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Epidemiological analysis of carried *Streptococcus pneumoniae* among healthy children attending communities

Ph.D. thesis

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1 LIST OF THE FREQUENTLY USED ABBREVIATIONS

AOM - Acute otitis media

CAP - Community acquired pneumonia

CBP - Choline-binding protein

CDC- Centers for Disease Control and Prevention

CPS - Capsular polysaccharide

DCC - Day care centre

EUCAST - European Committee on Antimicrobial Susceptibility Testing

GNRCS - German National Reference Centre for Streptococci

GR1 – Group-1; DCC groups with low-level vaccination rate

GR2 - Group-2; DCC groups with high-level vaccination rate (with PCV7)

GR3 - Group-3; toddlers groups with high-level vaccination rate (with PCV13)

IPD - Invasive pneumococcal disease

LytA- Autolysin

MDR - Multidrug resistant

MIC - Minimal inhibitory concentration

MLST - Multilocus sequence typing

NCSP - Non-classical surface protein

NIP - National Immunization Programme

NT- Non-typeable (serotypes)

OEK - National Epidemiological Centre in Hungary

PBP - Penicillin-binding protein

PCR - Polymerase chain reaction

PCV10 – 10-valent pneumococcal conjugate vaccine, Synflorix

PCV13 – 13-valent pneumococcal conjugate vaccine, Prevenar-13

PCV7 – 7-valent pneumococcal conjugate vaccine, Prevenar-7

PFGE - Pulsed-field gel electrophoresis

PMEN - Pneumococcal Molecular Epidemiology Network

PPS23 – 23-valent polysaccharide vaccine, Pneumovax 23

ST - Sequence type

WHO - World Health Organization

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2 INTRODUCTION

2.1. About Streptococcus pneumoniae in general

2.1.1. Historical outlook

The bacterium itself was discovered in 1880 by Sternberg, a US Army physician and by Louis J. Pasteur (1822–1895) independently, Pasteur named the bacteria *Microbe septicemique du salive* and Sternberg named it *Micrococcus pasteuri* than the first name *Diplococcus pneumoniae* was given (2-4). In 1886 Fränkel identified it as the causative agent of lobar bacterial pneumonia and not so long afterwards its pathogenic role was proved also in meningitis, endocarditis, arthritis and otitis media (5).

In the turn of the 20th century realms of immunology and vaccinology started using *Streptococcus pneumoniae* (pneumococcus) for fundamental researches. In 1909 Neufeld and Händel described different serotypes of pneumococci and observed that protection by immune serum was type-specific (2, 6). With the help of this finding they recognized the specific antisera and its role in therapy which resulted in the first effective treatment in the pre-antibiotic era against pneumococcal infections. This experience helped Oswald T. Avery and his colleague Michael Heidelberger in 1923 to determine the pneumococcal antigens that induced protective immunity as carbohydrates (2, 7).

The basements of molecular biology could be owed to *S. pneumoniae* as well. In 1928 Griffiths proved that pneumococci are capable of taking up large foreign DNA fragments from the surroundings (4).

In 1944 Avery together with Colin MacLeod and Maclyn McCarty demonstrated their favourite experiment with the help of different virulence of R (rough, non-capsulated) and S (smooth, capsulated) colonies and proved the chemical nature of the principle of transforming substance as DNA (2, 8). After the introduction of penicillin in 1940s, treatment of the pneumococcal infections seemed to be solved. Nevertheless, the first penicillin resistant strains appeared in 1960s, followed by multidrug resistant (MDR) strains, which still remained a problem (9).

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2.1.2. Morphology and culivation

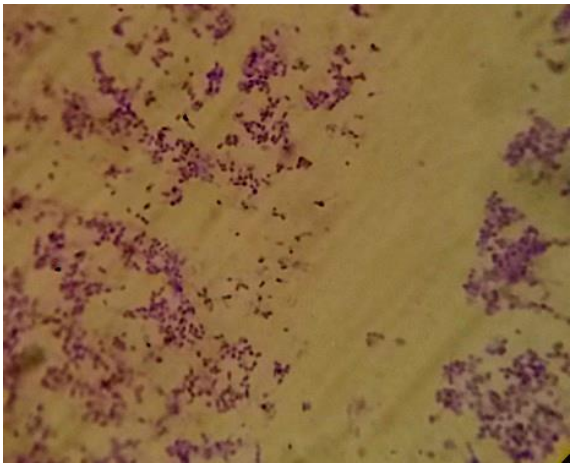

Figure 1. Microscopic picture of *S. pneumoniae* by A Tóthpál

S. pneumoniae is a Gram-positive coccus, sized 0.8-1.5 μm, forming pairs or short chains (**Figure 1**). Chain formation is more frequent when grown on solid medium. The bacterium can have pili and are encapsulated, with a large polysaccharide capsule.

S. pneumoniae is a fastidious, facultative anaerobe, bacterium, growing best on media supplemented with 5% blood and requires 5% to 10% CO_2 for incubation at 35-37°C. On blood agar it shows α-haemolysis and the colonies have special umbilicus morphology (**Figure 2**), due to autolytic activity (10).

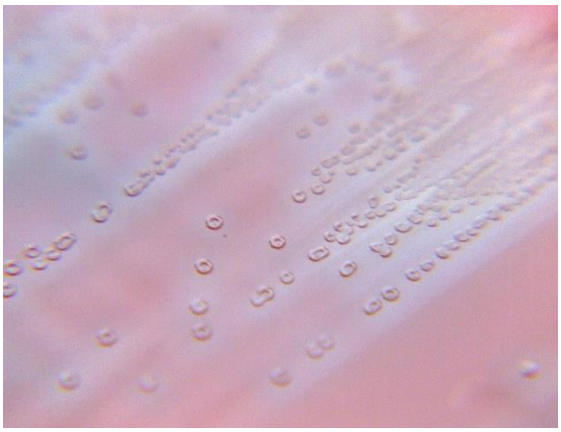

Figure 2. *S. pneumoniae* umbilicated colonies on blood agar by A Tóthpál and Á Ghidán

2.1.3. Identifiation

It is not an easy task to identify *S. pneumoniae*. As a respiratory pathogen, it can be found in the nasopharynx and in the sputum sample during infection, but sometimes the less fastidious normal microbiota members, such as the genetically related viridans Streptococci (for example *Streptococcus mitis*) can overgrow the pneumococci leading to problems in identification.

The classical diagnostic identification of pneumococci is based on colony morphology (α-haemolysis and characteristic colonies), optochin sensitivity - although optochin resistant strains were described (11) - and some biochemical activities, such as the lack of catalase enzyme, bile solubility (when the surface active bile salts provide full activity for the autolytic enzymes) or inulin degradation, for separation from the viridans Streptococci (12). Antigen detection is possible from sterile body fluids (e.g. in case of meningitis) with latex agglutination. Urinary antigen tests are also available. These are rapid immunochromatographic tests which detect the C polysaccharide cell

wall antigen common to all strains of *S. pneumoniae* and can be positive for weeks to several months after the infection(13).

For serotyping pneumococci, monovalent antisera and latex agglutination kits are also available (14, 15). The most reliable way for identification is using molecular biological methods, such as PCR for pneumococcal specific genes like *lytA* gene (1) *ply* gene (16) *pspA* gene (17), or detection of a conserved gene such as 16S rRNA (18).

2.1.4. Most important virulence factors

Properties that explain the pathogenic potential of *S. pneumoniae* include polysaccharide capsule, pili, different enzymes such as IgA1 protease, pneumolysin, autolysin and several surface-exposed proteins that mediate contact with components of host tissues and secretions (**Table 1**).

Table 1. List of virulence factors, only selected examples are shown (19)

Pneumococcal virulence factors and disease	Main role in colonization
Upper-airway colonization	
Capsule	Prevents entrapment in the nasal mucus, thereby allowing access to epithelial surfaces. Also inhibits effective opsonophagocytosis.
Phosphorylcholine	Binds to the epithelial surface of the human nasopharynx.
Choline-binding protein A	Binds to human secretory component on a polymeric Ig receptor during the first stage of translocation across the epithelium.
Neuraminidase	Act sequentially to cleave terminal sugars from human glycoconjugates, which might reveal receptors for adherence.
Hyaluronate lyase	Breaks down hyaluron-containing extracellular matrix components.

Pneumococcal adhesion and virulence A	Binds to fibronectin.
Enolase	Binds to plasminogen.
Competition in upper airway	
Bacteriocin (pneumocin)	Small antimicrobial peptide that targets members of the same species.
Respiratory-tract infection and pneumonia	
Pneumolysin	Cytolytic toxin that also activates complement. An important determinant of virulence in in vivo models of disease. Wide range of effects on host immune components at sub-lytic concentrations.
Pneumococcal surface protein A	Prevents binding of C3 onto pneumococcal surface. Also binds lactoferrin.
Autolysin A	Digests the cell wall, which results in the release of Ply.
Pneumococcal surface antigen	Component of the ABC transport system, which is involved in resistance to oxidative stress.
PiaA: pneumococcal iron acquisition	Component of the ABC transport system.
IgA1 protease	Cleaves human IgA1 immuneglobulin.

2.1.4.1. Capsule

Capsule (**Figure 3**) is the major virulence factors of pneumococci and it was the first non-protein substances shown to be antigenic in humans. Fred Neufeld described first the process to differentiate pneumococci into serotypes with the help of type-specific antisera and he also discovered that these type-specific antigens were carbohydrates, which are the basement of the capsule (2).

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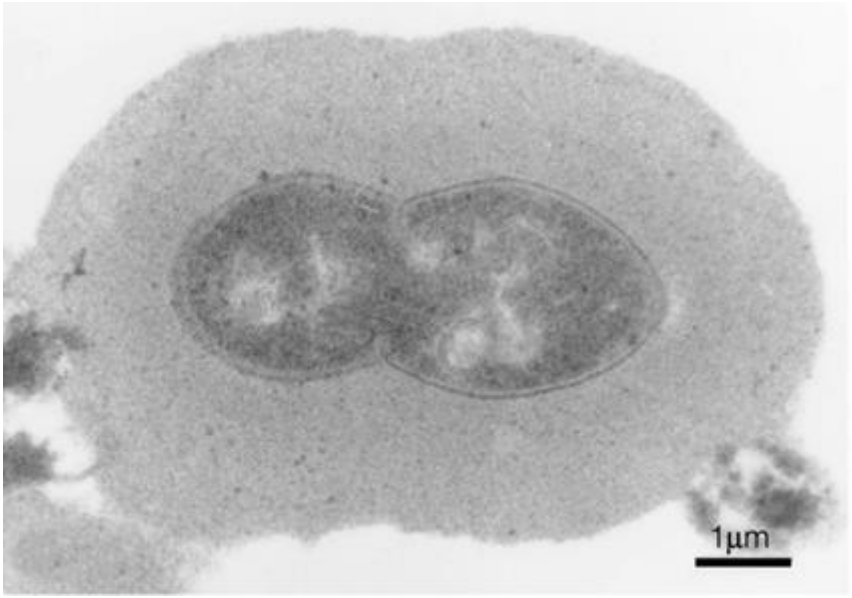

Figure 3. Immunoelectron microscopy of pneumococcal capsules showing an increased zone of capsular material in serotype 6B (20)

So far we know 94 different capsular polysaccharides (CPSs), which are distinguished by using a set of antisera that recognise the chemical differences in the capsules, therefore they are called serotypes (21). Expression of a capsule is important for survival in the blood and is associated with the ability of pneumococci to cause invasive disease. CPSs are built up from repeating oligosaccharide units and antibodies against these saccharides provide protection against pneumococcal disease. The most frequent monosaccharides in CPSs in different combinations are α/β-D-glucose, α/β-D-galactose, α/β-L-rhamnose, N-acetyl-α/β-D-glucosamine, N-acetyl-α/β-D-galactosamin, N-acetyl-β-D-mannosamine, N-acetyl- α-L-fucosamine and α/β-D-glucuronic acid (21).

There are significant differences between different serotypes. In addition to the above mentioned monosaccharides, α-L-fucose (serotype 19A), β-D-ribose (serotype 7, 19B, 19C), 2-acetamindo-4-amino-2,4,6-trideoxy-α-D-galactose (serotype 1) can be present. Comparison of the CPSs of serotype 6A and 6B shows that the only difference is the presence of 3-substituted D-ribitol in 6A and 4-substituted D-ribitol in 6B.

The capsular gene (*cps*) locus, responsible for the expression of CPSs - with the exception of serotypes 3 and 37 (22) - is located between *dexB* and *aliA* on the pneumococcal chromosome (23) and the total size of alternative coding DNA at this

one locus exceeds 1.8 Mbp. The regulatory and processing genes *wzg*, *wzh*, *wzd* and *wze* (also known as *cpsABCD*) are conserved with high sequence identity in all cases and are almost always in this gene order at the 5′ end of the *cps* locus (23). In most *cps* clusters, the fifth gene encodes the initial glucose phosphate transferase, WchA (also known as *cpsE*), responsible for linkage of an activated glucose phosphate to the lipid carrier. The polysaccharide polymerase *(wzy)* and flippase *(wzx)* genes are always present downstream together with a varying set of genes for glycosyl transferases, acetyl transferases, nucleotide diphosphate sugar biosynthesis and modifying enzymes (**Figure 4**). In the regions between the *cps* genes and the flanking *dexB* and *aliA* genes, there is almost always evidence of mobile genetic elements.

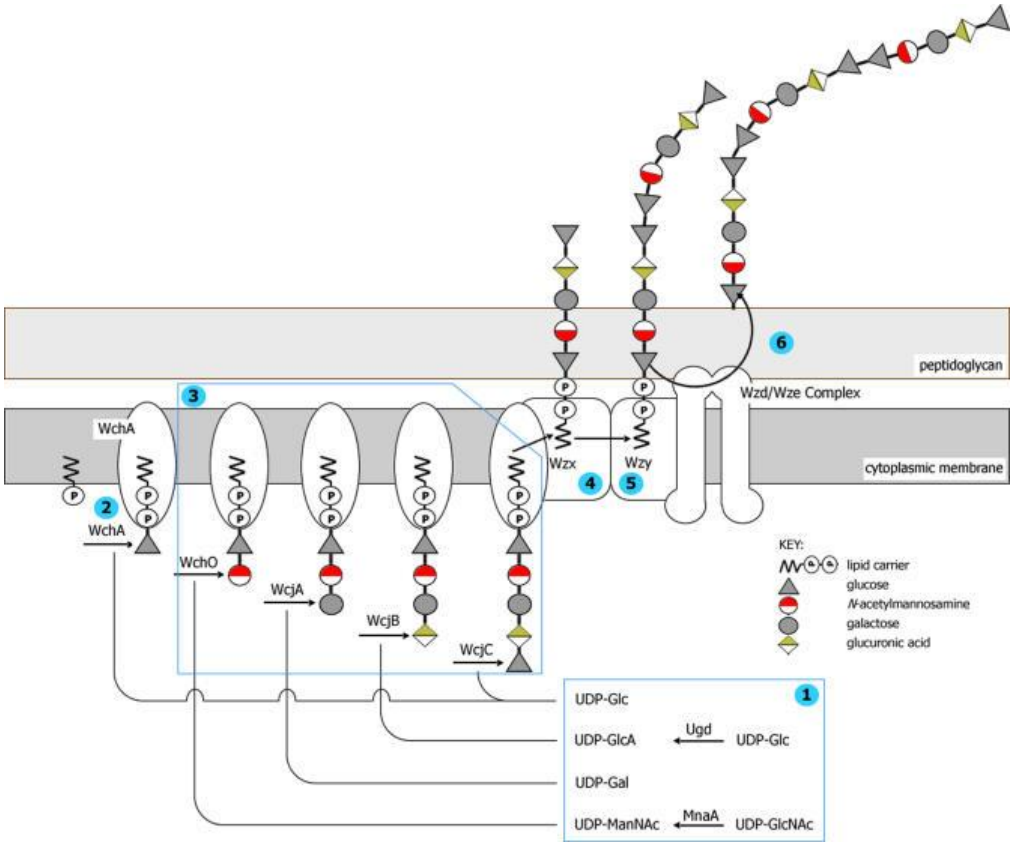

Figure 4. Pneumococcal capsular biosynthesis: Schematic representation of the biosynthesis of CPS by the Wzy-dependent pathway. The biosynthesis of the CPS of serotype 23F is represented (24)

Due to the natural transformability of the pneumococcus, horizontal recombination allows that one serotype can belong to different genotypes and a single genotype can express different capsular genes, i.e. different serotypes. This phenomenon is known as capsular switching (25).

S. pneumoniae capsule can affect several aspects of complement activity. These include preventing binding of both IgG (Immunoglobulin G) and CRP (C-reactive protein) to *S. pneumoniae* and thereby inhibiting classical pathway activity, reducing alternative pathway activity through unexplained mechanisms and decreasing the degradation of C3b bound to the bacterial surface to iC3b. The effects on C3b/iC3b deposition prevent phagocytosis of encapsulated bacteria, but data also suggest that the capsule inhibits phagocytosis mediated directly by IgG and by nonopsonic phagocytic receptors. The results clarify some of the mechanism by which the *S. pneumoniae* capsule could mediate immune evasion (26).

2.1.4.2. The presence of pilus

S. pneumoniae, like many other Gram-positive bacteria, has long filamentous pili extending on their surface through which they adhere to host cells. This may be the first of those virulence factors which are responsible for initial adhesion of the bacteria to host tissues during colonization and biofilm formation. The pilus of *S. pneumoniae* was first identified in 2006 (27).

Pilus genes can be found in a 12 Kb pathogenicity island called Pilus islet-1 (PI-1), encoding a positive transcriptional regulator (*rlrA*), the pilus-1 structural subunits (*rrgA*, *rrgB* and *rrgC*) and three pili-specific sortases. *RrgB* encodes the major component RrgB (28) which is strictly necessary for the pilus formation while the other two (RrgA, RrgC encoded by *rrgA* and *rrgC*) are ancillary proteins (29). PI-1 is present in about one-third of the clinical isolates (30) and its prevalence is higher among antibiotic non-susceptible strains (31).

Pilus subunits are immunogenic in humans (32) and were able to elicit a protective response when tested in mouse models of infection (33). Thus pili could be a good target to develop a new vaccine.

Recent studies demonstrated that pilus can be dedicated to DNA transformation in pneumococci (34). According to Laurenceau et al. the transformation pilus act as a "DNA-trap" to capture DNA in the environment(35). Their data clearly establish the existence and function of a transformation pilus on the surface of competent pneumococci.

2.1.4.3. Surface proteins

Many of the surface proteins are virulence factors that contribute to the pathogenesis of this organism. Three main groups of them have been identified in *S. pneumoniae*: around 50 lipoproteins, up to 18 peptidoglycan binding (LPxTG) consensus sequence-carrying proteins that are covalently linked via sortase to the cell wall peptidoglycan and up to 16 choline-binding proteins (CBPs). In addition to the three main groups of surface proteins, the cell wall of pneumococci is decorated with another cluster of proteins that lack classic leader peptide and membrane-anchoring motifs (**Figure 5**). These proteins are termed non-classical surface proteins (NCSPs) and they could also play a relevant role in subverting the physiological function of host-derived proteins (36).

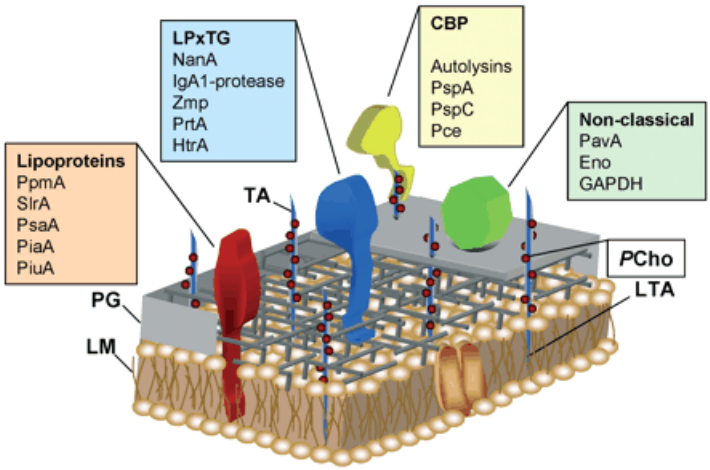

Figure 5. Schematic model of the pneumococcal outer cell wall and surface-exposed proteins. The pneumococcal cell wall consists of a phospholipid membrane (LM), peptidoglycan (PG) and teichoic and lipoteichoic acids (TA, LTA). An unusual component of the cell wall is phosphorylcholine (*P*Cho), which anchors the choline-binding proteins (CBPs) non-covalently on the cell wall. Virulence proteins of the different classes of pneumococcal surface proteins are depicted above (36)

Cell wall components

The cell wall of *S. pneumoniae* contains an unusually complex wall TA, which has identical repeating units as the membrane-anchored LTA (37). Although the structure of the pneumococcal peptidoglycan was found to resemble that of other characterized Gram-positive bacteria, the wall TA is complex and unusual and contains choline as a structural component. Thus pneumococcus is unique among prokaryotes due to an absolute requirement for choline in growth, which is incorporated as phosphorylcholine into the cell wall TA and the membrane LTA (38).

2.1.4.4. Most important enzymes

2.1.4.4.1. Pneumolysin

A key virulence factor of pneumococcus in colonizing the upper respiratory tract is the pneumococcal thiol-activated, membrane pore forming toxin, called pneumolysin (39). Pneumolysin is a 53 kDa protein composed of 471 amino acids. It is common to all serotypes and it can be thought of as a multi-effective factor for virulence following pneumococcal infection. At high levels it is lytic to all cells with cholesterol in the membrane. The lytic activity of this toxin can be inhibited by preincubation with cholesterol, consistent with the suggestion that membrane cholesterol is the receptor for this toxin (40). At lower, sublytic concentrations, which exist in the early stages of infection, the toxin also may cause a range of effects, including induction of apoptosis, activation of host complement and induce proinflammatory reactions in immune cells. At higher lytic concentrations, which may exist in the later stage of infection, the toxin may cause widespread direct cellular and tissue damage by virtue of its membrane pore forming properties (40-42).

2.1.4.4.2. Autolysin

S. pneumoniae has a special autolytic response that leads to the excessive lysis of cultures in vitro and causes the characteristic colony morphology called umbilicated shaped colonies. The main autolysin in the pneumococcus is N-acetyl-muramoyl-1-alanine amidase, commonly known as LytA, which encoded by the *lytA* gene. LytA causes lysis by cleaving the lactyl-amide bond that links the stem peptides and the

glycan strands of the peptidoglycan, resulting in hydrolysis of the cell wall. LytA orthologs are now known to be conserved throughout eubacteria and in many bacteriophages (43-45).

The *in vivo* function of the suicide LytA enzyme remains controversial. One hypothesis is that LytA mediates lysis to release other virulence factors such as pneumolysin (46).

Another theory suggests that LytA is released to lyse neighbouring non-competent pneumococcal cells (47). This would potentially facilitate genetic exchange between naturally competent pneumococcal populations that easily take up and incorporate DNA by homologous recombination.

A third possibility is that LytA mediates lysis to release proteins involved in immune evasion or cell wall components that may interfere with the host immune response (48).

From diagnostic point of view, *lytA* gene can be used for specific identification of *S. pneumoniae* (1).

It is also interesting that there is a highly polymorphic region in the *lytA* gene where two different families of alleles can be differentiated by PCR and restriction digestion. Morales et al proved that this polymorphic region arose from recombination events with homologous genes of pneumococcal temperate phages (45).

2.1.4.4.3. IgA protease

Antibodies of the immunoglobulin A (IgA) class react with capsular polysaccharides of *S. pneumoniae* and support complement-dependent opsonophagocytosis of the organism by phagocytes. IgA may provide both local defence against mucosal infection and activity in local tissues to prevent dissemination of the infection (49).

The IgA1 protease is one of the two to four large zinc metalloproteinase present in the pneumococcal genome (50). This protease is a polypeptide of about 1900 amino acids associated to the bacterium via N-terminal anchoring. The enzyme specifically cleaves the hinge regions of human IgA1, which dominates most mucosal surfaces and is the major IgA isotype in serum. This protease is expressed in all of the known pneumococcal strains and plays a major role in pathogen's resistance to the host immune response(51).

2.1.4.4.4. Other enzymes

Most of the human cell surfaces and secreted molecules are glycosylated. This glycosylation is often complex and involves a number of different sugar residues. Glycosylation serves a number of functions including recognition processes, cell-to-cell interactions and the binding and transport of positively charged molecules (52). Pneumococcus expresses a variety of enzymes: surface attached exoglycosidases: neuraminidase, β-galactosidase and N-acetylglucosaminidase, which sequentially remove terminal sugars common to many human glycoconjugates. Deglycosylation of host molecules may expose cell-surface receptors, inhibit mechanisms of clearance that require these glycoproteins, or provide nutrition for the organism (53).

2.2. Genetic background of Streptococcus pneumoniae

2.2.1. The position of pneumococcus in the *Streptococcaceae* family

Streptococci were first recorded in 1683 in van Leeuwenhoek's drawings of microscope images of the material removed from between his teeth (54). The main entry of streptococci into history was in 1879, when Louis Pasteur was studying puerperal fever. This was causing high mortality rates in maternity wards. Within the bodies of diseased women, he found rounded granules (microorganisms) arranged in the form of chains or strings of beads. He was convinced and it was later proven, that this was the cause of infections in women after childbirth (54).

This famous meaningful event belongs to Ignác Semmelweis, who was working at the largest maternity hospital in the world: the Vienna Maternity Hospital, which was divided into two clinics. Almost all the maternal deaths there were due to puerperal fever. The excess deaths in the first clinic were due to the routine procedures carried out in the courses attended by doctors and medical students. Each day started with postmortem examinations of women who had died of puerperal fever. Then, without washing their hands, the pupils went straight to the maternity wards to perform vaginal examinations. By contrast, the pupil midwives in the second clinic did not undertake either post-mortem examinations, nor routine vaginal examinations (55). In 1847 he introduced a system whereby the students were required to wash their hands in chloride of lime before entering the maternity ward. The result was dramatic. In 1848, the

maternal mortality rate in the first clinic fell compared to the rate in the second (midwives) clinic. Thus, streptococci were one of the first microbes to be identified as causing contagious disease and their existence led to the introduction of hygiene and aseptic practices into hospital wards (55).

There are now over 100 recognized species of streptococci. Historically, the classification of streptococci was based on the Lancefield scheme, which groups streptococcal strains according to the carbohydrate composition of cell wall antigens (56, 57). Such antigens, known as group-specific antigens or C substances, are either polysaccharides (as in groups A, B, C, E, F and G), teichoic acids (as in groups D and N), or lipoteichoic acid (as in group H). This approach has proved successful for the more pathogenic streptococci, but its widespread application is hindered by the fact that group-specific antigens for other species may be absent or shared between distinct taxa. The streptococci may also be organized into six groups (**Figure 6**) based on 16S rRNA gene sequences (10).

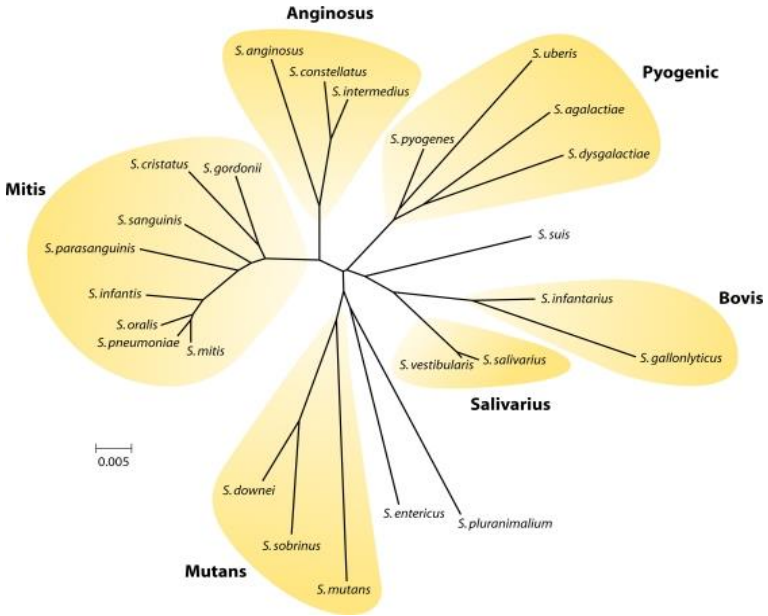

Figure 6. The pyogenic group of Strepotococci. It includes *Streptococcus pyogenes* (Lancefield group A), *Streptococcus agalactiae* and *Streptococcus uberis* (group B) and *Streptococcus dysgalactiae* (group C, G, or L). The mitis group comprises species almost all of which are isolated from the human oral cavity or nasopharynx. *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus gordonii* and *Streptococcus pneumoniae* are highly related and because of extensive horizontal gene transfer, the delineation of strains into these species is often blurred (10)

Although identification of streptococci is based on the current taxonomic standards using a combination of 16S rRNA gene sequence analyses, DNA-DNA hybridization, serologic and phenotypic data, 16S rRNA gene sequences of *S. mitis* and *S. oralis* are almost identical (> 99%) to *S. pneumoniae*, making the use of this information alone insufficient to distinguish these species.

2.2.1.1. Evolution of *Streptococcus pneumoniae*

Based on Kilian et al. research it is supposed that the immediate common ancestor of *S. pneumoniae, S. mitis* and *S. pseudopneumoniae* (pneumoniae-mitis-pseudopneumoniae) cluster was a bacterium with resemblance to the present-day pneumococcus with all the properties associated with virulence(58). One of these properties, the IgA1 protease evolved by gene duplication in response to emergence of the immunoglobulin A1 (IgA1) subclass in the common ancestor of man, chimpanzees and gorillas (59), which according to recent calculations existed 6 to 7 million years ago (60). While the pneumococcus lineage conserved the expression of both capsule production and IgA1 protease activity to ensure their ability to colonize in the presence of IgA1 antibodies (61), lineages evolving into a commensal life style with a more subtle relationship with the mucosal immune system and the host in general gradually lost both characters and achieved the colonization advantage of the capsule-deficient phenotype (62). This evolutionary model proposing that the pneumoniae-mitis-pseudopneumoniae cluster arose from a pneumococcus-like organism pathogenic to the immediate ancestor of hominoids is consistent with our inability to isolate *S. mitis*-like bacteria from a range of mammals including old and new world monkeys, pigs, dogs, sheep, cattle, rats and mice, while there is evidence of pneumococci causing infections in chimpanzees and other mammals (63-65).

2.2.2. Genotyping of pneumococcus

For a better taxonomic resolution 16S rRNA gene sequence analysis, multilocus sequence analysis (MLSA) (66), average amino acid identity (AAI) (67), Genome-to-genome distances (GGD) (68) and codon usage analysis (69) can be rarely used (70).

Currently, pulsed-field gel electrophoresis (PFGE) (71, 72) and multilocus sequence typing (MLST) (66) are the gold standards for genotyping of pneumococci.

Advantages of PFGE are that it has good typeability, reproducibility and resolving power. In addition, the costs for materials and equipment are relatively low and handling of the equipment is easy. However, it is laborious and time consuming and may yield ambiguous results if not performed by a well-trained technician. PFGE is quite useful for local epidemiology and it has also been used for global epidemiology once standardized (73).

MLST is a DNA sequence-based method that relies on PCR amplification and sequencing of internal fragments of 7 housekeeping genes [**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)] (73). For allele assignment, each sequence is compared to all known alleles which are available at an online database (http://spneumoniae.mlst.net/). Different sequences are assigned different allelic numbers. The 7 assigned allele numbers form an allelic profile or sequence type (ST). MLST is expensive; therefore many laboratories cannot afford to use it routinely. However, it has the advantages of being reproducible, unambiguous, portable allowing intra-laboratory comparisons and suited to create international databases. For *S. pneumoniae*, MLST has a good resolving power being useful for local and global epidemiology. Furthermore, in contrast to PFGE, MLST does not always require a culture and can sometimes be directly performed on samples containing bacterial DNA such as cerebrospinal fluid (74).

In 1992 conserved repeated sequences, named BOX elements, were identified in the genome of the pneumococcus (75). The genome contain 115 and 127 BOX elements, respectively. BOX elements consist of 3 different subunits, BoxA, BoxB and BoxC. The function and origin of BOX elements are unknown; however, they may be involved in regulating the expression of virulence-associated genes (75). A multiple-locus variable number tandem repeat analysis (MLVA) scheme based on BOX typing was introduced in 2005 (76). It analyses 16 BOX loci that are PCR-amplified in single PCR reactions and products are analysed by agarose gel electrophoresis. A website

(www.mlva.eu) providing a database in which profiles can be compared has been created (76).

2.2.3. Serotyping of pneumococcus

S. pneumoniae serotyping was developed at the beginning of the 20th century using panels of specific anti-sera produced in animals and directed against polysaccharides of the pneumococcal capsule (14). For several decades the traditional capsular swelling test, the Quellung reaction was the gold standard method (77). To perform traditional agglutination assays, growth of *S. pneumoniae* on culture media is required. However, culture is often negative if patients received antibiotics before sampling of blood, cerebrospinal fluid or other biological fluids. In recent years, immunological assays based on ELISA (Enzyme-linked immunosorbent assay) or latex agglutination have been shown to work directly on clinical specimens (78). PCR-based serotyping using primers that amplify serotype-specific sequences are also widely used method for serotyping (79, 80).

2.3. Clinical aspects

S. pneumoniae is a major cause of morbidity and mortality worldwide, particularly in young children, individuals with chronic cardiopulmonary disease, the elderly and immunocompromised such as HIV+. In splenectomised patients, where the encapsulated organisms are the most virulent pathogens, pneumococcus is the most important, but *Haemophilus influenzae* and *Neisseria meningitidis* are also significant (81). *S. pneumoniae* causes wide variety of infections including mucosal infections, sinusitis and otitis media, eye infections, pneumonia, arthritis, pericarditis, peritonitis and severe invasive infections such as meningitis and septicaemia (**Figure 7**). According to CDC, ~1 million children below 5 years of age die yearly in pneumococcal infections (**Figure 8**) (82-84). The normal habitat for pneumococci is the nasopharynx, mostly of small children (85). Colonization precedes pneumococcal disease and colonized individuals serve as a reservoir for horizontal spread of the bacterium in the community (85).

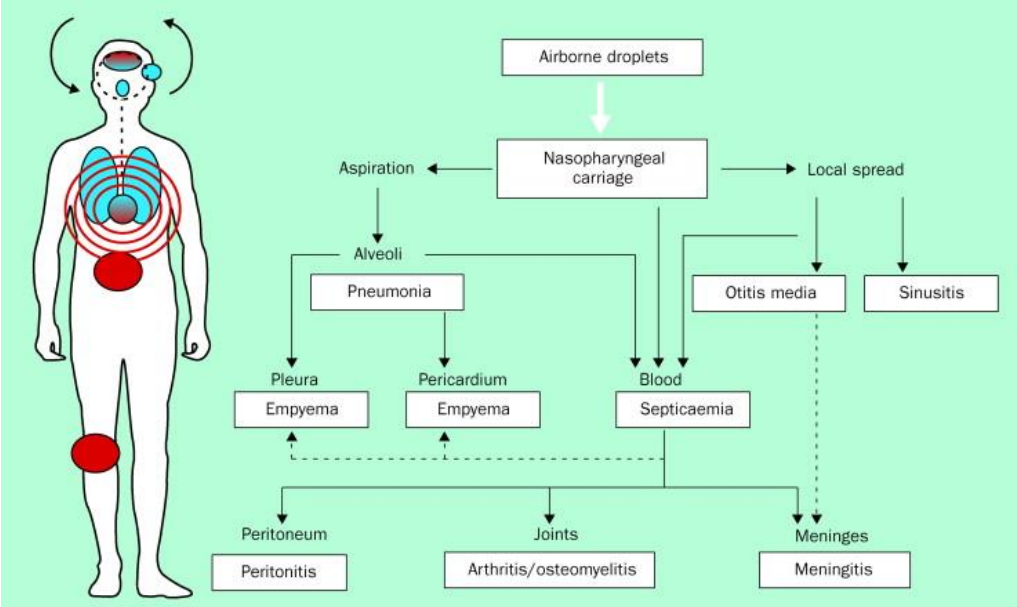

Figure 7. Pathogenic route for *S. pneumoniae* infection. Organs infected through the airborne and haematogenic routes are depicted in blue and red, respectively (85).

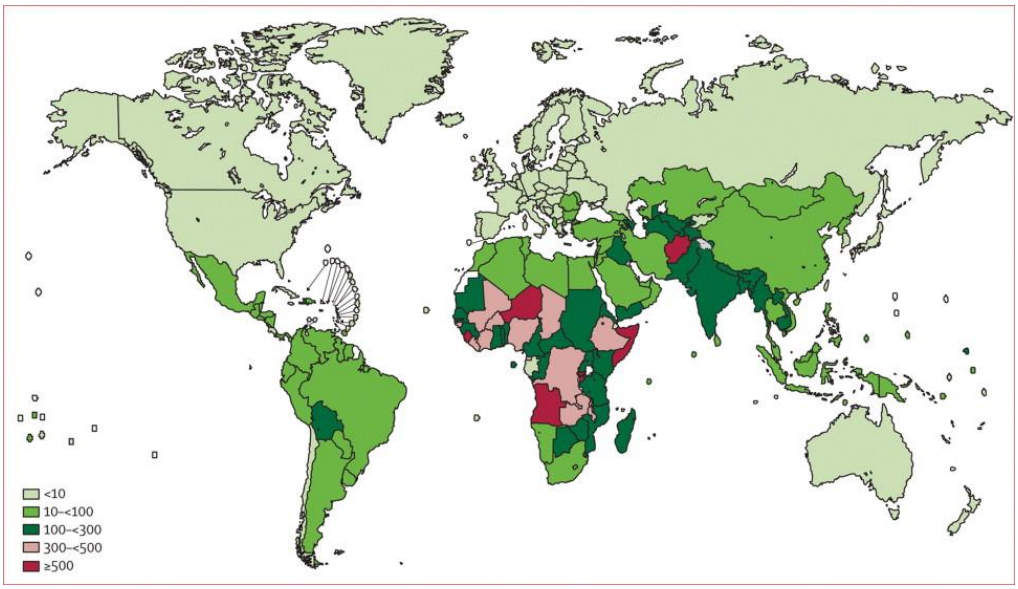

Figure 8. Pneumococcal mortality rate. Pneumococcal deaths in children aged 1–59 months per 100000 children younger than 5 years (HIV-negative pneumococcal deaths only). Colours show different incidences (84)

2.3.1. Carriage

The main biological niche for *S. pneumoniae* is the upper respiratory tract of humans where it colonizes the mucosal surfaces lining the nasopharynx (86). Disease occurs when resident organisms from the upper respiratory tract gain access to normally sterile spaces in the middle ear, lung, or bloodstream and therefore colonization is the initial step in the pathogenesis of all pneumococcal disease (85, 87). The carriage rate is highest in young children, who most likely carry pneumococci in the nasopharynx at least one time and are the primary source for its spread within a community (86).

Before the wide use of conjugate vaccines four serogroups 6, 14, 19 and 23 were referred to as 'pediatric serotypes' and they were among the most commonly carried ones in children and at the same time caused the most cases of pneumococcal acute otitis media (88, 89).

Although conjugate vaccines against *S. pneumoniae* reduce carriage of the serotypes contained in the vaccine, there is little or no impact in most populations on the overall prevalence of pneumococcal carriage as other serotypes become more common (90, 91). This process, known as serotype replacement is exemplified by increased carriage and disease prevalence caused by serotype 19A, observed in several populations following the introduction of seven-valent pneumococcal conjugate vaccine (PCV7) (91), (92).

2.3.1.1. The impact of carriage

Gray et al. investigated carriage and disease in 82 infants followed from birth up to 2 years of age. In this study they sampled the nasopharynx of children monthly for the first 6 months of life and at 2–3-month intervals thereafter until 2 years of age and collected information about any disease episodes experienced by the children. By correlating acquisition episodes and carriage periods of pneumococcus with culture-confirmed pneumococcal disease, they concluded that pneumococcal infection, most of which (28/31) was acute otitis media (AOM), is mainly associated with a newly acquired serotype. In particular, although prolonged duration of carriage was common, 74% of infections were caused by serotypes found less than a month before the illness. Carriage itself was not found to pose risk for disease and the authors hypothesized the

role of prolonged carriage as one of the protective factors against pneumococcal disease (93).

McCool et al. performed an experimental pneumococcal colonization in humans. Many of the participants developed a mild rhinorrhea without other symptoms. Together, these observation provide suggest that initiation of pneumococcal colonization is an inflammatory rather than a quiescent process (94).

2.3.2. Diseases caused by *Streptococcus pneumoniae*

2.3.2.1. Otitis media

Otitis media (OM) is the most frequently reported paediatric bacterial infection, with approximately 80% of children experiencing an episode by the age of three years (95). At the beginning of the 20th century, group A *Streptococcus* (GAS) was the most common pathogen leading to complications in AOM, but it is now rare in the Western world. A 'new' triad of AOM pathogens has emerged in the last century – *S. pneumoniae*, non-encapsulated *H. influenzae* and *Moraxella catarrhalis* (96, 97) - with the ability to cause long-term hearing damage (98). AOM can be caused also by a number of different viruses (99).

Some pneumococcal serotypes (i.e., serotypes 3, 5, 1, 12F, 19F, 19A) seem to have a higher AOM disease potential once carriage is established, since in countries where the pneumococcal conjugate vaccines (PCV7, and later PCV13) is widely used, non-vaccine serotypes account for a more significant proportion of this disease. Among them an increase in serotypes 6C, 22F, 23B, 35B and nontypeables (NT) was observed after PCV13 vaccination (88, 100).

2.3.2.2. Eye infections

S. pneumoniae is found rarely in normal conjunctivae (0–0.3%) (101), but obstruction of the nasolacrimal duct predisposes to ascendant colonisation and infection of the lacrimal system and conjunctivae with the normal nasopharyngeal commensals including this bacterium (102). Therefore pneumococcus is typically among the top three most commonly isolated species from cases of bacterial keratitis, an infection of the cornea of the eye (103, 104). Pneumococcal keratitis can be a sight-threatening

infection if left untreated or if treatment is delayed. Corneal ulceration occurs during the course of the infection and often results in an opaque scarification of the corneal surface after the infection is cleared. The clinical picture of pneumococcal keratitis is a central yellowish or greyish white ulcer associated with infiltrates, folds in Descemet's membrane and hypopyon. Spread in an irregular fashion produces a ‘serpiginous ulcer’ (Ulcus serpens corneae) with the advancing edge of the ulcer being undermined (103).
In case of endophthalmitis *S. pneumoniae* is an infrequent causative agent, being isolated from 2.2–13.6% of cases (102, 105).

2.3.2.3. Pneumonia

S. pneumoniae remains the leading microbial aetiology of community acquired pneumonia (CAP) (106). It occurs most frequently in patients least well equipped to handle the effects of the disease, namely, the very young or the very old, the immunosuppressed or chronically ill. The development of pneumococcal pneumonia results from translocation of pneumococci from the nasopharynx to the lung through aspiration and possibly blood-borne dissemination (107) (**Figure 7**). In adult invasive and non-invasive pneumococcal CAP, a study from UK (between 2008 and 2010) showed that the most common serotypes implicated were 14, 1, 8, 3 and 19A (108). According to CDC the use PCV7 since 2000 and PCV13 since 2010 among children in the United States has reduced pneumococcal infections directly and indirectly among children, and indirectly among adults. Approximately, 20%–25% of IPD cases and 10% of community-acquired pneumonia cases in adults aged ≥65 years are caused by PCV13 serotypes and are potentially preventable with the use of PCV13 in this population (109).
A new French study from the post PCV13 era demonstrated 32% decrease in CAP cases in children younger than 2 years. These data suggest a strong impact of PCV13 on CAP, pleural effusion and documented pneumococcal pneumonia, particularly cases due to PCV13 serotypes (110).

2.3.2.4. Invasive pneumococcal diseases (meningitis, sepsis)

World Health Organization (WHO) estimates that approximately 1 million children die each year of invasive pneumococcal disease (IPD) such as meningitis and sepsis (84). The seven most common serotypes causing IPD in the era prior to PCV introduction included 1, 5, 6A, 6B, 14, 19F and 23F (111). Serotype 14 was the most common serotype accounting for 12%–29% of IPD (111). After the introduction of PCV7 vaccine the rate of all IPD cases dropped by almost 70%, as it happened in the US (112). The highest incidence of IPD generally occurs among children aged 6–11 months, at the very same age when the incidence of pneumococcal acquisition is high (113). It is also shown that serotypes 3, 6B, 14, 23F can cause more severe meningeal inflammation than serotypes 1, 5, 9 and 7F (114). A recently published study from Israel conclude the effect of PCV7 and PCV13 vaccination on IPD (115). In total, a 63% reduction of all-serotype IPD episodes was observed. They found that the rate of IPD caused by serotype 1, 3, 5, 7F and 19A increased by 47% in children <5 years after PCV7 vaccination and after PCV13 vaccination an overall 70% reduction of IPD caused by these serotypes was observed. On the other hand the rate of IPD caused by non-PCV13 serotypes increased by 54% when comparing the PCV7 and the pre-PCV periods in children <5 years. Of all non-PCV13 disease, serotypes 12F, 15B/C and 33F were found to be the most common serotypes.

Other studies from Europe observed almost the same, that PCV13 introduction significantly decreased the cases of IPD, pneumonia and otitis media despite potential non-PCV13 serotype replacement (116, 117).

2.4. Prevention of pneumococcal diseases

2.4.1. History of vaccination

The first anti-pneumococcal serum appeared in the H.K. Mulford catalogue (where it remained into the 1940s) in 1895 (118). Early studies of antiserum therapy of pneumococcal infections came in the 1910s (6). The first specific record is about a whole-cell heat-treated pneumococcal vaccine (‘Pneumo-Bacterin’) licensed in the USA in 1909, with manufacturers such as H.K. Mulford Co., Eli Lilly & Company and Parke, Davis & Co (3). From 1942 to 1945, Heidelberger and MacLeod took advantage of the

preceding developments in polysaccharide technology to develop and test a pneumococcal polysaccharide vaccine at the US Army Air Force Technical School (119). With this finding it was proved that purified CPS can be used as active immunogens in adult humans and clinical efficacy against pneumonia was shown but the approach was abandoned with the availability of antibiotics. As the limitations of antibiotics therapy were realized, vaccination with pneumococcal CPS was revived in the 1960s through the efforts of Robert Austrian (120). A 14-type mixture of the most prevalent types was selected for vaccination in 1977 and increased to 23 types in 1983 (**Table 2**). This vaccine is licensed and recommended for adults >65 y and younger subjects with conditions, such as asplenia, that predispose to pneumococcal infection. Pneumococcal polysaccharide vaccines are poorly immunogenic in children younger than 2 years of age, who are at high risk of invasive pneumococcal disease (121). To improve the immune response to the capsular polysaccharide in young children, third-generation vaccines in which capsular polysaccharides are conjugated to one of several different proteins were developed and tested (122). However, the technology limits the number of serotypes that can be included and infections caused by non-included types are a substantial problem in some populations (91).

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Table 2. Pneumococcal vaccines distributed since 1977 (3)

MANUFACTURERS (TRADE NAMES)	**VALENCE**: SEROTYPES INCLUDED	**DATE INTRODUCED, INITIAL REGION**
PNEUMOCOCCAL POLYSACCHARIDE VACCINES		
Merck Sharp & Dohme (MSD) (Pneumovax™) Lederle Laboratories (Pnu-Imune™) Institut Mérieux (Imovax Pneumo 14™)	**14-valent:** 1, 2, 3, 4, 5, 6A, 7F, 8, 9N, 12F, 18C, 19F, 23F, 25F	November 1977, USA August 1979, USA February 1981, France
Smithkline Beecham (Moniarix™)	**17-valent:** 1, 2, 3, 4, 6A, 7F, 8, 9N, 11A, 12F, 14, 15F, 17F, 18C, 19F, 23F, 25	1980s, Europe
Merck Sharp & Dohme (MSD) (Pneumovax™ 23) Lederle Laboratories (Pnu-Imune™ 23) Institut Mérieux (Pneumo 23™) Chengdu Institute Of Biological Products	**23-valent:** 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F	USA July 1983, USA_1987, Europe 2005, China
PNEUMOCOCCAL CONJUGATE VACCINES		
Wyeth Laboratories (Prevnar™ Or Prevenar™) Now Pfizer	**7-valent:** 4, 6B, 9V, 14, 18C, 19F, 23F	February 2000 for infants, USA and Europe
Glaxo Smith Kline (GSK) (Synflorix™)	**10-valent:** 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	March 2009 for infants: Europe
Pfizer (Prevenar™ 13)	**13-valent:** 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F	February 2010 for infants, late 2011 for adults, Europe, Australia, USA

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2.4.2. Available vaccines nowadays

2.4.2.1. Polysaccharide vaccine

Pneumovax®23 (PPV23, MSD) has been recommended since the mid-1980s for use in adults ≥65 years and in younger adults who have any risk conditions (asplenia or splenic dysfunction, various malignancies, immunosuppression, asthma etc.) (123). There is considerable debate regarding the efficacy of PPV23 in preventing non-invasive pneumococcal pneumonia, although the general belief is that there is evidence of protection against IPD, at least in healthy young adults and in the healthy older population (123). Its use in children is limited as polysaccharides are poorly immunogenic in infants and children under the age of 2 years (124).

2.4.2.2. Conjugate vaccines (PCV)

Lack of efficacy of PPV23 in neonates and infants <2 years of age led to the development of PCVs. While PPV23 elicits a T-cell independent, humoral immune response, covalent conjugation of capsular polysaccharides to a carrier protein activates a T-cell dependent antibody response in the setting of mucosal immunity and immunological memory (125) (126). Mucosal immunity mediates nasopharyngeal carriage, thus being associated with, among other factors, herd protection. This has been confirmed in many studies in which the use of childhood PCV was associated not only with a sustained decrease in IPD and declines in US hospitalisations for pneumonia in children, but also significant decreases in IPD in older adults (125) (127). Prevenar-7 (PCV7) contains capsular polysaccharides from seven *S. pneumoniae* serotypes conjugated to CRM_{197}, a non-toxic mutant of diphtheria toxin. When PCV7 was licensed, those serotypes (4, 6B, 9 V, 14, 18C, 19 F and 23 F) caused the majority of invasive pneumococcal infections in the US (128) and were also associated with antibiotic resistance (129). PCV7 was included in the National Immunization Programmes (NIP), or was recommended for routine vaccination, in a number of European countries, between 2006 and 2008 (**Table 3)** (87).

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Table 3. PCV7 adoption in NIP or universal vaccination recommended by health authorities in European countries (87)

[1] Limited recommendation in 2003 with 3 + 1 administration schedule

[2.] PCV7 was recommended regionally prior to the date of inclusion in the NIP

[3.] PCV vaccination recommendation is regional

COUNTRY	INTRODUCTION IN NIP OR UNIVERSAL RECOMMENDATION	SCHEDULE
BELGIUM	Jan 2007	2 + 1
CYPRUS	Sep 2008	3 + 1
DENMARK	Oct 2007	2 + 1
FRANCE	May 2006	2 + 1
GERMANY	Jul 2006	3 + 1
GREECE	Jan 2006	3 + 1
HUNGARY	Oct 2008, mandatory since July 2014	2 + 1
IRELAND	Sep 2008	2 + 1
ITALY	Dec 2006	2 + 1
LUXEMBOURG	Feb 2003	3 + 1
NETHERLANDS	Jun 2006	3 + 1
NORWAY	Jul 2006	2 + 1
SPAIN (MADRID)[3]	Jan 2007	3 + 1
SWITZERLAND	Jul 2006	2 + 1
TURKEY	Jan 2009	3 + 1
UK	Sep 2006	2 + 1

PCVs are effective not only in small children, but also in elderly. CAPiTA study (Community-Acquired Pneumonia Immunization Trial in Adults) shows that adults 65 years and older who received PCV13 had 45.56% fewer first episodes of vaccine-type CAP, 45% fewer episodes of non-bacteraemic vaccine-type CAP, and 75% fewer episodes of vaccine-type IPD compared to the placebo group (http://www.empr.com/detailed-data-from-prevnar-13-capita-study-released/article/337871/). Therefore vaccination in this group is also suggested.

Some emerging serotypes are associated with increased resistance to antibiotics and higher propensity for invasive disease, especially serotype 19A. Additionally, the efficacy of PCV7 in preventing pneumonia or mucosal disease such as AOM is less pronounced than its ability to reduce bacteremic disease. To address these issues, several expanded valency (so-called 2nd generation, **Table 4**) PCVs have been licensed (13-valent Prevenar®, Pfizer; 10-valent with 3 different protein carriers Synflorix™, GSK Biologicals), or are currently under investigation (15-valent PCV, Merck). The expanded serotype coverage of these vaccines may further reduce IPD rates in the US and Western Europe, but the complexity and cost of these vaccines will undoubtedly continue to rise.

Table 4. Pneumococcal serotypes included in the licensed pneumococcal conjugate vaccines (87)

VACCINE	MANUFACTURER	SEROTYPES	INDICATED AGE
PCV7 (PREVENAR)	Pfizer	4, 6B, 9V, 14, 18C,19F, 23F	< 5 years >60 years
PCV10 (SYNFLORIX)	GSK	4, 6B, 9V, 14, 18C,19F, 23F, 1, 5, 7F,	< 5 years
PCV13 (PREVENAR 13)	Pfizer	4, 6B, 9V, 14, 18C,19F, 23F, 1, 3, 5, 6A, 7F, 19A	< 5 years >50 years

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2.4.2.3. New perspectives in vaccination

2.4.2.3.1. Protein-based pneumococcal vaccines

Polyhistidine triad protein D (PhtD) has been described as a promising vaccine candidate for use against pneumococcal infections (130). These surface-exposed antigens are characterised by the presence of five to six histidine triad motifs and are highly conserved amongst pneumococcal strains and could elicit protective immunity in mouse models of pneumococcal disease against a number of pneumococcal strains (131).

Other strategy involves the use of one or more pneumococcal protein antigens common to all serotypes, to provide cheap, non-serotype-dependent protection. In one study, the protective efficacy of immunization of mice was evaluated with PdB (a pneumolysin toxoid), PspA, PspC (CbpA), PhtB and PhtE in an invasive-disease model (132).

Based on several in vitro assays Giefing et al. preselected 18 novel candidates for animal studies and 4 of them showed significant protection against lethal sepsis. Two vaccine candidates, protein, required for cell wall separation of group B streptococcus (PcsB) and serine/threonine protein kinase (StkP), were found to be exceptionally conserved among clinical isolates. Therefore a vaccine containing PcsB and StkP is intended for the prevention of infections caused by all serotypes of pneumococcus in the elderly and in children (133).

Alexander et al. immunized mice with a genetically engineered toxoid version of pneumolysin, which was derived from a serotype 2. Immunized mice had significantly increased levels of anti-pneumolysin antibodies. And they have shown protection against nine tested pneumococcal serotypes too (134).

2.4.2.3.2. Whole cell vaccine

Malley and Anderson investigated killed cells of a noncapsulated strain, which expose many such common antigens. Given to mice intranasally, this vaccine elicits antibody-independent, CD4+ T lymphocyte-dependent accelerated clearance of pneumococci of various serotypes from the nasopharynx mediated by the cytokine IL-17A. Given by injection, the killed cell vaccine induces bifunctional immunity: plasma antibodies

protective against fatal pneumonia challenge, as well as IL-17A–mediated nasopharyngeal clearance (135).

Recent study shows, that live and heat-killed *Lactobacillus casei* enhanced the antigen-specific immune response when administered nasally conjugated with a pneumococcal antigen (pneumococcal protective protein A: PppA). This type of vaccines can be a safe and effective strategy for the prevention of pneumococcal infections and opens new possibilities of application of dead lactobacilli as adjuvants in vaccine formulations against other pathogens (136). It is also proved that this vaccine is safety in liver, kidney, pulmonary and systemic levels (137).

In a study by Lu et al. whole-cell pneumococcal vaccine was examined as an injectable vaccine using the currently approved aluminum adjuvant. A pneumococcal strain was engineered to be capsule negative, autolysin negative and to express a nontoxic mutant pneumolysoid. The adjuvant $Al(OH)_3$ strongly increased immune responses in mice injected with the chemically inactivated cells. The immunized mice were protected against nasal colonization and sepsis with different pneumococcal serotypes (138).

Roche et al. constructed live attenuated mutants of *S. pneumoniae* containing deletions in genes encoding three of major virulence determinants including capsular polysaccharides, pneumolysin and *pspA*. The attenuated strains were not able to cause disease but retained the ability to colonize the upper respiratory tract. Nasal colonization by live attenuated vaccine strains was used to immunize mice. A single intranasal administration of live attenuated vaccine without adjuvant induced both systemic and mucosal protection from intranasal challenge with a high dose of the parent strain. Colonization by live attenuated *S. pneumoniae* is an effective vaccine strategy that may offer broad protection against pneumococci (139).

2.4.2.3.3. DNA vaccines

DNA vaccines are easy to manufacture, have a low cost and are stable in transportation. These properties make DNA vaccines ideal for implementation in developing countries. Vectors encoding the complete N-terminal regions of PspAs elicited humoral responses in mice and cross-reactivity was mainly restricted to the same family (140).

Nasal delivery of naked plasmid DNA induces only weak immune responses, which may be due to the degradation of naked DNA at mucosal surfaces (141). Therefore, for

construction of effective DNA vaccines, an appropriate DNA delivery system is demanded. Chitosan is a natural biodegradable polysaccharide derived from chitin that possesses biocompatibility and mucoadhesion properties. Mice were immunized intranasally with chitosan-DNA nanoparticles expressing PsaA (chitosan-*psaA*). Compared to levels in control mice immunized with naked DNA containing *psaA* gene or chitosan-backbone vector without *psaA* gene, systemic and mucosal immune responses against PsaA were elevated in mice immunized with chitosan-*psaA*. In addition, fewer pneumococci were recovered from the nasopharynx of mice vaccinated with chitosan-*psaA* than for the control groups following intranasal pneumococcal challenge. These findings suggested that chitosan-DNA nanoparticles expressing pneumococcal major antigens could be developed to prevent pneumococcal infections (142).

2.5. Antibiotic treatment

The discovery of penicillin by Alexander Fleming initiated a success story of antimicrobial compounds unmatched by any other antibiotic. This β-lactam antibiotic has long been the mainstay against pneumococcal infections, but its efficacy is threatened by the rapid dissemination of penicillin-nonsusceptible clones worldwide (4, 143).

2.5.1. Antimicrobial resistance of *Streptococcus pneumoniae*

2.5.1.1. Development of resistance

The emergence of penicillin-resistant and multidrug-resistant pneumococcal strains has become a global concern. Since 1960s, penicillin-resistant strains have been found in various parts of the world with increasing frequency. Resistance to non-β-lactam antibiotics such as chloramphenicol, tetracycline, erythromycin, clindamycin, rifampin and trimethoprim-sulfamethoxazole has been reported (9, 144). Penicillin-resistant and multidrug-resistant pneumococci are known to be restricted worldwide to a few serogroups, namely 23, 6, 19, 9 and serotype 14, which were particularly associated with carriage and disease in children in the pre-vaccination era (9, 145).

Molecular studies have shown that the penicillin-resistant and multidrug-resistant pneumococcal populations are highly dynamic and that resistance is a combination of the spread of resistant clones, the acquisition and loss of resistance genes within those clonal lineages and the spread of resistance genes to new lineages (73).

2.5.1.2. Resistance mechanism

2.5.1.2.1. β-lactam resistance

Resistance to β-lactam antibiotics in clinical isolates of *S. pneumoniae* is mediated by mosaic genes encoding altered penicillin-binding proteins (PBPs; a family of enzymes involved in peptidoglycan metabolism) with lower antibiotic binding affinities than their native versions (146, 147). While *S. pneumoniae* contains six PBPs, variants of PBP2x, PBP2b and PBP1a are considered the most relevant for resistance and the acquisition of low-affinity PBP2x and PBP2b variants is a necessary first step for the acquisition of PBP1a variants that confer high-level resistance to β-lactams (148). Pneumococci have a dedicated system for the acquisition of exogenous DNA from the environment and the mosaic gene structure of low-affinity PBPs is the result of interspecies gene transfer events involving closely related streptococcal species (149).

The presence of other, non-PBP contributors has also been reported. For example, the cell wall of penicillin-nonsusceptible isolates is often highly enriched in branched-chain muropeptides, a phenomenon that has been linked to mosaic alleles of the *murM* gene (150, 151). Furthermore, mutations in a peptidoglycan *N*-acetylglucosamine (GlcNAc) deacetylase (152), a peptidoglycan *O*-acetyltransferase (153), a putative glycosyltransferase (154), a serine threonine kinase (155), a histidine protein kinase that is part of a two-component signal-transducing system (156) and a phosphate ABC transporter (157) have all been implicated in resistance to β-lactams. Finally, the selection of a nonsense mutation in a putative iron permease in penicillin-resistant *S. pneumoniae* has recently been shown to decrease susceptibility to bactericidal antibiotics, including penicillin (158).

2.5.1.2.2. Macrolide resistance

Resistance to erythromycin in *S. pneumoniae* was first detected in 1967 in the United States and subsequently worldwide (9, 159, 160). Macrolide resistance is mediated by two main mechanisms: target modification due to a ribosomal methylase encoded by the *erm* (erythromycin ribosome methylase) gene, which confers high-level resistance to macrolides, lincosamides and streptogramin B (MLS_B types) usually carried by transposable elements (160) and the second one the efflux transport system associated with the *mef* gene (160).

The widely predominant *erm* gene is *ermB* gene although this is not the only representative of the *erm* gene class in pneumococci. The presence of an *ermA* gene has been reported in some strains where it conferred cross-resistance to erythromycin and clindamycin (161).

Resistance with the help of efflux pump appears expressed at moderate levels, with erythromycin MICs of between 1 and 64 μg/ml (generally between 8 and 32 μg/ml). Because the 16-membered macrolides, the lincosamides and the streptograminB antibiotics are not substrates of the pump, these antimicrobial agents remain active, even after induction with erythromycin. Resistance to erythromycin combined with susceptibility to clindamycin, whether the cells are induced or not induced by erythromycin, defines the M resistance phenotype. The *mef* gene is also transferable among pneumococci (162). Of the two variants of the *mef* gene, *mefA* was originally found in *Streptococcus pyogenes* (163) and *mefE* was originally found in *S. pneumoniae* (164). *MefA* and *mefE* are 90% identical at the nucleotide level.

2.5.1.2.3. Resistance to other antibiotics

The genetic basis of sulfonamide resistance in *S. pneumoniae* was demonstrated to be 3- or 6-bp duplications within *sulA*, the chromosomal gene encoding dihydropteroate synthase (165).

The tetracycline resistance is a result of the acquisition of one of the two genes, *tetM* and *tetO*, both of which encode ribosome protection proteins (166, 167). Pneumococcal resistance to erythromycin and tetracycline is frequently associated with

the insertion of the *ermB* gene into the transposons of the Tn*916* or Tn*917* family (Tn*6002*, Tn*2010*, Tn*3872*, Tn*1545* and Tn*6003*) that contains the *tet*M gene (168).

2.5.1.2.4. Successful pneumococcal clones

The Pneumococcal Molecular Epidemiology Network (PMEN) was established in 1997 under the auspices of the International Union of Microbiological Societies with the aim of standardizing the nomenclature and classification of resistant pneumococcal clones worldwide. In the beginning, 16 national and international antibiotic-resistant pneumococcal clones were identified using PFGE, BOX-PCR and MLST (73). PBP profiles and macrolide resistance determinants were also described. The system used for the nomenclature of clones is as follows: country$^{\text{first identified serotype}}$-sequential numbering in network-subsequent described serotype. For example, in Spain^{23F}-1, Spain is the country in which the clone was first identified (based on publication), 23F is the serotype of the clone first identified and 1 is the clone number. Several publications have shown that members of a single clone may express different capsular polysaccharides. In the case of capsular switching, the nomenclature is, e.g., as follows: in Spain^{23F}-1-19F, 19F indicates a serotype 19F variant of the Spain^{23F}-1 clone. The global increase of highly penicillin-resistant and multidrug-resistant pneumococci appears, in large part, due to the spread of individual highly resistant pneumococcal clones. This phenomenon has been particularly highlighted by the worldwide spread of the serotype 23F pneumococcal clone (Spain^{23F}-1), first identified in Spain in the early 1980s and resistant to penicillin, chloramphenicol and tetracycline (and sometimes erythromycin) (169).

A clone of multidrug-resistant serotype 19A strains first identified in Hungary (Hungary^{19A}-6) (170) has been described in the Czech Republic and Slovakia (171). A second, highly penicillin-resistant clone of serotype 19A (CSR^{19A}-10) and a serotype 14 clone (CSR^{14}-11) have also been described in Slovakia and the Czech Republic (171).

MLST studies have shown that isolates of the Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3 and Spain^{14}-5 clones either have identical allelic profiles or differ from the typical profile at only one of the seven loci (172).

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2.6. Pneumococcus data from Hungary

2.6.1. Serotyping data of pneumococci in Hungary

Serotype of *S. pneumoniae* can only be determined officially by the reference laboratory in National Epidemiological Centre (OEK) and for research use by us at the Semmelweis University, Institute of Medical Microbiology. Since April 2012 it is mandatory for all of the laboratories in Hungary to send the invasive sample to OEK. Before that only every third laboratory sent the sample for further investigation. Therefore the serotype and antibiotic results don't represent the total amount of IPD cases until April 2012.

Publications before approximately 2010 showed the same "paediatric" serotypes (such as 14, 23F, 6B, 19F) to be most frequent (173, 174), except in very early studies (1988-1994) where serotype 19A was found most frequently in the early cohort, representing approximately 50% of the isolates (175, 176).

In the latest years, as the vaccination rate of children in the whole population increased, earlier common serotypes (such as serotype 14) have nearly disappeared, especially from the paediatric specimens (92, 177, 178). According to data form OEK in 2012, 27.2% of the isolated IPD cases belonged to serotype 3, followed by serotype 1, 6B and 19A respectively and 61% of them would have been covered by PCV13. Two third of these samples obtained from age above 50, therefore it is crucial to vaccinate this group (179).

In Hungary, this current PhD is the first study providing wide-scale epidemiological data on carried *S. pneumoniae*, with several hundreds of pneumococcal isolates having been serotyped.

2.6.2. Antibiotic resistance data of pneumococci in Hungary

The resistance in the early publications, where 19A isolates were part of a highly resistant clone might have been overestimated especially to penicillin, since that time only very few or no penicillin resistant strains have been found by other authors. In other hand the macrolide resistance has been relatively stable for decades, around 30-40% (170, 175, 176).

Before wide vaccination, results from Dobay et al. (173) in 2010 showed low penicillin resistance (1–2%) but high intermediate level rates (37–38%) and very high erythromycin resistance (37–41%) among invasive pneumococci. They found a strong correlation between age and resistance, with higher penicillin and macrolide resistance rates in the very young and rather old age groups. It is well known that the burden of pneumococcal diseases is greater at the extremities of life and especially in the young, the incidences of resistance are higher. The most prevalent serotypes in this study in descending order were 14, 6A, 6B, 23F, 3, 19F and 11A. These represented the formerly prevalent paediatric serotypes except 11A (89, 177, 180, 181).

According to OEK data in the period 2005-2008, 3-5.3% of the IPD strains were resistant to penicillin and 24.5 – 32 % to erythromycin. In 2009 the penicillin resistance increased to 4.9% and in 2010 it was 5.3%. The reason could be the increasing rate of non PCV7 type serotype 19A and 3 which dominated that time among IPD patients. From 2011 we see some decrease and in 2013 only 1.8% of the isolates were resistant to penicillin. A similar decline could also be observed in erythromycin resistance. In 2009 20.6%, in 2010 24.5% in 2011 16%, in 2012 18.3% and in 2013 only 15.3% of the strains were resistant to it. (http://oek.hu/oek.web?to=1048&nid=505&pid=1&lang=hun). It seems that the newly emerging, non-vaccine serotypes are more sensitive to antibiotics.

2.6.3. Vaccine situation in Hungary

In Hungary, PCV7 was introduced in 2005, but in the first 3 years only very few children obtained vaccination due to a high price and a low awareness. In October 2008, PCV7 became freely available for children under the age of 2 years in the framework of a pneumococcus surveillance programme and in April 2009 it was included in the national immunisation programme as a recommended, but not obligatory vaccine. In September 2010, PCV13 replaced PCV7 in Hungary. The vaccine uptake is monitored nationwide by the OEK. By August 2009 (just 4 months after wide-spread usage started), already 72.2% of the children were vaccinated with at least the first portion and by the end of the year this rate increased to 89.1% (182). For those children born between January and December 2010, 92.8% received the first vaccination, 90.7% the basic immunisation (two portions) and 89.5% the booster (183). The 2011 and 2012

data show even higher vaccination rates: 94.6% / 93.1% / 92.4% (2011) and 94.8% / 93.6% (2012), as seen in **Table 5** (http://epa.oszk.hu/00300/00398/) (183, 184).

From 1st July 2014 PCV13 was made a mandatory childhood vaccine. The 2+1 vaccination scheme is applied in Hungary, the children receive the vaccine at 2, 4 and 12 months of age.

Table 5. Yearly data of the vaccinated children in Hungary. PCV1: first dose from the PCV7 and from 2010 from PCV13, PCV2: second dose from the same vaccine (183, 184)

		PCV1		**PCV2**		**Booster**	
Year	**Number of new-born in the year**	**Number of vaccinated children**	**%**	**Number of vaccinated children**	**%**	**Number of vaccinated children**	**%**
2012	87 612	83 076	**94.8**	82 040	**93.6**	no data available	
2011	85 373	80 725	**94,6**	79 484	**93,1**	78 605	92,4
2010	87 805	81 454	**92,8**	79 614	**90,7**	78 305	89,5
2009	85 520	76 233	**89,1**	73 789	**86,3**	71 949	84,6

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3 OBJECTIVES

The objective of the study presented here was to provide epidemiological insights into the dynamism of carried pneumococci among children attending communities, for the first time in Hungary. We tried to analyse the antimicrobial resistance, serotype distribution, genetic diversity and the spread of certain clones.

Since October 2008 PCV7 became free of charge, the number of the vaccinated children increased sharply, which was expected to influence the serotype distribution in carriage and also in invasive infections. We aimed to follow this serotype replacement and the epidemiology of these new prevailing serotypes after the introduction of PCV7 and compare it to the non-vaccinated population.

In 2010 PCV7 was replaced by PCV13, to be more effective against the newly emerging non-PCV7 serotypes. We also collected samples from children vaccinated with PCV13, to reveal the efficacy of the new vaccine. We wanted to determine the antibiotic resistance, serotype distribution of these new serotypes, to determine their relation to the same serotypes observed in GR2 (PCV7 vaccinated population) and to compare our isolates to the internationally wide-spread resistant pneumococcal clones and examine their presence in the country.

Additionally, with this study we try to predict which serotypes will be dominant after PCV13 vaccination. Based on the serotype and genetic background we can prognose the invasiveness of a certain serotype.

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4 MATERIALS AND METHODS

4.1 Permissions

4.1.1 Parents' permission

Questionnaires were sent to nurseries and playgroups at different parts of Hungary. Informed consent obtained from the parents was a condition for enrolment. Besides informing the parents about the purpose of the study, data were asked about the children's vaccination status, siblings, history of recurrent otitis media or other severe infections and later we expanded the questionnaires (from GR2) with the age of the children, exposure to passive smoking in the family and earlier antibiotic treatment (see Appendix 1).

4.1.2 Ethical permission

The Regional and Institutional Committee of Science and Research Ethics of Semmelweis University authorised our research by giving us the ethical permission. The number of it: TUKEB 4-3/2009 (see Appendix 2).

4.2 Bacterial strains

4.2.1 Origin of the strains

After approval, nasal samples were taken from the children's both nostrils. The samples were collected at different nurseries that can be found in various parts of Hungary (**Figure 9**). Collection time occurred over a five-year period, from March 2009 until April 2013. All together 2485 samples were collected from 48 different nurseries and day care centres (DCCs). These originated form 15 different villages and cities around the country.

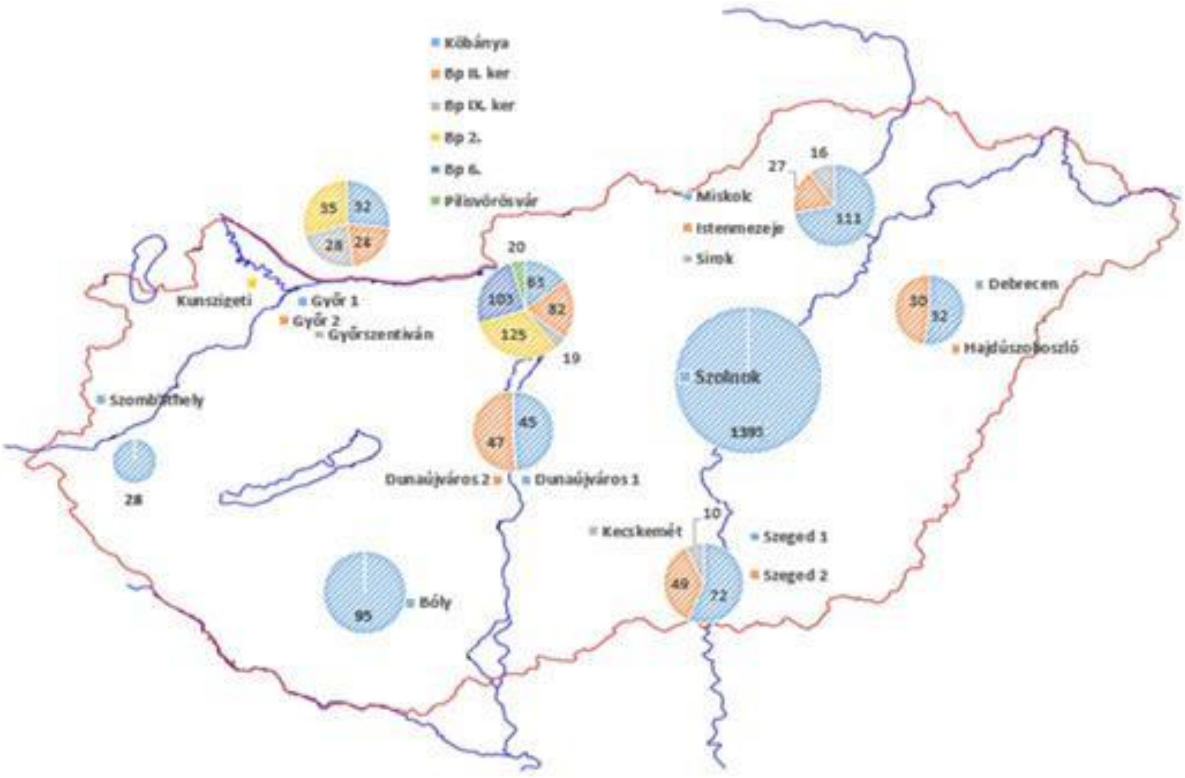

Figure 9. Number of screened children from the different nurseries

4.2.2 Specimen collection and growth media

Samples were taken with sterile cotton swabs, inserted into transport tubes containing active charcoal agar (Transwab, Medical Wire & Equipment, Corsham, UK). After collection, samples were transported to the microbiology laboratory within 24 hours and inoculated onto Columbia blood agar plates (Mast Diagnostica, Bootle, UK). All media were prepared in our own culture medium kitchen from powder according to the manufacturer's instructions. The inoculated plates were incubated overnight at 37 °C in 5% CO_2 atmosphere (Jouan IG150 thermostate).

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4.3 Identification

4.3.1 Conventional microbiological methods for identification

After overnight incubation the cultivated nasal samples were examined. Umbilicated colonies with α-haemolysis were subcultured and further tested for optochin sensitivity (5 μg discs, Mast Diagnostica, Bootle, UK). Although some optochin resistant strains were described we studied only the optochin sensitive strains. Optochin positive (inhibition zone ≥14mm) strains were stored at -80°C on cryobeads (Mast Diagnostica, Bootle, UK) (11).

4.3.2 LytA PCR

To avoid the false positive results we performed a genetic identification procedure in the optochin sensitive strains. The presence of the *lyt*A gene, which is responsible for the autolysin production, can give us almost 100% confidence, as this is a widely accepted identification method (1). The primers used for LytA PCR are in **Table 6**. The amplification temperatures and the length of the time (=cycling parameters) are shown is **Table 7**. The amplified fragment length is 318 bp. For more information about PCR see chapter 2.5.

Table 6. Primers and amplified fragment length size in LytA PCR (1) for: forward primer rev: reverse primer bp: base pair

Primer		**Sequence**	**Amplified fragment length** (bp)
*lyt*A	for	5’ CAA CCG TAC AGA ATG AAG CGG 3’	**318**
	rev	5’ TTA TTC GTG CAA TAC TCG TGC G 3’	

Table 7. PCR cycling conditions for the amplification of *lyt*A (1)

Repeat	Temperature	Time
1x	94°C	3 min
30x	94°C 54°C 72°C	1 min 1 min 30 s
1x	72°C	10 min

4.4 Serotyping

As it is known, pneumococci have 94 currently known serotypes. In order to detect the serotypes of distinct isolates we can use several methods. For example the Neufeld capsule swelling reaction and slide agglutination with serotype specific antisera, latex agglutination with polyvalent antisera pool, PCR reaction designed for capsule encoding genes and also sequencing these genes help in the accurate serotype determination.
During our research the combination of latex agglutination and PCR method was used for serotyping and several strains were sent to the German National Reference Centre for Streptococci (GNRCS, Aachen) and to the Hungarian National Center for Epidemiology (OEK, Budapest) for further serotyping.

4.4.1 Serotyping by latex agglutination

Determination of the serogroups was done by the Pneumotest Latex Kit (Statens Serum Institut, Copenhagen, Denmark) using antisera. According to the manufacturer's instructions, after overnight incubation 1 colony was selected from the blood agar plate and on the provided white card it was mixed together with a droplet (10 µl) of latex antisera and saline. A positive reaction is indicated by an agglutination appearing within 5 to 10 seconds. If the reaction time exceeds 30 seconds, false positive reactions may occur.

First pools A, B, C, D, E, F, G, H, I, should be used. If it is positive with one of them, a second pool set P, Q, R, S, T has to be used. This chessboard application is an accurate system for identifying pneumococcal serotypes or serogroups (**Figure 10**).

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 10. The chessboard arrangement of the Pneumotest-Latex kit. The letters beside the numbers state possible types within a group

For example if an isolate is positive with A and T, it means that the strain is serotype 2, but positivity with B and P results only serogroup 19. Therefore serotype determination within serogroups was performed by PCR or special factor antisera for certain serotypes.

4.4.2 Serotyping by PCR

The factor determination was done by PCR using primers described by the CDC (185), or by others (186) (**Table 8)**.

Table 8. Primers and amplified fragment length size in serotype PCR for: forward primer, rev: reverse primer bp: base pair

Primer for serotypes		Sequence	Ref.	Size (bp)
1	for	5'- GGA GAC TAC TAA ATT GTA ATA CTA ACA CAG CG -3'	(186)	**99**
	rev	5'- CAA GGA TGA ATA AAG TAA ACA TAT AAT CTC -3'		
3	for	5'- TTG TTT TTT GTC TTT ATT CTT ATT CGT TGG -3'	(186)	**818**
	rev	5'- TAC TGA GAA CCT TCT GCC CAC CTT AGT TGC -3'		
4	for	5'- CTG TTA CTT GTT CTG GAC TCT CGT TAA TTG G -3'	(186)	**430**
	rev	5'- GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G -3'		
5	for	5'- GAG ACG TCT TTG GGG CAT AA -3'	Own design	**366**
	rev	5'- GCG GAA ACG ATG AGA AGA AG -3'		
6 A/B/C/D	for	5'- CGA CGT AAC AAA GAA CTA GGT GCT GAA AC -3'	(186)	**220**
	rev	5'- AAG TAT ATA ACC ACG CTG TAA AAC TCT GAC -3'		
7C/B/40	for	5'- CTA TCT CAG TCA TCT ATT GTT AAA GTT TAC GAC GGG A- 3'	(185)	**260**
	rev	5'- GAA CAT AGA TGT TGA GAC ATC TTT TGT AAT TTC- 3'		
7F	for	5'- TGA CTG CAA GTG TTT CAA TGG -3'	Own design	**528**
	rev	5'- CGT TTC CAA AAA TTC CTC CA -3'		
9V	for	5'- AGA GGA GTT CAA TCG CCA GA -3'	Own design	**242**
	rev	5'- ATC GGT TCC CCA AGA TTT TC -3'		
10A	for	5'- GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC- 3'	(185)	**628**
	rev	5'- GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C- 3'		
10F/10C /33C	for	5'- GGA GTT TAT CGG TAG TGC TCA TTT TAG CA- 3'	(185)	**248**
	rev	5'- CTA ACA AAT TCG CAA CAC GAG GCA ACA- 3'		
12/44/46	for	5'- GCA ACA AAC GGC GTG AAA GTA GTT G- 3'	(185)	**376**
	rev	5'- CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC- 3'		
11A/F/D	for	5'- CGA AAT ATC GCC ATT CAT CA -3'	Own design	**379**
	rev	5'- TCA ACA GCA ACT GTG CCA CT -3'		
13	for	5'- ACG ACT TGG AAG TGC TGC TT -3'	Own design	**308**
	rev	5'- CCAAAAACAAAATCGCTGGA -3'		
14	for	5'- GTC TGT TTA TTC TAT ATA CAA AGA GGC TCC -3'	(186)	**268**
	rev	5'- GCA TTG CTA CAA TCG CTA TAC TAG ATA TGC -3'		
15A/F	for	5'- CAT TTG CAC CCT GAC TTC AC -3'	Own design	**409**
	rev	5'- GTC CCG CAA ACT CTG TCC TA -3'		

15B/C	for	5’- TGT TCA AAG AGG CGC TAA TG -3’	Own design	**493**
	rev	5’- TGT TCT GAT TCC TGC TCC AA -3’		
16F	for	5’- GAA TTT TTC AGG CGT GGG TGT TAA AAG- 3’	(185)	**717**
	rev	5’- CAG CAT ATA GCA CCG CTA AGC AAA TA- 3’		
18C	for	5’- GCC GTG GGA AGC TTA TTT TT -3’	Own design	**285**
	rev	5’- CCT GCC TAA AGG CAA CAA TG -3’		
19A	for	5’- GTT AGT CCT GTT TTA GAT TTA TTT GGT GAT GT -3’	(186)	**478**
	rev	5’- GAG CAG TCA ATA AGA TGA GAC GAT AGT TAG -3’		
19F	for	5’- CAC CTA ATT TTA ATA CTG AGG TTA AGA TTG C -3’	(186)	**408**
	rev	5’- CAT AGG CTA TCA GAA TTT TAA TAA TAT CTT GC -3’		
20	for	5’- ATC AGG AAT ACG CCA ATC AA -3’	Own design	**195**
	rev	5’- ATC GGT AAT GCA AAG CCA AC -3’		
23A	for	5’- GAT TTG GAG CGG ATC GAT TA -3’	(185)	**823**
	rev	5’- AAT GGG TAA TGG AGG GGA GT -3’		
23B	for	5’- CCA CAA TTA G CG CTA TAT TCA TTC AAT CG- 3’	(185)	**199**
	rev	5’- GTC CAC GCT GAA TAA AAT GAA GCT CCG -3’		
23F	for	5’- GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC -3’	(186)	**384**
	rev	5’-CAC AAC ACC TAA CAC ACG ATG GCT ATA TGA TTC -3’		
24A/B/F	for	5’- GCT CCC TGC TAT TGT AAT CTT TAA AGA G- 3’	(185)	**99**
	rev	5’- GTG TCT TTT ATT GAC TTT ATC ATA GGT CGG- 3’		
25F/25A /38	for	5’- CGT TCT TTT ATC TCA CTG TAT AGT ATC TTT ATG- 3’	(185)	**574**
	rev	5’- ATG TTT GAA TTA AAG CTA ACG TAA CAA TCC- 3’		
33F	for	5’- TCC CCA ACG GTT TAT GTG TT -3’	Own design	**171**
	rev	5’- CAA TGC AAG GCT CAA TAC CA -3’		
35B	for	5’- GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG -3’	(185)	**677**
	rev	5’- CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G- 3’		
35F/47F	for	5’- GAA CAT AGT CGC TAT TGT ATT TTA TTT AAA GCA A- 3’	(185)	**517**
	rev	5’- GAC TAG GAG CAT TAT TCC TAG AGC GAG TAA ACC- 3’		
42/35A/ 35C	for	5’- TCC CTT TTT CAG ACG TAG CC -3’	Own design	**492**
	rev	5’- TCC CTT TTT CAG ACG TAG CC -3’		

4.5 Polymerase chain reaction

To identify pneumococci itself and pneumococcal serotypes and to determine macrolide resistance genes we used polymerase chain reaction (PCR).

4.5.1 DNA template preparation

After an overnight incubation on blood agar plates, a loopful of bacteria (5 μl) was boiled in 200 μl sterile nuclease free distilled water for 15 minutes (99°C in the PCR machine). Then the suspension was vortexed and centrifuged for 2 minutes at 7000 rpm (Hermle Z 160M centrifuge) and 2 or in some cases 5 μl of the supernatant was used in the PCR reaction as template.

4.5.2 PCR mix

In our study we used both simple and multiplex PCR. For simple PCR (final volume 50 μl) the PCR mix consisted of the following components, as seen in **Table 9.** Multiplex PCR is detailed in the *Erm*/*Mef* PCR chapter.

Table 9. PCR mix for simple PCRs

Ingredients	**1 mix (μl)**
dH_2O	9.35
5×cc GoTaq Green buffer	5
$MgCl_2$ (25mM)	2
Nucleotide mix (10mM)	0.5
Primer forward (5 pmol/μl)	0.5
Primer reverse (5 pmol/μl)	0.5
Taq polymerase	0.15
DNS template	2
Total volume	20

We also used DreamTaq Green PCR Master Mix (Thermo Scientific, USA), which is a ready-to-use solution containing DreamTaq DNA polymerase, optimized DreamTaq buffer, $MgCl_2$ and dNTPs. The mix is supplemented with two tracking dyes and a density reagent that allows for direct loading of the PCR product on the gel. Ingredients are listed in **Table 10**.

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Table 10. PCR mix with DreamTaq master mix for simple PCR according to the manufacturer's recommendation

Ingredients	**1 mix (µl)**
DreamTaq Green PCR Master Mix (2)	25 µl
Primer forward	0.1-1.0 µM
Primer reverse	0.1-1.0 µM
DNS template	10 pg – 1 µg
Water, nuclease-free	to 50 µl

4.5.3 PCR cycle

The PCR cycling conditions generally included a 3 minute-long hot start at the denaturation temperature (94-95ºC), followed by 30 or 35 cycles of the actual PCR reaction (parameters varied from reaction to reaction, see the detailed description at the individual PCR methods) and a final extension for 5 or 10 minutes at the extension temperature (72ºC). The PCR machines used were Corbett Research PCR machine controlled by Hewlet Packard PDA.

4.5.4 Gel running and analysis

1.5 % agarose gel was prepared with 0.5x TEA buffer (for solutions see the end of this chapter). Because GoTaq Buffer and DreamTaq master mix both contained a loading dye, the samples could directly be loaded in the gel. The running time was 36 minutes at 100 V. Four µl of the DNA ladder with blue-orange loading dye (Promega UK Ltd, Southampton, UK) was run together with the samples. Depending on the PCR product size, either the 100 bp or the 1 kb ladder was used as molecular weight marker (Promega). The gels were stained with GelRed Nucleic Acid Gel Stain (Biotium, USA) and a photo image was made (Cannon Camera) and retouched by Photoshop programme (Adobe Photoshop CS4, USA).

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4.6 Antimicrobial susceptibility testing

For determining the MIC (minimal inhibitory concentration) we used both E-test and agar dilution method. Our strains were tested for penicillin, cefotaxime, imipenem, erythromycin, clindamycin, levofloxacin, moxifloxacin, tetracycline and vancomycin. Some of these antibiotics can not be administered to children, however, as the pneumococci colonising the children's nasopharynx could be the subsequent causative agents of infections in adults, it was important to determine the sensitivity also to these drugs. Erythromycin is usually not used *per os* for respiratory infections, but it is a good indicator antibiotic for the macrolide group.

4.6.1 Susceptibility testing by disc diffusion and E-test

For some strains we used E-test strips (Liofilchem, Roseto, Italy) for MIC determination. Strains were suspended in physiological saline to reach 0.5 McFarland, determined by VITEK Densichek (Biomérieux, Marcy l'Etoile, France) densitometer. Bacterial suspensions were inoculated onto 5% horse blood Columbia agar plates. Two strips of E-test was positioned onto the agar plate and cultivated overnight at 37°C, in 5% CO_2 thermostat. Next day the result was evaluated: where the elliptic inhibition zone crossed the E-test strip we could read the MIC (**Figure 11**).

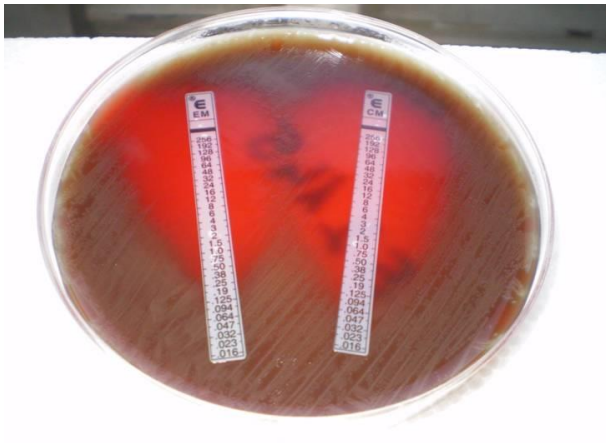

Figure 11. E-test with erythromycin and clindamycin. Where the elliptic inhibition zone crosses the strip, we can read the MIC photo by A Tóthpál

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4.6.2 Susceptibility testing by agar dilution

In order to measure MIC in a high number of strains at the same time, susceptibility testing was performed by agar dilution method. First the bacteria were grown overnight on Columbia (Mast Diagnostica, Bootle, UK) blood agar plates. Next day the strains were suspended in physiological saline to reach 0.5 McFarland standard, which is equivalent to 10^7 CFU/mL. Then 10 µl of each suspension was inoculated on Columbia agar plates (containing 5% horse blood, 20 mg/l NAD and the diluted antibiotic) using a multipoint inoculator (187) (AQS Manufacturing Ltd., Southwater, UK), resulting 10^5 CFU/spot. The plates were incubated overnight at 37°C in 5% CO_2 (**Figure 12a, 12b**). The sensitivity and resistance percentages of the isolates were determined based on the breakpoints recommended by the 2014 EUCAST guidelines (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_4.0.pdf). The control strain for sensitivity tests was *S. pneumoniae* ATCC 49619.

Figure 12a. Agar dilution method. Each spot represents a different pneumococcal isolate. On the left, all strains could grow at the given low concentration of the antibiotic, while on the right, some strains were inhibited already by the higher concentration.

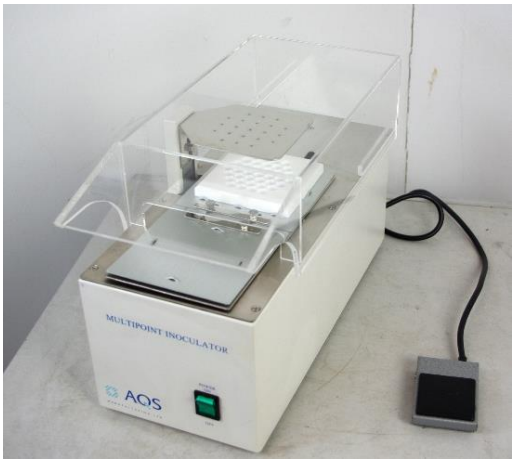

Figure 12b. Multipoint inoculator Photo by A Tóthpál

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4.7 Determination of the macrolide resistance mechanisms

4.7.1 Detection of the presence of *erm* and *mef* genes by PCR

The presence of four resistance determinants: *ermB, ermA, ermTR* and *mefE/A* was tested by PCR. The primers used are listed in **Table 11**. The *mef* primer pair was designed to the common nucleotide sequence regions of *mefE* and *mefA*, so could amplify both genes. The PCR cycling conditions in all cases were as listed in **Table 12**.

Table 11. Primers and amplified fragment length size in *erm/mef* PCR, for: forward primer, rev: reverse primer, bp: base pair

Primers		**Sequences**	**size (bp)** (ref)
ermB	for	5'- GAA AAA GTA CTC AAC CAA ATA- 3'	**639** (188)
	rev	5'- AGT AAC GGT ACT TAA ATT GTT TAC- 3'	
ermA	for	5' -TCT AAA AAG CAT GTA AAA GAA - 3'	**645** (188)
	rev	5'- CTT CGA TAG TTT ATT AAT ATT AGT -3'	
ermTR	for	5'- ACA GAA AAA CCC GAA AAA TAC G- 3'	**679** (189)
	rev	5'- TGG GAT AAT TTA TCA AGA TCA G- 3'	
mef	for	5'- AGT ATC ATT AAT CAC TAG TGC- 3'	**348** (188)
	rev	5'- TTC TTC TGG TAC TAA AAG TGG- 3'	

Table 12. PCR cycling conditions for the amplification of macrolide resistance genes

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

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4.7.2 Distinction between *mefE* and *mefA*

The two genes are easily distinguishable by restriction digestion (190, 191). The amplified 348 bp region of the *mef* gene was digested with the *Bam*HI restriction enzyme, which generated two fragments of 281 and 65 bp in size in *mefA*, but none in *mefE*. For 10 μl PCR product, 1 μl FastDigest™ (FD) *Bam*HI enzyme (ThermoScientific, Waltham, Massachusetts, USA) was added in 2 μl FD buffer and nuclease-free distilled water resulting 30 μl reaction mix (**Table 13**). The mix was then incubated at 37°C for 30 minutes and the samples were run in a 1.5% agarose gel (Sigma-Aldrich, USA).

Table 13. Digestion solution for distinction between *mefE* and *mefA*

Mix	
PCR product	10 μl
*Bam*HI enzyme	1 μl
Fast digest Buffer	2 μl
Nuclease free dH_2O	17 μl
Total	30 μl

4.8 Pulsed-field gel electrophoresis (PFGE)

PFGE was used to discover the genetic relatedness of certain strains. For the preparation of chromosomal DNA we followed the method described by Hall et al. with some modifications (71).

4.8.1 Preparation of chromosomal DNA

The strains were grown on 5% Mueller Hinton blood agar overnight. The bacterium cells were suspended in 0.2 ml of CS buffer, depending on growth density then the

suspension was mixed with an equal volume of 2% chromosomal grade low melting agarose (Bio-Rad, USA), prepared freshly with sterile distilled water and dispensed into plug molds. After solidification, plugs were first incubated at 37°C for 3 hours in 2 ml of Lysis 1 buffer and subsequently overnight at 55°C in 2 ml of Lysis 2 buffer (**Table 14**). The plugs were then washed three times for 30 minutes each at 4°C with TE buffer and either cut into half and processed to the digestion step, or stored at 4°C until required.

Table 14. Composition of Lysis 1 and Lysis 2 buffer, used to purify the prepared plugs containing whole chromosomal DNA

	For 1 plug		**For 1 plug**
Lysis 1	3 ml EC lysis buffer 150 µl lysozyme (20 µg/ml) 2 µl mutanolysine 20 µl RNase A (25 µg/ml)	**Lysis 2**	3 ml ES lysis buffer 20 µl proteinase K (20 mg/ml)

4.8.2 Digestion

Half of the plugs used in the reaction were digested with 30 U of *Sma*I (Promega, Madison, USA) in buffer SA (**Table 15**) at 25°C for 4 hours.

Table 15. Digestion solution for the purified plugs

Mix	**for 1 Plug**
*Sma*I enzime	1.5 µl
10×cc SA Buffer	15 µl
dH_2O	133.5 µl
Total	150 µl

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4.8.3 Gel running

The plugs were directly placed into the pockets of the 1% pulsed-field certified agarose gel (Bio-Rad, USA) prepared with 0.5xTBE and covered with the remaining amount of the molten gel in order to obtain a smooth surface. The fragments were separated in a CHEF-DR® II apparatus (Bio-Rad, USA) at 6 V/cm for 21.5 hours at 14°C, in 2 blocks. **Table 16** shows the precise settings of gel running. The No34O3 λ ladder PFGE marker was used as molecular weight control (New England Biolabs Ltd., Hitchin, Hertfordshire, UK), included in 3 lanes of every gel. The ATCC 49619 strain was used as inner control.

Table 16. Settings of PFGE gel running

BLOCK1	Initial time : 5s Final time : 15s Running time : 10 h Voltage : 6 V / cm (= 200V)
BLOCK2	Initial time : 15s Final time : 60s Running time : 11 h Voltage : 6 V / cm (= 200V)

4.8.4 Gel analysis

The gels were stained with GelRed (Biotium, USA) and a photo image made by Cannon camera. The analysis of the PFGE profiles (normalisation, gel comparison and dendrogram creation) was performed with the Bionumerics program (version 2.5; Applied Maths BVBA, Sint-Martens-Latem, Belgium). The definition of a PFGE genotype was at least 90% identity in the dendrogram created by the unweighted pair group method using arithmetic averages (UPGMA) and the different bands similarity coefficient, with 1.0% band position tolerance.

4.9 Multi-locus sequence typing (MLST)

Multi-locus sequence typing (MLST) was also performed for ten isolates. Well defined sections of seven housekeeping genes were amplified by PCR except for recP, where the recP reverse primer was modified by us to 5'- TGCCAACCGTAGGCATTGTAAC-3'(**Table 17**) (66).

The products were purified by the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sent for sequencing to the BIOMI Ltd., Gödöllő, Hungary. The sequences at each of the seven loci were then compared with the sequences of all of the known alleles at those loci. Sequences that were identical to the sequence of a known allele were assigned the same allele number, whereas those that differed from the sequence of any known allele, even at a single nucleotide site, were assigned new allele numbers. The assignment of alleles at each locus was carried out using the software available at the pneumococcal MLST website (http://spneumoniae.mlst.net/). The alleles at each of the seven loci define the allelic profile of each isolate as well as their sequence type. The pneumococcal MLST scheme uses the following seven house-keeping genes: 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) (66).

Table 17. Primers and amplified fragment length size for the PCR amplification of internal fragments of these genes PCR, rev: reverse primer, for: forward primer, bp: base pair

Primers		**Sequence**	**Amplified fragment length (bp)**
aroE	for	5'-GCC TTT GAG GCG ACA GC-3'	479
	rev	5'-TGC AGT TCA (G/A)AA ACA T(A/T)T TCT AA-3'	
gdh	for	5'-ATG GAC AAA CCA GC(G/A/T/C) AG(C/T) TT-3'	659
	rev	5'-GCT TGA GGT CCC AT(G/A) CT(G/A/T/C) CC-3'	
gki	for	5'-GGC ATT GGA ATG GGA TCA CC-3'	626
	rev	5'-TCT CCC GCA GCT GAC AC-3'	
recP	for	5'-GCC AAC TCA GGT CAT CCA GG	572
	rev	5'- TGCCAACCGTAGGCATTGTAAC-3'	
spi	for	5'-TTA TTC CTC CTG ATT CTG TC-3'	560
	rev	5'-GTG ATT GGC CAG AAG CGG AA -3'	
xpt	for	5'-TTA TTA GAA GAG CGC ATC CT-3'	572
	rev	5'-AGA TCT GCC TCC TTA AAT AC-3'	
ddl	for	5'-TGC (C/T)CA AGT TCC TTA TGT GG-3'	514
	rev	5'-CAC TGG GT(G/A) AAA CC(A/T) GGC AT-3'	

4.10 Whole genome sequencing (WGS)

Two pneumococcal isolates (serotype 19A) were sent for whole genome sequencing for the CellCall Ltd, Budapest, Hungary. After sample processing with the Ion Torrent system (LifeTechnolgies™), data were analysed by the MATLAB® software constructed by the company. Full analysis of the data is in process, only the MLST sequences were extracted, providing MLST results for a further 2 isolates in addition to the other 10 islates described in chapter 2.9.

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4.11 Detection of the presence of pili in pneumococcus

To detect the presence of pili with PCR, the following primers were used (27, 192) (**Table 18**). **Table 19** shows the PCR cycle used during amplification.

Table 18. Primers and amplified fragment length size for the pilus PCR, rev: reverse primer, for: forward primer bp: base pair

Primers		**Sequence**	**Amplified fragment length** (bp)
rrgC	for	5'- GCT CTG TGT TTT TCT CTT GTA TCC- 3'	1056
	rev	5'- ATC AAT CCG TCC TCG CTT GTT ATT TTT A- 3'	

Table 19. PCR cycling conditions for the amplification pilus gene

Repeat	**Temperature**	**Time**
1x	94ºC	3 min
30x	94ºC 55ºC 72ºC	30 sec 30 sec 30 sec
1x	72ºC	5 min

4.12 Statistical analysis

We used the Chi-squared distribution and the Fisher exact test (for small sample size). We used the *p* value as a significance level for rejecting the null hypothesis of no correlation if it was < 0.05.

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4.13 Solutions

All chemicals were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). The ingredients were dissolved in distilled water, the pH adjusted where necessary and further distilled water added to the final volume. All solutions were then autoclaved.

50x TAE buffer: 2M TRIS, 1M EDTA, 5.71 v/v % glacial acetic acid

CS buffer: 10 mM TRIS, pH 7.6 and 1M NaCl; pH=8.0

EC lysis buffer: 1M NaCl, 100mM EDTA, 6 mM TRIS, 0.5% Brij58, 0.2% deoxycholate, 0.5% N-lauroyl sarcosine; pH=8.0

ES buffer: 0.5M EDTA, 1% N-lauroyl sarcosine; pH=8.8

TE buffer: 10mM TRIS, 1mM EDTA; pH=8.0

10x TBE buffer: 45mM TRIS, 2mM EDTA, 45mM boric acid

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5 RESULTS

5.1 General information about the isolates

All together 2485 nasal samples were collected from children attending nurseries or DCCs. The gender ratio was 1284 (52.3%) boys and 1171 (47.7%) girls. (From the first DCC – 30 children - we did not receive the gender and other information). From the 2485 children, 1684 (68.6%) had siblings and from that 1488 (88.4%) attended communities (nursery, DCC, or school). From 2485 children 427 (17.4%) had acute otitis media previously in their life.

5.1.1 Carried isolates

All together 838 pneumococci were isolated from the 2485 children, thus the overall carriage rate is 33.7%. The gender ratio among the carriers was 435 (51.9%) boys and 388 (46.3%) girls. Three children carried 2 different serotypes, therefore the number of carried strains is 841.

Carriers were arranged into 3 groups based on the time when the samples were collected, which correlates with the children's vaccination status. DCC groups with low-level vaccination (GR1) were screened before October 2010 and PCV7 vaccination rate was 14.0%. DCC groups with high-level vaccination (GR2) were screened between October 2010 and June 2012, with an average of 43.9% vaccination rate with PCV7. Nursery groups (GR3) were screened between March 2013 and April 2013. These children were vaccinated already with PCV13 and the vaccination rate was 84.5%.

5.2 GR1 in general

Between February 2009 and March 2010, 15 different DCC populations with 631 children were screened in different parts of Hungary (**Table 20a, Table 20b**). From the 631 children, 313 (52.1%) were boys and 288 (47.9%) were girls and 30 unknown (The

detailed data from nursery in Hajdúszoboszló are missing therefore the genders and other background information are missing in the case of these 30 children). From the 631 children, 216 were carrying pneumococcus, thus the carriage rate was 34.2%. The gender ratio among the carriers was 104 (51.7%) boys, 97 (48.3%) girls (p = 0.9062) and 15 were of unknown gender.

From the 631 children 488 (77.3%) had siblings and 415 (85.0%) attended communities. From the carriers 150 (69.4%) had siblings (p = 0.9810) and 136 (90.1%) attended communities. In total 113 (17.9%) children had AOM, 30 (13.9%) among carriers (p = 0.2138). All together 112 (17.8%) were definitely vaccinated with PCV7, among the carriers 35 (16.2%). The average vaccination status was 14%, which varied in the different DCCs. We could isolate 218 strains from the 216 carriers, because 2 children carried 2 different serotypes of the bacterium.

Table 20a. Summarized information about nurseries belonging to GR1

Date of collection	Day care centres	Number of children	Number of carriers	Carriage rate (%)	Number of vaccinated carriers	Number of vaccinated non-carriers	Total number of vaccinated children	Vaccination rate (%)
GR1								
Feb 2009	Hajdúszoboszló	30	15	50	0	0	0	0
March 2009	Győrszentiván	28	15	53.57	3	2	5	17.80
March 2009	Kunsziget	35	10	18.57	0	0	0	0
April 2009	Győr-Prohászka	32	12	37.50	3	2	5	16.20
May 2009	Győr-Brunszvik	24	8	33.3	0	2	2	8.33
Oct 2009	Debrecen	32	11 (12)	34.38	0	0	0	0
Dec 2009	Pilisvörösvár	20	5	25	1	3	4	25
Jan 2010	Budapest-2	82	31	34.15	5	18	23	28.04
Jan 2010	Budapest-1	63	17	26.98	8	15	23	36.50
Feb 2010	Szeged-A (Sz)	49	15	28.57	1	4	5	10.20
Feb 2010	Kecskemét	10	2	20	0	1	1	10
Feb 2010	Szeged-B (X)	72	22	30.56	10	11	21	29.17
March 2010	Sirok	16	7	43.75	0	1	1	6.25
March 2010	Istenmezeje	27	11	40.74	0	1	1	3.70
March 2010	Miskolc	111	35 (36)	31.53	4	17	21	18.92
Total		**631**	**216 (218)**	**mean: 34.2**	**35**	**77**	**112**	**mean: 14.0**

Table 20b. Summarized information about nurseries belonging to GR1

Day care centres	Number of boys	Number of girls	Number of carrier boys	Number of carrier girls	Number of all siblings	Number of all siblings attending communities	Number of carrier's siblings	Number of carrier's siblings attending communities	Number of AOM	Number of carrier's AOM
GR1										
Hajdúszoboszló	NO	DATA	-	-	-	-	-	-	-	-
Győrszentiván	13	15	7	8	19	13	9	3	3	1
Kunsziget	19	16	8	2	21	16	5	3	5	0
Győr-Prohászka	17	15	4	8	24	27	9	10	7	4
Győr-Brunszvik	9	15	3	5	17	13	8	7	5	1
Debrecen	14	18	4	7	27	32	8	10	5	0
Pilisvörösvár	6	14	3	2	17	14	4	4	5	1
Budapest-2	47	35	12	19	63	63	27	22	22	7
Budapest-1	32	31	11	6	45	39	13	11	16	6
Szeged-A (Sz)	28	21	10	5	35	31	10	10	7	0
Kecskemét	8	2	1	1	10	7	2	2	0	0
Szeged-B (X)	34	38	11	11	63	69	18	21	10	2
Sirok	14	2	6	1	13	12	6	5	3	2
Istenmezeje	12	15	5	6	22	21	8	9	2	0
Miskolc	60	51	19	16	72	58	23	19	23	6
Total	**313**	**288**	**104**	**97**	**448**	**415**	**150**	**136**	**113**	**30**

5.3 GR2 in general

Between October 2010 and June 2012, 25 different DCC populations with 1627 children were screened in different parts of Hungary (**Table 21a, Table 21b**). From the 1627 children, 850 (52.2%) were boys and 777 (47.8%) were girls. From the 1627 children, 528 were carrying pneumococcus thus the carriage rate was 32.5%. The carriers' gender ratio was 286 (54.2%) boys and 242 (45.8%) girls ($p = 0.2816$)**.** From 1627 children, 1100 (67.6%) had siblings and 958 (87.1%) attended communities. From the 528 carriers 396 (75.0%) had siblings ($p = 0.0013$) and 335 (84.6%) attended communities. 300 (18.4%) children had AOM, among carriers 102 (19.3%) had it before ($p = 0.5995$).

All together 776 (47.7%) children were definitely vaccinated with PCV7 and 274 (51.9%) among the carriers. The average vaccination status was 43.9%, which varies in the different DCCs. We could isolate 529 strains from the 528 carriers, because 1 child carried 2 different serotypes of the bacterium.

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Table 21a. Summarized information about nurseries belonging to GR2

Date of collection	Day care centres	Number of children	Number of carriers	Carriage rate (%)	Number of vaccinated carriers	Number of vaccinated non-carriers	Total number of vaccinated children	Vaccination rate (%)
GR2								
Oct 2010	Szombathely	28	10	35.71	2	1	3	10.71
Dec 2010	Dunaújváros-1	47	21	44.68	7	2	9	19.15
Dec 2010	Dunaújváros-2	45	20	44.44	8	4	12	26.67
Feb 2011	Budapest 9. kerület	19	6	31.57	4	5	9	47.37
Dec 2011	Bóly	95	45	47.37	24	23	47	49.47
Feb 2012	Szolnok-Hétszínvirág	123	46	37.4	25	35	60	48.78
Feb 2012	Szolnok-Gézengúz	39	16	41.02	10	19	29	74.36
Feb 2012	Szolnok-Kacsa	29	8	27.58	3	5	8	27.58
Feb 2012	Szolnok-Százszorszép	51	14	27.45	7	17	24	47.05
Feb 2012	Szolnok-Csemetekert	26	15	57.69	7	1	8	30.77
Feb 2012	Szolnok-Kertvárosi	21	5	23.80	2	3	5	23.80
Feb 2012	Szolnok-Munkácsy	124	29	23.38	14	52	66	53.22
Feb 2012	Szolnok-Eszterlánc	49	14	28.57	7	13	20	40.81
Feb 2012	Szolnok-Nyitnikék	51	22	43.15	11	17	28	54.90
Feb 2012	Szolnok-Hold	63	26	41.26	20	22	42	67.54
Feb 2012	Szolnok-Kolozsvári	94	26	27.66	14	34	48	51.06
March 2012	Szolnok-Tesz-Vesz	35	9 (10)	18.97	1	1	2	7.20
April 2012	Szolnok-Manóvár	58	11	18.97	7	29	36	62.07
April 2012	Szolnok-Ligeti	75	23	30.67	12	33	45	60.00
May 2012	Szolnok-Zengő	109	39	35.78	22	37	59	54.13
May 2012	Szolnok-Rózsa	45	14	31.11	6	13	19	42.22
May 2012	Szolnok-Csicsergő	44	12	27.27	7	16	23	52.27
May 2012	Szolnok-Szapári	83	30	36.14	19	30	49	59.03
June 2012	Szolnok-Szivárvány	124	32	25.80	13	30	43	34.68
June 2012	Szolnok-Pitypang	150	35	23.33	22	60	82	54.66
Total		**1627**	**528 (529)**	**mean: 32.5**	**274**	**502**	**776**	**mean: 43.9**

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Table 21b. Summarized information about nurseries belonging to GR2

Day care centres	Number of boys	Number of girls	Number of carrier boys	Number of carrier girls	Number of all siblings	Number of all siblings attending communities	Number of carrier's siblings	Number of carrier's siblings attending communities	Number of AOM	Number of carrier's AOM
GR2										
Szombathely	14	14	4	6	23	24	8	8	4	0
Dunaújváros-1	21	24	11	10	27	26	12	12	9	5
Dunaújváros-2	23	24	9	11	33	27	14	13	11	8
Budapest 9. kerület	4	15	0	6	14	12	4	2	5	2
Bóly	50	45	27	18	70	58	35	27	31	15
Szolnok-Hétszínvirág	69	54	27	19	50	47	36	31	21	16
Szolnok-Gézengúz	21	18	7	9	22	15	11	6	8	1
Szolnok-Kacsa	16	13	2	6	21	18	14	10	4	2
Szolnok-Százszorszép	29	22	10	4	36	31	20	19	7	1
Szolnok-Csemetekert	17	9	10	5	17	15	8	7	7	4
Szolnok-Kertvárosi	9	12	3	2	16	16	3	3	4	0
Szolnok-Munkácsy	65	59	17	12	98	89	25	22	25	6
Szolnok-Eszterlánc	23	26	6	8	36	34	9	8	9	1
Szolnok-Nyitnikék	24	27	10	12	39	29	16	11	12	6
Szolnok-Hold	30	33	17	9	40	37	18	18	6	2
Szolnok-Kolozsvári	53	41	12	14	65	53	11	11	14	4
Szolnok-Tesz-Vesz	19	16	6	3	29	28	8	8	4	0
Szolnok-Manóvár	33	25	6	5	34	29	5	3	5	0
Szolnok-Ligeti	37	38	15	8	51	45	20	17	14	6
Szolnok-Zengő	48	61	15	24	66	56	20	14	22	5
Szolnok-Rózsa	22	23	8	6	35	30	14	13	7	0
Szolnok-Csicsergő	21	23	5	7	33	29	10	8	9	1
Szolnok-Szapári	46	37	16	14	56	47	23	20	18	3
Szolnok-Szivárvány	75	49	23	9	87	83	26	26	16	7
Szolnok-Pitypang	81	69	20	15	102	80	26	18	28	7
Total	**850**	**777**	**286**	**242**	**1100**	**958**	**396**	**335**	**300**	**102**

5.4 GR3 in general

In March 2013 and April 2013, 227 children were screened in different districts of Budapest attending 8 different nurseries (**Table 22a, Table 22b**). From the 227 children 121 (53.3%) were boys and 106 (46.7%) were girls and 94 were carrying pneumococcus

in total, thus the carriage rate was 41.4%. The carriers' gender ratio was 45 (47.9%) boys and 49 (52.1%) girls (p = 0.1678).

From the 227 children, 136 (59.9%) had siblings and 115 (84.6%) attended communities. From the 94 carriers, 56 (59.6%) had siblings (p = 0.6739) and 47 (83.9%) attended communities. Fourteen (6.2%) children had AOM, among carriers only 4 (4.3%) (p=0.4980). All together 193 (85.0%) and among the carriers 80 (85.1%) children were definitely vaccinated with PCV13. The average vaccination status was 84.5%, which varies in the different groups.

Table 22a. Summarized information about nurseries belonging to GR3

Date of collection	Day care centres	Number of children	Number of carriers	Carriage rate (%)	Number of vaccinated carriers	Number of vaccinated non-carriers	Total number of vaccinated children	Vaccination rate (%)
GR3								
March 2013	Törökvész	32	6	18.75	2	24	26	81.13
March 2013	Varsági	23	12	52.17	9	10	19	82.60
April 2013	Hűvösvölgyi	23	14	60.87	11	6	17	73.91
April 2013	Pasaréti	47	21	44.68	18	17	35	74.47
April 2013	Csemetelak	13	4	30.77	4	5	9	69.23
April 2013	Manófészek	27	13	48.15	13	14	27	100
April 2013	Micimackó	36	15	38.89	14	20	34	94.44
April 2013	Ficánka	26	9	34.62	9	17	26	100
Total		**227**	**94**	**mean: 40.9**	**80**	**113**	**193**	**mean: 84.5**

Table 22b. Summarized information about nurseries belonging to GR3

Nurseries	Number of boys	Number of girls	Number of carrier boys	Number of carrier girls	Number of all siblings	Number of all siblings attending communities	Number of carrier's siblings	Number of carrier's siblings attending communities	Number of AOM	Number of carrier's AOM
Törökvész	16	16	2	4	18	14	3	2	3	0
Varsági	12	11	5	7	16	14	8	7	2	1
Hűvösvölgyi	12	11	5	9	15	14	10	9	1	1
Pasaréti	28	19	14	7	34	29	16	13	3	2
Csemetelak	7	6	2	2	9	7	1	1	2	0
Manófészek	14	13	4	9	18	17	9	8	0	0
Micimackó	19	17	9	6	10	6	3	2	1	0
Ficánka	13	13	4	5	16	14	6	5	2	0
Total	**121**	**106**	**45**	**49**	**136**	**115**	**56**	**47**	**14**	**4**

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5.5 Serotype distribution of the carried isolates

In the case of pneumococcus, one of the most important information is the serotypes. As it is known certain serotypes have high invasive potential (193). Therefore the PCV7 and PCV13 vaccines contain the purified capsule of these most invasive strains. Since the vaccination rate has significant effect on the serotype distribution, it has to be taken into consideration during data analysis. Nurseries and DCCs were sorted into 3 groups based on the average vaccine uptake among the attending children. GR1 had low vaccination rate and the children received PCV7. GR2 had high vaccination rate, also with PCV7. GR3 had very high vaccination rate, but they were vaccinated with the new PCV13.

5.5.1 Serotype distribution in GR1

From the 218 isolated strains 14.2% (n=31) belonged to serotype 11A, 11D or 11F (we could not distinguish the factor with PCR, however, 11A is the most common carried serotype among serogroup 11 (194). 11A/D/F was followed by the so called paediatric serotypes, 6B (n=25), 23F (n=22), 19F (n=21), 14 (n=20) (**Figure 13**). Before wide spread vaccination, these serotypes were the most common causative agents of invasive pneumococcal infections in small children all around the word (195). Therefore these serotypes were included in PCV7.

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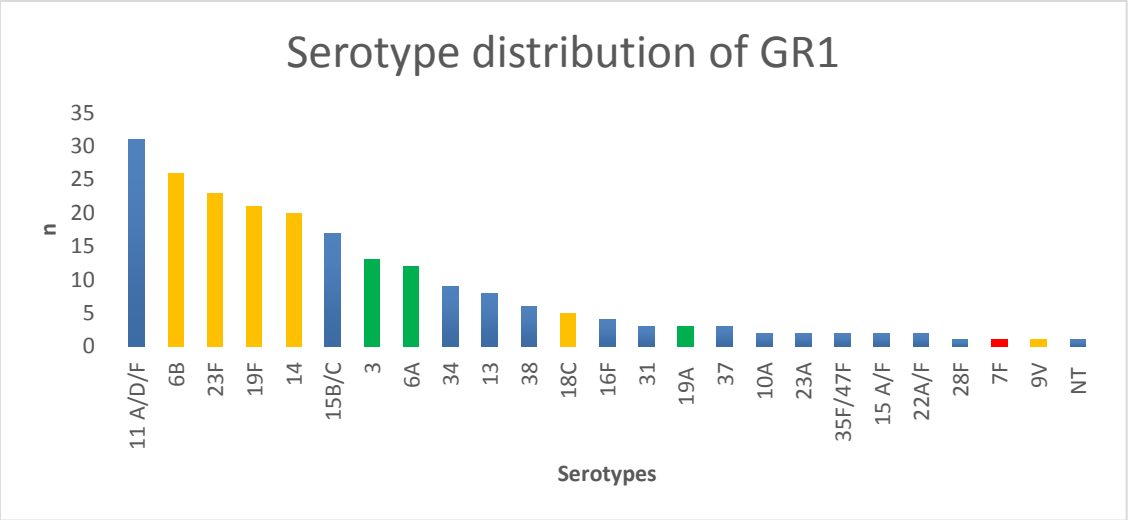

Figure 13. Yellow column: PCV7 serotypes, red column: additional serotypes in PCV10, green column: additional serotypes in PCV13, blue column: non-PCV7 serotypes, NT: non typeable isolates, n= number of isolates

5.5.1.1 Vaccine coverage

The yellow columns on **Figure 13** show the serotypes included in PCV7. All together 89 strains belonged to this group, thus the vaccine coverage of PCV7 was 44.5%. It means that 44.5% of the carried strains could have been prevented by the vaccine PCV7. The coverage of PCV10 would be only slightly more, 44.9% and for PCV13 it would be 57.8%.

5.5.2 Serotype distribution in GR2

The prevalence of the more frequent serotypes in ranking order among the 529 strains in GR2 was the following: 19A (n=60), 23A (n=49), 15B/C (n=49), 11A/D/F (n=45), 24A/B/F (n=40), 35F/47F (n=39) 3 (n=36), 6A (n=28) and altogether 30 different serotypes were identified (**Figure 14**). All NT strains are truly non-typable as determined by the GNRCS.

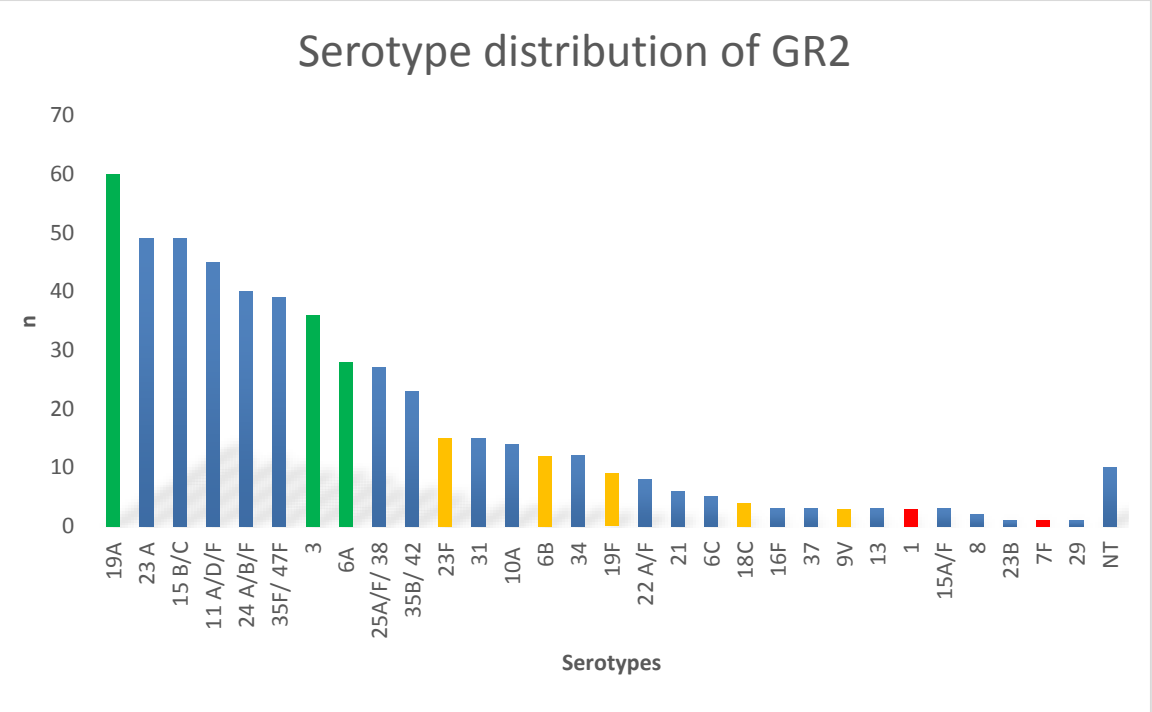

Figure 14. Yellow column: PCV7 serotypes, red column: additional serotypes in PCV10, green column: additional serotypes in PCV13, blue column: non-PCV7 serotypes, NT: non typeable isolates, n= number of isolates

5.5.2.1 Vaccine coverage

The calculated vaccine coverage for the 529 strains is 8.1% for PCV7, 8.9% for PCV10 and 32.3% for PCV13.

5.5.3 Serotype distribution in GR3

The prevalence of serotypes of GR3 in ranking order was the following: 11A/D/F (n=18), 15B/C (n=15), 35F/47F (n=15), 23B (n=14), 23A (n=13), 10A (n=6), 6C (n=5), 19A (n=2), 24 (n=2), 8 (n=1), 19F (n=1), 16F (n=1) and 21 (n=1) (**Figure 15).**

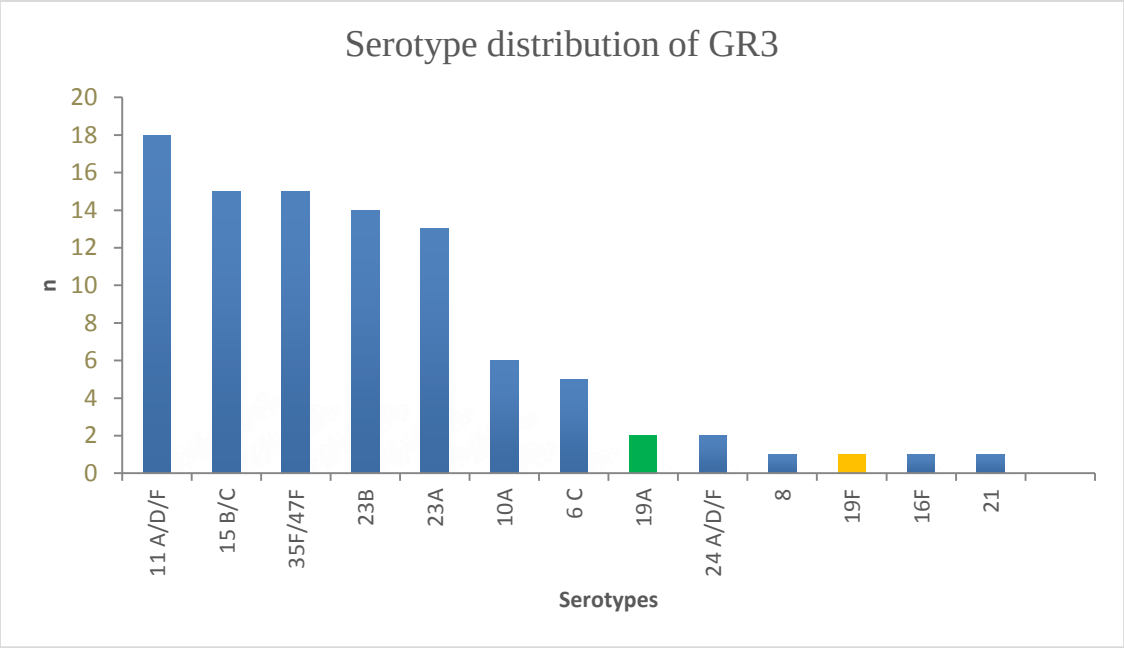

Figure 15. Yellow column: PCV7 serotypes, green column: additional serotypes in PCV13, blue column: non-PCV7 serotypes, n= number of isolates

5.5.3.1 Vaccine coverage

The calculated vaccine coverage of PCV7 was 1.1% (n=1), of PCV10 it was the same 1.1% (n=1) and of PCV13 it was 3.2% (n=3).

5.6 Antibiotic susceptibility

In GR1 the antibiotic sensitivity of the strains to penicillin, cefotaxime, imipenem, erythromycin, clindamycin, levofloxacin, and vancomycin was determined by the agar dilution method and by E-test. In GR2 telithromycin, moxifloxacin, and tetracycline was added to the above mentioned antibiotics and in GR3 telithromycin and imipenem was not determined any more. The susceptibility and resistance rates were determined using the breakpoints recommended by the EUCAST guidelines (The European Committee on Antimicrobial Susceptibility Testing – EUCAST: http://www.eucast.org/clinical_breakpoints/).

5.6.1 Antibiotic susceptibility in GR1

The isolates were usually very sensitive to antibiotics (**Table 23**). The highest penicillin MIC was 2 mg/L (serotype 19A and 6B), hence, according to the newest EUCAST breakpoints, none of the strains fell into the resistant category (>2 mg/L), but 24.2% were intermediate and the majority belonged to serotype 23F (n=15), 6A (n=10) 19F (n=8) and 6B (n=4). Among fluoroquinolones, 6.7% resistance was observed for levofloxacin. Macrolide resistance had the highest resistance ratio, 21.4% for erythromycin. Fifteen strains had low-level resistance to erythromycin (4–16 mg/L) and were sensitive to clindamycin (M phenotype), 9 of them carried the *mefE* gene. These strains had elevated penicillin MICs (0.25–1 mg/L) except for 2 strains. Twenty-nine strains had high-level resistance (>256 mg/L) for both erythromycin and clindamycin (MLSB phenotype); 16 of these had the *ermB* gene alone, while 5 carried the *ermB* and the *mefE* genes together. Half of these had lower penicillin MICs (0.008–0.25 mg/L). The vast majority of the macrolide resistant strains belonged to serotypes 19F (n = 14) and 6A (n = 12), with an additional few strains of 6B, 23F 11A/D/F, 19A, 18C and 31.

Table 23. Antibiotic susceptibility of GR1 (n= number of strains tested to each antibiotic)

	MIC range	**MIC_{50}**	**MIC_{90}**	**S %**	**I %**	**R %**
Penicillin (n=211)	≤0.015-2	0.015	0.5	75.8	24.2	**0**
Cefotaxime (n=207)	≤0.015-2	0.03	0.25	95.7	4.3	**0**
Imipenem (n=192)	≤0.015-2	0.03	0.125	99	-	**1**
Erythromycin (n=206)	0.03-≥512	0.25	≥256	77.7	0.9	**21.4**
Clindamycin (n=204)	0.047-≥256	0.25	≥128	85.3	-	**14.7**
Levofloxacin (n=209)	0.38-4	1	2	93.3	-	**6.7**
Vancomycin (n=190)	0.25-2	1	1	100	-	**0**

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5.6.2 Antibiotic susceptibility in GR2

In GR1 only one single isolate (serotype 19A) was resistant to penicillin, but only at the lowest level (MIC=4 mg/L), while 77.9 % of all strains were fully susceptible (**Table 24**). No resistance was observed to cefotaxime, imipenem, vancomycin and moxifloxacin. On the other hand, 21.8% of the isolates were resistant to erythromycin. Ten strains out of these showed the M phenotype (low-level resistance to erythromycin, MIC=4–16 mg/L, and sensitivity to clindamycin); these all had the *mefE* gene and five of these were serotype 6A. The majority, however, showed the MLSB type (high-level resistance to both) and serotype 19A was dominant (54.0%) in this group; all serotype 19A strains were *ermB* positive. Seven isolates possessed *ermB* and *mefE* together (four strains serotype 19A, three strains serotype 19F). In the case of telithromycin, the strains could be divided clearly into two distinct groups. Those that had a telithromycin MIC ≤0.03 mg/L were all macrolide sensitive and none of them were serotype 19A. On the other hand, those with an MIC ≥0.06 mg/L contained all the macrolide resistant isolates, M types and MLSB types as well. Tetracycline resistance was found to be 8.0% and was always associated with erythromycin and clindamycin resistance. Half of the tetracycline resistant strains were 19A (n=11). The isolates belonging to the leading serotype 11A/D/F were very sensitive to penicillin (most of them had MIC=0.015 mg/L) and also to macrolides, but they had the highest levofloxacin MICs (4 mg/L). Out of the 12 levofloxacin-resistant strains (MIC=4 mg/L), eight were serotype 11A/D/F. Serotype 19A isolates were (with a very few exceptions) macrolide resistant and most of them had a penicillin MIC≥0.25 mg/L. Many of the serotype 6B strains had high-level macrolide resistance, while the 6A strains were mostly sensitive, or had the M phenotype, with a resulting low-level macrolide resistance. Isolates with elevated MICs to penicillin (intermediate category) had serotypes 19A, 19F, 6A, 6B, 23A, 23F and 35B/42F. Serotypes 3, 15A/F, 15B/C, 24A/B/F, 35F/47F and most of the “minor” types as well were fully sensitive to all tested antibiotics, with especially low MIC values.

Table 24. Antibiotic susceptibility of GR2 (n= number of strains tested to each antibiotic)

	MIC range	MIC_{50}	MIC_{90}	S %	I %	R %
Penicillin (n=485)	≤0.015-4	0.015	0.50	77.9	21.9	**0.2**
Cefotaxime (n=486)	≤0.015-1	0.015	0.25	97.9	2.1	**0**
Imipenem (n=182)	0.004-5	0.004	0.06	100	-	**0**
Erythromycin (n=482)	0.06-256	0.125	256	77.8	0.4	**21.8**
Clindamycin (n=483)	0.06-128	0.125	128	81	-	**19**
Telithromycin (n=182)	0.015-2	0.03	0.125	95.6	2.7	**1.6**
Levofloxacin (n=485)	0.06-4	1	1	97.5	-	**2.5**
Moxifloxacin (n=470)	0.06-0.5	0.25	0.25	100	-	**0**
Vancomycin (n=437)	0.06-2	0.5	0.5	100	-	**0**
Tetracycline (n=276)	0.25-64	0.25	0.5	92	-	**8**

5.6.3 Antibiotic susceptibility in GR3

None of the strains were resistant to penicillin, though we could detect 20.7% intermediate resistance (**Table 25**). No resistance was observed to cefotaxime, levofloxacin, vancomycin and moxifloxacin. On the other hand, 21.7% (n=20) of the isolates were resistant to erythromycin. Four strains out of these showed the M phenotype; but we could not detect any of the *mef* genes. The majority showed the MLSB type (high-level resistance to both) and serotype 23A was dominant (50.0%) in this group. Eight isolates possessed *ermB* gene (half of them were serotype 23A). The leading serotype in this group was 11A/D/F too, also very sensitive to penicillin (most of them had MIC ≤ 0.015 mg/L) and to the other tested antibiotics. The serotype 15 B/C isolates were also very sensitive to the tested antibiotics except one strain with 0.125 mg/L penicillin MIC. Three of the serotype 35F/47F strains had intermediate penicillin resistance and one strain had high-level macrolide resistance. Serotype 23B strains were

mostly sensitive, only one strain had the M phenotype, with a resulting low-level macrolide resistance (8 mg/L). Isolates with elevated MICs to penicillin (intermediate category) had serotypes 23A (n=9), 35F/47F (n=3), 6C (n=2), 19A (n=2), 15B/C (n=1) and 11 FAD (n=1). Almost 90% of these strains were highly resistant to erythromycin, clindamycin and tetracycline.

Table 25. Antibiotic susceptibility of GR3 (n= number of strains tested to each antibiotic)

	MIC range	**MIC_{50}**	**MIC_{90}**	**S %**	**I %**	**R %**
Penicillin (n=92)	≤0.015-4	0.03	0.5	79.3	20.7	**0**
Cefotaxime (n=92)	≤0.015-1	0.015	0.25	100	0	**0**
Erythromycin (n=92)	0.06-256	0.125	256	77.2	1.1	**21.7**
Clindamycin (n=92)	0.06-128	0.125	128	82.6	-	**17.4**
Levofloxacin (n=92)	0.06-4	0,5	1	100	-	**0**
Moxifloxacin (n=92)	0.06-0.5	0.25	0.25	100	-	**0**
Vancomycin (n=92)	0.06-2	0.5	1	100	-	**0**
Tetracycline (n=92)	<0.25-64	<0.25	32	82.6	-	**17.4**

5.7 Macrolide resistance mechanisms

5.7.1 *Erm* and *mef* genes in GR1

We could find *ermB* gene in 24 strains showing erythromycin resistance. These were serotype 6A (n=7) 19F (n=6) 6B (n=5) 23F (n=3) 19A (n=2) 14 (n=1). In 16 strains we could detect the *mefE* genes resulting low level resistance in 10 cases. Among these 16 strains, 7 strains carried *ermB* genes simultaneously (**Figure 16**).

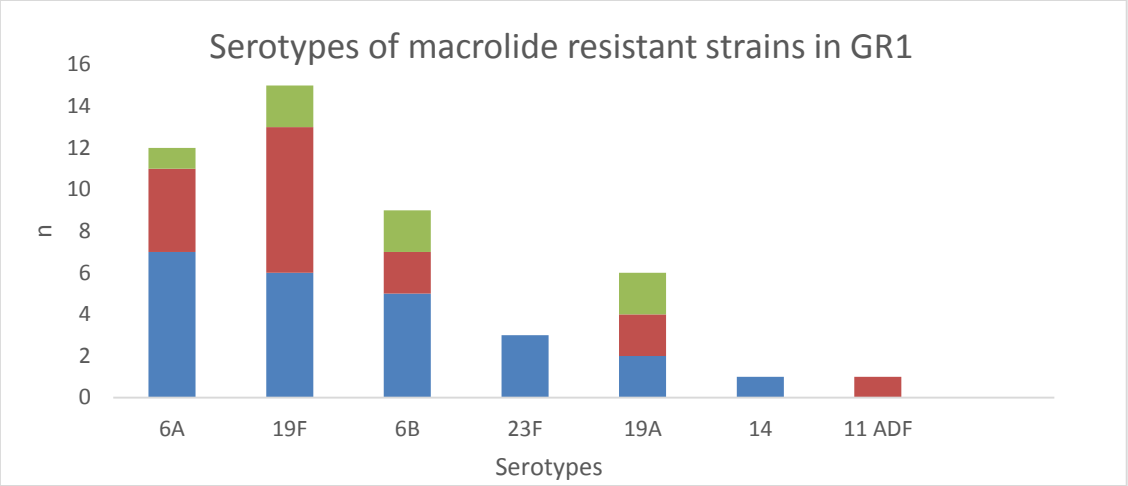

Figure 16. Detected resistance genes among macrolide resistant strains in GR1. Blue column: total *ermB* positive strains, red column: total *mef* positive strains, green column: *ermB* and *mef* gene detected together among the total *ermB* and *mef* positive strains, n= number of isolates

5.7.2 *Erm* and *mef* genes in GR2

In GR2 we detected the *ermB* gene in 91 strains, 64.8% of them were serotype 19A. Except for 2 cases, it resulted in high-level erythromycin and clindamycin resistance. In 16 strains *mefE* gene was observed (only in 2 cases of 19A) which showed M phenotype. In 5 cases we found *ermB* and *mefE* genes simultaneously, among 19A and 19F serotypes (**Figure 17**).

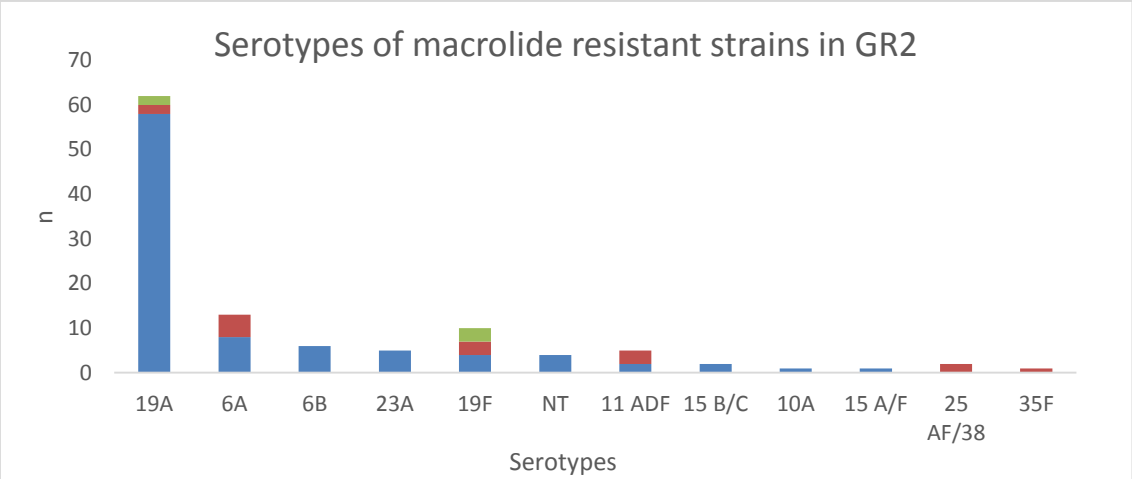

Figure 17. Detected resistance genes among macrolide resistant strains in GR2. Blue column: total *ermB* positive strains, red column: total *mef* positive strains, green column: *ermB* and *mef* gene detected together among the total *ermB* and *mef* positive strains, n= number of isolates

5.7.3 *Erm* and *mef* genes in GR3

In GR3 only 8 strains carried the *ermB* gene. This resulted in high-level resistance to erythromycin and clindamycin and half of them belonged to serotype 23A (**Figure 18**). None of the strains carried *mefE* gene.

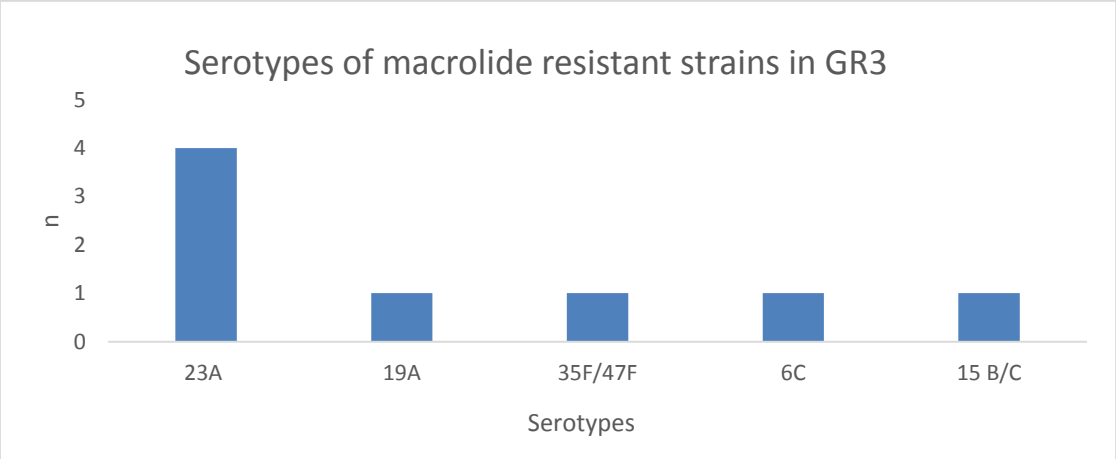

Figure 18. Detected resistance genes among macrolide resistant strains in GR3. Blue column: total *ermB* positive strains, n= number of isolates

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5.8 Pilus positive strains

The presence of pili was determined in the carried isolates in GR1 and GR2. The results show very important and interesting association between pilus positivity and antibiotic resistance and/or serotypes.

5.8.1 Pilus positive strains in GR1

Out of the 218 strains, we could detect pilus by PCR in 25 (11.5%) cases by PCR (**Figure 19**). Sixteen cases of the isolates showed intermediate resistance to penicillin, 14 isolates were highly resistant to erythromycin and clindamycin; in 7 cases the *ermB* gene and in 8 cases the *mef* gene was detected. The p value shows a very strong association between pilus positivity and antibiotic resistance ($p = 1.68*10^{-5}$). This shows that the piliated serotypes belong to the most prevalent serotypes of GR1, to the "paediatric" serotypes. All the 19F strains had high-level resistance to erythromycin and clindamycin. Five from them had intermediate resistance to penicillin and based on the PFGE patterns, 4 of them were similar to the PMEN Taiwan^{19F}-14 clone (see **Figure 23** in Chapter 5.9.2). Three strains each among 6B and 6A were resistant to macrolides and clindamycin. Interestingly both 19A strains in GR1, had pili and resistance to macrolides and clindamycin.

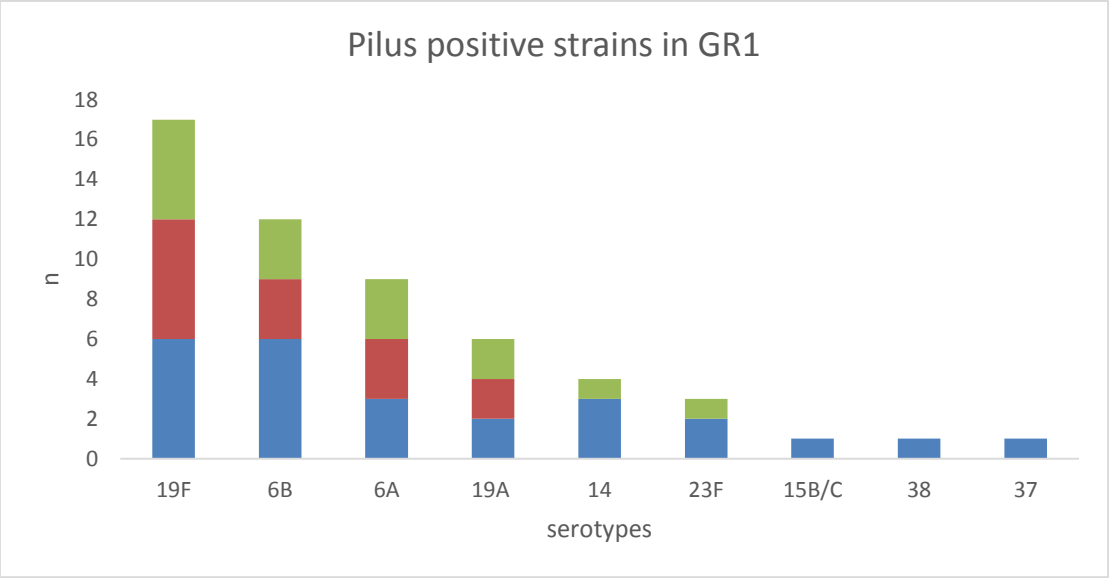

Figure 19. Serotype distribution of pilus positive strains in GR1. Blue column: Pilus positive strains, red column: erythromycin resistant strains among pilus positive strains, green column: penicillin intermediate resistant strains among pilus positive strains, n= number of isolates

5.8.2 Pilus positive strains in GR2

Out of 529 strains, we could detect pilus in 102 (19.3%) cases (**Figure 20**). In 27 cases the strains had intermediate resistance to penicillin and also 27 isolates were highly resistant to erythromycin and clindamycin (25 *ermB* positive and 3 *mefE* positive). We found a strong association (p-value $=6*10^{-8}$) between serotype 24 A/B/F and pilus positivity. Surprisingly these strains were sensitive to all tested antibiotics. Serotype 6A was the second most common piliated serotype. Among the 15 strains, 8 had high-level resistance to erythromycin and clindamycin and these carried the *ermB* gene. As in GR2 the prevalence of 19A serotype increased, presence of pilus positivity increased too and all of them were highly resistant to erythromycin and clindamycin.

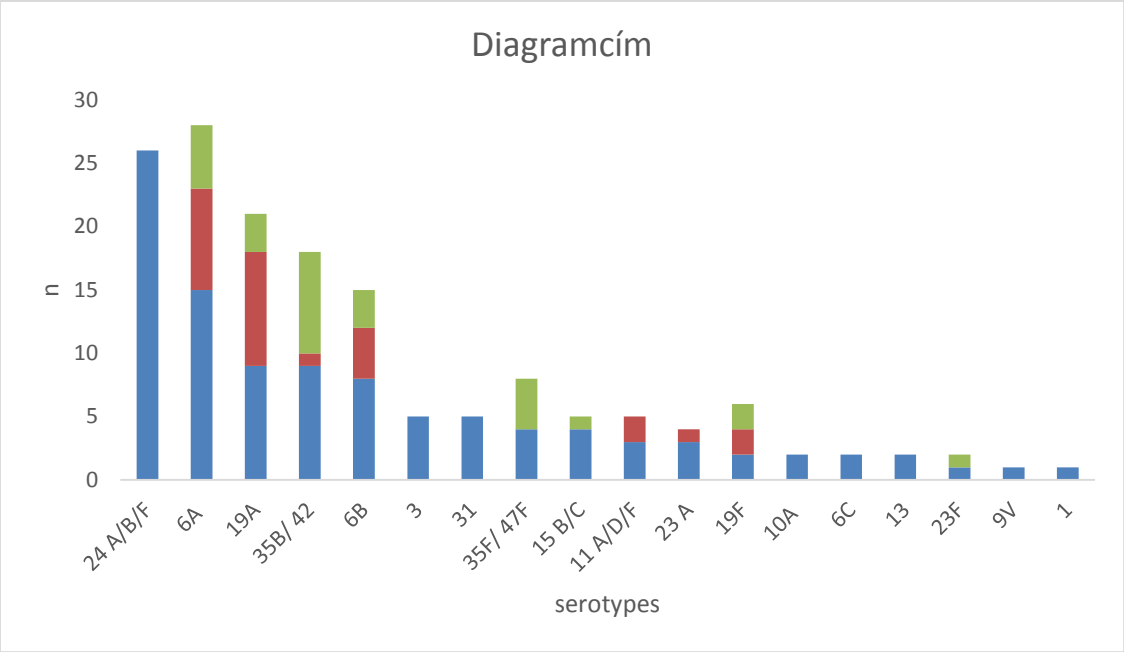

Figure 20. Serotype distribution of pilus positive strains in GR2. Blue column: pilus positive strains, red column: erythormycin resistant strains among pilus positive strains, green column: penicillin intermediate resistant strains among pilus positive strains, n= number of isolates

5.9 PFGE pattern of certain distinquished serotypes

The clonality among certain serotypes was examined with PFGE. Gels were analysed using the Bionumerics software (Bio-Rad), with the Jaccard coefficient and the unweighted pair group method with arithmetic mean (UPGMA) dendrogram type.
Among all serotypes, 19A was the most interesting due to its emergence after PCV7 vaccination and also its antibiotic resistance.

5.9.1 PFGE pattern of serotype 19A

The digested genome patterns of 19A (**Figure 21**) showed that although all isolates were related to one another, sub-clones could be identified. These PFGE clusters shared similar penicillin susceptibility rates. For instance, the isolates fully susceptible to penicillin formed a distinct group (cluster A). Another larger cluster comprised of isolates with intermediate penicillin MICs (cluster B). Interestingly, the single penicillin

resistant strain in the collection (L477) had an identical PFGE pattern to these isolates. Additionally, the four isolates possessing *ermB + mefE* together, were clustered into a separate small PFGE clone (cluster C). In order to compare the 19A strains of the current study to the previously frequent PMEN clone Hungary^{19A}-6 (73, 196), which typically was resistant to penicillin, we have included one representative isolate of that clone (strain 21646, isolated in 2001; last lane on **Figure 21**) in the PFGE analysis. As it is obvious the new 19A isolates differ greatly from this older clone.

In GR3 out of the 94 isolates, only two were serotype 19A. These 2 isolates (G53 and G69) had similar PFGE pattern to some of those obtained at the nurseries in GR2, as seen in **Figure 22**. These strains all had intermediate penicillin MICs (0.25-1 mg/L).

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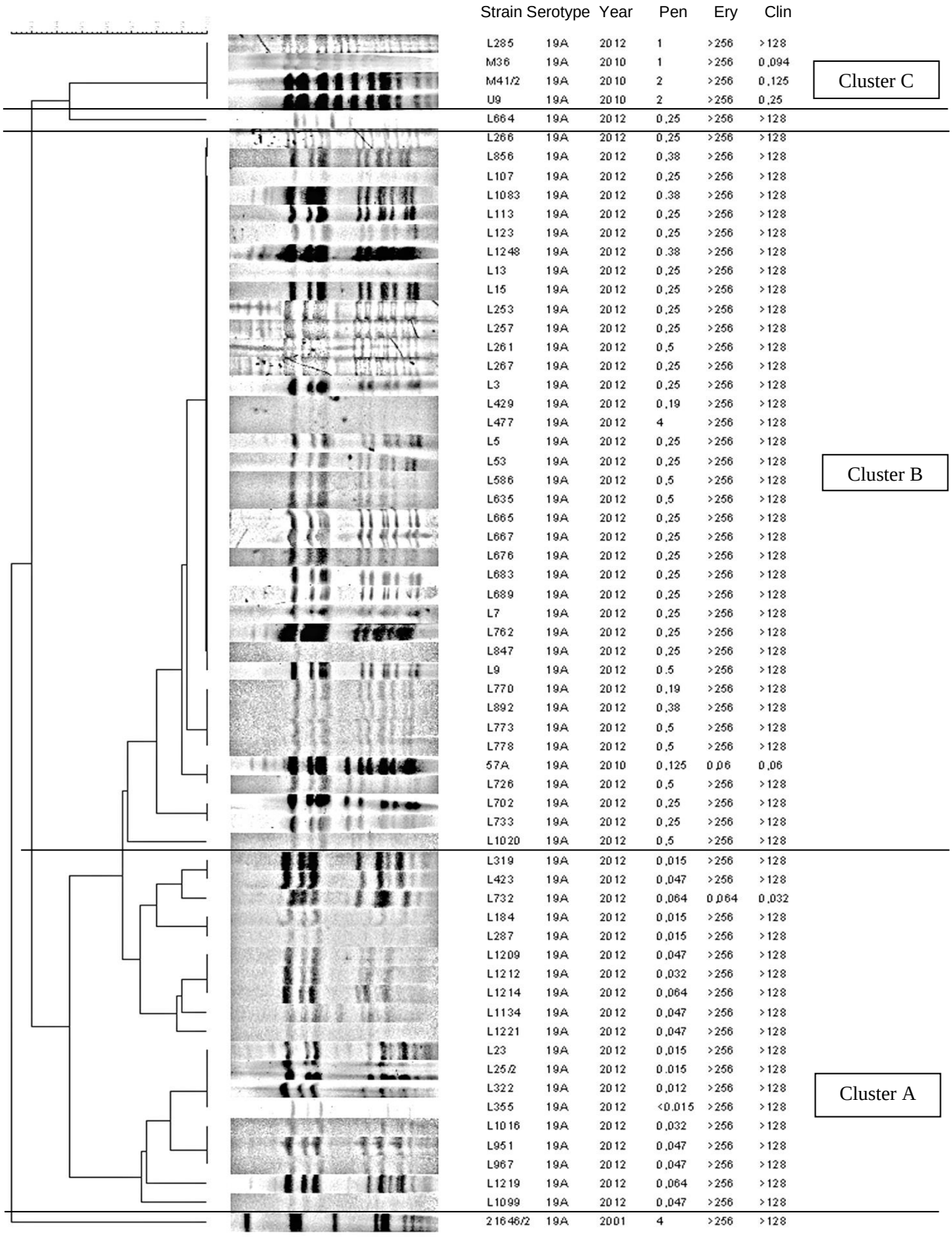

Figure 21. PFGE pattern of serotype 19A isolates

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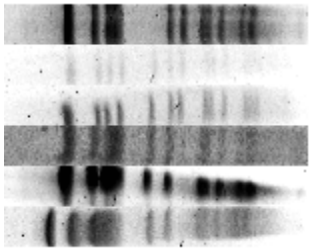
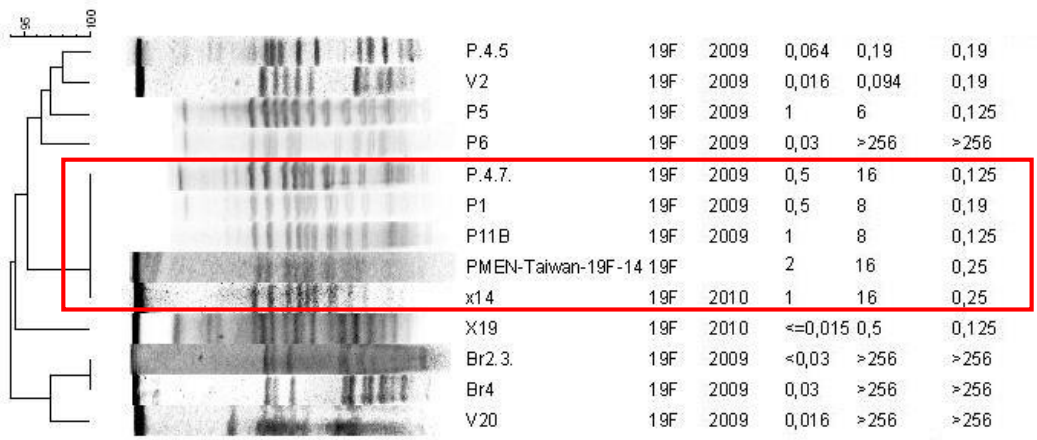


Strain		Year	Pen	Ery	Clin
L9	19A	2012	0.5	>256	>128
G53	19A	2014	1	>256	>128
G69	19A	2014	1	>256	>128
L726	19A	2012	0,5	>256	>128
L702	19A	2012	0,25	>256	>128
L733	19A	2012	0,25	>256	>128

Figure 22. PFGE pattern of the 19A serotypes from GR3, compared to other 19A isolates found in GR2

5.9.2 PFGE pattern of serotype 19F



Strain	Serotype	Year	Pen	Ery	Clin
P.4.5	19F	2009	0,064	0,19	0,19
V2	19F	2009	0,016	0,094	0,19
P5	19F	2009	1	6	0,125
P6	19F	2009	0,03	>256	>256
P.4.7.	19F	2009	0,5	16	0,125
P1	19F	2009	0,5	8	0,19
P11B	19F	2009	1	8	0,125
PMEN-Taiwan-19F-14	19F		2	16	0,25
x14	19F	2010	1	16	0,25
X19	19F	2010	<=0,015	0,5	0,125
Br2.3.	19F	2009	<0,03	>256	>256
Br4	19F	2009	0,03	>256	>256
V20	19F	2009	0,016	>256	>256

Figure 23. PFGE pattern of serotype 19F isolates

Digestion pattern on **Figure 23** shows similarities of some tested 19F isolates. The red square indicates the isolates (from two different nurseries in Szeged and Győr) which were identical to the PMEN-Taiwan^{19F}-14 clone. This group shows intermediate resistance to penicillin and low-level resistance to erythromycin.

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5.9.3 PFGE pattern of serotype 11 A/D/F

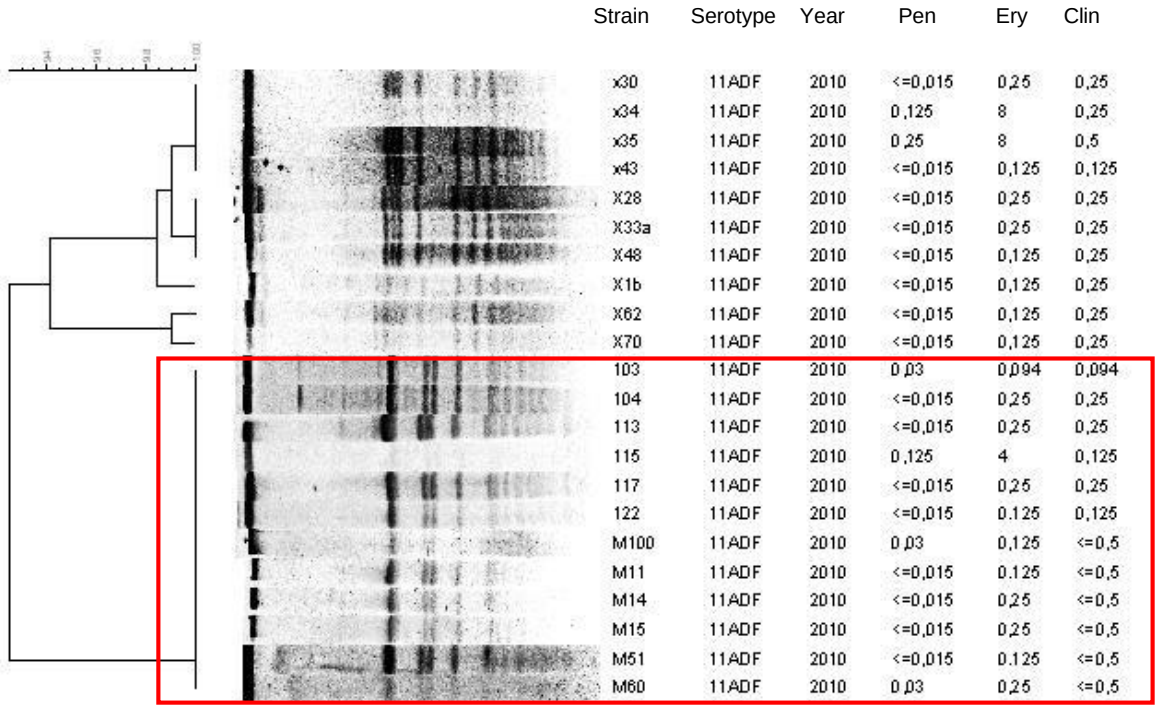

Figure 24. PFGE pattern of serotype 11 A/D/F isolates

Figure 24 shows 2 distinct clones among serotype 11 A/D/F. Strains in the first group were identical to each other (red square, isolates from 2 different nurseries, Miskolc and Budapest). The other group showed close relation but not full similarity, although the isolates originated from the same nursery in Szeged.

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5.9.4 PFGE pattern of serotype 15 B/C

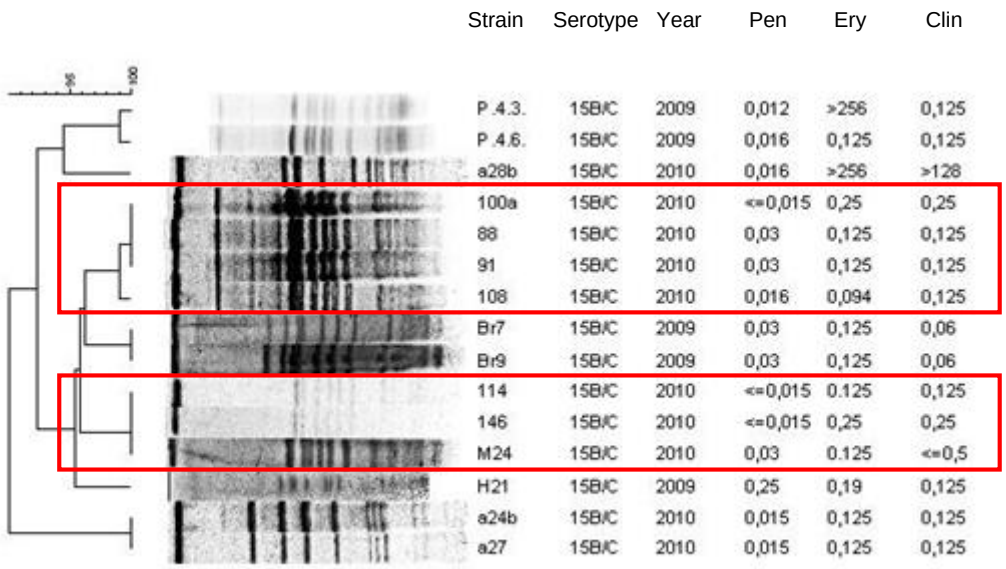

Figure 25. PFGE pattern of serotype 15B/C isolates

Serotype 15 B/C strains showed clonality within the same nursery (red squares) but no similarities were found between the strains from different nurseries (**Figure 25**). Except in one case, when samples from two different nurseries showed the same pattern (M24, 114 and 116, from Miskolc and Budapest, respectively).

5.9.5 PFGE pattern of serotype 3 and 37

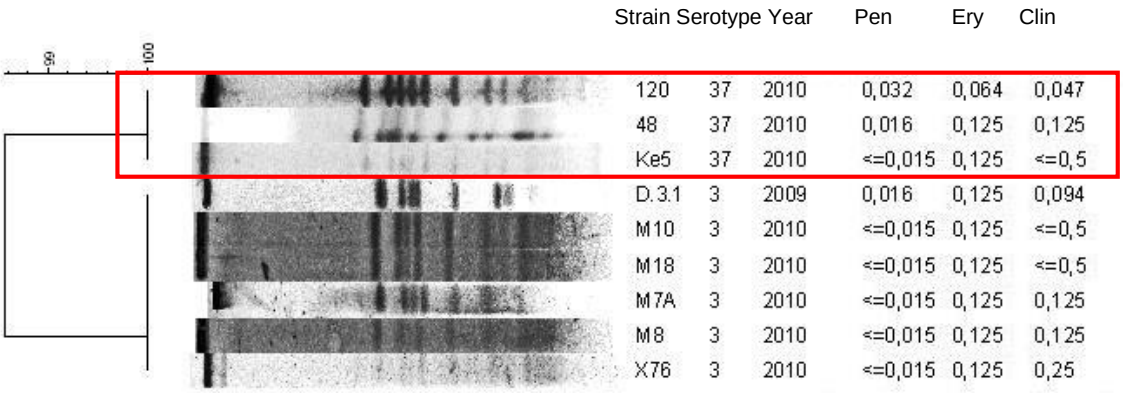

Figure 26. PFGE pattern of serotype 3 and 37 isolates

On this pattern (**Figure 26**) we can see that all serotype 3 isolates were identical (from different nurseries in Miskolc, Szeged, Debrecen), meanwhile serotype 37 which shows similarly mucoid colonies macroscopically, differs from serotype 3, but identical to each one another (red square, from different nurseries in Kecskemét and Budapest).

5.10 MLST results of representative serotype 19A isolates

For MLST analysis, a few isolates were selected from each PFGE cluster of 19A serotype (**Figure 21**). The MLST was determined for five isolates within PFGE cluster A (L23, L1214, L1134, L319 and L732, all penicillin sensitive, **Figure 21**) and four of these had identical sequence types (ST-8430). The fifth isolate had a completely different ST (ST-97), differing in 6 loci, but this isolate was sensitive to macrolides, unlike the rest **(Table 26).** The MLST was determined also for five isolates within PFGE cluster B (L477, L770, L762, 57A and L1020) and again, four belonged to the same ST (ST-319), but the only macrolide sensitive isolate (57A) was different (ST-1611), in this case differing in 3 alleles. Regarding PFGE cluster C, MLST was done for one representative isolate (M41) and this proved to be ST-320. Additionally, isolate L664, which showed a dissimilar PFGE pattern, proved to have an unrelated sequence type (ST-1757).

Based on the MLST data, we could also confirm that the current isolates are different from the Hungary19A-6 PMEN clone. The latter one belongs to the ST-268 clone (http://spneumoniae.mlst.net/sql/pmensts.asp?clone=Hungary%2019A-6&st=268), while our isolates had different sequence types.

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Table 26. MLST results of the representative serotype 19A isolates

Isolate	PFGE cluster	Penicillin MIC	Erythromycin MIC	Clindamycin MIC	Sequence type
L23	A	0.015	>256	>128	ST-8430
L1214	A	0.064	>256	>128	
L1134	A	0.047	>256	>128	
L319	A	0.015	>256	>128	
L732	A	0.064	0.064	0,032	ST-97
L477	B	4	>256	>128	ST-319
L770	B	0.19	>256	>128	
L762	B	0.25	>256	>128	
L1020	B	0.5	>256	>128	
57A	B	0.125	0.06	0.06	ST-1611
L664	none	0.25	>256	>128	ST-1757
M41/2	C	2	>256	0.125	ST-320
21646	PMEN	4	>256	>128	ST-268

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6 DISCUSSION

6.1 Carriage rate

The average carriage rate throughout the whole study was 33.7%. This figure correlates well with the literature data reported from different countries (197-199). We detected double carriage only in 3 occasions. This low co-colonisation rate might be the result of the culture-base technique we applied. Several papers suggest that co-colonisation is often underestimated and to reveal the real rates, more sophisticated methods such as multiplex PCR or microarray should be used (200, 201). We didn't find any correlation between carriage and AOM in all of the 3 groups.

6.1.2 Carriage rate in GR1

In the first group we could detect an overall carriage rate of 34.2%. The international published rates vary within a wide range between a surprisingly low 8.6%, observed in an Italian study (involving 2800 children) (197) up to 66.0% in Russia (733 children) (198), or 78.4% in Norway (573 children) (199), or even higher in some Asian and African countries (85). Highly similar results to ours were observed among Turkish children (37.2%, n = 301, aged <6 years) (202). Detecting pneumococci beside the often very dense normal flora of the children required careful examination; ignoring it might lead to an apparent unsuccessfulness in determining the real carriage rate in many studies.

6.1.3 Carriage rate in GR2

The carriage rate in this group with high-level PCV7 vaccination was 32.5%. This figure is a bit smaller than observed in GR1, but not significantly and it suggests that the conjugate vaccines have only a small, but non-significant effect on the carriage rate (**Figure 27**); they rather influence the distribution of the carried serotypes. Studies from France and Japan also observed the same, after the introduction of conjugate

vaccination the overall carriage rate remained unchanged (203, 204). In this group we detected double carriage only in one case.

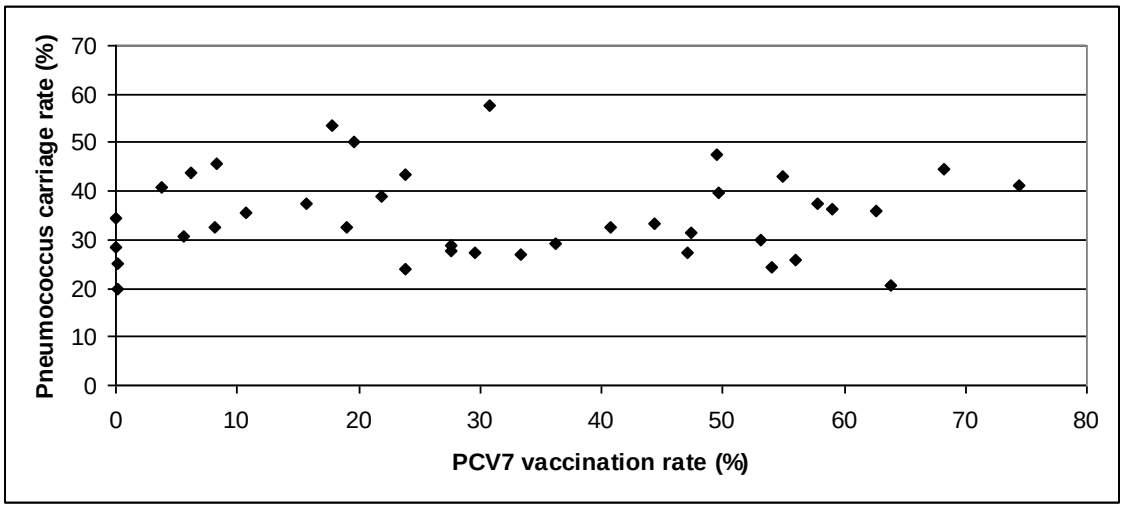

Figure 27. Pneumococcal carriage rate in the individual nursery groups in GR2, in relation to the vaccination rate of each group

We found a slightly but not significantly (p=0.28**)** higher colonisation rate in males, 54.17% versus 45.83% in girls; this was also found among Brazilian adolescents (205). As these authors have also suggested, a higher pneumococcal carriage rate may be one of the reasons leading to the observations that male gender is a definitive risk factor for pneumonia (206). The highest level of carriage was observed at 3 years of age (43.3 %), which declined to 18.4% by 6 years of age (**Figure 24**). This is in accordance with the results of a large-scale review, where the peak incidence was identified also at 3 years (85). After that age, colonisation steadily declines to below 3 % by the age of 19 years (205). The 20–40-year-old adults are least effected by pneumococcal infections, disease burden starts increasing above 50 years, but the source is not any more the self-carriage, rather small children in the close environment.

6.1.4 Carriage rate in GR3

The carriage rate in GR3, after the PCV13 vaccination, was 41.4%. This is significantly (p=0.008) higher than in GR2 and GR1; but lower than it was found for example in England (47.7%) (207), where they did not find any differences in carriage after

vaccination with PCV13 compared to their earlier studies. We can explain the difference in carriage rate with the increase of the surrogate non-PCV13 serotypes and the average lower age since the samples in this group derived from children aged mostly 1-2 years, where the carriage rate is the highest (208). We didn't find significantly differences in the colonisation rate in boys or girls.

6.2 Serotype distribution and vaccine coverage

6.2.2 Serotype distribution in GR1

The majority of the strains in GR1 belonged to the usual so called "paediatric serotypes" (6A/B, 14, 19F, 23F) (85, 209, 210), but interestingly serotype 9V was totally absent. Their disease causing potential is very high, these serotypes cause 70%–88% of IPD in young children in the United States and Canada, Oceania, Africa and Europe and <65% in Latin America and Asia (128). On the other hand, the clear emergence of some rare serotypes (especially 15B, but also 11A and 13) were observed, despite the relative low vaccination status of children at the time of the study. These newly emerging serotypes were almost all completely sensitive to antibiotics, unlike the paediatric serotypes, therefore colonisation with them does not mean a comparable danger, even if they spread further and cause infection.

6.2.3 Serotype distribution in GR2

In GR2 we found striking differences in serotype distribution. While in the pre-vaccination era, the well-known paediatric serotypes were still dominant (14, 19F, 23F, 6A, 6B in ranking order); these were rapidly replaced by others. For example, although serotype 14 has been the leading type before, it fully disappeared as soon as children became vaccinated with PCV7. The frequency of 19F, 23F and 6B also decreased significantly ($p=5.2*10^{-7}$, $p=1.3*10^{-5}$ and $p=4.7*10^{-8}$, respectively) but on the other hand, the previously absent serotypes 19A and 23A significantly emerged ($p=2.7*10^{-6}$, $p=4.4*10^{-6}$), competing for the second position. Unfortunately serotype 19A, which became a leading serotype, was shown to have high invasive disease potential and antibiotic resistance capacity and has often been reported worldwide as an emerging

serotype after the implementation of PCV7 (211-213). For instance, van Gils et al. reported that nasopharyngeal acquisition of serotype 19A was significantly higher in children who completed the 2+1 vaccination schedule with PCV7, compared to the unvaccinated ones (214). As a consequence of this robust serotype rearrangement, the vaccine coverage rates declined sharply (from GR1 to GR2), only 8.1% would be covered by PCV7 and 32.3% by PCV13.

We can place our results into a more complex frame if we compare them to the national and international IPD cases, as pneumococcal infections typically originate from carriage. Van Hoek et al. have investigated the disease potential of the different serotypes based on more than 23000 IPD isolates from England (215). They found that serotype 11A, which was frequently found in our GR2 group too, despite its usual antibiotic sensitivity, was found to be associated with the highest mortality in the age group of 5–64 years, along with serotype 31 (rare in our collection) (215). In their study serotypes 3 and 1 caused empyema in the highest proportion, while they were rarely associated with meningitis.

We have to compare our results to other (non-carriage) Hungarian data covering more or less the same time period. The only available data from the post PCV7 era derived from the OEK, where serotypes of invasive specimens have been routinely determined since 2008. Between January 2008 and August 2012, they received 545 invasive specimens (mostly from adults) from 67 hospitals all over the country (216). The dominating serotype was type 3 (~27 %) for the whole period, but there were changes in frequency of other serotypes over time. In the earlier years of the study, serotypes 14 and 19A were in the second place (6.6 % both). While serotype 19A retained its ranking (6.0%), serotype 1 emerged sharply during the last 1.5 years (6.6 %) and serotype 14 totally vanished, just as was observed in our GR2 group. During the time period overlapping with our study, the number of pneumococcal meningitis cases were as follows: n=49 (2009), n=48 (2010), n=62 (2011) and n=66 (2012). The mortality rate varied between 19.7 % (2012) and 27.1 % (2010) and with one exception, only adults (>30 years) died (179, 183).

6.2.4 Serotype distribution in GR3

In GR3 we detected a decline in the carriage of PCV13 serotypes. Serotype 19A, which became a dominant serotype after PCV7 vaccination in GR2, was observed only in 2 cases in GR3, however, these were clonally similar to some of the GR2 isolates.
Significantly emerging serotypes were 15B/C, 35F/47F, 6C and 23B. The same situation was observed in other countries after the replacement of PCV7 by PCV13, in case of IPD cases and carriage (115, 204, 207, 217). These studies showed continued reduction in carriage of both PCV7 and PCV13 serotypes.

6.3 Antibiotic susceptibility and resistance genes

6.3.2 Antibiotic susceptibility in GR1

Generally, antibiotic susceptibility was very high among GR1, except for macrolides. None of the isolates were resistant to penicillin or to other β-lactams and the erythromycin resistance was only 21.3%. Similar observations were made in other countries (198, 199). Before the introduction of conjugate vaccines, the colonisation of nasopharynx by antibiotic resistant strains were relatively frequent in young children, although it was usually restricted to certain (paediatric) serotypes, such as 6A and 6B, 9V, 14, 19A and 19F and 23F (197, 218-220). In GR1 the macrolide resistant strains belonged to serotypes 6A, 19F, 6B, 23F, 19A, 14 and 11 A/D /F. In 24 cases we could find the *ermB* genes resulting high-level resistance to macrolides and lincosamides. In 16 strains we detected the *mefE* genes resulting low level resistance in 10 strains. Among these 16 strains, 7 strains carried *ermB* and *mefE* genes simultaneously.

6.3.3 Antibiotic susceptibility in GR2

The isolates in this group were also rather sensitive to the drugs tested, except for the macrolides again. Only one isolate was resistant to penicillin (MIC=4 mg/L) and 21.6% were resistant to erythromycin.

Among macrolide resistant strains we detected the *ermB* gene in 91 cases and more than half of them were serotype 19A. Except 2 cases it resulted high-level erythromycin and clindamycin resistance. In 16 strains the *mefE* gene was observed, where 5 strains carried *ermB* and *mefE* genes simultaneously (among 19A and 19F serotypes). After the wide spread vaccination with PCV7 the number of serotype 19A strains became predominant. Unfortunately these isolates are all fully resistant to erythromycin and clindamycin and have elevated MICs to penicillin, as it was observed in other countries after PCV7 vaccination not only in carriage but unfortunately in invasive infections as well (168, 221, 222).

6.3.4 Antibiotic susceptibility in GR3

PCV13 was introduced to prevent invasive infections caused by world-wide rapidly emerging serotypes, such as the multi-resistant serotype 19A, which appeared after PCV7 vaccination. In GR3, where the children received already PCV13, the measured antimicrobial susceptibility among pneumococci was also relatively low; the isolates were generally sensitive to the tested drugs except to macrolides again. We didn't find any penicillin resistant strain, but intermediate resistance was found in 20.7% of the isolates, almost the same level was found in GR1 and GR2. In case of penicillin, 9.8% of the emerging serotype 23A showed intermediate resistance, followed by 3.3% of serotypes 35F/47F and 2.2% of serotypes 6C. In macrolide resistance we also obtained almost the same results as in GR1 and GR2 (21.7% to erythromycin and 17.4% to clindamycin), but here 12% of the 23A strains were highly resistant. The only two 19A strains found were also highly resistant to macrolides and lincosamides and were clonally related to those found in GR2. Among macrolide resistant strains, 8 had the *ermB* gene. This resulted in high level resistance to erythromycin and clindamycin and half of them belonged to serotype 23A. None of the strains carried the *mefE* gene.

6.3.5 Summarised antibiotic susceptibility

In general, our results found in the three groups suggest that although the average resistance did not change significantly due to the vaccinations, the serotypes changed, which carry the same resistance genes. In GR1, these serotypes were the earlier common "paediatric" serotypes, which are present in the PCV7 vaccine. After

vaccination the typically multi-resistant 19A serotype became dominant, whereas after PCV13 vaccination the "dangerous" 19A decreased and other, earlier uncommon serotypes started competing for its place, showing the same level of resistance, but less virulence. (**Figure 28, Figure 29, Figure 30**). The same observation was found in a study from the US (223). Another study from Italy vaccination showed higher resistance to antibiotics among carried pneumococci shortly after PCV13, where non-PCV13 serotypes accounted for 75.4% and 70.8% of the penicillin and erythromycin non-susceptible isolates, respectively (224).

The resistance rates observed in our study, however, are much lower compared to those of disease-causing strains in Hungary (173, 225). According to the nationwide reporting system data maintained by the OEK, penicillin resistance of clinical isolates has been varying between 1.5 and 5 % in the last 10 years (based on approximately 4,000 specimens annually) and the higher rates have been observed in hospitalised patients and invasive specimens (179). Regarding macrolide resistance of clinical isolates, although it shows a decreasing tendency over the last few years (from a previously decade-long stable ~40 %, it gradually fell to ~26 % between 2008 and 2013), it is still higher than that detected by us for the carried strains. Most of the macrolide resistant strains among IPD isolates belonged to serotype 19A, which increased after the widespread introduction of PCV7. All these findings are most likely related to the drastic rearrangement of serotypes occurring everywhere where vaccination was introduced.

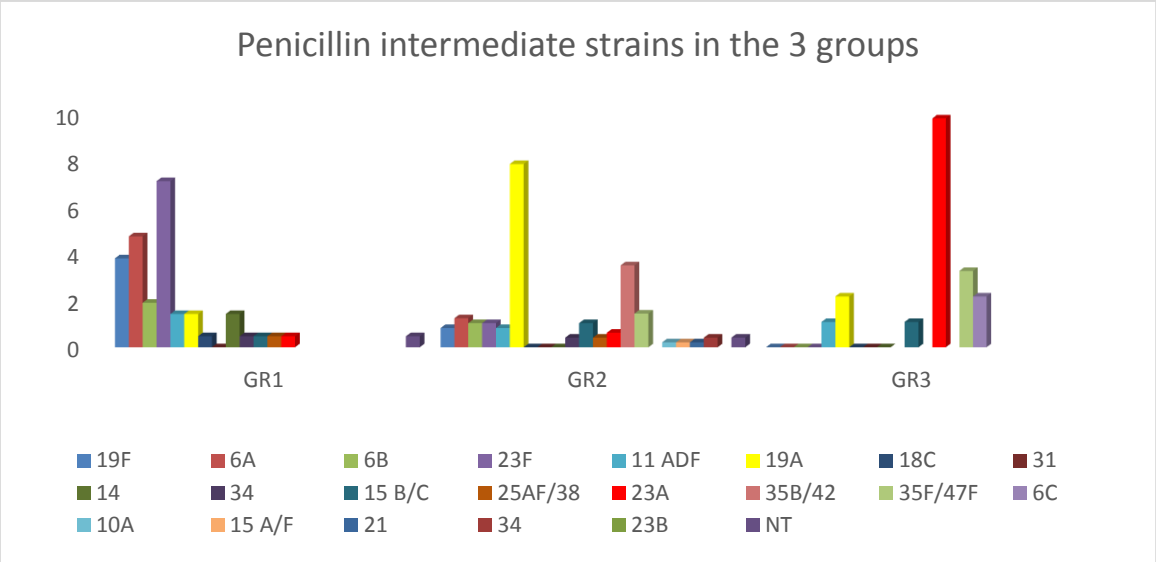

Figure 28. The penicillin intermediate resistant isolates. In GR1, decreased penicillin susceptibility was shared among the previously prevalent "paediatric' serotypes. In GR2, after PCV7 vaccination, the serotype distribution changed markedly and 19A (yellow column) became dominant. In GR3, after PCV13 vaccination, 19A decreased, meanwhile serotype 23A (red column) replaced it

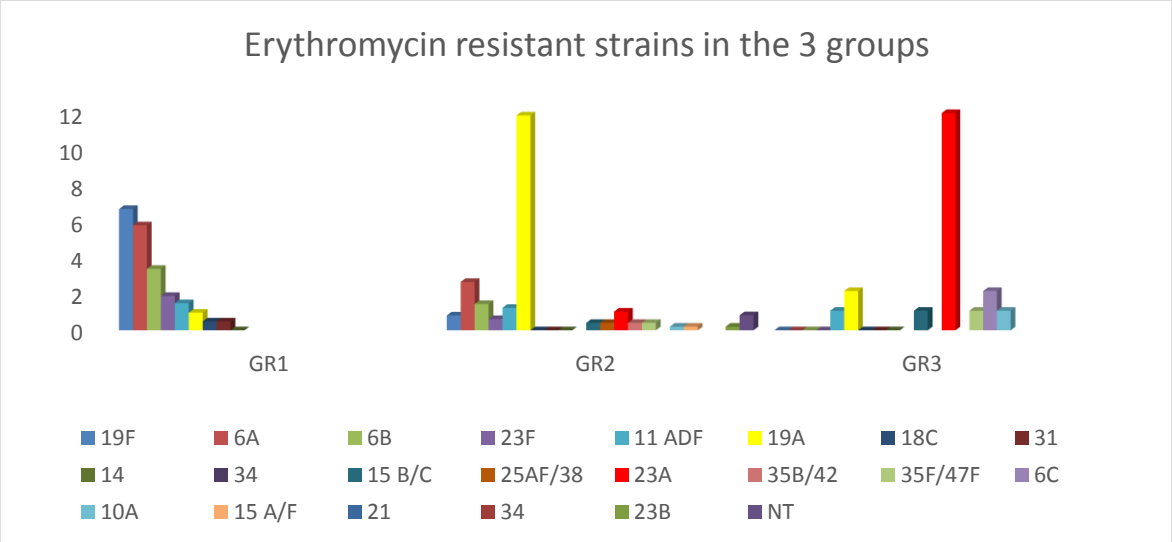

Figure 29. The erythromycin resistant isolates. In GR1, the "paediatric' serotypes were mostly responsible for erythromycin resistance. In GR2, after PCV7 vaccination, the serotype distribution changed markedly and 19A (yellow column) became most dominant. In GR3, after PCV13 vaccination, 19A decreased, meanwhile serotype 23A (red column) replaced it

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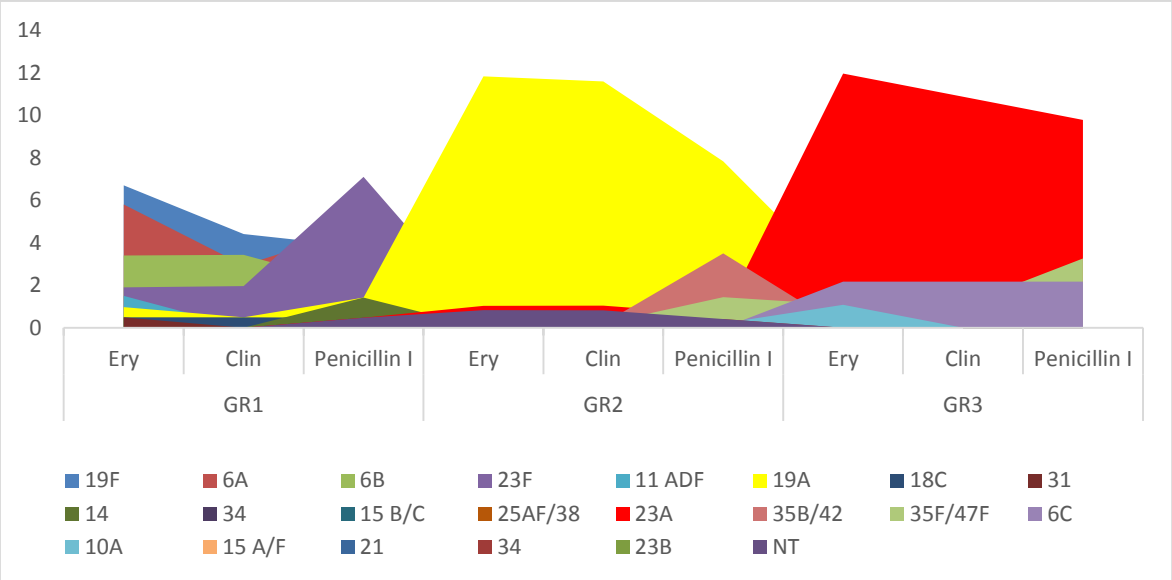

Figure 30. Serotype distribution of erythromycin^{R}, clindamycin^{R} and penicillin^{I} strains

On **Figure 30** the same results can be seen as on **Figures 28 and 29**, that in each group the general resistance did not change significantly, only the serotypes. In each group, because of the selective pressure of the vaccines, new serotypes emerged and probably acquired the resistance genes. In GR2 the dominant resistant serotype was 19A, while in GR3 it was 23A

6.4 Pilus positive strains in the 3 groups

In GR1, we could detect a strong association with pilus positivity and antibiotic resistance, high-level resistance measured in 64 % of the piliated strains. In GR2, we could detect a strong association between serotype 24 A/B/F and pilus positivity, despite that these strains were sensitive to all the tested antibiotics. Serotype 6A was the second most common piliated serotype and 8 out of the 15 strains were resistant to erythromycin and 5 showed intermediate resistance to penicillin.

These results need more investigations since recent studies could demonstrate that native pilus could be dedicated to DNA transformation in pneumococci. A model for DNA transformation is proposed whereby pilus assembly “drills” a channel across the thick cell wall that becomes transiently open by secretion of the pilus, providing the

entry port for exogenous DNA to gain access to DNA receptors associated with the cytoplasmic membrane (34).

Therefore, in an invasive infection, if a strain is piliated, under the selective pressure of antibiotic treatment, the strain can easier acquire resistance genes from the environment and so develop resistance and/or escape mechanisms, such as serotype switch with the help of the pili. This suggests that piliated strains need more attention and combination of a pilus-based vaccine with the current serotype-based vaccines would probably be useful to avoid the development of higher level antibiotic resistance.

6.5 PFGE results of the 3 groups

To compare the clonality of the isolates we have to handle the results of all the 3 groups all at once. Than we can get information about the clonality of strains isolated at different times and distant places, to investigate if there is any widespread clonal strains in the country.

Obviously, within a DCC group we could detect quite often, that the same serotype showed the same PFGE pattern, thus they belonged to the same clones, providing evidence for strong clonal spread between the children in the same group. This can be easily explained by the close contact between children during the time spent together in the given community.

On several occasions we could detect the same clone in different DCCs in different parts of Hungary. For instance, in case of serotype 11 ADC we detected the same PFGE clone in Miskolc and in Budapest. Similarly, in case of serotype 19F, we could detect the same clone in Szeged and in Győr and interestingly these clones were identical to the PMEN Taiwan ^{19F}-14 clone, which has been a widespread clone on the earth (73, 226, 227) and resistant to erythromycin and non-susceptible to penicillin, just like our strains. This strain was found already earlier among invasive isolates in Hungary (227).

Serotype 15B/C was shown to be clonal in the same group, but genetically distinct strains were found in different DCCs, except for one clone which was found in 2 different DCC. The PFGE pictures of serotype 3 strains were identical and different from serotype 37, which phenotypically is very similar to serotype 3 because of the large mucoid colonies.

The most important PFGE examination was conducted among serotype 19A isolates. Since this serotype became highly frequent after vaccination and was highly resistant to macrolides, we wanted to know whether they belong to any worldwide spread PMEN clone, especially the famous resistant Hungary^{19A}-6 clone (73). We found that all 19A strains were more or less clonal, but PFGE could identify some smaller clusters, which showed strong relation to penicillin sensitivity levels. It is clear from the PFGE patterns and MLST results that none of them is identical to the previously dominant, highly resistant and virulent Hungarian 19A clone. Some of our 19A isolates (PFGE cluster C), showed elevated penicillin MICs, high-level erythromycin resistance, and possessed the *ermB* + *mefE* genes together.

6.6 MLST results of representative serotype 19A isolates

We could detect the presence of 2 major sequence types, ST-319 and ST-8430. Both STs showed a very good correlation with the PFGE clustering.

In the MLST database (http://spneumoniae.mlst.net/), 9 hits were found for ST-319 (serotype 19A = 6, serotype 19F = 3), 5 strains from Germany, 2 from Poland and 1 each from Greece and Spain. Five of these derived from invasive infections and two from the nasopharynx. They were all penicillin intermediate, like our isolates.

In the case of ST8430, only one strain was found in the MLST database (serotype 19A), also isolated in Germany and sensitive to penicillin.

Among our isolates with the highest level of penicillin non-susceptibility and macrolide resistance, possessing the *ermB* + *mefE* genes together, we identified ST-320 as well, which is a famous multiresistant pneumococcal clone circulating all over the world. For instance, ST-320 was first detected in 2005 in Barcelona, 4 years after the introduction of PCV7 and 5-6 years later it was responsible for nearly 3/4 of the serotype 19A isolates (228). In Alaska, ST-320 was also detected first only 6-7 years after the introduction of PCV7, but soon they represented 13% of all 19A isolates (229). All these ST-320 isolates also carried the *ermB* + *mefE* genes together and were multi-resistant (221) suggested that ST-320 probably developed from its ancestor Taiwan^{19F}-14 PMEN clone (ST-236) through a recombination event between a strain belonging to this clone and a serotype 19A strain (221), under the pressure of the use of PCV7. The isolates of this new ST-320 were shown to have outcompeted Taiwan^{19F}-14 (ST-236)

(230). Very interestingly isolates belonging to the Taiwan^{19F}-14 clone were also found among our carried isolates in GR2 in Győr and Szeged, so either a genetic recombination occurred also in Hungary leading to the development of ST-320, or members of the ST-320 clone have spread to Hungary.

6.7 Strengths and limitations of this thesis

This is the first study that reports important epidemiological data about pneumococci involved in carriage in Hungary. The main strength of the present study is the examination of a large number (several thousand) of samples spanning over a 5-year period to determine the serotype distribution and antimicrobial resistance pattern according to (based on) the vaccination state. Studies of short duration risk over- or underestimating serotype coverage due to inability to take the periodicity of serotypes into account (231).

The other major strength of this study is that it provides serotyping data for several hundreds of pneumococci; serotyping data were rarely available in Hungary (involving only a very few strains) until the National Center for Epidemiology (OEK) started serotyping invasive isolates in 2008, however, no data were presented before from carried isolates.

Nonetheless, our study has also some limitations. First, the number of isolates in GR3 was less than in GR2 or in GR1, therefore more studies are necessary in the future within this age category. We have already contacted several new nurseries in different cities in Hungary and will continue the survey, but we wanted to provide early information about the effect of PCV13.

Another limitation is that in GR2 the majority of the samples were taken from Szolnok, since all of the local nurseries took part in this study (organised well by the head of all DCCs in Szolnok), while only some DCCs participated in other cities. However, as our data are in very good correlation with other European studies (regarding changes in serotype distribution and antibiotic susceptibility after PCV vaccination), we feel that this could still represent the general situation in Hungary.

A further limitation is the relative inability of the applied methods to determine the actual rate of co-colonization with multiple serotypes.

As this is a cross-sectional study, duration of carriage, known to vary according to the serotype, was not taken into account.

It was also a limitation that direct culturing of the nasal swab has some disadvantages, namely it may lead to false-negative results, as we analysed viable cells only.

Finally, the large amount of unknown vaccination status of children, where the parents did not know the answer, can influence the calculation of vaccination rate, therefore it might have been underestimated in GR1 (although the yearly vaccination rates reported by OEK could give some grasps).

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7 CONCLUSIONS

In this study we compared three groups of children with low-level (GR1) and high-level (GR2) vaccination rates with PCV7 and the third group was vaccinated with PCV13 (GR3); each group involved several hundreds of children. It was clear that, although global carriage rate hardly decreased, the serotype distribution almost completely changed. In GR1 the earlier world-wide common, resistant, invasive serotypes were found to be dominant (6B, 23F, 19F, 14), but serotype 11A/D/F started emerging already. The carriage rate of the PCV7 and PVC13 serotypes was 44.5% and 57.8%, respectively. In this group the intermediate resistance to penicillin was 24.2% and we did not find any fully resistant strain. In case of erythromycin resistance we found that 21.4% of the strains showed high-level resistance. Most of these strains carried the *ermB* gene which results high macrolide resistance.

In GR2, after PCV7 vaccination, the earlier common serotypes decreased or vanished (such as serotype 14) and completely new types (e.g. 23A, 24F) emerged. Among the PCV13-non-PCV7 serotypes, the frequency of serotypes 3 and 6A remained stable compared to GR1, while the number of serotype 19A strains increased to a great extent in GR2 after PCV7 vaccination. These emerging 19A isolates are fully resistant to erythromycin and clindamycin and have elevated MICs to penicillin. Most of these strains carried *ermB* gene. In this group the carriage of PCV7 serotypes decreased dramatically to 8.1% and that of PCV13 serotypes to 32.3%. Only one strain was found to be resistant to penicillin, but only with 4 mg/L MIC.

Our preliminary results in GR3, observed during surveying children <3 years old, having received PCV13 with high coverage rates, show that the prevalence of serotype 19A decreased to 2.1 % and serotype 3 was not detected at all among these strains. The prevalence of PCV7 serotypes further decreased to 1.1% and 3.2% prevalence were observed for PCV13 types. The antibiotic sensitivity of these strains didn’t change significantly compared to GR1 and GR2, intermediate resistance to penicillin was 20.7% and resistance to macrolides was 21.7% and most of the macrolide resistant strains carried *ermB* gene. The only two serotype 19A isolates showed identical PFGE pattern to the previous 19A isolates found in GR2, which belonged to ST-319.

If we summarise the serotype changes in association with PCV vaccination, the following conclusions could be drawn. In case of 6B, 23F, 19F, 14 and 13, we could detect significant decrease after PCV7 vaccination. On the other hand, in case of 19A, 23A, 35F/47F 24 A/B/F and 35B/42, we could detect significant increase after PCV7 vaccination. Following PCV13 vaccination, a significant decrease was detected in case of serotypes 3 and 19A, while the rates of serotypes 15B/C, 35F/47F, 6C and 23B showed an increase **(Figure 31**).

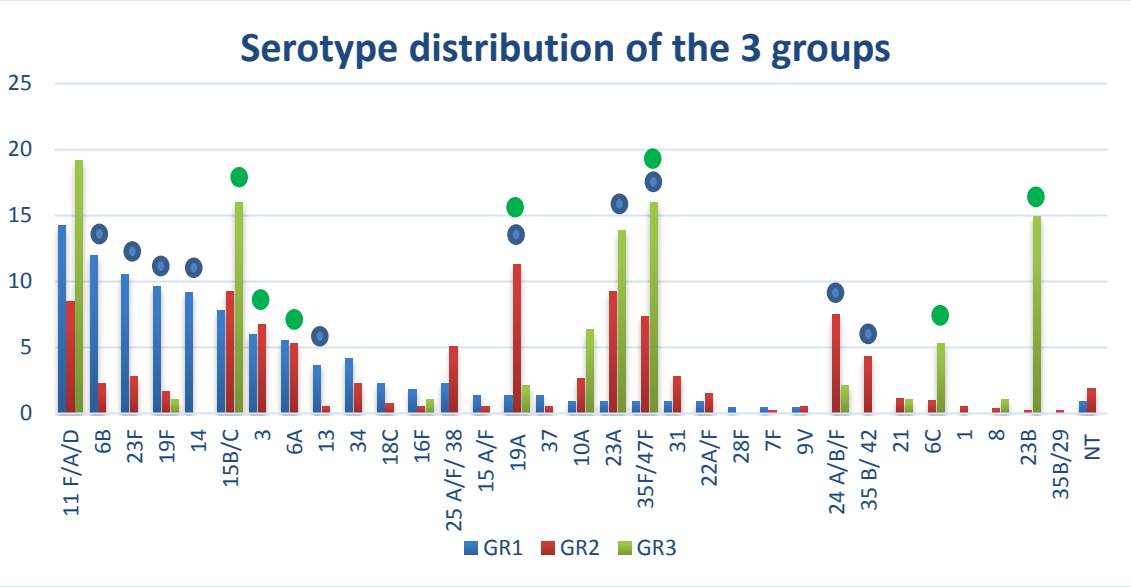

Figure 31. Serotype distribution in GR1, GR2 and GR3. Blue circle shows significant changes between GR1 and GR2, after PCV7 vaccination ($p < 0.05$). Green circle shows significant changes between GR2 and GR3 after PCV13 vaccination ($p < 0.05$)

We observed that pilus positive strains have a strong correlation with antibiotic susceptibility, namely elevated penicillin MICs and/or macrolide resistance. This is interesting as some studies suggest the role of pili in DNA uptake providing antibiotic resistance.

It was obvious from the PFGE examinations that children attending to the same group are likely to carry the same clone of a serotype. We were able to compare our isolates to well defined internationally widespread resistant clones and define the presence in Hungary in case of 19F in GR2. In case of 19A the strains were quite clonal, but PFGE

could identify some smaller clusters, which showed strong correlation to penicillin sensitivity levels. It is clear from the PFGE patterns and MLST results that none of the strains is identical to the previously dominant, highly resistant and virulent Hungarian 19A clone. Among 19A strains, with the help of MLST, we could detect the presence of 2 major sequence types, ST-319 and ST-8430. Both STs showed a very good correlation with the PFGE clustering. We also identified ST-320, which is a famous multi-resistant pneumococcal clone circulating all over the world.

As a conclusion we can say that after PCV7 vaccination the pneumococcal serotypes showed a dramatic re-arrangement. The previously dominating 'paediatric' serotypes disappeared or decreased greatly in prevalence, while formerly absent serotypes emerged rapidly. Serotype 19A appeared to have large importance due to its fitness, antibiotic resistance and invasive capacity. According to OEK data in Hungary and other studies from Europe and USA, it is clear that following vaccination with PCV7, not only the carriage of this serotype increased sharply, but its prevalence in invasive infections in children and also as a large number in elderly where it is likely to cause severe pneumonia and invasive infections. Therefore it was crucial to introduce PCV13 into the vaccination programme, to prevent the spread of this violent serotype. Studies after the introduction of PCV13 vaccination could detect a decline of 19A serotype again. Based on the preliminary results of our GR3, we could also observe the strong decrease of this serotype. We hope this tendency will further continue now that PCV13 was made a mandatory vaccine in Hungary. It remains important to monitor the pneumococcal carriage, to investigate the epidemiology of infections and vaccine effectiveness and predict the next rising serotypes, as long as the vaccines are serotype-based and a possible new generation, non-capsule dependent (whole cell, or protein) vaccine is not available.

The novel observations that could be concluded based on this thesis are the following:

1) Vaccination with PCV7 lead to a dramatic re-arrangement in pneumococcal serotypes. The rate of previously prevalent 'paediatric' types decreased or vanished (e.g. serotype 14) and other, formerly absent serotypes emerged.

2) The most important emerging serotype after PCV7 was 19A, which is multi-resistant and has high invasive capacity and caused worries in most countries in the post-PCV7 era.
3) After the introduction of PCV13, serotype 19A seems to decline again and the other PVC13-PCV7 serotype, serotype 3 was not detected any more among the youngest children.
4) There was no significant difference in carriage between genders, however, a bit more males were found to be carriers.
5) The overall antibiotic resistance of the carried strains did not change during the 3 examined period, but different serotypes were involved in resistance; while the 'paediatric' serotypes dominated in GR1, 19A alone took over in GR2 and it was replaced by 23A in GR3.
6) Based on the PFGE and MLST data, the highly prevalent serotype 19A isolates were genotypically unrelated from the world-wide prevalent PMEN Hungary^{19A}-6 clone, however, other PMEN clones were detected among our 19F strains.
7) PFGE is a useful tool in the determination of the genetic relatedness of the pneumococcal isolates within a country of the size of Hungary; further refinement of the clustering was possible with the MLST method.
8) The PFGE data showed that clonal spread of the isolates as well as horizontal transfer of resistance genes together contribute to the dynamic serotype changes observed in the post-PCV era.
9) Very strong correlation could be established between pilus positivity and antibiotic resistance (penicillin and macrolide non-susceptibility) and/or serotypes (especially serotype 24 A/B/F).
10) Both PCV7 and PCV13 proved to be very successful in the elimination of the vaccine-types, meanwhile the PCV10-PCV7 serotypes (1, 5 and 7F) were never prevalent among the carried pneumococci. We suggest that vaccination with PCV13 should definitely be continued in Hungary.

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8 SUMMARY

Invasive diseases due to *S. pneumoniae* are estimated to be responsible for more than one million deaths per year. *S. pneumoniae* can be part of the commensal flora of the upper respiratory tract, mostly in small children. Though colonization with pneumococci is mostly asymptomatic, it remains the common source of pneumonia, bacterial meningitis, bacteraemia and otitis media in children and in elderly, too. The pneumococcus is antigenically diverse: 94 different capsular serotypes have been described. Unfortunately, since the pneumococcal vaccines contains only a few (7, 10, 13 or 23) capsular antigens, the selective pressure applied by vaccination lead to increased carriage of non-vaccine serotypes. The replacement of these serotypes may favour the emergence of successful strains. The impact of these processes will depend on the ability of the replacing strains that cause disease, which is largely unknown.

In this study 2485 children were screened and 838 strains were isolated. Around 22% of the tested strains showed intermediate resistance to penicillin and the macrolide resistance was around 21%. We could detect a radical serotype rearrangement after PCV7 vaccination (which is widely used since October 2008). The previous dangerous serotypes decreased due to the vaccination, but unfortunately non-vaccine serotypes increased such as 19A, which is highly resistant to erythromycin and clindamycin, has elevated MICs to penicillin and high potential to cause invasive infections. PCV7 was replaced by PCV13 also in Hungary in 2010. This vaccine contains 6 additional capsular antigens compared to PCV7 and protects also against serotype 19A. As expected, a decrease of 19A was observed in the group where PCV13 were used. Among these children we could detect an increase of earlier rare, uncommon serotypes such as 23B or 6C.

In summary, we can say that the prevalence data in asymptomatic carriage can be used as a prognosis for the serotypes that will increase after vaccination and would be likely to cause infections. Also, since there are geographical differences, it is very important to know how the serotype replacement will occur after vaccination in different countries. We still don’t know whether these newly emerging serotypes found after PCV13 vaccination will be dangerous and/or invasive. Therefore we have to carry on the continuous follow up of carriage estimating the effect of vaccination within Hungary.

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9 ÖSSZEFOGLALÁS

A *S. pneumoniae* több mint egymillió halálos kimenetelű invazív fertőzésért felelős évente. Gyakran megtalálható főleg egészséges gyerekek felső légútjaiban. Bár ez a hordozás általában tünetmentes, mégis nagy jelentőséggel bír, mivel ez lehet a leggyakoribb forrása a lobáris pneumonia, bakteriális meningitis, bacteraemia, és otitis media kialakulásáért gyerekekben és idősekben egyaránt. A faj antigenitása nagyon változékony: eddig 94 szerotípust ismerünk a tok antigén alapján. Az elérhető pneumococcus ellenes oltások ebből csak néhányat (7-et, 10-et, 13-at vagy 23-at) tartalmaznak, ezért szelektív hatásuk jelentős az oltott populációkban. Így az oltást követően a nem-vakcina típusok gyakorisága mindenhol megnőtt.

Kutatásunk során összesen 2485 gyerek orrmintáját gyűjtöttük be, és 838 hordozott törzset találtunk. Átlagosan 22%-uk volt mérsékelten érzékeny penicillinre, és 21%-uk makrolid rezisztens. Drasztikus szerotípus kicserélődést figyelhettünk meg a PCV7 oltást követően. Szerencsére a korábban elterjedt, veszélyes és invazív megbetegedésért felelős szerotípusok száma jelentősen lecsökkent, de sajnos a nem vakcina-szerotípusú törzsek, első sorban a 19A, nagyarányú megnövekedését figyeltük meg. Ez a törzs sajnos gyakran multi-rezisztens, mérsékelten érzékeny penicillinre és gyakran okoz súlyos invazív kórképeket. Ezért 2010-ben Magyarországon is felváltotta a PCV7 oltást az újonnan kifejlesztett PCV13. Ez az oltóanyag, a PCV7-hez képest, további 6 szerotípussal bővült, és védelmet nyúlt a 19A okozta kórképek ellen is. Emiatt a PCV13 oltást követően számítani lehetett a 19A szerotípus prevalenciájának csökkenésére, ahogy ez be is következett. Természetesen ez után is tapasztalható volt, hogy még újabb nem-PCV13 típusú törzsek kerültek előtérbe, mint pl. a 23B, vagy 6C.

Összegzésként elmondható, hogy a tünetmentes hordozók szűrésével előre jelezhető, hogy az oltás után mely szerotípusok válnak gyakorivá és ebből kifolyólag később felelőssé a pneumococcus okozta fertőzésekért. Mivel területi különbségek is léteznek a különböző szerotípusok elterjedését tekintve, minden országban fontos a szerotípusváltás nyomon követése. Mivel még nem tudjuk, hogy a PCV13 oltás után megjelenő új szerotípusok mennyire válnak veszélyessé, folytatnunk kell a hordozók szűrését és ezzel megjósolni az oltás további hatását Magyarországon.

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11 LIST OF PUBLICATIONS

List of publications

Related to the thesis

Papers

Tóthpál A, Kardos S, Laub K, Nagy K, Tirczka T, van der Linden M, Dobay O. (2014) Radical serotype rearrangement of carried pneumococci in the first 3 years after intensive vaccination started in Hungary. Eur J Pediatr. September 2014, DOI: 10.1007/s00431-014-2408-1 **IF: 1.983**

Tóthpál A, Laub K, Kardos S, Nagy K., Dobay O. (2014) Invazív és hordozott *Streptococcus pneumoniae* izolátumok vakcina lefedettsége Magyarországon. Lege Artis Medicinae, 24:(4) pp. 180-185.

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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

Gyermek 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 Prevenar oltást kapott-e igen nem nem tudom

Gyermekem Pneumovax oltást kapott-e igen nem nem tudom

Szokott-e középfülgyulladása lenni igen nem

Volt-e már súlyos tüdőgyulladása, agyhártyagyulladása vagy középfülgyulladása? (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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Appendix 1. Parent's permission. Qestions with bold font were asked later, only in GR2

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Dr. Dobay Orsolya
egyetemi adjunktus
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Budapest

Tisztelt Adjunktus Asszony!

A Semmelweis Egyetem Regionális, Intézményi Tudományos és Kutatásetikai Bizottsága a 2012. január 30-án megtartott ülésén a *„Pneumococcus orrban való hordozásának felmérése közösségbe járó egészséges (6 év alatti) kisgyerekek körében"* című kutatáshoz megküldött **protokollmódosítás (2011. január 19) – Staphylococcus aureus baktériumra is kiterjeszteni a kutatást –, Szülői tájékoztató és Beleegyező nyilatkozat (2012. február 24.) módosítását a Bizottság engedélyezte.**

A bizottság fenti döntését az egészségügyről szóló 1997. évi CLIV. törvény, az emberen végzett orvostudományi kutatásokról szóló 23/2002. (V.9.) és a 31/2009. (X.20.) EüM rendelet alapján hozta.

A kutatási engedély 2015. június 30-ig érvényes.

Budapest, 2012. március 19.

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Dr. Sótonyi Péter
egyetemi tanár
a TUKEB elnöke

~~Mellékletek az ülésen résztvevők névsora.~~
Kérem, a fenti TUKEB számra minden esetben hivatkozni.

Appendix 2. Ethical permission

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