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Vaccine-driven serotype-rearrangement is seen with latency in clinical isolates: Comparison of carried and clinical pneumococcal isolates from the same time period in Hungary

Eszter Kovács [a], Judit Sahin-Tóth [a], Adrienn Tóthpál [a], Katalin Kristóf [b], Mark van der Linden [c], Tamás Tirczka [d], Orsolya Dobay [a,*]

[a] Institute of Medical Microbiology, Semmelweis University, Nagyvárad tér 4, H-1089 Budapest, Hungary
[b] Institute of Laboratory Medicine, Semmelweis University, Üllői út 78/a, H-1082 Budapest, Hungary
[c] German National Reference Center for Streptococci, Department of Medical Microbiology, University Hospital RWTH Aachen, Pauwelsstrasse 30, D-52074 Aachen, Germany
[d] National Public Health Institute, Albert Flórián út 2-6, H-1097 Budapest, Hungary

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ABSTRACT

Young children – the main asymptomatic carriers of pneumococcus – are often the source of pneumococcal infections. PCV13 replaced PCV7 in 2010 in Hungary and it became a mandatory vaccine in 2014. In this work we surveyed the effect of vaccination in three groups: in healthy children under 7 years; in children of the same age but infected with pneumococcus (P1); in older patients (P2) who were very likely not vaccinated.

Nasal swabs were taken from 522 healthy children to screen pneumococcal carriage between March 2015 and May 2016. In the same time period, 146 clinical isolates were collected, mainly from mucosal infections. Serotypes, antibiotic susceptibility and clonality of the isolates was determined and compared.

The carriage rate was 39.1%. Regarding carriage, the serotype distribution showed the total disappearance of serotypes 3 and 6A compared to former Hungarian studies. The prevalence of PCV13 serotypes was only 5.8% represented by three serotypes (19F, 19A, 9V). Of note, serotype 19A (a very resistant and invasive type) also decreased significantly. In the patient groups, PCV13 prevalence was higher: 17.5% (P1) and 32.6% (P2). Although serotype 3 was present in P1 (7.9%), the leading serotype was 23B (22.2%), a non-vaccine type (NVT). P2 showed the most diverse serotype distribution, but serotype 3 was predominant here (15.7%). Pneumococcal isolates from the patients were more resistant towards the tested antibiotics compared to those from carriers.

PCV13 seems to be highly successful in reducing the prevalence of vaccine serotypes. The serotype-rearrangement can be seen also among clinical isolates, albeit somewhat later in time. Fortunately, the replacing serotypes are less invasive and less resistant, but, most worrisome, serotype 19F can be found again with increased frequency among carriage isolates and mucosal infections. Further surveillance is needed to carefully monitor such successful, antibiotic resistant "refugees".

1. Introduction

Streptococcus pneumoniae is a causative agent of respiratory infections and invasive pneumococcal disease mainly in children <5 years, immune-compromised patients and in the elderly [1]. Since its natural habitat is the human nasopharynx, asymptomatic carriers are major reservoirs of infection [2]. Pneumococcal carriage contributes to horizontal spread of this pathogen mainly in crowded places like hospitals and day-care centres (DCCs) [3,4]. Young children have the highest colonization rate, up to 55% of the children are pneumococcus carriers in the age of 3 years [2]. Therefore, an important goal is to prevent nasopharyngeal colonization with different vaccines.

Pneumococcal polysaccharide vaccine (PPV) has been available since 1982 containing purified polysaccharides from 23 of the currently known 94 serotypes. Polysaccharides are not able to induce

Abbreviations: DCC, day-care centre; PPV, pneumococcal polysaccharide vaccine; PCV, pneumococcal conjugated vaccine; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; MIC, minimum inhibitory concentration; MLSB, macrolide, lincosamide and streptogramin B resistance phenotype; M type, macrolide resistance phenotype; NVT, non-vaccine type; NT, non-typeable.

* Corresponding author.
E-mail addresses: kristof.katalin@med.semmelweis-univ.hu (K. Kristóf), mlinden@ukaachen.de (M. van der Linden), dobay.orsolya@med.semmelweis-univ.hu (O. Dobay).

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a good immune response in children <2 years due to their immature immune system. In the case of pneumococcal conjugated vaccines (PCVs) the capsular polysaccharide is conjugated to a highly immunogenic protein which induces a T-cell dependent immune response also in young children. PCVs have an effect on both pneumococcal disease as well as on carriage, so infection can be controlled at the basic level [5].

In Hungary, PCV7 became available in 2005 and in 2008 vaccination became freely available for children <2 years. In 2009, vaccination was officially recommended; it was replaced by PCV13 in 2010, which was then made mandatory in July 2014 in a 2 + 1 scheme [6,7]. From 2009 onwards, the vaccination rate has quickly raised to >80% and now is close to 100%, according to the latest available data [8,9].

The aim of this study was to demonstrate vaccine efficacy both in carriers and in patients in the same time period in Hungary, and to monitor serotype replacement six years after the implementation of PCVs. Hungary is a good model for this type of monitoring, as it is a country with a very strict vaccination policy, unlike some other European countries which have a significant level of vaccine sceptics.

2. Materials and methods

2.1. Study population

In this work, 206 pneumococcal carriage isolates from healthy children and 146 clinical isolates from patients with manifest infections were analysed, all deriving from the same time period, March 2015 to May 2016. The carriage isolates derived from screening 522 children (1–6 years old) from Budapest and from two towns in Hungary, attending DCCs and nurseries. Parents were informed about the purpose of the study and their written permission was a condition for enrolment. Besides, questions were asked about the children's vaccination status, siblings, history of recurrent otitis media, pneumonia, meningitis and passive smoking. The number of the ethical permit is TUKEB 4-4/2009, issued by the Regional and Institutional Committee of Science and Research Ethics of Semmelweis University, Budapest. The clinical isolates derived from the Central Laboratory of Semmelweis University, Budapest.

2.2. Specimen collection

Samples were collected from both nostrils of the carrier children with sterile cotton swabs. The samples were transported to the laboratory on active charcoal containing transport media (Transwab, Medical Wire & Equipment, Corsham, UK) and immediately inoculated onto Columbia blood agar plates. Samples were incubated overnight at 37 °C in 5% CO_2 atmosphere. The clinical specimens were obtained from the Institute of Laboratory Medicine, Semmelweis University, Budapest, as pure cultures of pneumococci on blood agar plates, on the day of isolation and routine laboratory identification.

2.3. Identification of pneumococci

The colonies showing typical pneumococcal morphology (α-haemolysis, flat colonies collapsed in the middle or mucoid appearance) were subcultured and further tested for optochin sensitivity (5 μg discs, Mast Diagnostica, Bootle, UK). PCR detection of the *lytA* (autolysin) gene was carried out on all strains [10]. It is worth to note – since it can lead to false results – that we had one *lytA* negative strain which proved to be a pneumococcus (serotype 11A), and in contrast, there were three suspicious *lytA* positive isolates, which belonged to other species [10,11]. The confirmed strains were stored at −80 °C on cryobeads (Mast Diagnostica) for further testing.

2.4. Serotyping

Serogroup determination was done with the Pneumotest-Latex kit (Statens Serum Institut, Copenhagen, Denmark) and the factor determination was done by PCR using primers described by the CDC [12] or others [13]. Several difficult strains were serotyped at the German National Reference Centre for Streptococci (GNRCS), Aachen, or at the National Public Health Institute, Budapest.

2.5. Genotyping

To get information about the clonality of the strains, pulsed-field gel electrophoresis (PFGE) was used. Chromosomal DNA was prepared and then digested by the *Sma*I enzyme (Sigma, Budapest) [14]. Separation was performed with the following pulse times: 5–15 s (block-1) and 15–60 s (block-2) for 20.5 h at 14 °C. PFGE profiles were analysed with the BioNumerics software version 2.5 (Applied Maths, Sint-Martens-Latem, Belgium). Multilocus sequence typing (MLST) was also performed on four isolates, selected from the PFGE dendrogram. According to the instructions of the MLST website [15] – with a modification in the *recP* reverse primer [16] – internal fragments of seven housekeeping genes (*aroE, gdh, gki, recP, spi, xpt, ddl*) were amplified by PCR and the products were purified by the QIAquick PCR purification kit (Qiagen, Germany). The products were sequenced by BIOMI Ltd, Gödöllő, Hungary. The allele sequences were compared to the MLST database and sequence types were determined.

2.6. Antibiotic susceptibility testing

The antibiotic susceptibility of the strains to penicillin, cefotaxime, imipenem, erythromycin, clindamycin, levofloxacin, moxifloxacin and vancomycin was determined using the agar dilution method and an A400 multipoint inoculator (AQS Manufacturing Ltd., Southwater, UK) on Mueller-Hinton blood agar plates. Plates were incubated at 37 °C in 5% CO_2 atmosphere. ATCC 49619 was used as control strain. The susceptibility and resistance rates were determined using the breakpoints suggested by the EUCAST guidelines [17]. Two of the three HC strains had very low MIC values (sensitive anyway) and one of them belonged to the intermediate category by this way (resistant as a meningitis case). Macrolide resistant strains were checked for the presence of *erm* and *mef* genes [18,19]. *Mef(E)* and *mef(A)* were distinguished by restriction digestion: there is no restriction site for *Bam*HI enzyme in the *mef (E)* gene, but it generates two fragments in the case of the *mef(A)* [18,20].

3. Results

3.1. Study population data

Among the 522 symptomless children, 204 (39.1%) were carriers, and most of them were 2–3 years old. In the case of two girls, double carriage was detected (all non-PCV serotypes: 31 + 23B and 15C + 23A) therefore the total number of the isolates was 206. In some carrier children pneumococci were obtained in nearly pure culture, while sometimes only a few colonies were found among the normal flora.

The clinical pneumococci were derived mostly from mucosal illnesses: bronchitis, acute otitis media, pneumonia, sinusitis maxillaris acuta, conjunctivitis. The distribution of the 146 specimens

was as follows: nasal swab n = 81, tracheal/bronchial/BAL/sputum n = 41, ear n = 9, conjunctiva n = 9, pleura/urine/abscess n = 1 each, haemoculture n = 3. The age of patients varied between 0 and 89 years, however, the vast majority belonged to the age groups <4 years (n = 49) and >50 years (n = 56; average 31.9 years). To be able to make a more direct comparison with the carriers, patients were divided into two age groups: 63 isolates came from patients <7 years (P1; 27 males, 36 females) and 83 isolates ≥7 years (P2; 45 males, 38 females).

3.2. Risk factors

Some important risk factors were investigated based on the questionnaires filled in by the parents (Table 1). Answers were missing in the case of 2 carriers and 2 non-carriers, so the data were based on 202 and 316 answers. The p-value was calculated by the chi square test. We screened almost an equal number of boys (n = 256) and girls (n = 262) but there was no significant correlation regarding gender and carriage. Even in the patients-group, the genders were equalised (72 males, 74 females), although there was a clear shift from female dominance in P1 towards male dominance in P2. Having siblings or previous infections like otitis media, pneumonia and meningitis did not affect the carriage either. However, passive smoking seemed to correlate negatively with pneumococcal carriage.

3.3. Serotype distribution and PCV13/PPV23 coverage

Vaccination status was only known for the carrier children. With a very few exception, all of them were born earliest in 2010, when PCV13 has already succeeded PCV7. Their vaccination rate was high (81.8%) even according the questionnaires, but it should be noted that some parents were not sure about the vaccination status of their own children and we counted only the definite "yes" answers. According to the official reports, the PCV vaccination rate was in the range of 92.8–98.5% in the study time frame [21,9]. Accordingly, the PCV13 coverage in the carried group was as low as 5.8%, whereas it was 17.5% in P1 and 32.5% in P2. PPV23 coverage showed a similar tendency with 20.9%, 39.7% and 60.2%, respectively. The prevalence of the different serotypes in ranking order and vaccine coverage data are shown in Table 2 and Figs. 1 and 2. In a few occasions, clear differences were observed in serotype prevalence between the different study groups, these are highlighted in Table 3. Serotype 3 was absent among carriers, whereas it was the leading type among older patients. On the other hand, serotype 15B was the most frequent in carriage and appeared less among the clinical isolates. Serotypes 11A and 19F showed similar prevalence in the three different groups (Table 3).

If we split P2 into further two age categories: patients between 7 and 49 years and >50 years, the following observations could be

Table 1
Correlation between possible risk factors and pneumococcal carriage.

Risk factor	Number of carriers (n = 202)	Number of non-carriers (n = 316)	p-value
Gender male	98 (38.3%)	158 (61.7%)	0.742 (NS)
Gender female	104 (39.7%)	158 (60.3%)	
Having siblings	126 (38.8%)	199 (61.2%)	0.891 (NS)
Not having siblings	76 (39.4%)	117 (60.6%)	
Otitis media in the past	24 (32.0%)	51 (68.0%)	0.966 (NS)
No otitis media	178 (40.2%)	265 (59.8%)	
Pneumonia or meningitis in the past	7 (33.3%)	14 (66.7%)	0.587 (NS)
No pneumonia / meningitis	195 (39.2%)	302 (60.8%)	
Antibiotic exposure in the past two weeks	49 (36.0%)	87 (64.0%)	0.409 (NS)
No antibiotics	153 (40.1%)	229 (59.9%)	
Passive exposure to smoking	49 (31.6%)	106 (68.4%)	0.024 (S)
No passive smoking	153 (42.1%)	210 (57.9%)	

Table 2
Serotype distribution in the different pneumococcal groups.

	Carriers <7y		Patients <7y		Patients 7-49y		Patients >50y	
		n (%)		n (%)		n (%)		n (%)
Number of different serotypes	23	206 (100)	19	63 (100)	15	27 (100)	24	56 (100)
Frequent non-PCV13 serotypes	15B	30 (14.6)	23B	14 (22.2)	15A	3 (11.1)	11A	8 (14.3)
	11A	22 (10.7)	15B	6 (9.5)	NT	3 (11.1)	15A	4 (7.1)
	24F	19 (9.2)	24F	5 (7.9)	11A	2 (7.4)	31	3 (5.4)
	35F	17 (8.3)	11A	5 (7.9)	9N	2 (7.4)	10A	3 (5.4)
	15C	15 (7.3)	21	5 (7.9)			23B	3 (5.4)
	23A	14 (6.8)					35B	3 (5.4)
	10A	11 (5.3)					NT	3 (5.4)
PCV13 serotypes	19F	10 (5.9)	3	5 (7.9)	3	4 (14.8)	3	9 (16.1)
	19A	1 (0.5)	19F	3 (4.8)	19F	4 (14.8)	19F	3 (5.4)
	9V	1 (0.5)	19A	2 (3.2)	19A	1 (3.7)	19A	1 (1.8)
			23F	1 (1.6)	1	1 (3.7)	23F	1 (1.8)
							6A	1 (1.8)
							6B	1 (1.8)
							9V	1 (1.8)
PCV13 coverage		12 (5.8)		11 (17.5)		10 (37.0)		17 (30,4)
PPV23 coverage		43 (20.9)		25 (39.7)		18 (66.7)		33 (58.9)

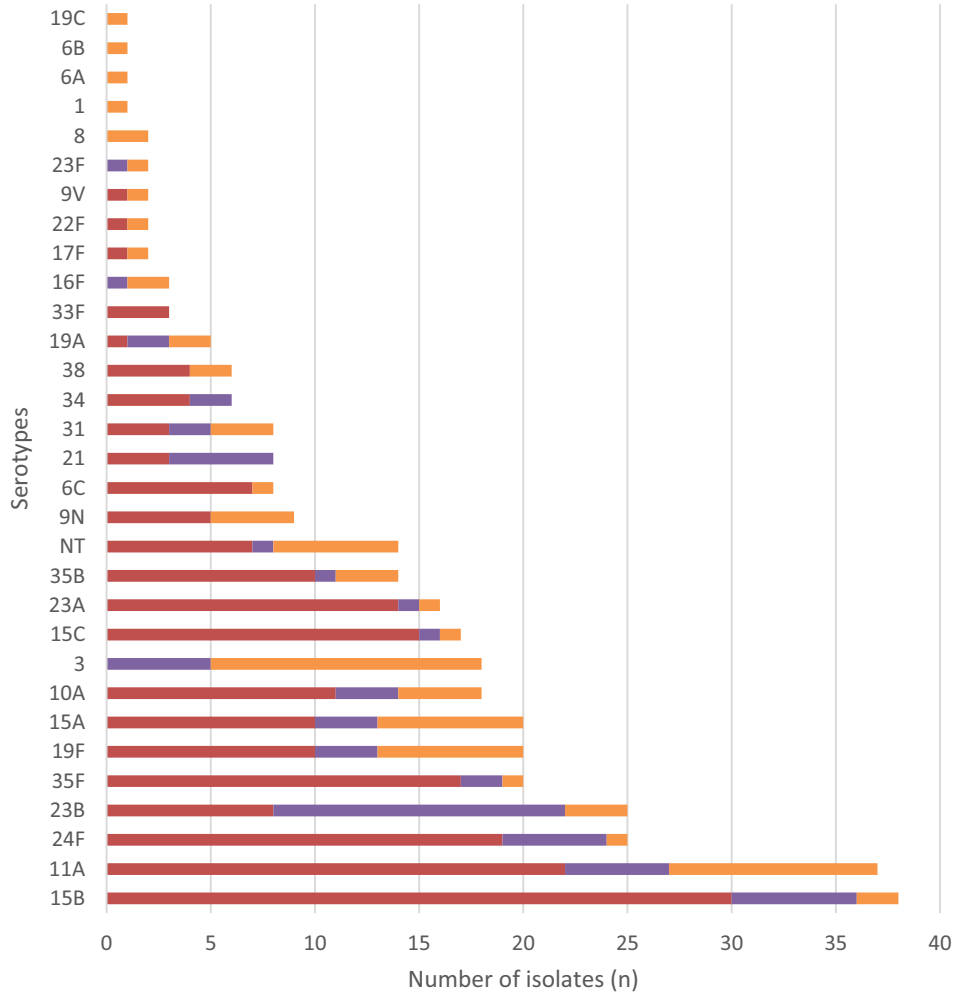


Fig. 1. Combined serotype distribution in the three pneumococcal groups. Red columns (■), carriers <7 years; indigo columns (■), patients <7 years (P1); orange columns (■), patients >7 years (P2); PCV13 serotypes are: 19A, 19F, 3, 9 V, 23F, 1, 6A, 6B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

made. Serotype 11A appeared to be more frequent in the >50y group compared to the 7-49y group, meanwhile an opposite observation can be made for serotype 15A and serotype 19F; serotype 3 showed no significant differences.

3.4. Antibiotic susceptibility

Full susceptibility was observed for imipenem and vancomycin throughout the study. Carriage isolates were less resistant to antibiotics compared to the clinical ones, and isolates from younger patients showed higher susceptibility rates compared to those from older patients (Table 4). This tendency was observed for all tested antibiotics. Penicillin resistant isolates were not found. The level of penicillin intermediate isolates was only 13.1% among carriage isolates, 17.5% among isolates in P1 and the largest proportion (25.3%) was found among isolates in P2. Similarly, the percentage of levofloxacin resistance was 0% among carriage isolates, 4.8% among isolates in P1 and 8.4% among isolates in P2. The major resistance problem could be seen (as always with pneumococci) regarding the macrolides: erythromycin resistance was 17.0%, 19.0% and 28.9% in the three groups, respectively.

Among the 72 erythromycin resistant isolates, *erm(B)* was detected in 38 cases (high MIC values for both erythromycin and clindamycin; MLSB-phenotype), *mef(E)* in 29 cases (lower erythromycin MIC values {8–16 mg/L} and clindamycin sensitivity; M-phenotype), and both genes in three cases (one strain each of serotype 19A, 19F and non-typeable) in addition to the previous two groups.

The serotype-specific resistance rates are shown in Supplementary Table 1. In summary, almost all serotype 3, 10A, 23A, 23B, 24F, 35B and 35F isolates were sensitive to all tested drugs, meanwhile the major serotypes contributing to macrolide resistance were: 11A (always M type), 19F, 15A, 15C and 19A (MLSB type).

3.5. Clonality

We found isolates with the same PFGE profiles among carried and clinical isolates, belonging to the same serotypes (e.g. 11A, 19A, 19F; Fig. 3). Serotype 3 showed the lowest level of diversity (Fig. 4), in contrast with serotype 19A.

The PFGE results of serotype 19F isolates revealed several different lineages with similar antibiotic sensitivity patterns each (Fig. 5). One representative of each of the four large clusters (indicated with bold face and underlining) were chosen for MLST analysis. Cluster 1 with classic MLSB phenotype but penicillin susceptibility belonged to ST179. Strains in cluster 2 were ST180, fully susceptible to all tested antibiotics. Isolate 57103 (see Fig. 5), which was both phenotypically and genotypically different

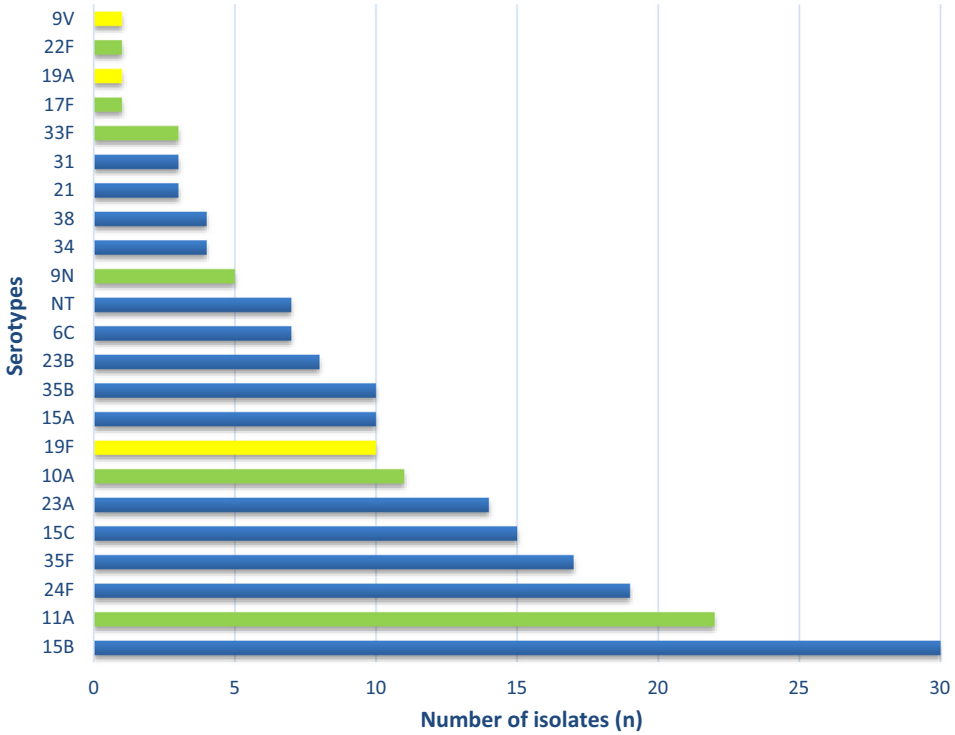


Fig. 2A. Serotype distribution among carriers <7 years. Yellow columns (), PCV13 serotypes; green columns (), additional PPV23 serotypes; blue columns (), NVTs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

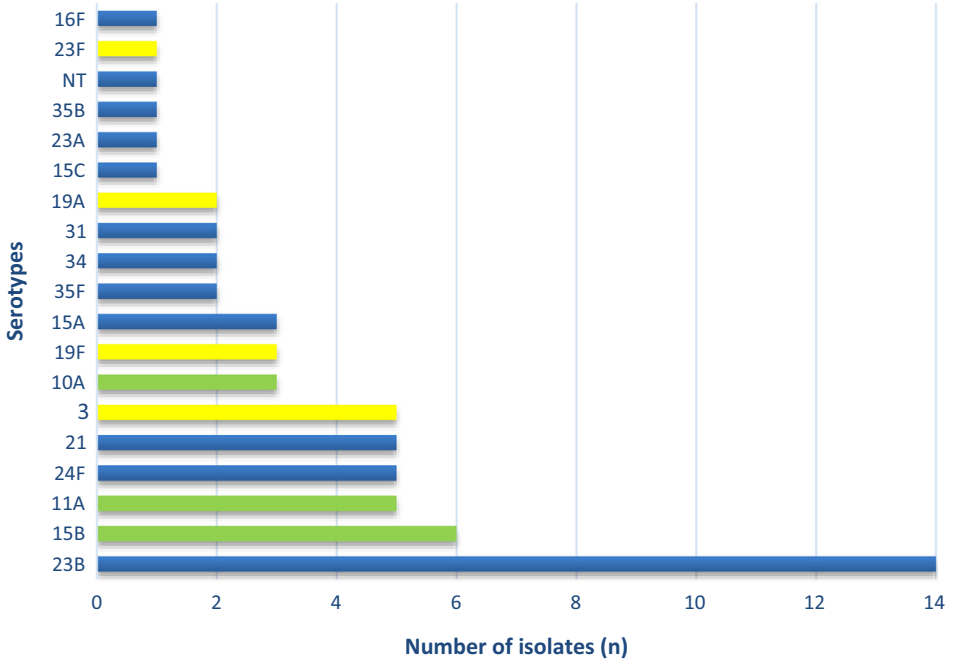


Fig. 2B. Serotype distribution among patients <7 years. Yellow columns (), PCV13 serotypes; green columns (), additional PPV23 serotypes; blue columns (), NVTs.

from the others, proved to be a member of the famous ST320 clone, characterised by elevated penicillin MIC and high resistance to erythromycin and clindamycin. Very interestingly, one of our serotype 19A isolates also belonged to ST320, with the same resistance pattern. Both ST320 isolates expressed *erm(B) + mef(E)* genes together. Finally, cluster 4 isolates with elevated penicillin MIC values and M phenotype belonged to ST651.

4. Discussion

In this study we compared three different groups of isolates. The first group represents isolates from asymptomatic carriers with a high PCV-13 vaccination rate (nearly 100%). The isolates in the second group (P1) derived from patients of the same age (<7 y), probably with a similarly high-level vaccination. The third

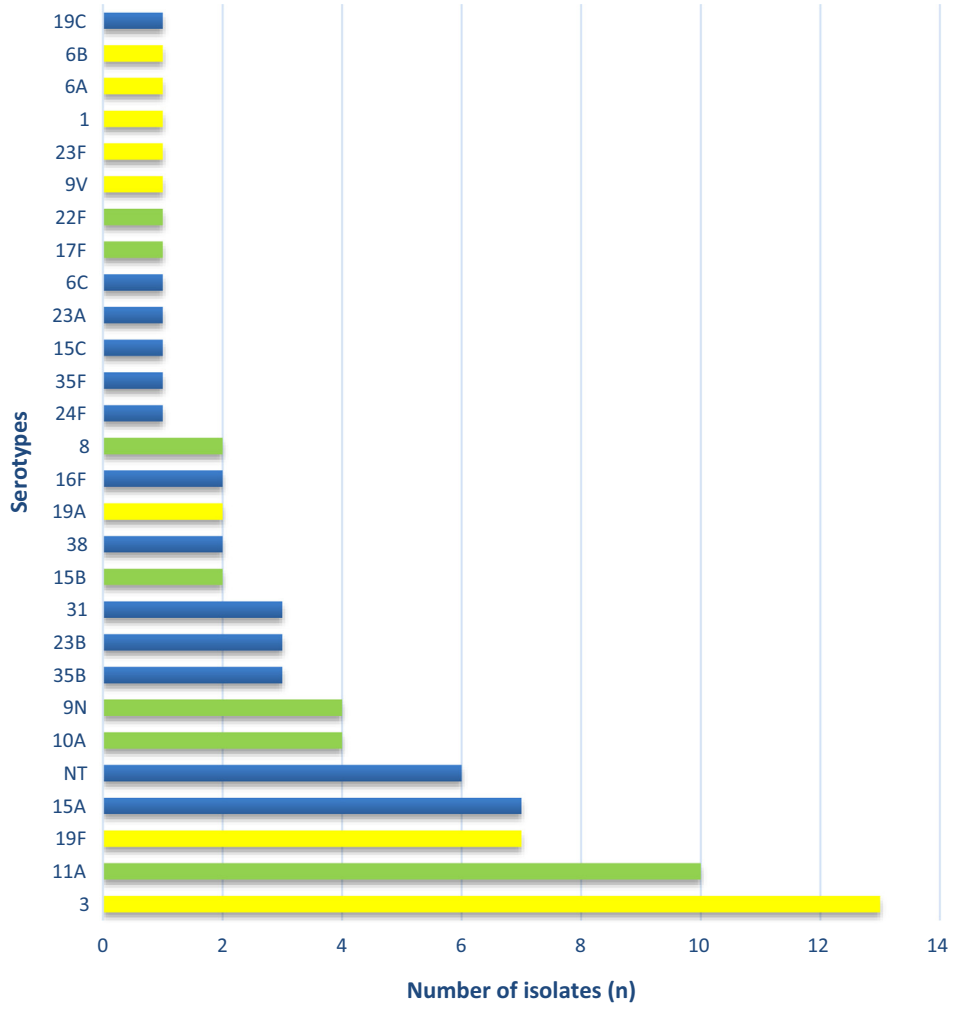


Fig. 2C. Serotype distribution among patients >7 years. Yellow columns (□), PCV13 serotypes; green columns (□), additional PPV23 serotypes; blue columns (□), NVTs.

Table 3
Most significant differences in serotype prevalence among the different study groups.

Serotype	Carriers	Patients <7y (P1)	Patients >7y (P2)
3	0%	7.9%	15.7%
15B	14.6%	9.5%	0.2%
19F	4.9%	4.8%	8.4%
11A	10.7%	7.9%	12.1%

group (P2) consisted of older patients (>7 y), with low-level vaccination rate.

Surveillance of *Streptococcus pneumoniae* carriage is suitable to monitor the immediate effect of PCVs. In our previous studies [22,23] we have screened children aged 3–6 years, attending DCCs and observed somewhat lower carriage rates (34.1% in 2009–2010 {screening 634 children} and 32.5% in 2011–2012 {screening 1628 children}). In the current project also younger children (1–7 years) were included, and as carriage peaks at 2–3 years of age, the carriage rate was accordingly higher: 39.1%. Based on these combined data, we can conclude that PCVs do not have an effect on the overall carriage rate itself, but very much on the serotype distribution. Similar observations were made also by others [24–26]. Pneumococcus carriage rates in small children vary widely in other countries from 14% in Turkey [27], 22.6% in Brazil [28] to 70.1% in Iceland [29], and marked serotype replacement by NVTs after PCV implementation is always detected.

Co-carriage of serotypes was detected in two occasions in this study: 31 + 23B and 15C + 23A, all serotypes being NVTs. A probable explanation for the relatively frequent detection of 23A and 23B as co-colonisers is their easy morphological detectability (thickness of capsule) [30,31]. Hence, multiple carriage can be underestimated using only culture based methods [32–34].

Usually studies investigating risk factors find that passive smoking contributes to a higher prevalence of pneumococcal carriage and to a higher rate of otitis media in children and invasive pneumococcal disease in adults [35–38]. Interestingly, here we found a negative association between exposure to passive smoking and pneumococcal carriage. Such an inverse association was also observed among Belgian infants in DCCs [39].

Following serotype rearrangement among carried pneumococci over time in Hungary, very clear tendencies can be seen. Beside the continuous decrease of the PCV7 serotypes (many of them – such as 14 or 18C – having completely disappeared), the most remarkable event was the rise and fall of serotype 19A. Whereas in the pre-PCV era (2009–2010) serotype 19A represented only a small proportion of all strains (1.4%), it quickly became the leading type (11.5%) in the post-PCV7 era (2011–2012) [16]. Finally, in the current study (2015–2016), its prevalence decreased again to 0.5%. Furthermore, further two PCV13 types (6A and 3) have completely disappeared from carriage by now, although they had represented approximately 5–6% of all isolates in the years before 2012. This is very important, as all three serotypes have a high invasive disease

Table 4
Antibiotic susceptibility of the carried and clinical isolates.

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

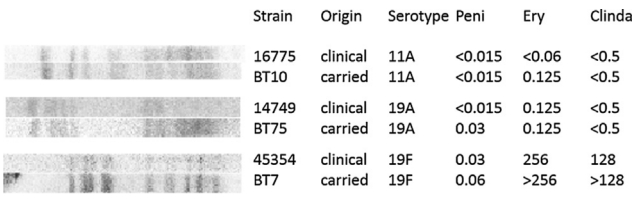


Fig. 3. Similar or identical PFGE restriction patterns of clinical and carried isolates among serotypes 11A, 19A and 19F.

potential, whereas the majority of the replacing serotypes have a low invasive potential [40–44]. In addition, the antibiotic resistance of serotype 19A is outstanding among pneumococci. All these data mirror the strong and beneficiary effect of PCV13 on carriage.

Whereas pneumococcal carriage isolates respond fast to the conjugated vaccines (due to the direct protection), the clinical isolates need more time to show the same changes [45]. This is well reflected in the different levels of PCV13 coverage in this study: it was 5.8% among carriers, 17.5% among patients <7y (P1) and 31.8% among patients >7y (P2). If we look at the three PCV13 types individually, we see that whereas serotype 19A was generally present at very low rates (0.5% in carriers, 3.2% in P1 and 2.4% in P2), serotype 3 was detected with increasing rates among the three groups (0%, 7.9% and 15.7%, respectively), and finally only one single serotype 6A isolate was found, only in P2. Serotype 3, which has been the leading serotype among invasive pneumococci in Europe in the post-PCV period, has withdrawn to the second position by

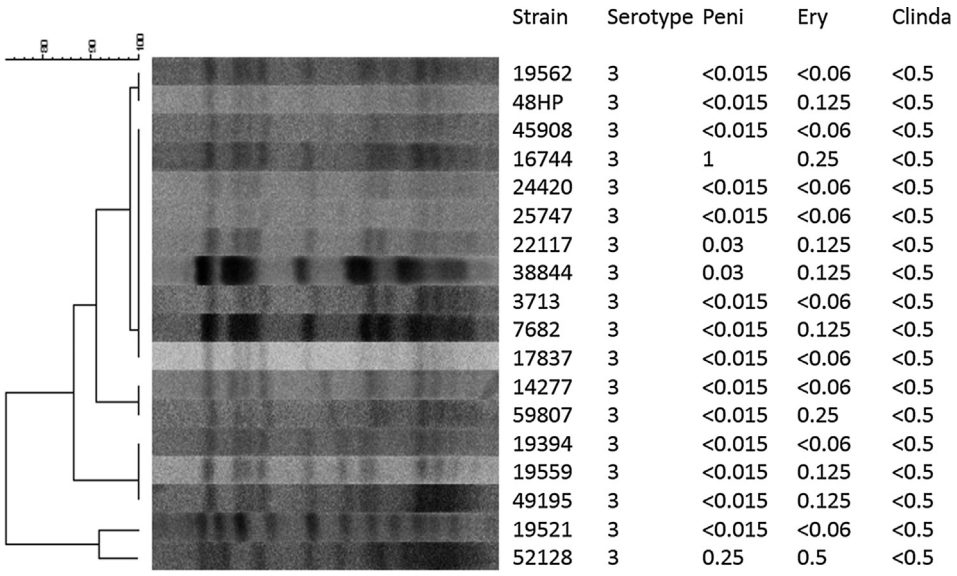
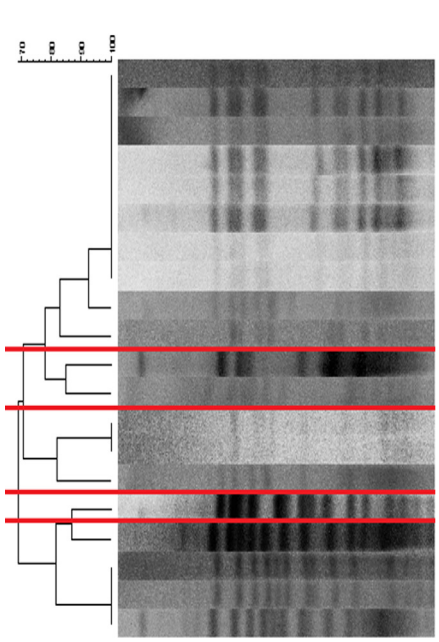


Fig. 4. PFGE dendrogram of serotype 3 clinical isolates.



Strain	Serotype	Peni	Ery	Clinda	
45354	19F	0.03	256	128	
BT7	19F	0.06	>256	>128	
BT8	19F	0.06	256	>128	
BT104	19F	0.06	>256	>128	Cluster 1
BT112	19F	0.06	>256	>128	
BT115	19F	0.06	>256	>128	
BT122	19F	0.06	>256	>128	
BT123	19F	0.06	>256	>128	
8431	19F	<0.015	>256	>128	
11204	19F	0.03	256	>128	
11406	19F	<0.015	<0.06	<0.5	
PP273	19F	0.03	0.125	<0.5	
PP266	19F	0.03	0.25	<0.5	Cluster 2
PP272	19F	0.03	0.25	<0.5	
18124	19F	0.06	1	<0.5	
57103	19F	1.5	>256	>128	Cluster 3
38198	19F	1	8	<0.5	
18017	19F	1	8	<0.5	Cluster 4
24536	19F	2	8	<0.5	
23535	19F	1	8	<0.5	

Fig. 5. PFGE dendrogram of serotype 19F strains; four different clusters based on MLST analysis.

2015, according to the latest ECDC report. It is now overtaken by serotype 8. Serotype 3 is found predominantly in the elderly [44,46]. We can hopefully declare the beginning of a successful eradication of this serotype among carriers, and a decrease also among clinical isolates.

The most frequent replacing NVTs among the carried isolates were 15B (14.6%), 11A (10.7%) and 24F (9.2%), while serotype 23B dominated in group P1 with 22.2%, followed by 15B (9.5%). In P2, serotype 3 was still most prevalent (15.7%), and the most frequent NVT was 11A (12.1%).

Surprisingly, serotype 19F still contributes to the remaining relatively high prevalence of the PCV13 types. In Hungary, prevalence of 19F has had an interesting fluctuation in carriage. In our screening study [16] conducted in 2009–2010, i.e. in the pre-PCV7 era (the average PCV7 vaccination of the enrolled children was only 16.4%), 19F was present at 9.6% (n = 21 out of 218 specimens). As the PCV7 vaccination rate increased (46.0% in average, screening performed between 2010 and 2012), significantly lower 19F rate (1.7%, n = 9 out of 530 specimens) was found ($p = 5.0 \times 10^{-7}$). Now, despite the fact that the PCV13 vaccination rate was close to 100%, 19F has reached 4.9% again (n = 10 out of 206 specimens; p = 0.015). A similarly high prevalence was found among the clinical specimens of this study (4.8% in P1 and 8.4% in P2). On the other hand, based on previous Hungarian data, prevalence of 19F in invasive isolates gradually decreased from around 8–13% in the pre-PCV period [45,47], to below 1% by 2016 [48], [unpublished data]. Apparently, 19F could conserve its position better in non-invasive specimens. Parallel to our findings, 19F also remained frequent in the paediatric populations in St. Louis and Alaska [49,50]. One possible explanation for this persistence might be that the highest vaccination failure rate was observed for cases caused by serotype 19F [51]. Alternatively, a newly emerging and genetically different clone of 19F might have successfully filled the vacated niche caused by the disappearance of other vaccine types [52].

Capsular switching resulting in different serotypes belonging to the same sequence types is a well-known phenomenon. In this work we identified one 19A and one 19F isolate as members of the ST320 clone. Both isolates expressed *erm(B)* and *mef(E)* genes which is characteristic for members of Taiwan^{19F}-14, a well-known international clone [53]. This clone was the ancestor of ST320 in the pre-PCV era, but after PCV7 implementation a capsular switch event lead to the current dominance of 19A within ST320 [54,55].

Serotype 11A is also of importance. It is frequent both among clinical and carried isolates and it seems to be one of the most successful serotypes after PCV vaccination. Even in the pre-PCV era it had been ranking high: it was the leading type already in 2009–2010 with 14.7% among carriers and has conserved its position ever since [16]. In the current study, 11A (10.7%) was the second most frequent after serotype 15B. It was present with 7.9% and 12.1%, respectively, in P1 and P2 as well. It is often expressing low-level resistance to macrolides (M type), hence it can potentially lead to treatment failures. Furthermore, 11A has the second highest serotype-associated mortality according to ECDC data [56].

The carried isolates in this work were generally more (or fully) susceptible to the tested antibiotics compared to clinical isolates. This has been observed in all our previous studies as well [16,23,45]. In addition, higher rates of resistance were measured among older patients (P2) than among children (P1). For instance, erythromycin resistance was 28.9% in P2, while only 19.0% in P1. Similarly, moxifloxacin resistance was 14.5% versus 7.9%.

These figures correlate well with the nationwide clinical data (non-invasive specimens), reported for the same period by the National Public Health Institute. On the other hand, whereas we found no penicillin resistant isolates (not even among the clinical ones), 1.8–4.3% resistance among non-invasive isolates in 2015-2016 was reported nationwide [57].

5. Conclusions

In summary, we can state that PCV13 has a strong effect on most serotypes included in the vaccine, but that these changes were seen with some delay in clinical isolates. For instance, serotypes 3 and 6A have completely disappeared from carriage in Hungary by now, but serotype 3 is still the leading type among patients of older age. On the other hand, of concern, serotype 19F seems to re-emerge slowly in carriage.

The continuous and dynamic rearrangement of serotypes due to vaccine pressure requires not just close monitoring of the changes, but also shows the necessity of the development of new higher-valent vaccines. New 15-valent and 20-valent conjugated vaccines are in the pipeline, with the following additional planned serotypes: 8, 10A, 11A, 12F, 15B, 22F, 33F [58]. These developments include serotype 8, which is currently the most prevalent serotype among invasive infections in Europe; 15B, which is currently leading in carriage; and finally 11A, which has silently been around for a long time, regardless of PCVs, in both carriage and infections. Alternatively, serotype-independent, non-polysaccharide-based vaccines should also be considered to eliminate as many dangerous serotypes as possible.

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Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

EK, AT, OD and KK contributed to the overall design of the study. EK and AT participated in sample collection. EK, JST, AT performed experimental analysis. TT and MvdL contributed to serotyping of difficult strains. EK wrote the initial draft of the manuscript. AT helped editing figures and tables, OD reviewed and finalized the manuscript. All authors have read and approved the final manuscript.

All authors attest they meet the ICMJE criteria for authorship.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.11.026.

References

[1] Lynch JP, Zhanel GG. Streptococcus pneumoniae: epidemiology, risk factors, and strategies for prevention. Semin Respir Crit Care Med 2009;30:189–209.
[2] Bogaert D, De Groot R, Hermans PW. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis 2004;4:144–54.
[3] De Lencastre H, Tomasz A. From ecological reservoir to disease: the nasopharynx, day-care centres and drug-resistant clones of Streptococcus pneumoniae. J Antimicrob Chemother 2002;50(Suppl S2):75–81.
[4] Shi ZY, Enright MC, Wilkinson P, Griffiths D, Spratt BG. Identification of three major clones of multiply antibiotic-resistant Streptococcus pneumoniae in Taiwanese hospitals by multilocus sequence typing. J Clin Microbiol 1998;36:3514–9.
[5] Pletz MW, Maus U, Krug N, Welte T, Lode H. Pneumococcal vaccines: mechanism of action, impact on epidemiology and adaption of the species. Int J Antimicrob Agents 2008;32:199–206.
[6] National Center for Epidemiology. Pneumococcal vaccination in Europe. Epinfo 2009;16:141–8 [in Hungarian].
[7] National Center for Epidemiology. Methodological letter of the National Center for Epidemiology about the vaccines in 2014. Epinfo. 2014; 21 (special issue) [in Hungarian].
[8] National Center for Epidemiology. Pneumococcal vaccination data in Hungary as in August 2009. Epinfo 2009;16:487–92 [in Hungarian].
[9] National Center for Epidemiology. Accomplishment of vaccines in 2015. Epinfo 2016;23:369–81 [in Hungarian].
[10] Nagai K, Shibasaki Y, Hasegawa K, Davies TA, Jacobs MR, Ubukata K, et al. Evaluation of PCR primers to screen for Streptococcus pneumoniae isolates and beta-lactam resistance, and to detect common macrolide resistance determinants. J Antimicrob Chemother 2001;48:915–8.
[11] Simões AS, Tavares DA, Rolo D, Ardanuy C, Goossens H, Henriques-Normark B, et al. lytA-based identification methods can misidentify Streptococcus pneumoniae. Diagn Microbiol Infect Dis 2016;85:141–8.
[12] Centers for Disease Control and Prevention. List of oligonucleotide primers used in 41 conventional multiplex PCR assays for pneumococcal serotype deduction of 71 serotypes. https://www.cdc.gov/streplab/downloads/pcr-oligonucleotide-primers.pdf; 2014 [accessed May 2018].
[13] Brito DA, Ramirez M, de Lencastre H. Serotyping Streptococcus pneumoniae by multiplex PCR. J Clin Microbiol 2003;41:2378–84.
[14] Dobay O, Rozgonyi F, Hajdú E, Nagy E, Knausz M, Amyes SG. The relationship between serotypes and PFGE genotypes in isolates of Streptococcus pneumoniae from Hungary. Clin Microbiol Infect 2005;11:673–6.
[15] Streptococcus pneumoniae MLST website. https://pubmlst.org/spneumoniae/; 2010 [accessed May 2018].
[16] Tóthpál A, Laub K, Kardos S, Tirczka T, Kocsis A, van der Linden M, et al. Epidemiological analysis of pneumococcal serotype 19A in healthy children following PCV7 vaccination. Epidemiol Infect 2016;144:1563–73.
[17] The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 8.0. http://www.eucast.org/clinical_breakpoints/; 2018 [accessed May 2018].
[18] Dobay O, Rozgonyi F, Amyes SG. Molecular characterisation of Hungarian macrolide-resistant Streptococcus pneumoniae isolates, including three highly resistant strains with the mef gene. Int J Antimicrob Agents 2005;25:488–95.
[19] Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother 1996;40:2562–6.
[20] Oster P, Zanchi A, Cresti S, Lattanzi M, Montagnani F, Cellesi C, et al. Patterns of macrolide resistance determinants among community-acquired Streptococcus pneumoniae isolates over a 5-year period of decreased macrolide susceptibility rates. Antimicrob Agents Chemother 1999;43:2510–2.
[21] National Center for Epidemiology. The present situation and results of the pneumococcus surveillance in Hungary as in October 2012. Epinfo 2012;19:521–8 [in Hungarian].
[22] Tóthpál A, Kardos S, Laub K, Nagy K, Tirczka T, van der Linden M, et al. Radical serotype rearrangement of carried pneumococci in the first 3 years after intensive vaccination started in Hungary. Eur J Pediatr 2015;174:373–81.
[23] Tóthpál A, Kardos S, Hajdú E, Nagy K, Linden M, Dobay O. Nasal carriage of Streptococcus pneumoniae among Hungarian children before the wide use of the conjugate vaccine. Acta Microbiol Immunol Hung 2012;59:107–18.
[24] Spijkerman J, Prevaes SM, van Gils EJ, Veenhoven RH, Bruin JP, Bogaert D, et al. Long-term effects of pneumococcal conjugate vaccine on nasopharyngeal carriage of S. pneumoniae, S. aureus, H. influenzae and M. catarrhalis. PLoS One 2012;7:e39730.
[25] Tocheva AS, Jefferies JM, Rubery H, Bennett J, Afimeke G, Garland J, et al. Declining serotype coverage of new pneumococcal conjugate vaccines relating to the carriage of Streptococcus pneumoniae in young children. Vaccine 2011;29:4400–4.
[26] Fleming-Dutra KE, Conklin L, Loo JD, Knoll MD, Park DE, Kirk J, et al. Systematic review of the effect of pneumococcal conjugate vaccine dosing schedules on vaccine-type nasopharyngeal carriage. Pediatr Infect Dis J 2014;33(Suppl 2): S152–60.
[27] Arvas A, Çokuğraş H, Gür E, Gönüllü N, Taner Z, Tokman HB. Pneumococcal nasopharyngeal carriage in young healthy children after pneumococcal conjugate vaccine in Turkey. Balkan Med J 2017;34:362–6.
[28] Neves FPG, Cardoso NT, Snyder RE, Marlow MA, Cardoso CAA, Teixeira LM, et al. Pneumococcal carriage among children after four years of routine 10-valent pneumococcal conjugate vaccine use in Brazil: the emergence of multidrug resistant serotype 6C. Vaccine 2017;35:2794–800.
[29] Sigurdsson S, Erlendsdóttir H, Quirk SJ, Kristjánsson J, Hauksson K, Andrésdóttir BDI, et al. Pneumococcal vaccination: direct and herd effect on carriage of vaccine types and antibiotic resistance in Icelandic children. Vaccine 2017;35:5242–8.
[30] Mostowy RJ, Croucher NJ, De Maio N, Chewapreecha C, Salter SJ, Turner P, et al. Pneumococcal capsule synthesis locus cps as evolutionary hotspot with potential to generate novel serotypes by recombination. Mol Biol Evol 2017;34:2537–54.
[31] Weinberger DM, Trzciński K, Lu YJ, Bogaert D, Brandes A, Galagan J, et al. Pneumococcal capsular polysaccharide structure predicts serotype prevalence. PLoS Pathog 2009;5:e1000476.
[32] Ercibengoa M, Arostegi N, Marimón JM, Alonso M, Pérez-Trallero E. Dynamics of pneumococcal nasopharyngeal carriage in healthy children attending a day care center in northern Spain. Influence of detection techniques on the results. BMC Infect Dis 2012;12:69.
[33] Turner P, Hinds J, Turner C, Jankhot A, Gould K, Bentley SD, et al. Improved detection of nasopharyngeal cocolonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. J Clin Microbiol 2011;49:1784–9.

[34] Wyllie AL, Wijmenga-Monsuur AJ, van Houten MA, Bosch AA, Groot JA, van Engelsdorp Gastelaars J, et al. Molecular surveillance of nasopharyngeal carriage of Streptococcus pneumoniae in children vaccinated with conjugated polysaccharide pneumococcal vaccines. Sci Rep 2016;6:23809.
[35] DiFranza JR, Aligne CA, Weitzman M. Prenatal and postnatal environmental tobacco smoke exposure and children's health. Pediatrics 2004;113:1007–15.
[36] Uhari M, Mäntysaari K, Niemelä M. A meta-analytic review of the risk factors for acute otitis media. Clin Infect Dis 1996;22:1079–83.
[37] Greenberg D, Givon-Lavi N, Broides A, Blancovich I, Peled N, Dagan R. The contribution of smoking and exposure to tobacco smoke to Streptococcus pneumoniae and Haemophilus influenzae carriage in children and their mothers. Clin Infect Dis 2006;42:897–903.
[38] Nuorti JP, Butler JC, Farley MM, Harrison LH, McGeer A, Kolczak MS, et al. Cigarette smoking and invasive pneumococcal disease. Active Bacterial Core Surveillance Team. N Engl J Med 2000;342:681–9.
[39] Wouters I, Van Heirstraeten L, Desmet S, Blaizot S, Verhaegen J, Goossens H, et al. Nasopharyngeal s. pneumoniae carriage and density in Belgian infants after 9 years of pneumococcal conjugate vaccine programme. Vaccine 2018;36:15–22.
[40] van Hoek AJ, Andrews N, Waight PA, George R, Miller E. Effect of serotype on focus and mortality of invasive pneumococcal disease: coverage of different vaccines and insight into non-vaccine serotypes. PLoS ONE 2012;7:e39150.
[41] Sugimoto N, Yamagishi Y, Hirai J, Sakanashi D, Suematsu H, Nishiyama N, et al. Invasive pneumococcal disease caused by mucoid serotype 3 Streptococcus pneumoniae: a case report and literature review. BMC Res Notes 2017;10:21.
[42] Lindstrand A, Galanis I, Darenberg J, Morfeldt E, Naucler P, Blennow M, et al. Unaltered pneumococcal carriage prevalence due to expansion of non-vaccine types of low invasive potential 8years after vaccine introduction in Stockholm, Sweden. Vaccine 2016;34:4565–71.
[43] Varon E, Cohen R, Béchet S, Doit C, Levy C. Invasive disease potential of pneumococci before and after the 13-valent pneumococcal conjugate vaccine implementation in children. Vaccine 2015;33:6178–85.
[44] European Centre for Disease Prevention and Control. Invasive pneumococcal disease. In: ECDC. Annual Epidemiological Report for 2015. Stockholm. https://ecdc.europa.eu/; 2017 [accessed May 2018].
[45] Tóthpál A, Laub K, Kardos S, Nagy K, Dobay O. Vaccine coverage of invasive and carried *Streptococcus pneumoniae* isolates in Hungary. LAM 2014;24:180–5.
[46] European Centre for Disease Prevention and Control. Annual Epidemiological Report 2016 – Invasive pneumococcal disease. Stockholm. http://ecdc.europa.eu/; 2016 [accessed May 2018].
[47] Tóthpál A, Laub K, Kardos S, Nagy K, Dobay O. Changes in the serotypes of Hungarian pneumococci isolated mainly from invasive infections: a review of all available data between 1988 and 2011. Acta Microbiol Immunol Hung 2012;59:423–33.
[48] National Center for Epidemiology. Streptococcus pneumoniae serotype distribution between 2013–2015 in Hungary. Microbiol Lett 2015;15:14–27 [in Hungarian].
[49] McFarland M, Szasz TP, Zhou JY, Motley K, Sivapalan JS, Isaacson-Schmid M, et al. Colonization with 19F and other pneumococcal conjugate vaccine serotypes in children in St. Louis, Missouri, USA. Vaccine 2017;35:4389–95.
[50] Gounder PP, Bruce MG, Bruden DJ, Singleton RJ, Rudolph K, Hurlburt DA, et al. Effect of the 13-valent pneumococcal conjugate vaccine on nasopharyngeal colonization by Streptococcus pneumoniae–Alaska, 2008–2012. J Infect Dis 2014;209:1251–8.
[51] Oligbu G, Hsia Y, Folgori L, Collins S, Ladhani S. Pneumococcal conjugate vaccine failure in children: a systematic review of the literature. Vaccine 2016;34:6126–32.
[52] Gounder PP, Bruden D, Rudolph K, Zulz T, Hurlburt D, Thompson G, et al. Re-emergence of pneumococcal colonization by vaccine serotype 19F in persons aged ≥5 years after 13-valent pneumococcal conjugate vaccine introduction-Alaska, 2008–2013. Vaccine 2018;36:691–7.
[53] Farrell DJ, Jenkins SG, Brown SD, Patel M, Lavin BS, Klugman KP. Emergence and spread of Streptococcus pneumoniae with erm(B) and mef(A) resistance. Emerg Infect Dis 2005;11:851–8.
[54] Tyrrell GJ. The changing epidemiology of Streptococcus pneumoniae serotype 19A clonal complexes. J Infect Dis 2011;203:1345–7.
[55] Beall B, McEllistrem MC, Gertz RE, Wedel S, Boxrud DJ, Gonzalez AL, et al. Pre- and postvaccination clonal compositions of invasive pneumococcal serotypes for isolates collected in the United States in 1999, 2001, and 2002. J Clin Microbiol 2006;44:999–1017.
[56] European Centre for Disease Prevention and Control. Surveillance of invasive bacterial diseases in Europe https://ecdc.europa.eu/; 2012 [accessed May 2018].
[57] National Bacteriological Surveillance Management Team. NBS Annual reports. National Center for Epidemiology, Budapest, Hungary. www.oek.hu; 2015-2016 [in Hungarian] [accessed May 2018].
[58] United States Patent Application. Immunogenic compositions comprising conjugeted capsular saccharide antigens and uses thereof. http://patents.com/us-20150202309.html; 2015 [accessed May 2018].