35.0)	80 (65.0)	**7.455×10^{-4} (S)**
S. aureus non-carriers n=457	-	-		128 (28.0)	329 (72.0)		238 (52.1)	219 (47.9)	
H. influenzae carriers n=152	-	-	-	-	-	-	96 (63.2)	56 (36.8)	**2.397×10^{-5} (S)**
H. influenzae non-carriers n=428	-	-		-	-		185 (43.2)	243 (56.8)	

*VT: vaccine type

*NVT: non-vaccine type

4.2. Risk factors

Various risk factors were analysed individually in each age group of asymptomatic children and we made a comparison with the cumulative data as well. As mentioned before, the questionnaires were missing in case of four children attending nurseries. Table 23 shows the age-specific results of the Fisher's exact test and Table 24 shows the cumulative results.

Based on our statistics, *M. catarrhalis* was not influenced by any of the investigated factors. Regarding gender, boys seemed to be colonized with *S. aureus* more frequently in the primary school group. Furthermore, having siblings was associated positively with *S. aureus* carriage in the summarized group. However, antibiotic exposure in the past two weeks affected negatively its carriage (cumulative data). Taking antibiotics also reduced *H. influenzae* carriage (but only in DCC group). Finally, passive smoking was negatively associated with *S. pneumoniae* carriage.

Table 23. Statistical analysis of the risk factors which may have an influence on carriage

Risk factor	Patho-gen	Nurseries (1-3y) n=332*		p-value	DCCs (3-6y) n=186		p-value	Primary school (6-13y) n=58		p-value
		Carriers (%)	NCs (%)		Carriers (%)	NCs (%)		Carriers (%)	NCs (%)	
Gender male	*S. p.*	78 (49.1)	81 (50.9)	1.000 (NS)	20 (20.6)	77 (79.4)	0.859 (NS)	3 (9.4)	29 (90.6)	0.620 (NS)
	S. a.	18 (11.3)	141 (88.7)	1.000 (NS)	29 (29.9)	68 (70.1)	1.000 (NS)	21 (65.6)	11 (34.4)	**0.017 (S)**
	M. c.	92 (57.9)	67 (42.1)	0.502 (NS)	34 (35.1)	63 (64.9)	0.454 (NS)	4 (12.5)	28 (87.5)	0.717 (NS)
	H. i.	61 (38.4)	98 (61.6)	0.132 (NS)	19 (19.6)	78 (80.4)	1.000 (NS)	0 (0.0)	32 (100.0)	-
Gender female	*S. p.*	84 (48.6)	89 (51.4)		20 (22.5)	69 (77.5)		1 (3.8)	25 (96.2)	
	S. a.	20 (11.6)	153 (88.4)		27 (30.3)	62 (69.7)		8 (30.8)	18 (69.2)	
	M. c.	107 (61.8)	66 (38.2)		36 (40.4)	53 (59.6)		5 (19.2)	21 (80.8)	
	H. i.	52 (30.1)	121 (69.9)		18 (20.2)	71 (79.8)		0 (0.0)	26 (100.0)	
Having siblings	*S. p.*	95 (49.0)	99 (51.0)	1.000 (NS)	31 (23.7)	100 (76.3)	0.330 (NS)	3 (5.8)	49 (94.2)	0.362 (NS)
	S. a.	21 (10.8)	173 (89.2)	0.728 (NS)	43 (32.8)	88 (67.2)	0.226 (NS)	26 (50.0)	26 (50.0)	1.000 (NS)
	M. c.	114 (58.8)	80 (41.2)	0.650 (NS)	51 (38.9)	80 (61.1)	0.621 (NS)	8 (15.4)	44 (84.6)	1.000 (NS)
	H. i.	63 (32.5)	131 (67.5)	0.483 (NS)	28 (21.4)	103 (78.6)	0.547 (NS)	0 (0.0)	52 (100.0)	-
Not having siblings	*S. p.*	67 (48.6)	71 (51.4)		9 (16.4)	46 (83.6)		1 (16.7)	5 (83.3)	
	S. a.	17 (12.3)	121 (87.7)		13 (23.6)	42 (76.4)		3 (50.0)	3 (50.0)	
	M. c.	85 (61.6)	53 (38.4)		19 (34.5)	36 (65.5)		1 (16.7)	5 (83.3)	
	H. i.	50 (36.2)	88 (63.8)		9 (16.4)	46 (83.6)		0 (0.0)	6 (100.0)	
Vaccinated with Prevenar 13	*S. p.*	139 (50.2)	138 (49.8)	0.302 (NS)	33 (21.7)	119 (78.3)	1.000 (NS)	1 (3.8)	25 (96.2)	0.620 (NS)
	S. a.	33 (11.9)	244 (88.1)	0.649 (NS)	47 (30.9)	105 (69.1)	0.683 (NS)	13 (50.0)	13 (50.0)	1.000 (NS)
	M. c.	163 (58.8)	114 (41.2)	0.452 (NS)	57 (37.5)	95 (62.5)	1.000 (NS)	3 (11.5)	23 (88.5)	0.495 (NS)
	H. i.	96 (34.7)	181 (65.3)	0.643 (NS)	31 (20.4)	121 (79.6)	0.816 (NS)	0 (0.0)	26 (100.0)	-

Not vaccinated with Prevenar 13	*S. p.*	23 (41.8)	32 (58.2)		7 (20.6)	27 (79.4)		3 (9.4)	29 (90.6)	
	S. a.	5 (9.1)	50 (90.9)		9 (26.5)	25 (73.5)		16 (50.0)	16 (50.0)	
	M. c.	36 (65.5)	19 (34.5)		13 (38.2)	21 (61.8)		6 (18.8)	26 (81.3)	
	H. i.	17 (30.9)	38 (69.1)		6 (17.6)	28 (82.4)		0 (0.0)	32 (100.0)	
Antibiotic exposure in the past two weeks	*S. p.*	46 (43.8)	59 (56.2)	0.239 (NS)	3 (9.7)	28 (90.3)	0.095 (NS)	0 (0.0)	6 (100.0)	1.000 (NS)
	S. a.	7 (6.7)	98 (93.3)	0.066 (NS)	7 (22.6)	24 (77.4)	0.394 (NS)	2 (33.3)	4 (66.7)	0.670 (NS)
	M. c.	65 (61.9)	40 (38.1)	0.632 (NS)	10 (32.3)	21 (67.7)	0.548 (NS)	2 (33.3)	4 (66.7)	0.231 (NS)
	H. i.	41 (39.0)	64 (61.0)	0.213 (NS)	1 (3.2)	30 (96.8)	**0.012 (S)**	0 (0.0)	6 (100.0)	-
No antibiotics	*S. p.*	116 (51.1)	111 (48.9)		37 (23.9)	118 (76.1)		4 (7.7)	48 (92.3)	
	S. a.	31 (13.7)	196 (86.3)		49 (31.6)	106 (68.4)		27 (51.9)	25 (48.1)	
	M. c.	134 (59.0)	93 (41.0)		60 (38.7)	95 (61.3)		7 (13.5)	45 (86.5)	
	H. i.	72 (31.7)	155 (68.3)		36 (23.2)	119 (76.8)		0 (0.0)	52 (100.0)	
Passive exposure to smoking	*S. p.*	35 (44.9)	43 (55.1)	0.440 (NS)	14 (18.2)	63 (81.8)	0.372 (NS)	2 (9.5)	19 (90.5)	0.615 (NS)
	S. a.	6 (7.7)	72 (92.3)	0.310 (NS)	26 (33.8)	51 (66.2)	0.418 (NS)	11 (52.4)	10 (47.6)	1.000 (NS)
	M. c.	47 (50.3)	31 (39.7)	1.000 (NS)	34 (44.2)	43 (55.8)	0.128 (NS)	4 (19.0)	17 (81.0)	0.710 (NS)
	H. i.	25 (32.1)	53 (67.9)	0.785 (NS)	17 (22.1)	60 (77.9)	0.578 (NS)	0 (0.0)	21 (100.0)	-
No passive exposure to smoking	*S. p.*	127 (50.0)	127 (50.0)		26 (23.9)	83 (76.1)		2 (5.4)	35 (94.6)	
	S. a.	32 (12.6)	222 (87.4)		30 (27.5)	79 (72.5)		18 (48.6)	19 (51.4)	
	M. c.	152 (59.8)	102 (40.2)		36 (33.0)	73 (67.0)		5 (13.5)	32 (86.5)	
	H. i.	88 (34.6)	166 (65.4)		20 (18.3)	89 (81.7)		0 (0.0)	37 (100.0)	
Total number of carriers and NCs	*S. p.*	162 (48.8)	170 (51.2)		40 (21.5)	146 (78.5)		4 (6.9)	54 (93.1)	
	S. a.	38 (11.4)	294 (88.6)		56 (30.1)	130 (69.9)		29 (50.0)	29 (50.0)	
	M. c.	199 (59.9)	133 (40.1)		70 (37.6)	116 (62.4)		9 (15.5)	49 (84.5)	
	H. i.	113 (34.0)	219 (66.0)		37 (19.9)	149 (80.1)		0 (0.0)	58 (100.0)	

*: Data of four children attending nursery were missing

NC, non-carrier

DCC, day-care centre

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Table 24. Cumulative table of correlation between carriage and possible risk factors

Risk factor	Pathogen	Screened children (1-13y) n=576* Carriers (%)	NCs (%)	p-value
Gender male	*S. p.*	101 (35.1)	187 (64.9)	0.794 (NS)
	S. a.	68 (23.6)	220 (76.4)	0.222 (NS)
	M. c.	130 (45.1)	158 (54.9)	0.156 (NS)
	H. i.	80 (27.8)	208 (72.2)	0.393 (NS)
Gender female	*S. p.*	105 (36.5)	183 (63.5)	
	S. a.	55 (19.1)	233 (80.9)	
	M. c.	148 (51.4)	140 (48.6)	
	H. i.	70 (24.3)	218 (75.7)	
Having siblings	*S. p.*	129 (34.2)	248 (65.8)	0.315 (NS)
	S. a.	90 (23.9)	287 (76.1)	**0.043 (S)**
	M. c.	173 (45.9)	204 (54.1)	0.136 (NS)
	H. i.	91 (24.1)	286 (75.9)	0.163 (NS)
Not having siblings	*S. p.*	77 (38.7)	122 (61.3)	
	S. a.	33 (16.6)	166 (83.4)	
	M. c.	105 (52.8)	94 (47.2)	
	H. i.	59 (29.6)	140 (70.4)	
Antibiotic exposure in the past two weeks	*S. p.*	49 (34.5)	93 (65.5)	0.763 (NS)
	S. a.	16 (11.3)	126 (88.7)	**0.001 (S)**
	M. c.	77 (54.2)	65 (45.8)	0.122 (NS)
	H. i.	42 (29.6)	100 (70.4)	0.272 (NS)
No antibiotics	*S. p.*	157 (36.2)	277 (63.8)	
	S. a.	107 (24.7)	327 (75.3)	
	M. c.	201 (46.3)	233 (53.7)	
	H. i.	108 (24.9)	326 (75.1)	
Passive exposure to smoking	*S. p.*	51 (29.0)	125 (71.0)	**0.030 (S)**
	S. a.	43 (24.4)	133 (75.6)	0.270 (NS)
	M. c.	85 (48.3)	91 (51.7)	1.000 (NS)
	H. i.	42 (23.9)	134 (76.1)	0.471 (NS)
No passive exposure to smoking	*S. p.*	155 (38.8)	245 (61.3)	
	S. a.	80 (20.0)	320 (80.0)	
	M. c.	193 (48.3)	207 (51.8)	
	H. i.	108 (27.0)	292 (73.0)	
Total number of carriers and NCs	*S. p.*	206 (35.8)	370 (64.2)	
	S. a.	123 (21.4)	453 (78.6)	
	M. c.	278 (48.3)	298 (51.7)	
	H. i.	150 (26.0)	426 (74.0)	

*: Data of four children attending nursery were missing

NC: non-carrier

4.3. Serotyping results and vaccination status

4.3.1. S. pneumoniae

Altogether, 208 pneumococcus carriers were identified, but in the case of two girls (from nursery and DCC), double carriage was detected (all non-PCV serotypes: 31 + 23B and 15C + 23A) therefore the total number of the isolates was 210. The PCV13 vaccination rate was high based on the questionnaires: 83.4% in nurseries, 81.7% in DCCs, 44.8% in primary school. As PPV23 is an optional vaccine, its frequency was much lower: 6.9%, 8.6% and 5.2%, respectively. PCV13 serotypes were hardly present in the three groups: 4.8% (19F, 19A), 9.8% (19F, 9V) and 25.0% (7F), while PPV23 serotypes were more prevalent: 34.5% (15B, 11A, 10A, 9N, 33F, 22F), 39.0% (11A, 15B, 10A, 17F) and 0.0% (Table 25). The leading serotypes were 15B (17.0%) in nurseries and 11A (26.8%) in DCCs. Out of the four isolates in primary school, two was serotype 37 and one of them was 7F which were missing from the other two groups. An increasing prevalence with age was observed in case of serotype 23B (2.4% in nurseries, 9.8% in DCCs and 25.0% among school children), serotype 11A (6.7% in nurseries vs. 26.8% in DCCs) and 6C (1.8% vs. 9.8%), while 15B and 24F showed decreasing tendencies (17.0% vs. 4.9% and 11.5% vs. 0.0%, respectively). Comparing nurseries and DCCs, both PCV13 (4.8% vs. 9.8%) and PPV23 (34.5% vs. 39.0%) coverage was higher in DCCs.

We divided the clinical specimens into two groups: deriving from patients <7 years (P1) and ≥7 years (P2) to make an easier comparison between carriers of the same age group and to monitor the patients (P2) who were probably not vaccinated against pneumococcus. This comparison can be found in Table 26. Even though there were only 83 isolates in P2, we identified 28 different serotypes, mostly non-vaccine types (NVTs, 38.6%) but PCV13 and PPV23 coverage was of a similar magnitude (32.5% and 28.9%). In P1, NVTs were absolutely dominant with 60.3%, whereas PCV13 and PPV23 serotypes distributed almost equally: 17.5% and 22.2%. The diversity of serotypes was smaller here in P1, comparable to that found among carriers (19 and 23 different types, respectively).

Carriers <7 years showed a spectacular tendency in vaccine coverage rate: only 5.8% for PCV13, 35.4% for PPV23 and 58.7% for NVT. 19F was represented in all three groups as a prominent PCV13 serotype, whereas serotype 3 was completely absent among carriers. On the other hand, type 3 was the leading serotype in older patients (15.7%)

explaining the high PCV13 coverage. Among PPV23 serotypes, 15B was the most frequent among carriers (14.6%) and in P1 (9.5%) whereas in P2 it had only 2.4%. Interestingly, 11A was the leading PPV23 serotype in P2 (12.0%) and it was the second leading in P1 (7.9%) and in carriers (10.7%). Absolutely different NVTs were specific in each group: 24F (9.2%), 35F (8.3%) 15C (7.3%) and 23A (6.8%) among carriers, 23B (22.2%) in P1, 15A (8.4%) and NT (7.2%) in P2, respectively. The serotype distribution in ranking order is depicted on Figure 11a, 11b and 11c for each category.

Table 25. PCV13, PPV23 and NVT serotype distribution among the three groups

Serotype	Nurseries (1-3y) n=165 (%)	DCCs (3-6y) n=41 (%)	Primary school (6-13y) n=4 (%)
PCV13 serotypes			
7F	0 (0.0)	0 (0.0)	1 (25.0)
9V	0 (0.0)	1 (2.4)	0 (0.0)
19A	1 (0.6)	0 (0.0)	0 (0.0)
19F	7 (4.2)	3 (7.3)	0 (0.0)
PPV23 serotypes			
9N	5 (3.0)	0 (0.0)	0 (0.0)
10A	9 (5.5)	2 (4.9)	0 (0.0)
11A	11 (6.7)	11 (26.8)	0 (0.0)
15B	28 (17.0)	2 (4.9)	0 (0.0)
17F	0 (0.0)	1 (2.4)	0 (0.0)
22F	1 (0.6)	0 (0.0)	0 (0.0)
33F	3 (1.8)	0 (0.0)	0 (0.0)
NVT serotypes			
6C	3 (1.8)	4 (9.8)	0 (0.0)
15A	10 (6.1)	0 (0.0)	0 (0.0)
15C	12 (7.3)	3 (7.3)	0 (0.0)
21	3 (1.8)	0 (0.0)	0 (0.0)
23A	14 (8.5)	0 (0.0)	0 (0.0)
23B	4 (2.4)	4 (9.8)	1 (25.0)
24F	19 (11.5)	0 (0.0)	0 (0.0)
31	0 (0.0)	3 (7.3)	0 (0.0)
34	5 (3.0)	1 (2.4)	0 (0.0)
35B	7 (4.2)	3 (7.3)	0 (0.0)
35F	16 (9.7)	1 (2.4)	0 (0.0)
37	0 (0.0)	0 (0.0)	2 (50.0)
38	4 (2.4)	0 (0.0)	0 (0.0)
NT	3 (1.8)	2 (4.9)	0 (0.0)
Total PCV13	8 (4.8)	4 (9.8)	1 (25.0)

Total PPV23	57 (34.5)	16 (39.0)	0 (0.0)
Total NVT	100 (60.6)	21 (51.2)	3 (75.0)

n: number of isolates

Table 26. Serotype comparison of carriers, patients <7 years and patients ≥7 years old

Serotype	carriers <7 y (n=206) (%)	patients <7 y (n=63) (%)	patients ≥7y (n=83) (%)
PCV13 serotypes			
1	0 (0.0)	0 (0.0)	1 (1.2)
3	0 (0.0)	5 (7.9)	13 (15.7)
6A	0 (0.0)	0 (0.0)	1 (1.2)
6B	0 (0.0)	0 (0.0)	1 (1.2)
9V	1 (0.5)	0 (0.0)	1 (1.2)
19A	1 (0.5)	2 (3.2)	2 (2.4)
19F	10 (4.9)	3 (4.8)	7 (8.4)
23F	0 (0.0)	1 (1.6)	1 (1.2)
PPV23 serotypes			
8	0 (0.0)	0 (0.0)	2 (2.4)
9N	5 (2.4)	0 (0.0)	4 (4.8)
10A	11 (5.3)	3 (4.8)	4 (4.8)
11A	22 (10.7)	5 (7.9)	10 (12.0)
15B	30 (14.6)	6 (9.5)	2 (2.4)
17F	1 (0.5)	0 (0.0)	1 (1.2)
22F	1 (0.5)	0 (0.0)	1 (1.2)
33F	3 (1.5)	0 (0.0)	0 (0.0)
NVT serotypes			
6C	7 (3.4)	0 (0.0)	1 (1.2)
15A	10 (4.9)	3 (4.8)	7 (8.4)
15C	15 (7.3)	1 (1.6)	1 (1.2)
16F	0 (0.0)	1 (1.6)	2 (2.4)
19C	0 (0.0)	0 (0.0)	1 (1.2)
21	3 (1.5)	5 (7.9)	0 (0.0)
23A	14 (6.8)	1 (1.6)	1 (1.2)
23B	8 (3.9)	14 (22.2)	3 (3.6)
24F	19 (9.2)	5 (7.9)	1 (1.2)
31	3 (1.5)	2 (3.2)	3 (3.6)
34	6 (2.9)	2 (3.2)	0 (0.0)
35B	10 (4.9)	1 (1.6)	3 (3.6)
35F	17 (8.3)	2 (3.2)	1 (1.2)
38	4 (1.9)	0 (0.0)	2 (2.4)
NT	5 (2.4)	1 (1.6)	6 (7.2)
Total PCV13	12 (5.8)	11 (17.5)	27 (32.5)
Total PPV23	73 (35.4)	14 (22.2)	24 (28.9)
Total NVT	121 (58.7)	38 (60.3)	32 (38.6)

n: number of isolates

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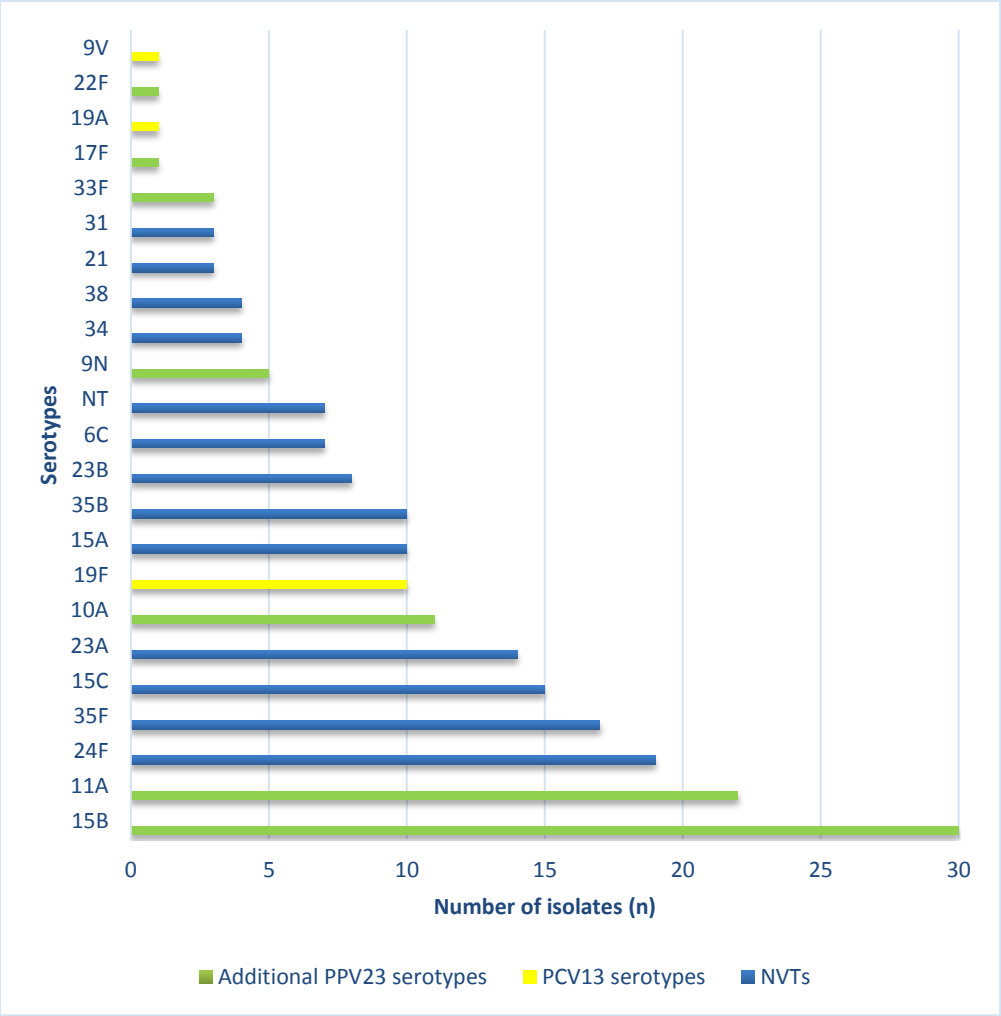

Figure 11a. Serotype distribution among carriers <7 years. Yellow columns, PCV13 serotypes; green columns, additional PPV23 serotypes; blue columns, NVTs.

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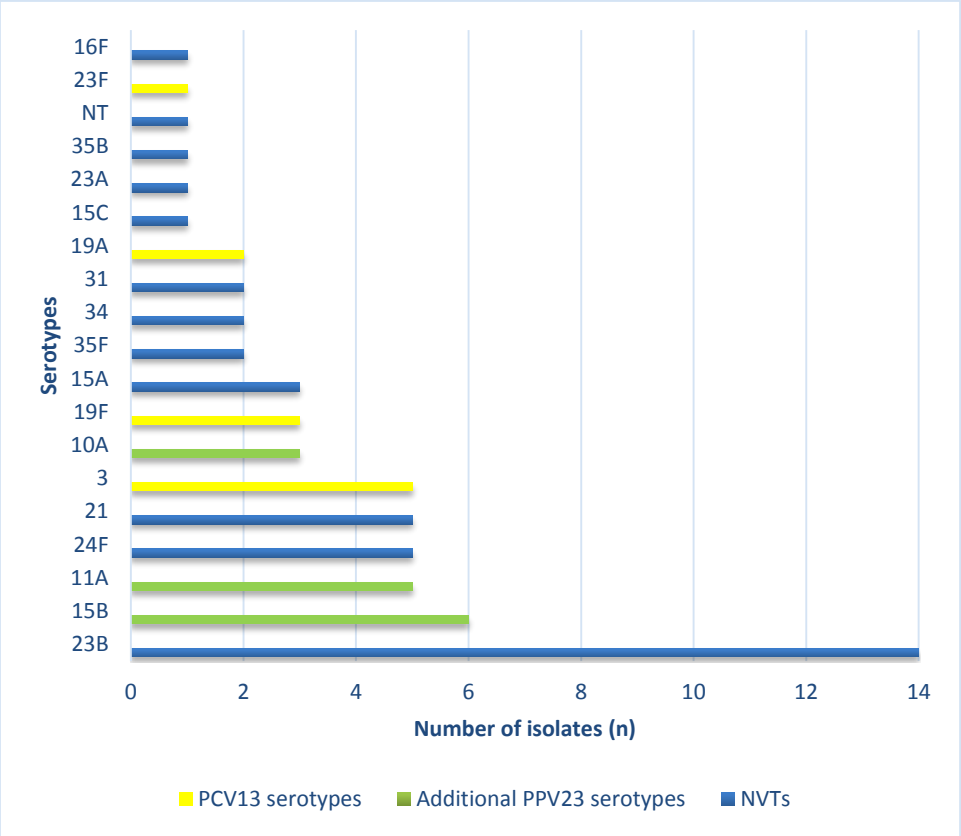

Figure 11b. Serotype distribution among patients <7 years. Yellow columns, PCV13 serotypes; green columns, additional PPV23 serotypes; blue columns, NVTs

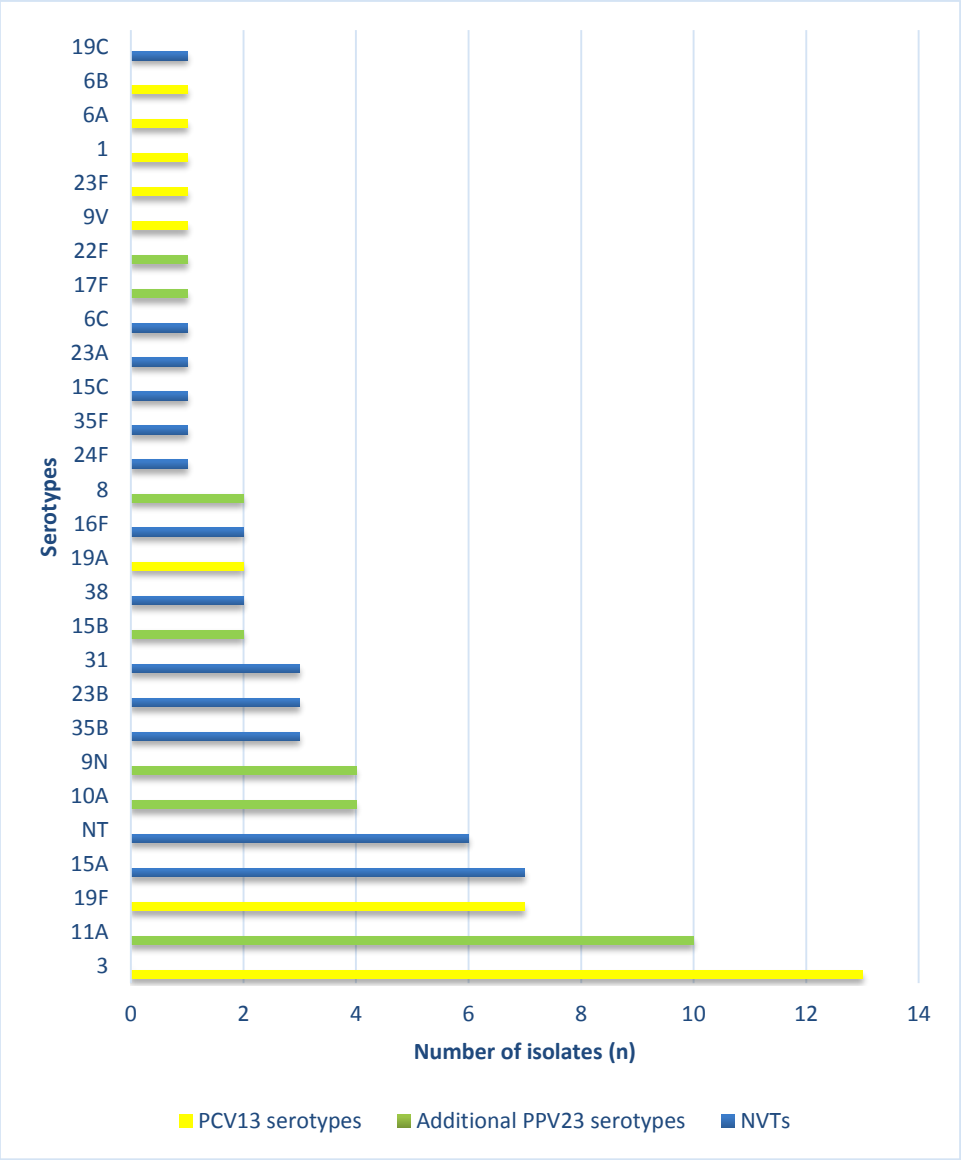

Figure 11c. Serotype distribution among patients ≥7 years. Yellow columns, PCV13 serotypes; green columns, additional PPV23 serotypes; blue columns, NVTs

4.3.2. H. influenzae

H. influenzae was not isolated in primary school children. In DCCs (n=37), 33 isolates (89.2%) were NTHi and 4 (10.8%) were serotype f. In nurseries (n=115), 110 (95.7%) NTHi were found, 3 (2.6%) f and 2 (1.7%) e. Type b was not found throughout the study.

4.3.3. M. catarrhalis

All isolates (n=9) deriving from primary school children belonged to serotype A. In DCCs (n=70), serotype A was represented in 68.6% (n=48), serotype B in 18.6% (n=13), serotype C in 2.9% (n=2) and 10.0% (n=7) were non-typeable (NT). Out of 202 isolates,

also serotype A was the most prevalent in nurseries with 89.1% (n=180), followed by B and C with 9.4% (n=19) and 1.0% (n=2). The remaining 1 isolate (0.5%) was NT.

4.4. Antibiotic susceptibility

4.4.1. S. pneumoniae

Full susceptibility was observed against imipenem and vancomycin, even among clinical isolates. Susceptibility results of isolates of carriers <7 years, P1 and P2 are compared in Table 27. In general, carriage isolates were the most susceptible and P2 isolates were the most resistant. This tendency was observed for all tested antibiotics. Penicillin resistant isolates were not found. Low level of intermediate resistance was observed: 13.1%, 17.5% and 25.3%. Regarding fluoroquinolones, 100% susceptibility was measured among carriers. In case of young patients, 4.8% of the isolates were resistant to levofloxacin, 7.9% to moxifloxacin. These percentages were almost doubled in P2 group: 8.4% and 14.5%. Erythromycin resistance was 17.5%, 19.0% and 28.9% in the three groups, respectively. The macrolide resistant isolates (n=36 among carriers, n=12 in P1 and n=24 in P2) were further checked for *erm(B)* and *mef* genes. *Erm(B)* was detected in 38 cases which showed MLSB phenotype: high MIC values for both erythromycin and clindamycin. *Mef(E)* was found in 29 cases which had lower MIC values (8–16 mg/L) for erythromycin and were susceptible to clindamycin, corresponding the classic M phenotype. In addition, both genes were present in three cases (one strain each of serotype 19A, 19F and NT). The serotype specific resistance distribution complemented with *erm(B), mef(E)* positivity is shown in Table 28. Erythromycin resistance was higher in the DCC group (29.3%) than in the nursery group (14.5%). This is due to the different serotype distribution because 11A was more prevalent in the DCC group (11/165 in nurseries vs. 11/41 in DCCs) and this type contributed significantly to erythromycin resistance. Besides 11A (always M phenotype), 19F, 15A, 15C and 19A (MLSB type) were the major macrolide resistant serotypes. Serotype 3, 10A, 23A, 23B, 24F, 35B and 35F isolates were sensitive mostly to all tested antibiotics. The four pneumococcus isolates from primary school were sensitive to all drugs except for one isolate (serotype 23 B) being intermediate resistant to penicillin.

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Table 27. Antibiotic susceptibility of pneumococcal isolates in three groups: carriers, patients <7 years and patients ≥7 years old

		MIC range	MIC_{50}	MIC_{90}	S (%)	I (%)	R (%)
Penicillin	Carriers <7y	<0.015-2	0.03	0.25	86.9	13.1	0.0
	Patients <7y	<0.015-2	<0.015	0.25	82.5	17.5	0.0
	Patients ≥7y	<0.015-2	<0.015	0.5	74.7	25.3	0.0
Cefotaxime	Carriers <7y	<0.004-1	0.03	0.125	96.1	3.9	0.0
	Patients <7y	0.008-2	0.03	0.25	95.2	4.8	0.0
	Patients ≥7y	<0.004-2	0.03	1	86.7	8.4	4.8
Imipenem	Carriers <7y	<0.004-0.5	0.015	0.06	100.0	0.0	0.0
	Patients <7y	0.008-2	0.06	0.5	100.0	0.0	0.0
	Patients ≥7y	<0.004-2	0.06	1	100.0	0.0	0.0
Erythromycin	Carriers <7y	<0.06->256	0.125	32	81.1	1.5	17.5
	Patients <7y	<0.06->256	0.125	256	81.0	0.0	19.0
	Patients ≥7y	<0.06->256	0.125	256	69.9	1.2	28.9
Clindamycin	Carriers <7y	<0.5->128	<0.5	<0.5	92.2	0.0	7.8
	Patients <7y	<0.5->128	<0.5	64	85.7	0.0	14.3
	Patients ≥7y	<0.5->128	<0.5	128	83.1	0.0	16.9
Levofloxacin	Carriers <7y	<0.5-2	1	2	100.0	0.0	0.0
	Patients <7y	<0.5->4	1	2	95.2	0.0	4.8
	Patients ≥7y	<0.5->4	1	2	91.6	0.0	8.4
Moxifloxacin	Carriers <7y	<0.03-0.5	0.25	0.25	100.0	0.0	0.0
	Patients <7y	0.125->0.5	0.25	0.5	92.1	0.0	7.9
	Patients ≥7y	<0.03->0.5	0.25	>0.5	85.5	0.0	14.5
Vancomycin	Carriers <7y	<0.125-1	0.5	1	100.0	0.0	0.0
	Patients <7y	0.25-1	0.5	1	100.0	0.0	0.0
	Patients ≥7y	<0.125-2	0.5	1	100.0	0.0	0.0

Table 28. Antibiotic susceptibility among the most frequent/important pneumococcal serotypes, including the detected macrolide resistance genes

Serotype (n)	Penicillin (n)	Cefotaxime (n)	Erythromycin (n)	Clindamycin (n)	*ermB*/*mefE*	Levofloxacin (n)	Moxifloxacin (n)
15B (38)	84.2% (32) S 15.8% (6) I	100.0% (38) S	86.8% (33) S 13.2% (5) R	86.8% (33) S 13.2% (5) R	n=5 *ermB*	100.0% (38) S	100.0% (38) S
11A (37)	94.6% (35) S 5.4% (2) I	100.0% (37) S	54.1% (20) S 45.9 (17) R	100.0% (37) S	n=17 *mefE*	100.0% (37) S	94.6% (35) S 5.4% (2) R
24F (25)	96.0% (24) S 4.0% (1) I	96.0% (24) S 4.0% (1) I	100.0% (25) S	100.0% (25) S	-	96.0% (24) S 4.0% (1) R	96.0% (24) S 4.0% (1) R
23B (25)	68.0% (17) S 32.0% (8) I	96.0% (24) S 4.0% (1) I	96.0% (24) S 4.0% (1) R	100.0% (25) S	n=1 *mefE*	100.0% (25) S	100.0% (25) S
19F (20)	75.0% (15) S 25.0% (5) I	85.0% (17) S 15.0% (3) I	25.0% (5) S 75.0% (15) R	45.0% (9) S 55.0% (11) R	n=10 *ermB* n=4 *mefE* n=1 *ermB*+*mefE*	95.0% (19) S 5.0% (1) R	95.0% (19) S 5.0% (1) R
15A (20)	65.0% (13) S 35.0% (7) I	100.0% (20) S	55.0% (11) S 45.0% (9) R	60.0% (12) S 40.0% (8) R	n=8 *ermB* n=1 *mefE*	100.0% (20) S	100.0% (20) S
35F (20)	100.0% (20) S	100.0% (20) S	100.0% (20) S	100.0% (20) S	-	100.0% (20) S	95.0% (19) S 5.0% (1) R
3 (18)	88.9% (16) S 11.1% (2) I	88.9% (16) S 5.6% (1) I 5.6% (1) R	100.0% (18) S	100.0% (18) S	-	94.4% (17) S 5.6% (1) R	88.9% (16) S 11.1% (2) R
10A (18)	100.0% (18) S	94.4% (17) S 5.6% (1) R	94.4% (17) S 5.6% (1) R	94.4% (17) S 5.6% (1) R	n=1 *ermB*	88.9% (16) S 11.1% (2) R	88.9% (16) S 11.1% (2) R
15C (17)	100.0% (17) S	100.0% (17) S	76.5% (13) S 23.5% (4) R	82.4% (14) S 17.6% (3) R	n=2 *ermB*	100.0% (17) S	100.0% (17) S
23A (16)	100.0% (16) S	100.0% (16) S	100.0% (16) S	100.0% (16) S	-	100.0% (16) S	100.0% (16) S
NT (14)	64.3% (9) S 35.7% (5) I	85.7% (12) S 14.3% (2) I	42.9% (6) S 57.1% (8) R	64.3% (9) S 35.7% (5) R	n=6 *ermB* n=1 *mefE* n=1 *ermB*+*mefE*	100.0% (14) S	100.0% (14) S
35B (14)	35.7% (5) S 64.3% (9) I	57.1% (8) S 35.7% (5) I 7.1% (1) R	92.9% (13) S 7.1% (1) I	100.0% (14) S	-	92.9% (13) S 7.1% (1) R	92.9% (13) S 7.1% (1) R
21 (8)	87.5% (7) S 12.5% (1) I	100.0% (8) S	100.0% (8) S	100.0% (8) S	-	87.5% (7) S 12.5% (1) R	75.0% (6) S 25.0% (2) R
19A (5)	60.0% (3) S 40.0% (2) I	80.0% (4) S 20.0% (1) I	40.0% (2) S 60.0% (3) R	40.0% (2) S 60.0% (3) R	n=2 *ermB* n=1 *ermB*+*mefE*	100.0% (5) S	100.0% (5) S

4.4.2. S. aureus

MRSA was not identified at all which was also confirmed by *mecA* negativity and cefoxitin screening. Regarding vancomycin, 100% sensitivity was measured. Table 29 shows the summarized antibiotic susceptibility data for *S. aureus, H. influenzae* and *M. catarrhalis*. Penicillin resistance was very high in all three groups: 73.7% in nurseries, 76.8% in DCCs and 82.8% in primary school, respectively. The second highest resistance rate was measured for erythromycin, but it was only 12.2%.

On the contrary, gentamicin resistance was absent only among the youngest children. Only one isolate appeared to be ciprofloxacin resistant (MIC = 2 mg/L) which was obtained from a child in the nursery group. Three isolates showed mupirocin resistance, all of them originated from DCCs in Pápa. This corresponds to a 2.4% prevalence in total, and 5.4% among the DCCs. As all of them expressed high level resistance (MIC >1024mg/L), all three possessed the *mupA* gene and none of them had point mutations in the *ileS* gene.

4.4.3. H. influenzae

Nine isolates in the nursery group (7.8%) and one from DCC (2.7%) were ampicillin resistant (Table 29). All 10 strains had a MIC value >8mg/L, but all were susceptible to amoxicillin/ clavulanic acid. This refers to beta-lactamase production which was confirmed by a positive nitrocefin disc test in every case. For cefotaxime and fluoroquinolons, 100% sensitivity was observed. Macrolide resistance was found in both groups, a bit higher percentage in case of DCCs: erythromycin^{R} 1.7% vs. 8.1%; azithromycin^{R} 8.7% vs. 10.8%. The highest resistance was detected for TMP/SMX (11.3% in the nurseries and 24.3% in the DCCs). To highlight the nine serotypeable isolates, all of them were fully susceptible to all tested antibiotics.

4.4.4. M. catarrhalis

In general, 100% sensitivity was documented in the case of amoxicillin-clavulanic acid, cefotaxime and moxifloxacin (Table 29). Additionally, primary school isolates were fully sensitive to all tested antibiotics. Only five isolates, all deriving from nurseries, were levofloxacin resistant, but only with low MIC values: 5mg/L (n=4) and 0.25mg/L (n=1). Non-susceptibility to macrolides and TMP/SMX was also below 5% and always with low MIC values.

Table 29. Summarized antibiotic susceptibility data for *S. aureus*, *H. influenzae* and *M. catarrhalis*

Bacteria (n) and antibiotics	MIC range (mg/L)	MIC50 (mg/L)	MIC90 (mg/L)	S (%)	I (%)	R (%)
S. aureus (n=123)						
Penicillin	<0.03-256	8	64	22.8	0.0	77.2
Oxacillin	0.06-0.5	0.25	0.5	100.0	0.0	0.0
Tetracycline	0.25->4	0.5	1	98.4	0.0	1.6
Erythromycin	0.19->256	0.19	>256	87.8	0.0	12.2
Clindamycin	0.094-1	0.094	0.25	99.2	0.0	0.8
Gentamicin	0.5-4	1	1	95.1	0.0	4.9
Ciprofloxacin	0.25-2	0.5	1	99.2	0.0	0.8
Mupirocin	<0.03->1024	0.06	0.06	97.6	0.0	2.4
Vancomycin	1-2	1	2	100.0	0.0	0.0
H. influenzae (n=152)						
Ampicillin	0.25->8	1	1	93.4	0.0	6.6
Amoxicillin-clavulanic acid	0.125-0.75	0.5	0.5	100.0	0.0	0.0
Cefotaxime	0.008-0.06	0.015	0.06	100.0	0.0	0.0
Levofloxacin	<0.004-0.125	0.008	0.015	100.0	0.0	0.0
Moxifloxacin	<0.004-0.5	0.03	0.06	100.0	0.0	0.0
Erythromycin	<0.25-32	8	16	96.7	0.0	3.3
Azithromycin	0.06-8	2	4	90.8	0.0	9.2
TMP/SMX	0.008->32	0.03	>32	85.5	0.0	14.5
M. catarrhalis (n=281)						
Amoxicillin-clavulanic acid	<0.06-0.38	0.094	0.25	100.0	0.0	0.0
Cefotaxime	<0.004-1	0.25	0.5	100.0	0.0	0.0
Levofloxacin	0.015-0.5	0.03	0.06	98.2	0.0	1.8
Moxifloxacin	0.06-0.25	0.06	0.125	100.0	0.0	0.0
Erythromycin	<0.125-16	0.25	0.25	96.1	2.5	1.4
Azithromycin	<0.06-8	0.06	0.06	97.9	0.4	1.8
TMP/SMX	0.015-2	0.125	0.25	98.9	0.4	0.7

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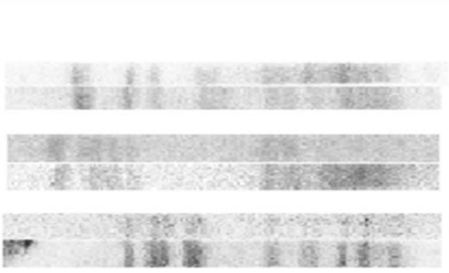
4.5. Genotyping results

4.5.1. S. pneumoniae

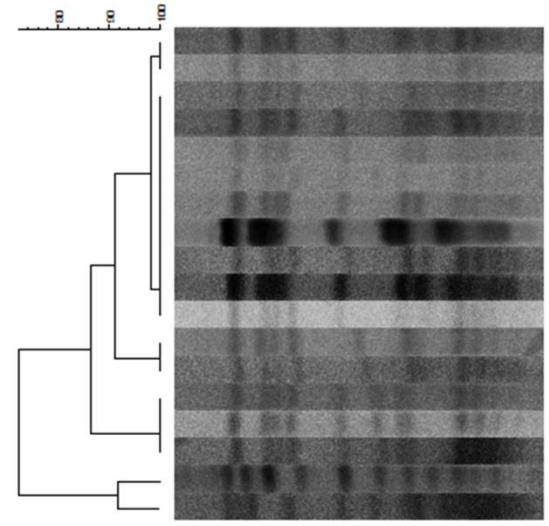
Interestingly, fully identical PFGE profiles of clinical and carried isolates were found in case of serotypes 11A, 19A and 19F, despite the fact that there was sometimes one year difference between the sampling dates of the similar pairs (Figure 12).



Strain	Origin	Serotype	Peni	Ery	Clinda	Date of isolation
16775	clinical	11A	<0.015	<0.06	<0.5	Apr-2015
BT10	carried	11A	<0.015	0.125	<0.5	Apr-2016
14749	clinical	19A	<0.015	0.125	<0.5	Apr-2015
BT75	carried	19A	0.03	0.125	<0.5	Apr-2016
45354	clinical	19F	0.03	256	128	Oct-2015
BT7	carried	19F	0.06	>256	>128	Apr-2016

Figure 12. Identical restriction pattern of clinical and carried isolates among serotypes 11A, 19A and 19F.

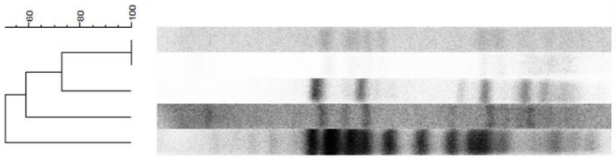
Regarding serotype 3, half of the isolates were indistinguishable (sharing the same pattern) and the rest of the clinical isolates were also closely or possibly related. Besides their general susceptibility to antibiotics, the two penicillin intermediate isolates (16744, 52128) did not belong to the same clone (Figure 13).



Strain	Serotype	Peni	Ery	Clinda	Date of isolation
19562	3	<0.015	<0.06	<0.5	May-2015
48 HP	3	<0.015	0.125	<0.5	2016
45908	3	<0.015	<0.06	<0.5	Oct-2015
16744	3	1	0.25	<0.5	Apr-2015
24420	3	<0.015	<0.06	<0.5	Jun-2015
25747	3	<0.015	<0.06	<0.5	Jun-2015
22117	3	0.03	0.125	<0.5	May-2015
38844	3	0.03	0.125	<0.5	Aug-2015
3713	3	<0.015	<0.06	<0.5	2016
7682	3	<0.015	0.125	<0.5	2016
17837	3	<0.015	<0.06	<0.5	Apr-2015
14277	3	<0.015	<0.06	<0.5	Mar-2015
59807	3	<0.015	0.25	<0.5	Dec-2015
19394	3	<0.015	<0.06	<0.5	Apr-2015
19559	3	<0.015	0.125	<0.5	May-2015
49195	3	<0.015	0.125	<0.5	Oct-2015
19521	3	<0.015	<0.06	<0.5	May-2015
52128	3	0.25	0.5	<0.5	Nov-2015

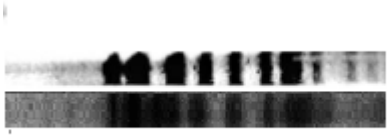
Figure 13. PFGE pattern of serotype 3 isolates

The restriction pattern of serotype 19A isolates was very variable (Figure 14). Only the above mentioned clinical and carried isolates were identical. The least related isolate (8754) had the same pattern as a previously isolated strain (M41/2), originating also from a carriage study in 2010 (37), which turned out to belong to the worldwide circulating resistant ST320 clone (Figure 15). Not only their *SmaI* restriction pattern but their MIC values were identical as well. In addition, isolate 8754 expressed *erm(B)* and *mef(E)* genes together.



Strain	Serotype	Peni	Ery	Clinda	Date of isolation
14749	19A	<0.015	0.125	<0.5	Apr-2015
BT75	19A	0.03	0.125	<0.5	Apr-2016
40874	19A	0.125	>256	>128	Sep-2015
34192	19A	<0.015	>256	>128	Jul-2015
8754	19A	2	>256	>128	Feb-2016

Figure 14. PFGE pattern of serotype 19A isolates

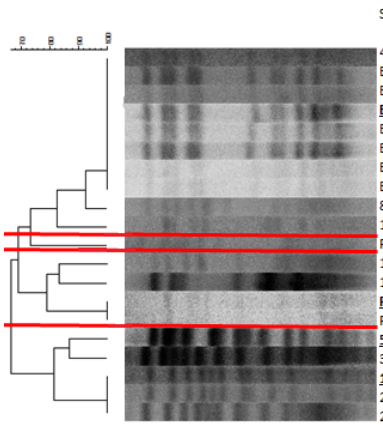


Strain	Serotype	Year	Peni	Ery	Clinda
M41/2	19A	2010	2	>256	>128
8754	19A	2016	2	>256	>128

Figure 15. Two ST320 isolates of serotype 19A

Genetic relatedness of serotype 19F is described on Figure 16. Their PFGE patterns showed three major clusters which corresponded well with their antibiotic sensitivity. The penicillin susceptible and high level macrolide and lincosamide resistant isolates formed the first and largest cluster, all of them deriving from Budapest. The second, smaller cluster comprised of fully susceptible isolates. Finally, cluster 3 contained only clinical isolates which had penicillin intermediate level resistance and low level erythromycin resistance, with one exception. Isolate 57103 (the only strain with elevated penicillin MIC and MLSB phenotype) stood alone in a separate subcluster. One representative of each three clusters and this latter strain (indicated with bold face and underlining) were chosen for MLST analysis. Based on this, cluster 1 strains belonged to ST179, cluster 2 to ST180. Isolate 57103 proved to be a member of the ST320 sequence type. Similarly to the previously mentioned ST320 isolate among serotype 19A isolates, 57103 also possessed *erm(B)* and *mef(E)* genes. The MLST type of the other cluster 3 isolate was ST651. ST651

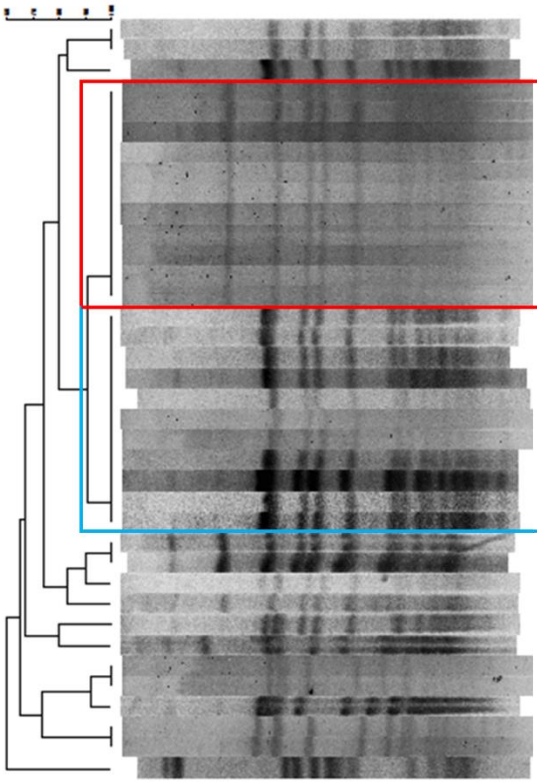
belongs to the same clonal complex as ST320 (CC-271) which means they differ only in one out of the seven housekeeping loci according to the BURST algorithm (211, 212).



Strain	Serotype	Peni	Ery	Clinda	Date of isolation	
45354	19F	0.03	256	128	Oct-2015	
BT7	19F	0.06	>256	>128	Apr-2016	
BT8	19F	0.06	256	>128	Apr-2016	
BT104	19F	0.06	>256	>128	May-2016	
BT112	19F	0.06	>256	>128	May-2016	Cluster 1
BT115	19F	0.06	>256	>128	May-2016	
BT122	19F	0.06	>256	>128	May-2016	
BT123	19F	0.06	>256	>128	May-2016	
8431	19F	<0.015	>256	>128	Feb-2015	
11204	19F	0.03	256	>128	Mar-2015	
PP273	19F	0.03	0.125	<0.5	Feb-2016	
18124	19F	0.06	0.125	<0.5	Apr-2015	
11406	19F	<0.015	<0.06	<0.5	Mar-2015	
PP266	19F	0.03	0.25	<0.5	Feb-2016	Cluster 2
PP272	19F	0.03	0.25	<0.5	Feb-2016	
57103	19F	1.5	>256	>128	Dec-2015	
38198	19F	1	8	<0.5	Aug-2015	
18017	19F	1	8	<0.5	Apr-2015	Cluster 3
24536	19F	2	8	<0.5	Jun-2015	
23535	19F	1	8	<0.5	2015	

Figure 16. PFGE dendrogram of serotype 19F strains

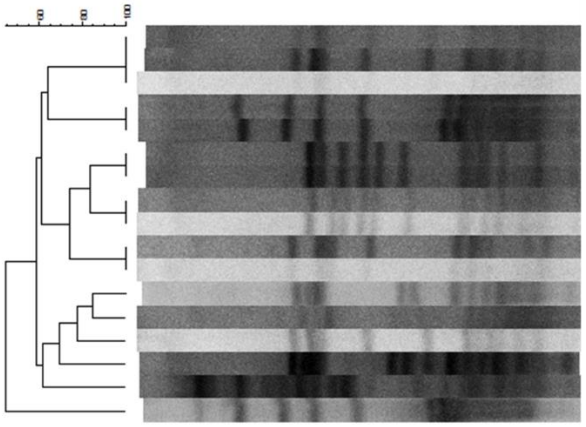
Figure 17 shows the digestion pattern of serotype 11A isolates. Two major clusters could be detected: The first one indicated with red square, containing isolates representing M phenotype, the second one in the blue square, comprising of sensitive isolates. The members of the first cluster derived from Pápa, whereas the second cluster included both clinical and carried isolates from Pápa and Budapest.



Strain	Serotype	Peni	Ery	Clinda	Date of isolation
16775	11A	<0.015	<0.06	<0.5	Apr-2015
BT10	11A	<0.015	0.125	<0.5	Apr-2016
38841	11A	<0.015	0.125	<0.5	Aug-2015
PP138	11A	0.06	32	<0.5	Nov-2015
PP178	11A	0.06	16	<0.5	Jan-2016
PP174	11A	0.06	32	<0.5	Jan-2016
PP64	11A	0.06	16	<0.5	Nov-2015
PP83	11A	0.06	32	<0.5	Nov-2015
PP79	11A	0.06	32	<0.5	Nov-2015
PP31	11A	0.03	16	<0.5	Nov-2015
PP69	11A	0.06	16	<0.5	Nov-2015
PP68	11A	0.06	32	<0.5	Nov-2015
PP92	11A	0.06	32	<0.5	Nov-2015
PP65	11A	0.06	32	<0.5	Nov-2015
20971	11A	<0.015	0.125	<0.5	May-2015
38380	11A	<0.015	0.125	<0.5	Aug-2015
48684	11A	0.06	<0.06	<0.5	Oct-2015
14699	11A	<0.015	0.25	<0.5	Apr-2015
28627	11A	0.03	0.125	<0.5	Jun-2015
PP38	11A	0.03	0.125	<0.5	Nov-2015
PP39	11A	0.03	0.125	<0.5	Nov-2015
BT172	11A	<0.015	0.125	<0.5	May-2016
BT71	11A	<0.015	<0.06	<0.5	Apr-2016
BT69	11A	0.03	0.25	<0.5	Apr-2016
BT196/2	11A	<0.015	0.125	<0.5	May-2016
59072	11A	<0.015	32	<0.5	Dec-2015
59068	11A	0.125	32	<0.5	Dec-2015
37735	11A	<0.015	0.125	<0.5	Aug-2015
38289	11A	0.06	16	<0.5	Aug-2015
25355	11A	<0.015	0.125	<0.5	Jun-2015
37278	11A	<0.015	16	<0.5	Aug-2015
PP76	11A	0.03	0.125	<0.5	Nov-2015
PP74	11A	0.03	0.125	<0.5	Nov-2015
38605	11A	<0.015	0.125	<0.5	Aug-2015
6/6/9	11A	0.03	16	<0.5	Dec-2015
6/6/10	11A	0.03	8	<0.5	Dec-2015
26768	11A	0.125	0.125	<0.5	2015

Figure 17. PFGE dendrogram of the serotype 11A pneumococcal isolates

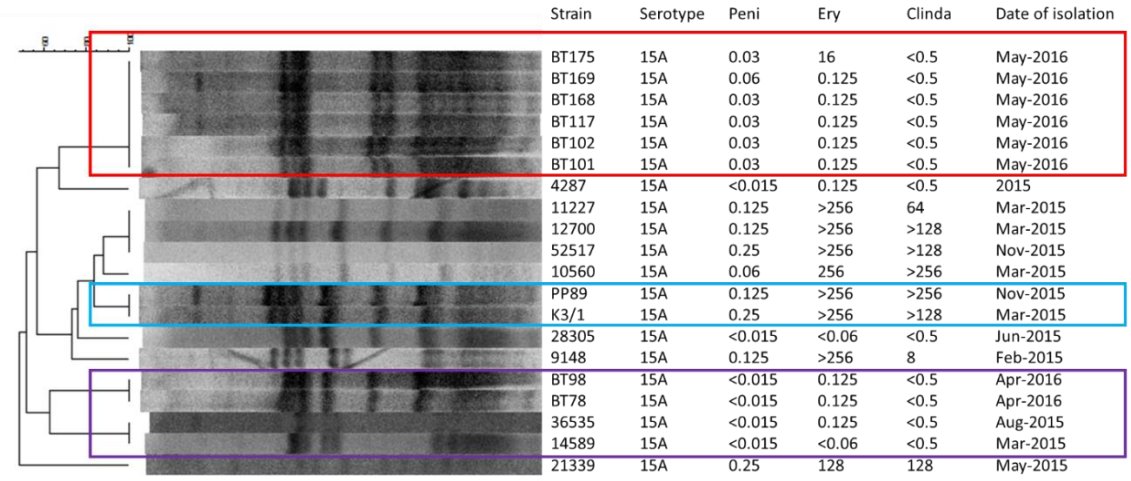
Serotype 15C isolates deriving from the same institutes showed close relatedness (e.g. BT189, BT87 and BT97 or PP55 and PP103) as it can be seen on Figure 18. However, sometimes isolates even from different cities had indistinguishable pattern (e.g. 6/3/2 and PP7 from Budapest and Pápa).



Strain	Serotype	Peni	Ery	Clinda	Date of isolation
BT189	15C	<0.015	0.125	<0.5	May-2016
BT87	15C	<0.015	<0.06	<0.5	Apr-2016
BT97	15C	0.03	0.125	<0.5	Apr-2016
6/3/2	15C	0.03	0.25	<0.5	Dec-2015
PP7	15C	0.03	0.125	<0.5	Nov-2015
BT129	15C	0.03	0.125	<0.5	May-2016
BT130	15C	0.03	0.125	<0.5	May-2016
BT62/1	15C	<0.015	0.125	<0.5	Apr-2016
BT100	15C	0.03	0.125	<0.5	Apr-2016
PP55	15C	0.03	32	1	Nov-2015
PP103	15C	0.03	32	1	Nov-2015
394	15C	<0.015	<0.06	<0.5	2016
PP170	15C	0.06	>256	<0.5	Jan-2016
BT89	15C	0.03	0.125	<0.5	Apr-2016
PP26	15C	0.03	0.125	<0.5	Nov-2015
PP13	15C	0.03	>256	>128	Nov-2015
49658	15C	<0.015	0.125	<0.5	Nov-2015

Figure 18. PFGE dendrogram of the serotype 15C pneumococcal isolates

Serotype 15A was a really diverse group (Figure 19). The biggest cluster (squared with red), consisting of carried isolates from Budapest, showed full susceptibility except for BT175 (M phenotype). PP89 and K3/1 (indicated with blue) were identical, however, they derived from Pápa and Székesfehérvár, respectively. Between the red and blue groups, four indistinguishable or closely related clinical isolates, having MLS_B phenotype, are found. The last group (indicated with purple) contained two pairs of related isolates each of carried (BT98, BT78) and clinical (36535, 14589) ones.



Strain	Serotype	Peni	Ery	Clinda	Date of isolation
BT175	15A	0.03	16	<0.5	May-2016
BT169	15A	0.06	0.125	<0.5	May-2016
BT168	15A	0.03	0.125	<0.5	May-2016
BT117	15A	0.03	0.125	<0.5	May-2016
BT102	15A	0.03	0.125	<0.5	May-2016
BT101	15A	0.03	0.125	<0.5	May-2016
4287	15A	<0.015	0.125	<0.5	2015
11227	15A	0.125	>256	64	Mar-2015
12700	15A	0.125	>256	>128	Mar-2015
52517	15A	0.25	>256	>128	Nov-2015
10560	15A	0.06	256	>256	Mar-2015
PP89	15A	0.125	>256	>256	Nov-2015
K3/1	15A	0.25	>256	>128	Mar-2015
28305	15A	<0.015	<0.06	<0.5	Jun-2015
9148	15A	0.125	>256	8	Feb-2015
BT98	15A	<0.015	0.125	<0.5	Apr-2016
BT78	15A	<0.015	0.125	<0.5	Apr-2016
36535	15A	<0.015	0.125	<0.5	Aug-2015
14589	15A	<0.015	<0.06	<0.5	Mar-2015
21339	15A	0.25	128	128	May-2015

Figure 19. PFGE dendrogram of the serotype 15A pneumococcal isolates

4.5.2. S. aureus

One of the *S. aureus* isolates could not be digested by *SmaI* enzyme consequently, hence only 122 PFGE patterns were available for comparison. High-grade diversity can be seen on Figure 20 with many smaller clusters comprising of 2-5 members. Identical isolates from the same town as well as from different towns were observed, two examples of them is marked with blue.

Attention should be payed to the three mupirocin resistant isolates from which two of them had the very same pattern and their antibiotic resistance pattern completely correlates with this partition (Figure 21). The third isolate (PP264) had a slightly modified PFGE and antibiotic profile (it was sensitive to erythromycin) but this was also in close relationship with the other two. It is supported by the information that all three isolates derived from Pápa, but the third one from a different DCC.

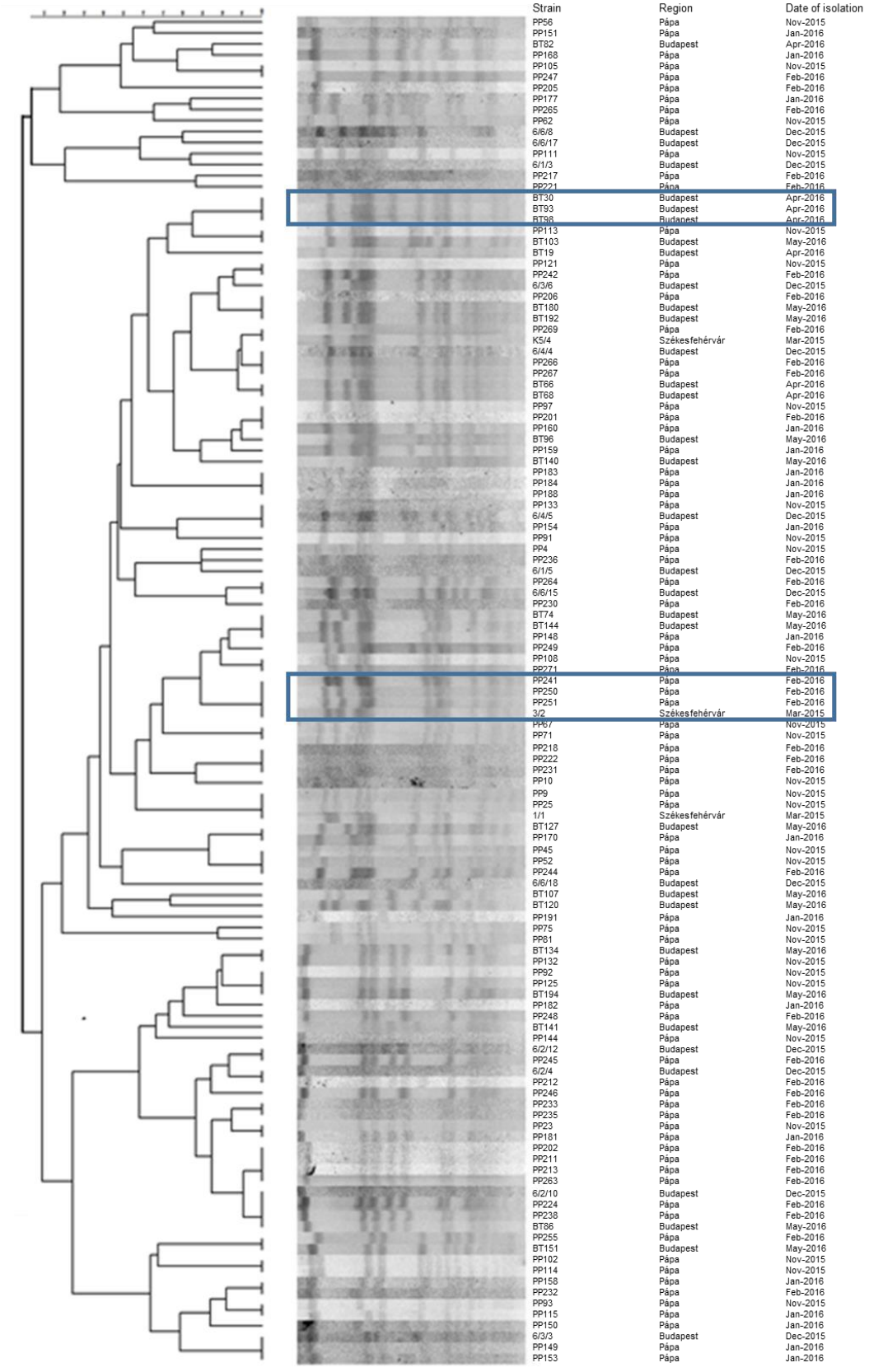
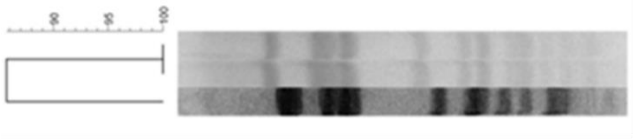


Figure 20. PFGE dendrogram of all *S. aureus* isolates from this study

DOI:10.14753/SE.2022.2609



Strain	Peni	Ery	Clinda	Date of isolation
PP159	4	>256	0.5	Jan-2016
PP160	8	>256	0.5	Jan-2016
PP264	4	2	0.5	Feb-2016

Figure 21. PFGE pattern of the three mupirocin resistant *S. aureus* isolates

4.5.3. H. influenzae

Out of the 143 isolates, two could not be digested by *Sma*I, hence only 141 strains were available for PFGE analysis. NTHi and typable isolates are shown on two separate figures. NTHi isolates differed a lot genetically, only a few major clusters could be determined (Figure 22). Within these clusters, closely or possibly related isolates could be found. Regarding the origin of isolates, the clusters are variable, isolates from the three different towns belonged to the same group. However, the absolute indistinguishable isolates always came from the same place except for one perfect match in the second squared group: identical isolates derived from Pápa and Budapest as well.

DOI:10.14753/SE.2022.2609

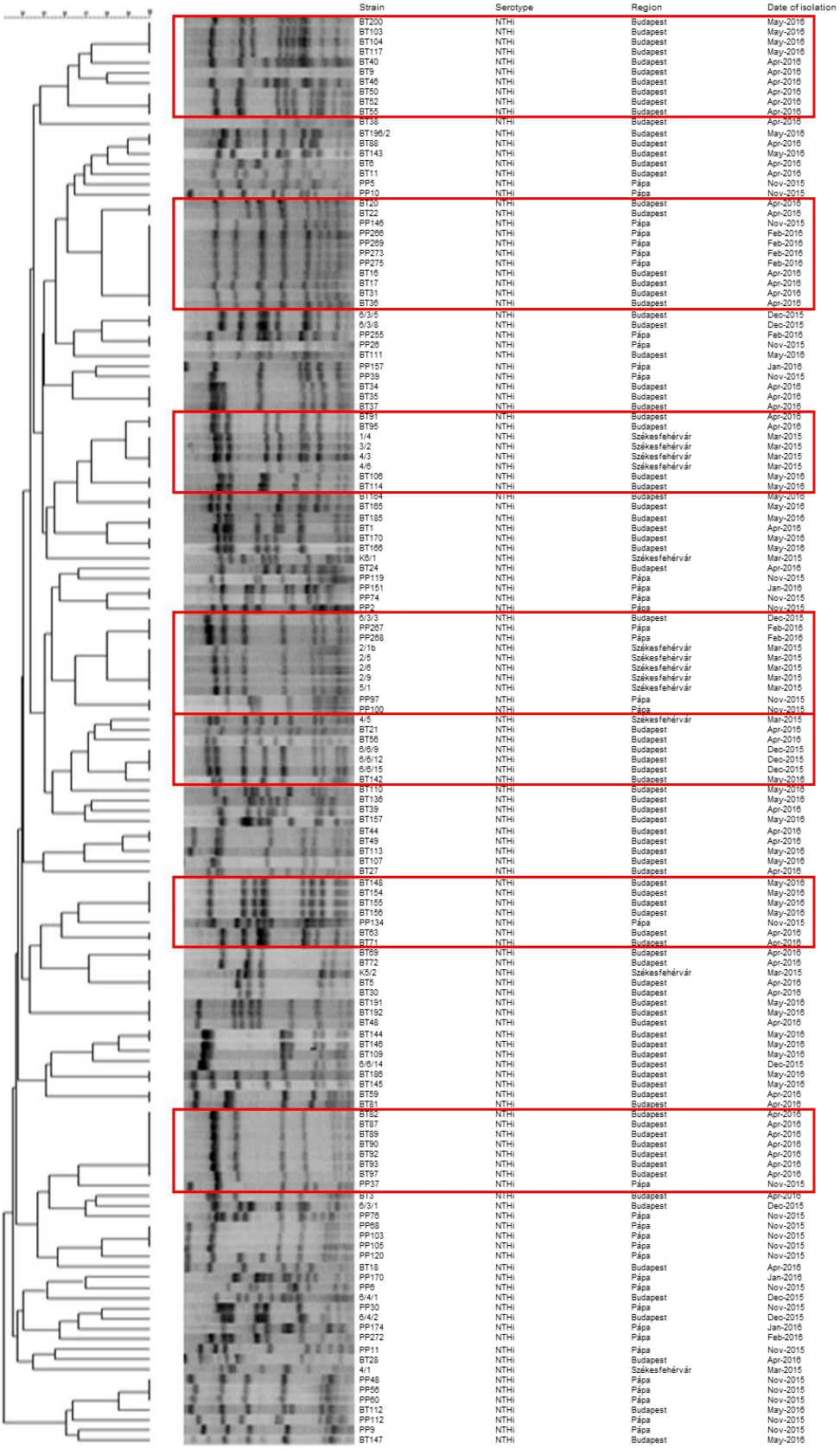
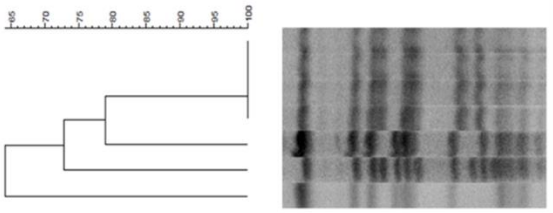
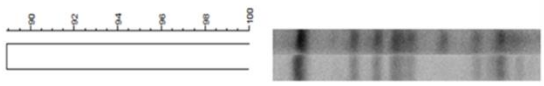


Figure 22. PFGE dendrogram of the NTHi isolates

Four of the seven *H. influenzae* serotype f isolates – originating from the same DCC – were indistinguishable (Figure 23), furthermore, their MIC values were the same. The other three strains were possibly related according to Tenover's criteria which were collected not only in different institutes but in different towns. The serotype e isolates differed only in one band so they were closely related (Figure 23).



Strain	Serotype	Ampi	AMC	TMP/SMX	Region	Date of isolation
PP257	f	1	0.75	0.25	Pápa	Feb-2016
PP259	f	1	0.75	0.25	Pápa	Feb-2016
PP263	f	1	0.75	0.25	Pápa	Feb-2016
PP264	f	1	0.75	0.25	Pápa	Feb-2016
PP17	f	0.5	0.5	0.125	Pápa	Nov-2015
2/8	f	0.5	0.5	0.125	Sz.fehérvár	Mar-2015
BT32	f	1	0.5	0125	Budapest	Apr-2016



Strain	Serotype	Ampi	AMC	TMP/SMX	Region	Date of isolation
BT115	e	0.25	0.5	0.03	Budapest	May-2016
BT25	e	0.25	0.125	0.03	Budapest	Apr-2016

Figure 23. PFGE pattern of the serotype f and e *H. influenzae* isolates

5. Discussion

5.1. Carriage rate

In our study, we had the opportunity to screen the carriage rates of four respiratory tract pathogens in different age groups. *S. aureus* was the only bacterium of which prevalence increased by age: 11.3% in nurseries, 30.1% in DCCs and 50.0% in primary school. On the contrary, the prevalence of the other three species decreased by age. A survey conducted in the Czech Republic among 3-6 years old children, had similar age-related observation: According to their results, children under 4 years tended to carry *S. pneumoniae* more frequently, while *S. aureus* colonization was higher among older children (>4 years). The carriage of *H. influenzae* and *M. catarrhalis* declined with age, similar to our data (213). In a Belgian study, younger age (under 4 years) was positively associated with *M. catarrhalis* and negatively associated with *S. aureus* carriage (214).

S. pneumoniae reaches the peak incidence during the first three years of life, based on the results of Bogaert et al (55% among 3 years old children). After a gradual decrease by age, carriage rate stabilizes at around 8% after the age of 10 years (215). Our study fits well with the literature: *S. pneumoniae* carriage peaked at 48.8% in 1-3 years old children, decreased to 21.5% among 3-6 years old children and further to 6.9% in the oldest age group.

Regarding *S. aureus*, a peak incidence was found at the age of 11 years (~50%) by Bogaert et al. which correlates well with our results (215, 216). In younger age groups, about 20-40% prevalence can be measured: 19.4% was reported in children under 5 years in Uganda (217), 13.5% in two years old children in the Netherlands (218), 35% in India (among children aged 1-6 years) (219).

Asymptomatic carriage data are available from previous Hungarian studies as well. Between 2009 and 2011, 878 DCC-attending healthy children were screened, *S. aureus* nasal carriage was found to be 21.3% (193). In 2012, 1390 children attending DCCs in Szolnok were tested. There, the overall carriage rate was notably higher: 34.1% (220). We measured 30.1% in our current survey.

Before PCV7 was introduced (in 2009-2010), 34.1% pneumococcal colonization was detected in Hungary and still 32.5% in 2011-2012 (early post-PCV7 era). This value dropped by 2015-2016 to 21.5% in the same age group (37).

Concluding from the previous Hungarian data, *S. aureus* carriage remained stable over the tested 7-year period, while *S. pneumoniae* decreased.

In case of *H. influenzae*, 35.5% carriage rate was observed among 0–6 years old children in Japan (221), 15.6% among preschool children (aged 5–6 years) in Turkey (222) and 24.9% in DCC children in the Czech Republic (213). Our corresponding percentages were 34.2% in nurseries and 19.9% in DCCs. No isolates were found in primary school. A Kenyan work reported also a very low prevalence in the age group of 10-19 years (4/104; 3.8%) (223).

Decreasing tendency was observed for *M. catarrhalis* carriage, 60.1%, 37.6% and 15.5% in nurseries, DCCs and primary school, respectively. Similar rates were observed in Japan in children <6 years old (58.1%) (221). In Turkey, 23.9% was reported among 6–10 years old children (224). 3-6 years old children were involved in the Czech and the Belgian surveys where 16.0% and 41.9% were found (213, 214).

Only a few European research groups reported about carriage rates in adulthood. A *S. aureus* nasal carriage rate of 27.3% was detected in Norwegian adults (225), and 37.4% in Switzerland (226). A study, conducted in Germany showed that it was still 28.5% among the elderly (≥65 years) (227). In the very same study, *H. influenzae* was found in only 1.9% of all participants and *S. pneumoniae* was not detected at all. Low colonization rate was also described among Swedish adults: 0.8% for *S. pneumoniae*, 2.7% for *H. influenzae* and 1.9% for *M. catarrhalis*, respectively (228).

Besides Europe, also low percentages were measured for *S. pneumoniae* and *H. influenzae* carriage among adults in Africa. *S. pneumoniae* rate decreased from 7.8% (20-29 years) further to 3.2% (30-49 years), but it slightly increased again above 50 years of age (4.7%). In the same age groups, 3.9%, 1.1% and 2.8% was measured for *H. influenzae* (223). Prevalence of *S. aureus* carriage was 28.8% among healthy adults (≥20 years) in Vietnam and 11.7% was measured for *M. catarrhalis* in Yemen (229, 230).

5.2. Effect of vaccination

5.2.1. Effect of PCVs on other species

The effect of PCVs on other members of the nasal flora can be estimated by long-term pneumococcal carriage surveillances. In the Netherlands, 11 months old, 24 months old children and their parents were followed-up: a significant decline in *S. pneumoniae* colonization was established when testing after 4.5 years post-PCV7 in all groups. Simultaneously, prevalence of *S. aureus, H. influenzae* and *M. catarrhalis* significantly increased. Seven years after the implementation of PCV7, pneumococcal carriage level was lower than at the beginning of the study but the ratios of the other three species started to decline from this point (231, 232). Official national support of vaccination with PCV7 resulted an elevated carriage of *H. influenzae* and *M. catarrhalis* among DCC children in Japan (221). Although *S. pneumoniae* carriage rate was not different after immunization with PCV7, *H. influenzae* and *M. catarrhalis* were more frequently isolated from the nasopharynx of children diagnosed with acute otitis media in the USA (233). It is a limitation of our study that we cannot assess the indirect effect of PCV13 vaccination regarding *H. influenzae* and *M. catarrhalis* as we do not have base-line data from the pre-PCV13 era in Hungary.

5.2.2. Effect of PCVs on *S. pneumoniae* serotype distribution

As described by many authors, PCV vaccines cause a marked shift in pneumococcal serotype distribution (231, 234, 235). The ratio of PCV13 serotypes clearly decreased in parallel to the increasing PCV vaccination rates in Hungary. Before the introduction of the conjugate vaccines, 57.3% was measured, later it dropped to 32.5% in the post-PCV7-pre-PCV13 era (37). In the current study, a further spectacular decrease was observed: the PCV13 serotype prevalence was only 9.8% in DCCs and 4.8% in nurseries. There were only four PCV13 serotypes (19F, 19A, 9V, 7F) which were present in the tested population and 19F was the most dominant (10/13). The frequently antibiotic resistant 19A serotype, which can pose a serious threat, was hardly present before PCV7 vaccination began (1.4%) then it rapidly became the dominant type (11.5%) in the post-PCV7 era (2011–2012) (37). After this rise, it seems to have fallen back by now to as low as 0.5% among carriers <7 years due to the extended coverage of PCV13 vaccine. Serotype 3, 6A, 6B or 23F have completely vanished by now. In the pre-PCV13 era, serotype 3 and 6A contributed significantly to the high PCV13 coverage, with a

prevalence of ~5-7% each (37). Their disappearance is very important, as they have a high potential to cause invasive diseases. On the contrary, the replacing new serotypes have a low invasive potential (236-239). PPV23 serotypes were more prevalent, out of them, 15B (17.0% in the nurseries) and 11A (26.8% in the DCCs) were the leading ones. According to the annual European Centre for Disease prevention and Control (ECDC) reports, 11A was one of the top ten IPD causing serotypes in the timeframe of our study (2015-2016) (240). By 2018, 11A was displaced from this ranking (27).

Clinical isolates had a higher PCV13 coverage: 17.5% in P1 and 32.5% in P2. This reflects well that PCV13 has a delayed effect on clinical isolates while the vaccine has a direct protecting effect on asymptomatic carriers resulting a faster serotype replacement (241). The most outstanding example is the presence of serotype 3 in P1 (7.9%) and P2 (15.7%). This serotype was ranking number one among IPD in Europe in 2014, but serotype 8 overtook the first place by 2015. Serotype 3 still kept the second place according to the latest available ECDC data. Serotype 3 is found predominantly in the elderly (27). 6A (1.2% in P2), 6B (1.2% in P2) and 23F (1.6% in P1 and 1.2% in P2) were also present among clinical isolates representing PCV13 serotypes.

23B dominated in P1 (22.2%) as a replacing NVT followed by 15B (9.5%). In P2, 11A was the most frequent NVT (12.0%) followed by 15A (8.4%). 23B and 15A are also found in the list of most common serotypes responsible for IPD (27).

Worryingly, 19F was represented in a bigger proportion both in the carried and clinical groups (4.9% in carriers, 4.8% in P1 and 8.4% in P2). It was unexpected based on the previous Hungarian results: in 2009–2010, i.e. in the pre-PCV7 era (when the average PCV7 vaccination of the enrolled children was only 16.4%), 19F was present at 9.6%. Between 2010 and 2012, as the vaccination rate increased to 46.0%, significantly lower 19F rate (1.7%) was found ($p = 5.0 * 10^{-7}$) (37). But it seems to creep back again. Similar results were found in the USA. In St. Louis, n=11/88 serotype 19F was identified, despite high vaccine uptake (86.7%). In Alaska, 19F ratio significantly increased among 5-17 years old children from 2008-2009 (0.3%) to 2011-2012 (4.8%). This leads us to conclude that certain serotypes, like 19F, can persist even among vaccinated children. One of the reason for this phenomenon can be a vaccine failure in cases of IPD (242). An alternative explanation could be the emergence of novel MLST genotypes (such as ST9074 in case

of 19F) among circulating pneumococcal isolates which have successfully filled the vacated niche caused by the disappearance of other vaccine types. Probably, the re-emergence of 19F is related more to pathogen-associated virulence factors rather than host immune response, because higher rates are confined to certain geographical regions. All ten carried 19F isolates in our surveillance derived from two institutes. It is also hypothesized that a higher antibody concentration is required for the protection against these new types (243).

5.3. *H. influenzae* serotype distribution

Hib was not found throughout our study. It is not surprising as Hib vaccine was implemented more than 20 years ago. In 2016, type b was only represented in 5.8% (half of the cases occurring in ≥25 years old patients), whereas 77.7% of *H. influenzae* invasive disease was caused by NTHi followed by serotype f (11.3%) and e (2.7%) in the European Economic Area (133). This ratio is mirrored in our carriage study: NTHi dominated both in nurseries (95.7%) and DCCs (89.2%). The remaining proportion was serotype f in DCCs (10.8%), whereas in the nurseries it was divided between serotype f (2.6%) and e (1.7%). Regarding carriage in other countries, where Hib was already implemented, serotype b also disappeared from carriage (221, 244). Camilli et al. identified only NTHi in children <6 years old in Italy in 2012. Besides serotype replacement, the epidemiology of *H. influenzae* invasive disease also changed in the affected age group. It means that currently adults suffer from invasive disease caused by *H. influenzae* rather than children (245).

In Hungary, 0-8 cases of Haemophilus-meningitis were reported annually between 2001 and 2014 (246). Serotypes are only known for a low number of cases (Table 30).

Table 30. Annually reported meningitis purulenta cases caused by *H. influenzae*

year	number of cases	serotypes (where known)
2000	13	
2001	5	
2002	5	
2003	2	
2004	3	
2005	2	
2006	0	

2007	2	b (n=1)
2008	6	f (n=2) NTHi (n=2)
2009	3	f (n=1)
2010	5	
2011	8	
2012	4	e (n=1) f (n=1)
2013	2	NTHi (n=2)
2014	3	

5.4. *M. catarrhalis* serotype distribution

According to Blakeway et al., A, B, C and NT serotypes are present usually in the following range: 60–75%, 20–30%, 2–6% and approximately 5%, respectively (190). This correlates well with our findings. Serotype A dominated in all three age groups (100% in primary school, 68.6% in DCCs and 89.1% in nurseries. Serotype B was the second most prevalent with 18.6% in DCCs and 9.4% in nurseries. Serotype C was represented in 2.9% and 1.0%, and finally 10.0% and 0.5% of the strains were NT.

5.5. Relationship between the investigated species

As the most common co-carried pair was *S. pneumoniae* and *M. catarrhalis*, not surprisingly there was a positive association between them, in accordance for instance with a Japanese publication (221). However, they found no correlation between *S. pneumoniae* - *H. influenzae* and *H. influenzae-M. catarrhalis* co-carriage, as we did in our study. It was described that *M. catarrhalis* is more often a co-infecting agent in otitis media besides S. *pneumoniae* and *H. influenzae* (247, 248). Their mutual co-colonization in the nasopharynx enhances the risk of otitis media development compared to a single infection (249). Synergistic interactions are assumed between these three bacteria: the β-lactamase produced by *M. catarrhalis* can protect susceptible S. *pneumoniae* and *H. influenzae* strains from β-lactam antibiotics (250-252). Another way of their collaboration is that outer membrane vesicles produced by *M. catarrhalis* can inhibit the complement-dependent killing of *H. influenzae* (253). Pneumococci can increase resistance of *M. catarrhalis* to macrolides in polymicrobial biofilms, while *H. influenzae* promotes *M. catarrhalis* presence via interspecies quorum signaling (250, 252).

A definite negative interaction was described by several authors between *S. pneumoniae* and *S. aureus* (254, 255). Several mechanisms can stand in the background: natural

competition for the niche, or the production of hydrogen peroxidase (H_2O_2) by *S. pneumoniae* which is lethal for *S. aureus* (256). This inverse relationship can be due to bacterial interference or a consequence of confounding effects like age, as *S. aureus* distribution shows a mirror image compared to that of *S. pneumoniae* (254). Regev-Yochay published an especially interesting negative association between PCV-7 VT strains and *S. aureus* co-carriage. In contrast, our results showed no difference in the co-carriage of *S. aureus* and VT or NVT pneumococci (15.4% versus 12.3%). Another confounding factor was demonstrated by Reddinger et al. First, it was shown that the two species can form a stable biofilm both individually and together. Single-species studies confirmed that both bacteria are capable to disperse from the biofilm in response to physiological changes. However, if the dual-species biofilm was exposed to influenza A virus infection, only *S. pneumoniae* could disperse from it whereas *S. aureus* dispersal was inhibited. Furthermore, an *in vivo* mouse model supported these findings. They infected the nasopharynx of mice artificially with the two bacteria and 48 hours later with influenza A virus. The majority of mice developed secondary pneumococcal pneumonia (62.5%), only 12.5% had staphylococcal pneumonia and pneumonia was always monomicrobial. Therefore, it could be concluded that *S. pneumoniae* reduces the pathogenic potential of *S. aureus* (257). In the previous Hungarian studies, also a negative relationship was published: examining altogether 2268 children attending DCCs, only 7.1% of the children proved to be double carriers (258).

In our study, *S. aureus* had a statistically significant negative correlation with *M. catarrhalis* and nearly significant with *H. influenzae* carriage. Pettigrew et al. and van den Bergh et al. reported negative association between *S. aureus* and *H. influenzae* among 6-36 months old children (259, 260). Moreover, van den Bergh et al. found also a negative association between *S. aureus* and *M. catarrhalis* (259).

Out data suggest that in the ever dynamic system of synergistic and competitive relationships among members of the microflora, three bacteria (*S. pneumoniae, H. influenzae* and *M. catarrhalis*) can easily share the niche, but all of them compete with *S. aureus*.

5.6. Risk factors

Significant association was found between male gender and *S. aureus* carriage in the primary school group. Males seemed to carry more frequently *S. aureus* in a U.S. survey with nearly 10000 participants (261). *S. aureus* was also more prevalent among males in Germany, though not significantly (227). In one of the former Hungarian *S. aureus* surveys, only one risk factor reached the level of significance: boys had a higher colonization rate (220). Summarizing all data, two more predictor variables influenced the carriage: having siblings had an enhancing effect while antibiotic exposure in the past two weeks had a negative effect. It is quite obvious that the more *S. aureus* colonized person lives in the same household, the bigger is the likelihood for a successful transmission (262). A Greek survey described that infants were more frequently colonized if they had many siblings or a carrier mother (263). Antibiotic exposure affected negatively the *S. aureus* and *H. influenzae* carriage in our study. Antibiotic use can support the selection and emergence of antibiotic resistant bacteria and it can also lead to the eradication of susceptible isolates (264-267).

Both active and passive smokers are at increased risk of respiratory infections (268, 269). In a former Hungarian paper, carriers of serotype 19A were exposed to passive smoking in a much higher percent (49.2%) than non-carriers (39.2%), however it was not statistically significant based on the Chi-square test (p=0.139) (37). Of note, the opposite effect of passive smoking was mentioned in a Belgian study where parental smoking was negatively associated with pneumococcal carriage of 6-30 months old infants (270).

5.7. Antibiotic sensitivity

5.7.1. *S. pneumoniae*

Among carried isolates, penicillin, cefotaxime and ciprofloxacin resistant isolates were not found. Higher values were measured in a Turkish work where 6–10 years old school children were tested: 7%, 7.5% and 1% were found to be resistant to the mentioned three antibiotics (224). Similarly, erythromycin and clindamycin resistance was higher with 42% and 34%, while our corresponding figures were 17.5% and 7.8%.

The results of the Czech study are rather similar to ours: no penicillin and cefotaxime resistance was found. They measured lower penicillin intermediate resistance (3.0%) compared to the current study (13.1%) (213).

The antibiotic susceptibility of carried isolates is typically lower compared to clinical strains. In the current study, a clear increasing gradient in resistance could be observed from the carriers towards P1 and P2 (carriers < P1 < P2) in case of macrolides and fluoroquinolones (Table 27). Furthermore, we compared our results to nationwide clinical data (inpatients and outpatients), based on several thousands of isolates each year and collected by the National Bacteriological Surveillance Management Team of the National Public Health Center in 2015-2016 (271). As seen on Figure 24, P1 and P2 results stand closer to inpatient and outpatient values. Not surprisingly, isolates deriving from inpatients showed higher resistance rates compared to outpatient isolates, with the exception of clindamycin. While we found no penicillin resistant isolates in any of the investigated groups, 2.5–4.3% resistance among non-invasive pneumococci was reported nationwide.

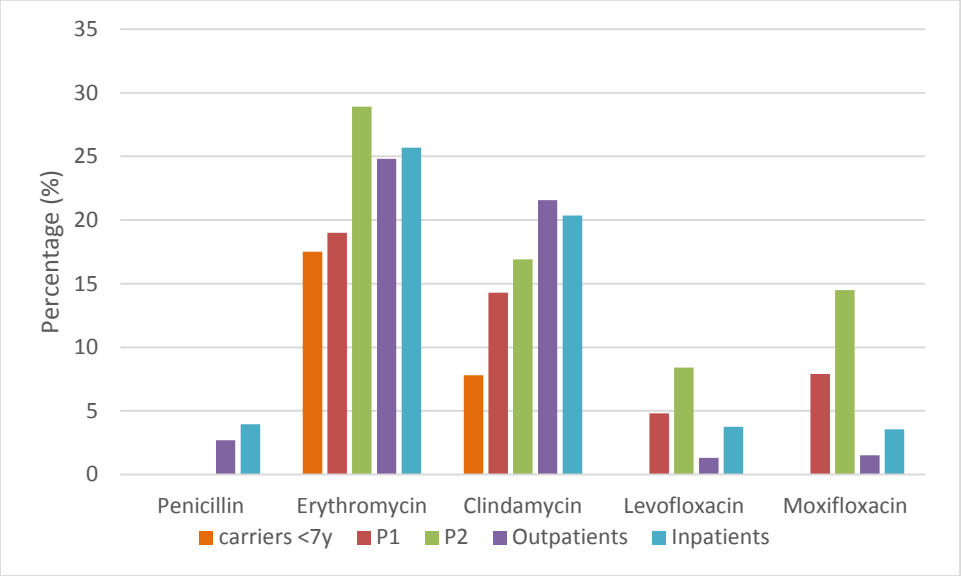


Figure 24. Antibiotic resistance rates of carried (n=206) versus clinical *S. pneumoniae* isolates from Hungary, in the period of 2015-2016.

P1 (n=63) and P2 (n=83) obtained in this study; outpatient and inpatient data taken from the annual resistance reports of the National Public Health Center

In a previous Hungarian study conducted between 2010 and 2012, carried isolates were also more sensitive than clinical isolates and also higher rates were observed among hospitalized patients (272). Comparing carried isolates from the period of 2010-2012 and from this study (2015-2016), we can detect a decrease in resistance to penicillin (0.3%

vs. 0.0%), clindamycin (17.3% vs. 7.8%) and levofloxacin (4.4% vs. 0.0%). These findings could probably be related to the drastic vaccine-driven serotype rearrangement, as resistance is serotype specific and the newly emerging serotypes are mostly more susceptible. PCVs were designed to include the most prevalent serotypes among invasive diseases, and some of these turned out to be associated with high resistance levels (such as 19A). Serotype-linked resistance is supported by the fact that in the DCC group, erythromycin resistance was significantly higher (29.3%) than in the nursery group (14.5%) which can be associated with the bigger proportion of 11A isolates at the DCCs, always displaying the M resistance phenotype.

5.7.2. *S. aureus*

In the current study, as always before, vancomycin resistance was not detected. On the other hand, whereas 100% mupirocin sensitivity was measured previously in our surveillance studies between 2009-2011 and in 2012 (193, 220), now three *S. aureus* isolates were resistant (2.4% of all strains). The penicillin resistance was somewhat lower now (only 77.2%) compared to the figures from the old studies (91-92%). We did not find any MRSA isolates in this study; in 2012, 4/476 isolates (0.8%) proved to be MRSA. The ciprofloxacin resistance was accordingly low (only 0.8%). Compared to the data measured in 2012, erythromycin resistance increased slightly (10.3% versus 12.2%) but clindamycin resistance dropped significantly (from 9.5% to 0.8%), which can be attributed to the high proportion of M type serotype 11A strains. Tetracycline resistance remained low (3.4% versus 1.6%).

Annual nationwide resistance rates of clinical strains are provided by the National Public Health Center (271) also for *S. aureus*, so we could make a comparison. Similarly to the pneumococci, the carried isolates displayed much lower resistance compared to the clinical ones, with the exception of mupirocin and gentamicin (Figure 25). For instance, erythromycin resistance of clinical outpatients *S. aureus* isolates was 22–24% in 2015-2016, while only 12.2% for our strains; or tetracycline resistance was 7.2–8.2% in outpatients, and only 1.6% in this study. According to the expectations, both oxacillin and ciprofloxacin resistance was significantly higher among inpatients, due to the higher prevalence of MRSAs.

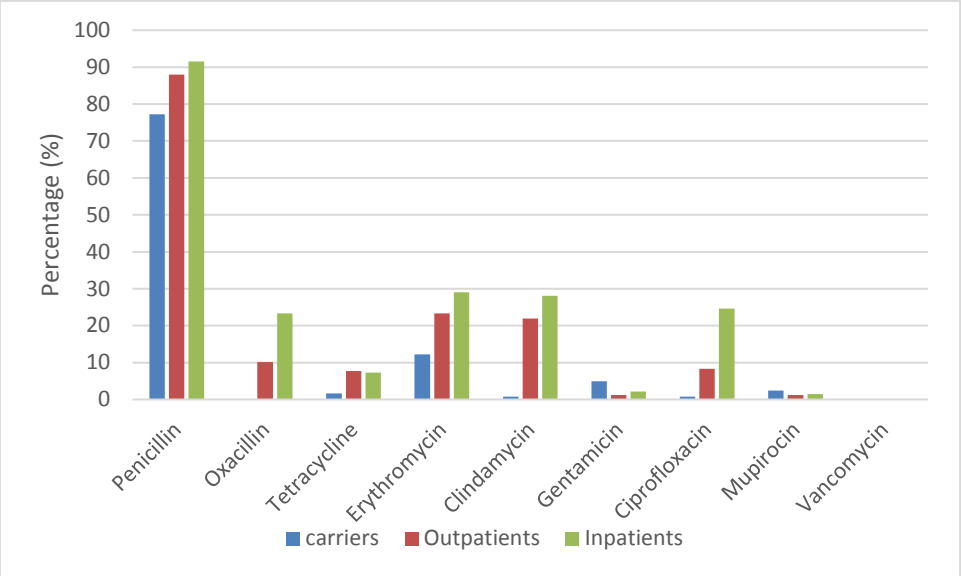


Figure 25. Antibiotic resistance rates of carried (n=123) versus clinical *S. aureus* isolates from Hungary, in the period of 2015-2016. Nationwide resistance data were obtained from the National Public Health Center annual reports.

Among children attending 16 DCCs in the Czech Republic, no MRSA was found either. Their erythromycin and clindamycin resistance rates were very low (2.8% and 0.0%, respectively) (213).

As a result of the increasing pressure to prevent MRSA infections - as mupirocin is the major agent used worldwide for nasal MRSA decolonization -, mupirocin resistance started emerging in several countries (273).

5.7.3. *H. influenzae*

Carried *H. influenzae* were less resistant compared to Hungarian clinical isolates from the same time period (Figure 26) (271). For instance, ampicillin resistance was 6.6% in this study, while it was twice as high for clinical isolates (13.8%). Levofloxacin, moxifloxacin and amoxicillin-clavulanic acid resistance were present only among disease causing strains (0.7% 0.7% and 5.8%). Only the TMP-SMX resistance found in our study was closer to the clinical values (14.5% versus 18.6%). Usually, β-lactam resistance in this bacterium is mediated by the production of β-lactamases, but an altered penicillin-binding protein can also result in a lower affinity to β-lactams. These are the so called BLNAR strains (274). In this study, none of the ten ampicillin-resistant isolates were BLNAR.

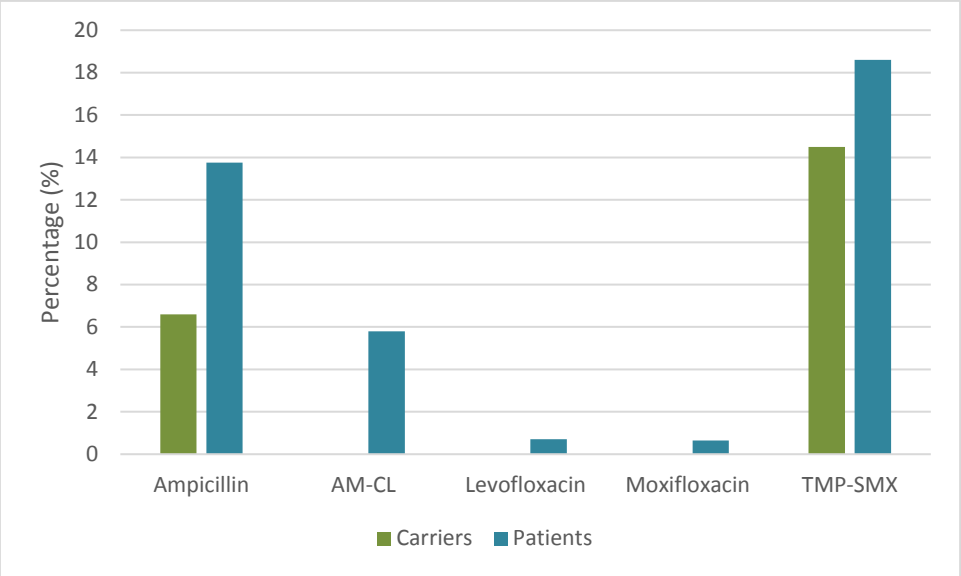


Figure 26. Antibiotic resistance rates of carried (n=152) versus clinical *H. influenzae* isolates from Hungary, in the period of 2015-2016. Nationwide resistance data were obtained from the National Public Health Center annual reports.

In the Czech surveillance, the authors published very similar figures to ours. The amoxicillin resistance was 4.6% in their study and all isolates were susceptible to amoxicillin-clavulanic acid and fluoroquinolones. TMP-SMX resistance rate was also very close to ours with 12.0% (213).

Torun et al. measured twice as high resistance rates for both ampicillin (12.9%) and TMP-SMX (28.6%) among asymptomatic children in Turkey compared to our values, (224). and 0.9% of their isolates were resistant to the ampicillin-sulbactam combination. Only fluoroquinolone susceptibility was 100%.

In parallel with the implementation of Hib vaccine, ampicillin and chloramphenicol resistance decreased in India where most invasive *H. influenzae* infections were caused by type b in the 1990s (275).

5.7.4. *M. catarrhalis*

The contrast between the resistance of carried and clinical *M. catarrhalis* isolates was even more marked than for the other species. The erythromycin and TMP-SMX resistance of our strains was only 1.4% and 0.7%, compared to the equivalent values of 11.3% and 13.8% in clinical isolates (Figure 27). Only levofloxacin resistance was a bit higher among carriers (1.8% versus 0.3%) but we detected no moxifloxacin resistance (271).

In the Turkish study in which 6–10 years old school children were screened, although all *M. catarrhalis* isolates were also susceptible to amoxicillin-clavulanic acid, but significantly higher TMP-SMX resistance was determined (20%), even higher than in the Hungarian clinical isolates (224).

M. catarrhalis strains isolated in the Czech DCCs showed slightly higher TMP-SMX resistance (5.3%) compared to our study, but none of the Czech isolates were erythromycin resistant (213).

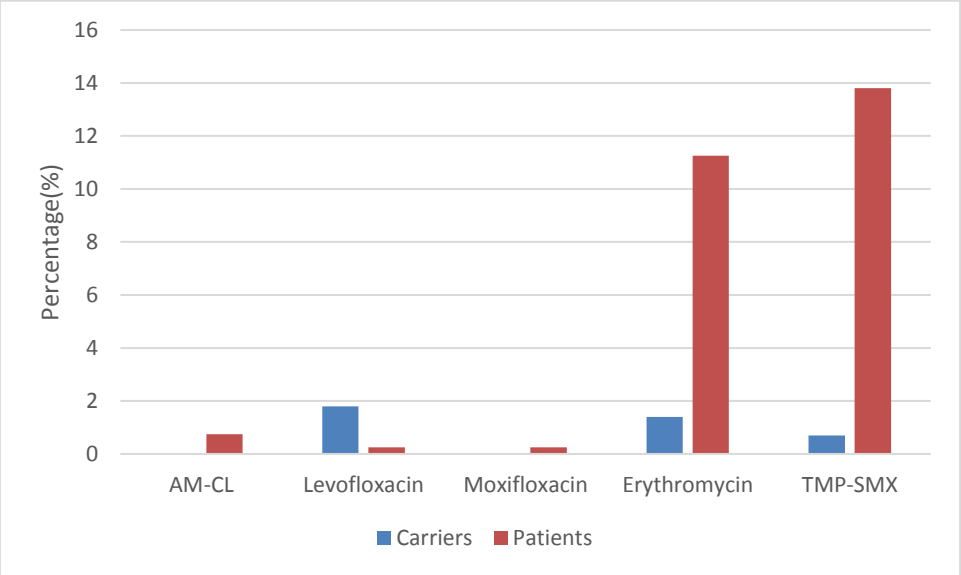


Figure 27. Antibiotic resistance rates of carried (n=281) versus clinical *M. catarrhalis* isolates from Hungary, in the period of 2015-2016. Nationwide resistance data were obtained from the National Public Health Center annual reports.

5.8. Genotyping results

5.8.1. *S. pneumoniae*

When comparing the PFGE restriction patterns of the isolates belonging to the same serotypes, identical patterns were detected among clinical and carried strains in case of serotypes 11A, 19A, 19F. Moreover, there was up to one year difference regarding time of isolation between the corresponding pairs. This means that some stable clones might circulate over a long time in the Hungarian population.

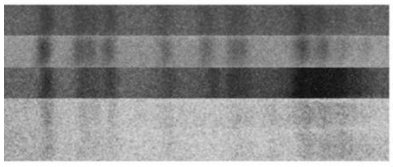
Serotype 3 was only present among patients but not among asymptomatic carriers. Their genetic diversity was really low, half of the isolates had indistinguishable banding pattern. This suggest that one dominant clone and its closely related variants circulate in the country.

Among the five 19A isolates, we found one belonging to the ST320 clone. Curiously, also among the 19F isolates we found one ST320 strain, as confirmed by MLST. Both of them had high-level erythromycin, clindamycin resistance and were penicillin intermediate resistant. Furthermore, both of them expressed *erm(B)* and *mef(E)* genes. These are characteristic features of members of the Taiwan^{19F}-14 clone (ST236), a well-known international, multiresistant linage which was the ancestor of ST320 in the pre-PCV7 era (276). According to Pillai et al. and Hsieh et al. a capsular switch event between the PCV7 type 19F and the PCV13 type 19A after PCV7 implementation (277, 278) has lead to the current dominance of 19A within ST320 (279, 280).

The incidence of invasive pneumococcal disease IPD due to serotype 19A increased from 0.8 to 2.5 cases per 100,000 population between 1998 and 2005 in the USA. Simultaneously, penicillin resistant 19A incidence increased from 6.7% to 35% among IPD cases. Of these, 73.5% belonged to the CC320 (281). In Barcelona, ST320 appeared first in 2005 among OM isolates and had a rapid expansion: during the period of 2002-2006, only 22.2% of 19A isolates belonged to ST320, while between 2007 and 2011 this number was 72.7% (282). Similarly, in Asian countries, ST320 dominance was observed during 2008 and 2009. Altogether 1637 *S. pneumoniae* isolates were collected in ten different countries in Asia and 91 proved to be serotype 19A. Out of these, 46 (51.1%) was ST320 deriving from Hong Kong, India, Korea, Malaysia, Saudi Arabia and Taiwan. ST320 isolates were mostly multidrug resistant and showed significantly higher resistance rates than other STs to cefuroxime, clindamycin, and TMP/SMX. Interestingly, it was predominant even in countries with no or low coverage of PCV7 indicating that that its emergence and dissemination was due to more than just vaccine selection pressure in Asian countries (283). Antibiotic overuse may explain the expansion of this multidrug resistant clone event before PCV7 introduction (284).

Another noteworthy clonal complex is CC180, which is mainly associated with serotype 3 (285). Controversially, serotype 3 ST180 is significantly associated with asymptomatic

carriage but also with high-case fatality rates when causing infection (286, 287). This phenomenon was already recognized in the early 20th century regarding serotype 3 (288). We had some serotype 19F ST180 strains, which - similarly to serotype 3 strains in general - were fully susceptible to all tested antibiotics (Figure 16). ST180 was already identified among 19F strains in Germany, however, it remained a predominantly a serotype 3 associated sequence type (289). We hypothesize that a capsular switching event might have occurred in the past between serotype 19F and serotype 3 – ST180 strains, which could explain the re-emergence of serotype 19F among carried isolates, despite the fact that it is a PCV-7 serotype. The identical PFGE banding pattern of some selected serotype 3 and 19F isolates supports this hypothesis (Figure 28), but it obviously requires further investigation.



Strain	Serotype	Peni	Ery	Clinda	Date of isolation
19394	3	<0.015	<0.06	<0.5	Apr-2015
19559	3	<0.015	0.125	<0.5	May-2015
49195	3	<0.015	0.125	<0.5	Oct-2015
PP266	19F	0.03	0.25	<0.5	Feb-2016
PP272	19F	0.03	0.25	<0.5	Feb-2016

Figure 28. Identical PFGE pattern of selected serotype 3 and 19F isolates, where the two 19F isolates belong to ST180 sequence type.

Not surprisingly, we could observe that in the same DCC/nursery, isolates belonging to the same serotype shared the same PFGE pattern (e.g. in case of 19F, 11A and 15A strains, larger identical groups were detected while among 15C strains only some pairs were identical). This is probably due to the close contact between the children and it also confirms frequent bacterial transmission among children. However, we have examples for the opposite as well, i.e. identical serotypes with unrelated pulsotypes. Identical clones were found sometimes in different cities, e.g. among 15A isolates deriving form Székesfehérvár and Pápa, or 15C isolates deriving form Budapest and Pápa.

5.8.2. *S. aureus*

Related strains were often present within the same DCC/nursery but also identical strains could be isolated far from each other both in time and place. Bonness et al. detected identical strains deriving from the same children's nose and hand, also among parents and

their children which provides an evidence that carriage on skin plays a major role in transmission (290).

The nearly identical pulsotype of the three mupirocin resistant strains can also allude to the clonal spread within the same community, or in the same town (in case of PP264).

5.8.3. *H. influenzae*

The capsulated types of *H. influenzae* are usually less diverse genotypically than the NTHi isolates, this is evidenced by MLST and ribotyping as well in the literature (291-294). This correlates well with our results, however, we had only seven serotypeable isolates. Among NTHi, a bigger genetic richness could be detected based on PFGE results, even within the same institute. That is comparable with the serotypeable isolates' profile as the four indistinguishable serotype f isolates derived from the same DCC and the other three from three different places and the two serotype e isolates derived from the same nursery but differed only in one band.

6. Conclusions

In this study, we have screened children belonging to three different age groups, for the carriage of four respiratory pathogen bacteria. In the same time period (2015-2016), clinical pneumococcal isolates were obtained as well. Due to the strict Hungarian vaccination policy – PCV13 and Hib are mandatory vaccines – we hardly found PCV13 serotype pneumococcal isolates: its prevalence was 4.8% in nurseries and 9.8% in DCCs, represented only by serotypes 19F, 19A and 9V. On the contrary, among patients the PCV13 coverage was higher with 17.5% (P1) and 32.5% (P2).

Among the clinical strains, we have found two ST320 isolates belonging to two different serotypes (19A and 19F), both of them expressing *erm(B)* and *mef(E)*.

Among *S. pneumoniae* isolates, penicillin intermediate resistance was observed: 13.1%, 17.5% and 25.3% among carriers, patients <7 y and patients >7 y, respectively. Compared to a previous Hungarian carriage study, the lower levels of resistance to certain antibiotics like penicillin, clindamycin and levofloxacin are probably due to the vaccine-driven serotype rearrangement as resistance is serotype specific and the newly emerging serotypes are mostly more susceptible.

Multiple carriage occurred more frequently without *S. aureus*. During statistical analysis, we found a significant negative association between *S. aureus* and *S. pneumoniae* or *M. catarrhalis*, and it was nearly significant with *H. influenzae.* On the other hand, positive association existed between *S. pneumoniae-H. influenzae*, *S. pneumoniae-M. catarrhalis* and *H. influenzae-M. catarrhalis.*

MRSA strains were not found during our survey. Among *H. influenzae* strains, NTHi dominated, only a few f and e serotypes were identified, but not b. Among *M. catarrhalis* strains, serotype A was the leading type in all age groups, followed by B and C.

Male gender was identified as a possible risk factor for *S. aureus* colonization as it was also published previously in Hungary. Passive smoking affected the *S. pneumoniae* carriage negatively, while antibiotic exposure seemed to reduce both *S. pneumoniae* and *H. influenzae* colonization.

Out of the three mupirocin resistant *S. aureus* isolates, two shared the very same PFGE pattern deriving from the same DCC. The third one with a slightly modified pattern, derived also from Pápa but from another DCC.

The following new findings could be established in this dissertation:

- This is the first study in Hungary investigating the asymptomatic carriage of *H. influenzae* and *M. catarrhalis*
- This is the first study in Hungary investigating the asymptomatic carriage of four respiratory pathogens together
- Carriage prevalence of bacteria changes along with the age of children
- Carriage prevalence of *S. aureus* shows an inverse pattern compared to the carriage of the other three investigated species
- The pneumococcal vaccination had caused a drastic serotype arrangement within the pneumococcal population, the most important changes being the following:
    - Serotypes 3 and 6A have completely disappeared from carriage in Hungary
    - Serotype 3 is still the leading serotype among elderly patients
    - Serotype 19A shows decreasing tendency while serotype 19F seems to re-appear
    - The replacing non-vaccine types (NVTs) have low invasive potential and lower proportion of antibiotic resistance
- The pneumococcal vaccination also had an effect on the prevalence of the other carried bacterial species
- The clinical pneumococcal isolates respond with delay to the selection pressure of conjugate vaccines
- No MRSAs were found among the carried *S. aureus* isolates
- All four species were more susceptible to the tested antibiotics compared to clinical isolates from Hungary in the same time period
- The two Gram-negative species were basically more sensitive than the two Gram-positive ones
- There were examples for the presence of isolates belonging to the same PFGE clone in different cities, indicating that certain clones have spread in a larger geographical area

- Some clones (e.g. pneumococcal ST320) could be detected already several years ago in previous carriage studies, indicating the long-term circulation of certain successful clones in Hungary

DOI:10.14753/SE.2022.2609

7. Summary

S. pneumoniae, S. aureus, H. influenzae and *M. catarrhalis* can cause a wide range of infections. However, they are often carried asymptomatically. Thus, carrier children are significant sources of infections in the surrounding population.

In this study, 580 asymptomatic children in three age categories were screened in Hungary in 2015-2016, and 210 *S. pneumoniae*, 123 *S. aureus*, 152 *H. influenzae* and 281 *M. catarrhalis* strains were isolated in total.

We observed a typical age-related carriage: *S. pneumoniae, H. influenzae* and *M. catarrhalis* were more prevalent in the first years of life and started disappearing with age, whereas *S. aureus* showed an inverse pattern.

Fortunately, some vaccines are already available against the most invasive types of these bacteria, namely PCV13, PPV23 and Hib. In Hungary, PCV13 and Hib are mandatory childhood vaccines. Due to the vaccination pressure, some non-vaccine types became predominant, indicating the need for further, higher-valent vaccines. The leading serotypes in carriage were 15B and 11A, the latter also being frequent among clinical isolates. Luckily, both serotypes are included both in the PPV23 vaccine, as well as the future 20-valent conjugated vaccine. *H. influenzae* serotype b was not found at all.

Among *S. pneumoniae* isolates, penicillin resistance was not detected. Only some penicillin intermediate resistance was observed. Regarding *S. aureus*, the increasing number of MRSA challenges worldwide the antibiotic treatment. Luckily, no MRSA isolates were found in our study. On the other hand, high-level mupirocin resistance was observed in three cases. As mupirocin is the major agent for nasal MRSA decolonisation, this can be of worry. *H. influenzae* and *M. catarrhalis* were typically sensitive to the tested antibiotics. Amoxicillin-clavulanic acid resistance was not detected at all.

It is always worth to follow epidemiological changes among carried isolates to predict emergence of disease causing types. Our results suggest that clinical pneumococcal isolates respond with a delay to the selection pressure of vaccination, concluding that the effects are reflected first in carriage. Continuous follow up on carriage is required to estimate long-term vaccine efficacy, to identify replacing types, their antibiotic resistance and to screen the successful clones, as well as the vaccines' effect on other species of the normal flora.

DOI:10.14753/SE.2022.2609

8. Összefoglalás

A *S. pneumoniae, S. aureus, H. influenzae* és *M. catarrhalis* sokféle fertőzést okozhat. Ennek ellenére gyakran tünetmentesen kolonizálják a légutak nyálkahártyáját, ami miatt a hordozó gyerekek jelentős forrásai lehetnek a környezetükben kialakuló fertőzéseknek. Vizsgálataink során három korcsoportba tartozó 580 tünetmentes gyerek orrmintáját gyűjtöttük be 2015-2016 során és összesen 210 *S. pneumoniae,* 123 *S. aureus*, 152 *H. influenzae* és 281 *M. catarrhalis* törzset izoláltunk.

A hordozás tipikus életkori függést mutatott: míg a *S. pneumoniae, H. influenzae* és *M. catarrhalis* gyakrabban kolonizáltak a fiatalabb korosztályban és később csökkent a gyakoriságuk, addig az *S. aureus* éppen fordított tendenciát mutatott.

Szerencsére a leginvazívabb típusok ellen már kifejlesztettek védőoltásokat (PCV13, PPV23, Hib). Magyarországon a PCV13 és a Hib kötelező gyerekkori védőoltások. A védőoltások hatására néhány nem vakcina típus vált meghatározóvá, ezáltal kijelölve az utat új, több szerotípust is magában foglaló vakcinák fejlesztésére. A hordozásban a 15B és a 11A voltak vezető helyen, az utóbbi a klinikai izolátumok között is dominált. Szerencsére mindkét szerotípus része mind a PPV23-nak, mind a nemsokára megjelenő új PCV20 oltásnak. A *H. influenzae* törzsek között egyáltalán nem találtunk b szerotípust. Penicillin rezisztens *S. pneumoniae* izolátumot nem találtunk, mérsékelt érzékenységű törzseket is csak kis számban. Az MRSA világszerte kihívást jelent az antibiotikum terápiát illetően. Szerencsére a jelen felmérésben egyáltalán nem találtunk MRSA-t. Ugyanakkor három mupirocin-rezisztens törzset is izoláltunk magas MIC értékekkel. Mivel a mupirocin az egyik fő dekolonizáló ágens MRSA ellen, ez aggodalomra adhat okot. A *H. influenzae* és *M. catarrhalis* izolátumok sokkal érzékenyebbek voltak a tesztelt antibiotikumokra. Amoxicillin-klavulánsav rezisztenciát egyáltalán nem detektáltunk.

Mindig megéri követni az epidemiológiai változásokat a hordozásban, hiszen így megjósolhatók a jövőbeli domináns, betegségeket okozó típusok. Az eredményeink azt sugallják, hogy a klinikai pneumococcus izolátumok késéssel reagálnak a védőoltások szelekciós hatására. Következetesen, a vakcinák hatása először a hordozásban mutatkozik meg. Folyamatos monitorozás szükséges a hosszú távú vakcina hatásosság megítéléséhez, fontos nyomon követni az új, domináns típusokat és a sikeres genetikai vonalakat, és meghatározni ezek antibiotikum rezisztenciáját valamint a vakcinák más, normál flóra fajokra gyakorolt hatását.

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10. List of publications

10.1. Publications related to the topic of the thesis

Kovács E., Sahin-Tóth J., Tóthpál A., Linden M., Tirczka T., Dobay O. (2020) Co-carriage of *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* among three different age categories of children in Hungary. PLOS ONE, 15:(2):e0229021

Kovács E., Sahin-Tóth J., Tóthpál A., Kristóf K., Linden M., Tirczka T., Dobay O. (2019) Vaccine-driven serotype-rearrangement is seen with latency in clinical isolates: Comparison of carried and clinical pneumococcal isolates from the same time period in Hungary. Vaccine, 37(1):99-108

10.2. Publications not related to the topic of the thesis

Sahin-Tóth J., Kovács E., Tóthpál A., Juhász J., Forró B., Bányai K., Havril K., Horváth A., Ghidán Á., Dobay O. (2021) Whole genome sequencing of coagulase positive staphylococci from a dog-and-owner screening survey. PLOS ONE, 16:(1):e0245351

Kovács E., Horváth A., Sahin-Tóth J., Kaptás Á., Huber A., Dobay O., Juhász E., Kristóf K. (2020) Tünetmentes meningococcus-hordozás felmérése Magyarországon egyetemisták és középiskolások körében. Gyermekgyógyászati Továbbképző Szemle, 25(4):14-16

Laub K., Tóthpál A., Kovács E., Sahin-Tóth J., Horváth A., Kardos S., Dobay O. (2018) High prevalence of *Staphylococcus aureus* nasal carriage among children in Szolnok, Hungary. Acta Microbiologica et Immunologica Hungarica, 65(1):59-72

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12. Appendix

Szülői beleegyezés a mintavételhez

Kérem a Tisztelt Szülőt, hogy nyilatkozzon a megfelelő szöveg aláhúzásával!

GYERMEKEM ORRÁNAK SZŰRŐVIZSGÁLATÁHOZ

HOZZÁJÁRULOK NEM JÁRULOK HOZZÁ

Gyermekem neve: …….

Gyermekem neme: fiú lány

Gyermekem életkora: ……

Testvére(i): van nincs

Ha van, hány testvére van: ……

Ha testvére van, ő közösségbe jár nem jár

ha igen, az: bölcsőde óvoda iskola felsőfokú int.
(kérjük aláhúzni)

Gyermekem kapott-e ***Prevenar*** oltást? igen nem nem tudom

Gyermekem kapott-e ***Pneumovax*** oltást? igen nem nem tudom

Szokott-e középfülgyulladása lenni? igen nem

Volt-e már súlyos tüdőgyulladása vagy agyhártyagyulladása? (A megfelelő aláhúzandó)

Kapott-e antibiotikumos kezelést a megelőző 2 hónapban, ha igen: mit, mikor és miért?

……

Volt-e a gyermek kórházban az elmúlt 3 hónapban? igen nem

Dohányoznak-e a családban? igen nem

Dátum:

Szülő aláírása:

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Tárgy: **„Pneumococcus orrban való hordozásának felmérése közösségbe járó egészséges (6 év alatti) kisgyerekek körében"** című kutatás kiterjesztése.
A kutatás címének megváltoztatása.

Tisztelt Adjunktus Asszony!

A Semmelweis Egyetem Regionális, Intézményi Tudományos és Kutatásetikai Bizottsága a 2015. szeptember 28-án megtartott ülésén a „Pneumococcus orrban való hordozásának felmérése közösségbe járó egészséges (6 év alatti) kisgyerekek körében" című kutatás **kibővítését további baktériumokra és szélesebb korosztályra való kiterjesztését (2015. szeptember 18.) engedélyezte.**

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