Wien Klin Wochenschr (2015) 127:691–695
DOI 10.1007/s00508-015-0721-y

Wiener klinische Wochenschrift
The Central European Journal of Medicine

Serotypes and genotypes of invasive pneumococci in the central part of Slovenia

Manica Müller Premru · Bojana Beović · Marko Pokorn · Vesna Cvitković Špik

Received: 3 September 2014 / Accepted: 19 January 2015 / Published online: 1 April 2015

Summary To investigate epidemiology of invasive pneumococcal disease (IPD) in the central part of Slovenia in a population with no routine pneumococcal vaccination, we carried out serotyping of isolates by sequential multiplex polymerase chain reaction (PCR) and genotyping by repetitive sequence-based PCR (rep-PCR) and some by multilocus sequence typing. IPD was confirmed in 134 (26.5 %) of 510 acutely ill patients, either by a positive blood culture or real-time PCR (rt-PCR). In 94 patients, isolates were available for typing (24 from blood and 70 from nasopharynx). They belonged to 12 different serotypes; the most prevalent were 14 (27.6 % isolates), 9V, 3 (12.7 % each), 7F (9.5 %), 19A, and 1 (7.4 % each) followed by 4, 6A/B, 19F, 23F, 18C, and 33F. Genotyping yielded 34 rep-PCR genotypes and 13 subtypes; six were found in serotype 14, one in 9V, four each in 3, 19A, and 6A/B, three each in 7F and 1, and two each in 4, 19F, 23F, and 18C. Serotype 9V was the most homogenous and 14 and 19A were heterogenous and had two divergent clonal groups each. The most common genotypes belonged to virulent widespread clones, like ST162, ST9, ST15, ST156, ST191, and ST1377; however, sporadic clones were also observed.

Keywords *Streptococcus pneumoniae* · Invasive disease · Serotypes · Genotypes

M. Müller Premru, PhD (✉) · V. Cvitković Špik, PhD
Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana,
Zaloška 4,
1000 Ljubljana, Slovenia
e-mail: manica.mueller-premru@mf.uni-lj.si

B. Beović, PhD · M. Pokorn, MD
Department of Infectious Diseases, University Medical Centre, Ljubljana, Slovenia

Introduction

Streptococcus pneumoniae is considered an important global pathogen that causes otitis media, pneumonia, and invasive pneumococcal disease (IPD). It is known to be a genetically diverse species [1]. Although serotype is important in determining invasiveness, virulence factors of the pneumococcus have now been found to vary according to serotype and genotype [2–4]. Jefferies et al. [5] observed that trends in incidence of IPD due to certain serotypes and clones are occurring also without vaccine introduction. There is substantial overlap between serotypes and genotypes that are colonizing and those that are recovered from invasive disease [1].

Pulsed-field gel electrophoresis (PFGE) is a reference method for typing different bacterial species, including pneumococci, but it is time-consuming, labor-intensive, and poorly reproducible [6]. Multilocus sequence typing (MLST) is a reproducible method that provides unambigous sequence types (ST), but has lower discriminatory power than PFGE and is also costly and time-consuming [7]. Repetitive sequence-based PCR (rep-PCR) uses primers that target noncoding repetitive sequences interspersed in bacterial genomes. The recently commercialized DiversiLab system is a rep-PCR technique offering semiautomation, standardization, and higher reproducibility compared with a manual technique. Most commonly, rep-PCR is used for typing gram-negative bacteria, but there are reports of typing enterococci, streptococci, and staphylococci as well [8, 9].

To investigate epidemiology of IPD in the central part of Slovenia in a population with no routine pneumococcal vaccination, we carried out serotyping of isolates by sequential multiplex PCR and genotyping by rep-PCR. The main rep-PCR genotypes were subsequently typed by MLST.

Patients, materials, and methods

In a prospective study during an 18-month period in 2011 and 2012, 510 acutely ill patients (325 children and 185 adults) with clinical signs of IPD were enrolled. IPD was confirmed in 134 (26.3 %) of them (88 children and 46 adults), either by a positive blood culture or positive real-time PCR (rt-PCR) from blood targeting *Streptococcus pneumoniae lytA* gene [10, 11], as was described in detail previously [12]. Among 134 patients with IPD, 94 (66 children and 28 adults) had isolates available for serotyping and genotyping. In 24 patients, isolates from blood were available (in 14 of them, 5 adults and 9 children, also isolates from nasopharynx). In the remaining 70 patients (in whom IPD was detected only by positive rt-PCR from blood), only isolates from nasopharynx were available for typing. Penicillin susceptibility of isolates was tested by E test according to Clinical Laboratory Standards Institute [13].

Serotyping *Streptococcus pneumoniae* by sequential multiplex PCR

Serotyping was performed using the method developed by Pai et al. [14] instead of Neufeld Quellung reaction. Briefly, in 5 sequential multiplex PCR reactions, 21 most frequent serotypes in our area (in the first reaction, 14, 1, 3, and 9V; in the second, 6A/B, 23F, 19A, 7F, and 10A; in the third, 19F, 4, 18, 22F, and 16F; in the fourth, 8, 33F, 15B, 38, and 17F; and in the fifth, 11A and 35F) were determined.

Genotyping

Genotyping was performed by semiautomated rep-PCR with the use of Diversilab System as already described [8]. Fifteen isolates (at least one of the most frequent rep-PCR genotype or subtype of each serotype) were additionally typed by MLST.

Semiautomated repetitive-sequence based PCR

Genomic DNA was extracted using the UltraClean microbial DNA Isolation kit (MoBio Laboratories), and rep-PCR was performed following the kit instructions. DNA fingerprint patterns were compared using the Pearson correlation coefficient determination in the DiversiLab v 3. 1 software that assesses both band position and band intensity and unweighted pair group method with arithmetic mean (UPGMA) algorithm to create dendrograms. Isolates with the similarity index of ≥ 97 % and no obvious band differences were indistinguishable or had the same genotype, those with one-band difference were related or subtypes, and those with <97 % similarity or two or more bands' difference had different genotype [8].

Multilocus sequence typing

MLST was performed using the method developed by Enright and Spratt [15] on at least one isolate within each serotype, especially if the most frequent rep-PCR genotype. Alleles and STs for MLST were assigned with reference to the *S. pneumoniae* MLST database [16].

Results

The 94 isolates from patients with IPD belonged to 12 different serotypes and were determined in four of five sequential multiplex PCR reactions. In the first PCR reaction, serotype was determined for 61 % isolates, and in the second, third, and fourth for additional 26, 12, and 1 % isolates, respectively.

The most frequent were serotypes 14 (27.6 % isolates), 9V and 3 (12.7 % isolates each), 7F (9.5 % isolates), 19A and 1 (7.4 % isolates each), followed by 4, 6A/B, 19F, 23F, 18C, and 33F. In children, the most prevalent serotypes were 14, 9V, 3, 7F, 1, 19A, and 6A/B, and in adults, serotypes 3, 14, 9V, 7F, 4, 19A, and 1 (Table 1).

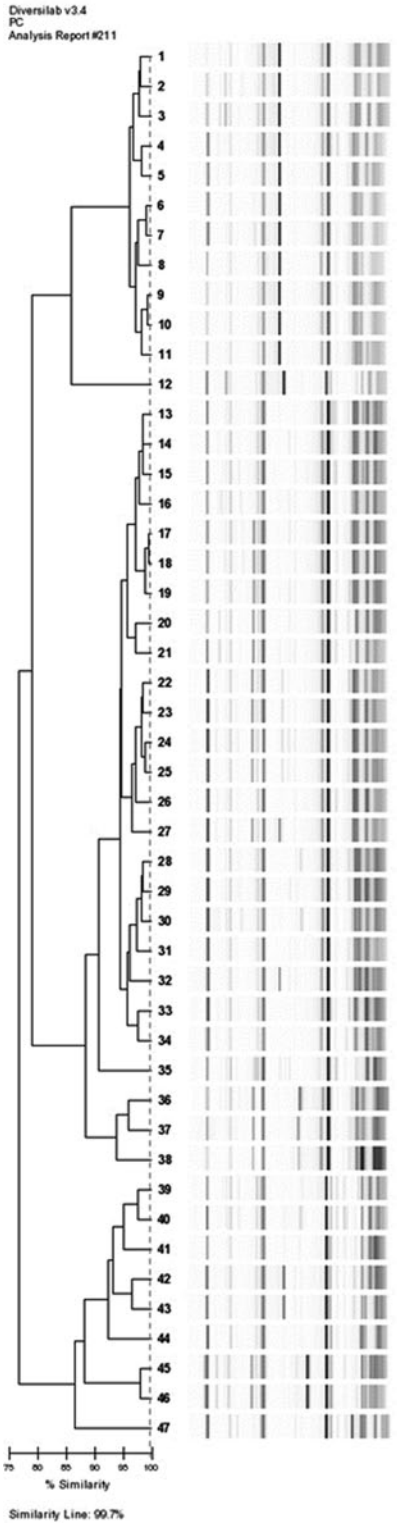
Genotyping by rep-PCR yielded 34 genotypes and 13 subtypes (together 47). In all, 6 genotypes were found in 26 isolates of serotype 14 (G18–23; 1 of them ST156, 1 ST9, 1 ST15), 1 genotype and 1 subtype in 12 isolates of serotype 9V (G17a, b; ST162), 4 in 12 isolates of serotype 3 (G4–7; 1 of them belonged to ST1377), 3 in 9 isolates of 7F (G14–16; 1 of them belonged to ST191), 4 in 7 isolates of serotype 19A (G26–29; 1 of them belonged to ST416), 3 in 7 isolates of serotype 1 (G1–3; 1 of them belonged

Table 1 Serotypes and genotypes of *Streptococcus pneumoniae* isolates from blood and nasopharynx (NP) in patients with IPD

Serotype	Total number of primoisolates	Children/ adults	Number of isolates from blood/NP	Number of genotypes (subtypes)
14	26 (27.6 %)	21/5	6/20	6 (4)
9V	12 (12.7 %)	8/4	3/9	1 (1)
3	12 (12.7 %)	6/6	1/11	4 (2)
7F	9 (9.5 %)	6/3	3/6	3 (0)
19A	7 (7.4 %)	5/2	1/6	4 (3)
1	7 (7.4 %)	5/2	1/6	3 (0)
4	5 (5.3 %)	2/3	4/1	2 (1)
6A/6B	5 (5.3 %)	5/0	3/2	4 (0)
19F	4 (4.2 %)	3/1	1/3	2 (0)
23F	4 (4.2 %)	3/1	1/3	2 (2)
18C	2 (2.1 %)	1/1	0/2	2 (0)
33F	1 (1.1 %)	1/0	0/1	1(0)
Total	94[a]	66/28	24[a]/70	34 (13)

[a]In 9 children and 5 adults, in whom serotype was determined from blood and nasopharynx, only serotype from blood is included

Table 2 Genotypes by rep-PCR and sequence types (ST) by MLST of isolates from blood or nasopharynx in patients with IPD (*isolates from blood)



genotype or subtype by rep-PCR	ST by MLST	Serotype	No.isolates
G7		3	1
G26b	ST416	19A	1*
G26a		19A	1
G29		19A	1
G9		4	1
G8a	ST205	4	3*
G8b		4	1*
G17b	ST162	9V	1*/1
G17a	ST162	9V	2*/8
G23a	ST156	14	4
G23b		14	1
G12		6A/B	1*
G21b		14	1
G21a		14	1
G28a		19A	1
G28b		19A	1
G27b		19A	1
G27a		19A	1
G30		19F	1
G4a		3	3
G4b		3	1
G5		3	1
G32c	ST36	23F	1*
G32b		23F	1
G32a		23F	1
G24		18C	1
G33		23F	1
G18a	ST9	14	5*/4
G18b		14	2
G18c		14	1*
G19	ST15	14	5
G20		14	1
G34		33F	1
G25	ST100	18C	1
G22		14	1
G15		7F	1*/2
G14	ST191	7F	2*/3
G16		7F	1
G1		1	2
G2		1	3
G3	ST306	1	1*/1
G10	ST473	6A/B	1*
G31	ST179	19F	1*/2
G11		6A/B	1*
G6a		3	2
G6b	ST1377	3	1*/3
G13		6A/B	2

to ST306), 2 in 5 isolates of serotype 4 (G8–9; 1 of them belonged to ST205), 4 in 5 isolates of 6A/B (G10–13; 1 of them belonged to ST473), 2 in 4 isolates each of serotype 19F (G30–31; 1 of them belonged to ST179) and 23F (G32–33; 1 of them belonged to ST36), 2 in 2 isolates of serotype 18C (G24–25; 1 of them belonged to ST100) and 1, G34 in 1 of serotype 33F (Tables 1 and 2). In all five adults and in seven of nine children, where both isolates from blood and nasopharynx were available, genotypes matched, but in two children, they did not.

Discussion

The most frequent serotypes in patients with IPD determined by multiplex PCR in the central region of Slovenia in the study period were serotypes 14, 9V, 3, 7F, 19A, and 1, followed by 4, 6A/B, 19F, 23F, 18C, and 33F. In children, the most prevalent serotypes were serotype 14 with 31.9 % isolates, followed by 9V, 3, 7F, 1, 19A, and 6A/B, and in adults, serotypes 3 with 21.4 % isolates, followed by 14, 9V, 7F, 4, 19A, and 1. Interestingly, serotype 6A/B was found only in children and all other serotypes in both children and adults. In the previous study, the most frequent IPD serotypes in children in the whole region of Slovenia in the period between 1993 and 2001 were 14, 1, 19F, 23F, 6B, 18C, 6A, 4, 7F, 19A, and 9V [17]. Serotype 3, which is not included in 7-valent pneumococcal conjugate vaccine (PCV-7), was most common in adults and found also in children in our study based on rt-PCR detection of IPD. This serotype deteriorates very quickly and is less commonly detected by blood culture than by rt-PCR, as already described by Selva et al. [18].

Relatively few articles utilizing Diversilab rep-PCR for pneumococci have been published. In the study by Harrington et al. [8], replicates of the same isolate that were known to be epidemiologically related had a similarity index of ≥ 97 % and no obvious band differences. Using these criteria, rep-PCR grouped isolates together with a discriminatory power equal to that of PFGE, except for minor genetic changes resulting in subtypes that would be detected by the two methods differently [8]. In our study, reproducibility of the method was controlled by analyzing isolates repeatedly and by using the same positive control in all runs.

Rep-PCR discriminated our isolates in 34 genotypes and further discriminated a portion of the isolates as 13 subtypes. There were between one and six genotypes and also subtypes inside serotypes. Serotype 9V was common and the most homogenous; all 12 isolates in this serotype belonged to genotype G17-ST162, the virulent widespread clone, known as pandemic clone Spain^{9V}-3. Serotype 14 was also common but heterogenous; it had two clonal groups, in the first were G18-ST9 (known as pandemic clone England^{14}-9) and G19-ST15, and in the second was G23-ST156. Similar was in serotype 19A with G26-ST416 and G29 in one group and G27 and G28 in another. G14-ST191 in serotype 7F and G6b-ST1377 in serotype 3 were also common (Table 2). All isolates except one belonged to serotypes contained in 13-valent vaccine.

Two adults with severe IPD had serotype 4 (one G8a-ST205 and one G8b) and two 9V G17a, b-ST162. All isolates of subtypes G23a (ST156) and G23b inside serotype 14 showed resistance to penicillin (minimal inhibitory concentration-MIC ≥ 0.06 μg/ml) according to criteria for meningitis, as described previously [5]. Resistance was also observed in serotype 19A and 19F.

Capsular type switching that can occur when there is a genetic exchange that alters the capsule, but the genomic content is largely maintained [5], was not observed, but some genotypes inside a certain serotype were genetically related to isolates of other serotype.

Among five adults, where both isolates from blood and nasopharynx were typed, isolates from nasopharynx were indeed representatives of blood isolates; however, this conclusion was not supported by results in two of nine children, due to frequent carriage of multiple strains of pneumococci [19].

The bacterial population in our study was heterogenous, indicating some more virulent worldwide clones and also some sporadic clones. Although capsular type may be more important for the invasiveness than the overall genotype, regions of genetic diversity are also important for the virulence. Further studies are required to examine the invasiveness of different clones of the same serotype.

Conflict of interest

The authors declare that there are no actual or potential conflicts of interest in relation to this article.

References

1. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. J Infect Dis. 2003;187:1424–32.
2. Silva NA, McCluskey J, Jefferies JM, et al. Genomic diversity between strains of the same serotype and multilocus sequence type among pneumococcal clinical isolates. Infect Immun. 2006;74:3513–8.
3. Linares J, Ardanuy C, Pallares R, Fenoll A. Changes in antimicrobial resistance, serotypes and genotypes in *Streptococcus pneumoniae* over a 30-year period. Clin Microbiol Infect. 2010;16:402–10.
4. Richter SS, Heilmann KP, Dohrn CL, Riahi F, Diekema DJ, Doern GV. Pneumococcal serotypes before and after introduction of conjugate vaccines, United States, 1999–2011. Emerg Infect Dis. 2013;19:1074–83.
5. Jefferies JM, Smith AJ, Edwards GF, McMenamin J, Mitchell TJ, Clarke SC. Temporal analysis of invasive pneumococcal clones from Scotland illustrates fluctuations in diversity of serotype and genotype in the absence of pneumococcal conjugate vaccine. J Clin Microbiol. 2010;48:87–96.
6. Tenover FC, Arbeit RD, Goering RV, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995;33:2233–9.
7. Boers SA, van der Reijden WA, Jansen R. High-throughput multilocus sequence typing: bringing molecular typing to the next level. PLoS One. 2012;7:e39630.
8. Harrington SM, Stock F, Kominski AL, et al. Genotypic analysis of invasive *Streptococcus pneumoniae* from Mali, Africa, by semiautomated repetitive-element PCR and pulsed-field gel electrophoresis. J Clin Microbiol. 2007;45:707–14.
9. Bourdon N, Lemire A, Fines-Guyon M, Auzou M. Comparison of four methods, including semi-automated rep-PCR, for the typing of vancomycin-resistant *Enterococcus faecium*. J Microbiol Methods. 2011;84:74–80.

10. Azzari C, Moriondo M, Indolfi G, et al. Molecular detection methods and serotyping performed directly on clinical samples improve diagnostic sensitivity and reveal increased incidence of invasive disease by *Streptococcus pneumoniae* in Italian children. J Med Microbiol. 2008;57:1205–12.
11. Resti M, Moriondo M, Cortimiglia M, et al. Community-acquired bacteremic pneumococcal pneumonia in children: diagnosis and serotyping by real-time polymerase chain reaction using blood samples. Clin Infect Dis. 2010;51:1042–9.
12. Cvitkovic Spik V, Beovic B, Pokorn M, et al. Improvement of pneumococcal pneumonia diagnostics by the use of rt-PCR on plasma and respiratory samples. Scand J Infect Dis. 2013;45:731–7.
13. CLSI. Performance standards for antimicrobial susceptibility testing; twenty-third informational supplement. CLSI document M100-S23. Wayne: Clinical and Laboratory Standards Institute; 2013.
14. Pai R, Gertz RE, Beall B. Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. J Clin Microbiol. 2006;44:124–31.
15. Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology.1998;144:3049–60.
16. http://spneumoniae.mlst.net/.
17. Paragi M, Kolman J, Kraigher A, Cizman M, Gubina M, Ribic H. Possibility of application of new pneumococcal conjugate vaccines in children in Slovenia. Vaccine. 2003;21:4708–14.
18. Selva L, Ciruela P, Esteva C, et al. Serotype 3 is a common serotype causing invasive pneumococcal disease in children less than 5 years old, as identified by real-time PCR. Eur J Clin Microbiol Infect Dis. 2012;31:1487–95.
19. Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nature Rev. 2008;6:288–301.