GENETIC SURVEY OF INVASIVE S. PNEUMONIAE SEROTYPES IN BULGARIA FOR A 5-YEAR PERIOD

I. Simeonovski, V. Levterova, M. Malcheva, T. Kantardjiev

National Centre of Infectious and Parasitic Diseases

ABSTRACT

Streptococcus pneumoniae colonises the mucosal lining of the upper respiratory tract and is an important cause of invasive infections affecting young children, adults over 65 years of age, the immunocompromised and individuals with chronic diseases. Recent studies have shown variations in virulence based on the high rate of pneumococcal recombination. PCR-based molecular methods are highly sensitive, specific and are becoming the preferred tool for quick and accurate diagnosis of bacterial meningitis which is required to be defined within 2-3 hours.

During the 5-year survey period (2013-2017), 202 materials received as cerebrospinal fluid samples and pneumococcal strains isolated from patients diagnosed with meningitis, were examined by Real-time PCR in the reference laboratory at NCIPD. Serotyping of *S. pneumoniae*-positive materials was performed with conventional multiplex PCR and Real-time PCR with primers for 41serotypes/serogroups.

There is a high incidence of *S. pneumoniae* serotypes not covered by the pneumococcal conjugate vaccine (PCV10) currently used in Bulgaria. It was found that all cases of meningitis caused by *S. pneumoniae* vaccine serotypes occurred in patients that were not vaccinated.

KEYWORDS:

serotyping, invasive *S. pneumoniae*, PCR

ADDRESS FOR CORRESPONDENCE:

Assoc. Prof. Viktoria Levterova,Ph.D.

Department of Microbiology

National Center

of Infectious and Parasitic Diseases

blvd.Yanko Sakazov 26,

Sofia 1504, BULGARIA

Tel: + 3592 944 6999 (290, 353)

E-mail: vlevterova@ ncipd.org

INTRODUCTION

Streptococcus pneumoniae is a Gram-positive, extracellular, opportunistic pathogen colonising the mucosal lining of the upper respiratory tract in humans (1, 2). Pneumococci are the leading cause of a wide range of invasive and non-invasive diseases (3). Non-invasive diseases are considered less severe and they are more widespread (4). One of the most common non-invasive diseases is otitis media in children. *S. pneumoniae* is the leading bacterial agent of community-acquired pneumonia, both in children and adults. It is a major cause of mortality, hospitalisation (5, 6) and invasive infections affecting young children, adults over 65 years of age, the immunocompromised and individuals with chronic diseases. The spread of *S. pneumoniae* outside its niche, the nasal epithelium, can cause invasive diseases such as pneumonia, bacteraemia, sepsis and meningitis (7).

The diagnosis of invasive pneumococcal diseases is a highly responsible task. In the routine work it is performed by isolation of the microorganism from blood cultures or other normally sterile body fluids – cerebrospinal fluid (CSF), pleural or synovial fluid.

PCR-based molecular methods are gaining popularity in the diagnosis of invasive pneumococcal infections. They are highly sensitive and very specific. Molecular techniques are extremely valuable and becoming the preferred tool for quick and accurate diagnosis of bacterial meningitis which is required to be defined within 2-3 hours as an essential part of the proper and adequate treatment of the patient.

Determination of the capsule serotype is most often used to monitor the administered pneumococcal vaccines.

The pneumococcal capsule has a polysaccharide composition and is defined as the major virulence factor involved in pathogenesis by various mechanisms. According to numerous studies characterising the capsule of *S. pneumoniae*, strains with thicker capsules such as serotype 3, 6A, 6B, 9N and 19F, have been associated with more aggressive invasive disease (8).

A small number of capsular serotypes cause invasive diseases in children and adults. Their prevalence has geographical and age specificities and may change depending on the administered vaccines (9). Recent studies have shown variations in virulence based on the high rate of pneumococcal recombination. In different clonal lines are observed differences in the gene content and phenotypic diversities, regardless of the capsular serotype (10).

PCR-based serotyping characterises the genes within the *cps* locus. Different serotypes have distinct construction of the genes within the cluster. The most important gene incorporated in the *cps* locus, the *wzy* gene, has multiple allotypes that determine the different construction of capsular polysaccharide chains. Approximately 95 pneumococcal serotypes are described so far (11, 12, 13, 14, 15, 16). The sequence of the *cps* locus and the location of the genes involved in biosynthesis and construction of the capsular polysaccharide chains, were determined in the last decade.

The introduced pneumococcal vaccines are an important measure for the prevention of bacterial meningitis in young children and adults.

MATERIALS

During the survey period from January 2013 to December 2017, the reference laboratory at NCIPD processed a total of 202 clinical materials sent from hospitals in the country for confirmation or determination of the etiological agent causing meningitis. Bacterial DNA isolation was performed from CSF samples and pneumococcal strains isolated from meningitis patients aged from 1 month to 83 years (Table 1).

Table 1. Number of analysed materials during the period 2013- 2017.

Year	2013	2014	2015	2016	2017
Number of analysed materials	39	41	55	30	37

Bacterial DNA was isolated using 5% Chelex 100 and Proteinase K, and stored at -20˚C.

The positive result for *S. pneumoniae* DNA in CSF, blood and/or pleural fluid samples was considered indicative of the presence of invasive pneumococcal disease.

METHODS

Isolation of bacterial DNA was carried out using 5% Chelex 100 and 20 mg/ml Proteinase K. 200 ml of the examined sample (CSF) was added to a 1.5 ml tube containing 150 µl 5% Chelex 100 and 6 µl proteinase K. The tube was placed in a thermoblock for 15 minutes at 56˚C and for another 15 minutes at 100˚C. After that the tube was allowed to cool for 5 minutes at room temperature and centrifuged for 5 minutes at 14,000 rpm. The supernatant containing the extracted DNA was transferred to a sterile 1.5 ml tube and the sediment was discarded.

The target genes used for identification of *S. pneumoniae* were *cpsA* and *lytA,* containing conserved sequences. Some of the samples showed positive result for *S. pneumoniae* DNA only by Real-time PCR. The strains received as culture isolates were serotyped by conventional multiplex PCR with 41 oligonucleotide primers used in 13 separate assays for deduction of 70 possible serotypes. The strains from the positive samples were serotyped by Real-time multiplex PCR with oligonucleotide primers used in 21 assays for deduction of 37 pneumococcal serotypes. In the conventional multiplex PCR all primers were targeting the *cpsA* gene which also served as an internal control of the assay (17).

RESULTS AND DISCUSSION

This study employed probe-specific primers and probes to screen for *S. pneumoniae* DNA in 202 materials received as CSF samples

and strains isolated from CSF of patients diagnosed with meningitis. Sixty-two materials gave positive result for *S. pneumoniae*.

The examination of CSF samples in this survey demonstrates that *Streptococcus pneumoniae* is the dominant pathogen of purulent bacterial meningitis in Bulgaria.

Multiplex PCR was used for determination of 41 serotypes/serogroups of *S. pneumoniae*. Electrophoresis results of DNA amplicons typed with conventional multiplex PCR are shown in Fig. 2.

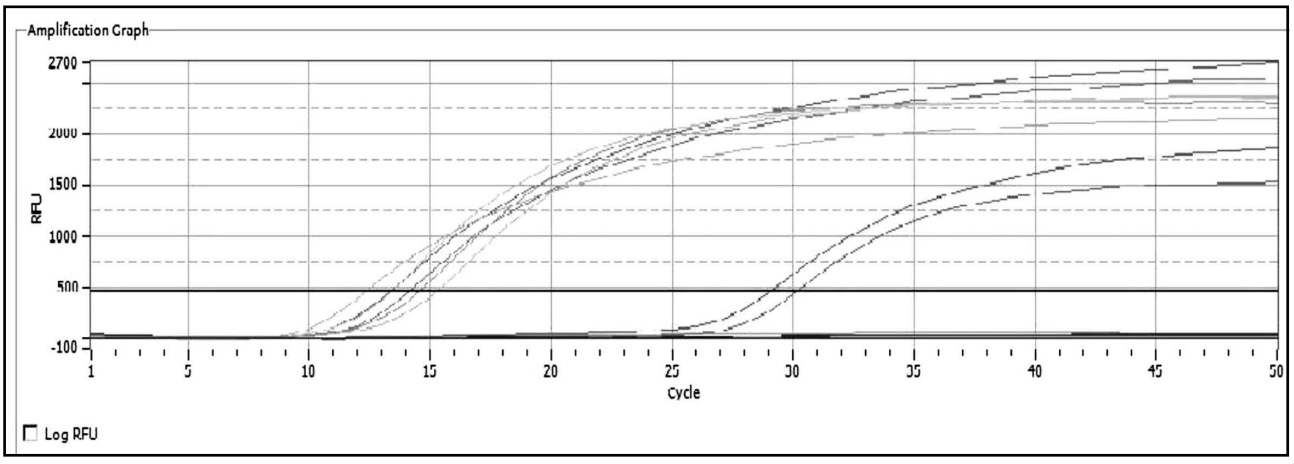

Figure 1. Real-time PCR results for identification of *S. pneumoniae*.

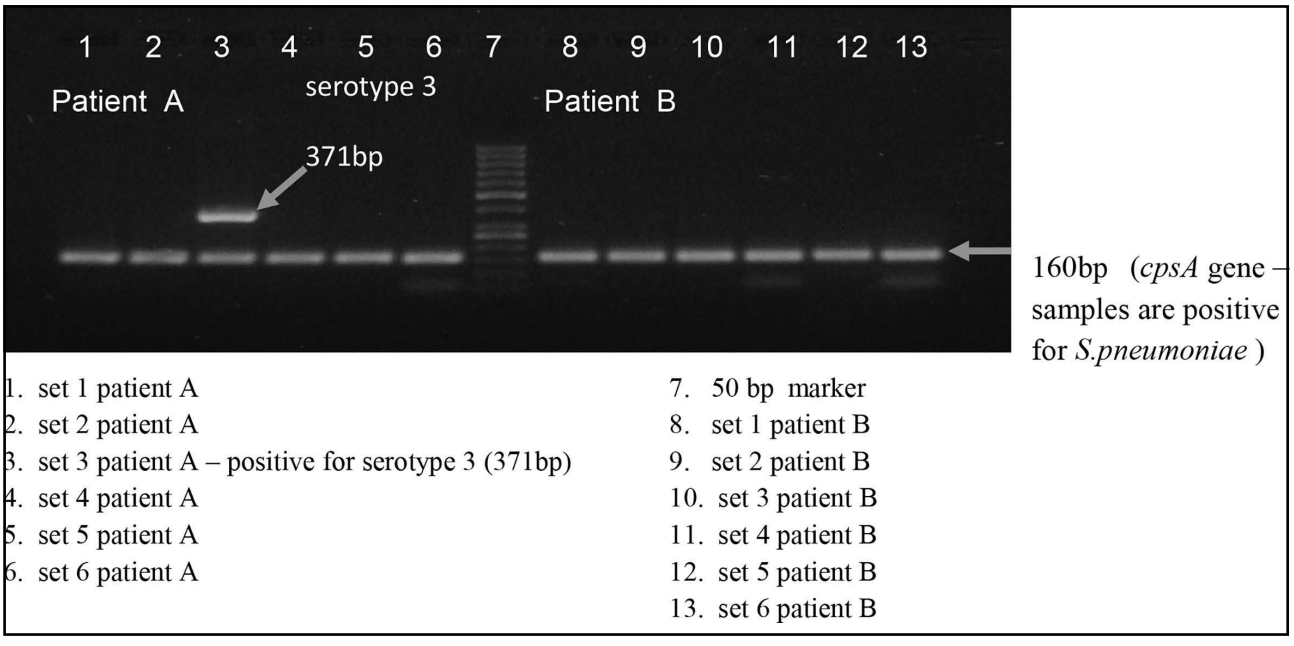

Figure 2. Electrophoresis results for samples of patients A and B tested by multiplex PCR with specific serogroup/serotype primers.

S. pneumoniae serotypes/serogroups identified in this study are presented in Table 2 and Fig. 3.

Table 2. Serogroup/serotype distribution of *S. pneumoniae*- positive samples.

Serogroup/serotype	Total number of positive samples	% of positive samples	Included in PCV10
3	13	21.0	
6A/B/C/D	4 (not vaccinated)	6.5	yes
7F/A	2 (not vaccinated)	3.2	yes
8	2	3.2	
9A/V	4 (not vaccinated)	6.5	yes

Serogroup/serotype	Total number of positive samples	% of positive samples	Included in PCV10
9N/L	3	4.8	
10A/D	3	4.8	
10B	1	1.6	
11A/D	1	1.6	
14	1 (not vaccinated)	1.6	yes
15B/C	4	6.5	
15A/F	1	1.6	
18C	5 (not vaccinated)	8.1	yes
19A	8	12.9	
19F	2 (not vaccinated)	3.2	yes
23F	1 (not vaccinated)	1.6	yes
24 A/B/F	1	1.6	
Nontypeable	6	9.7	

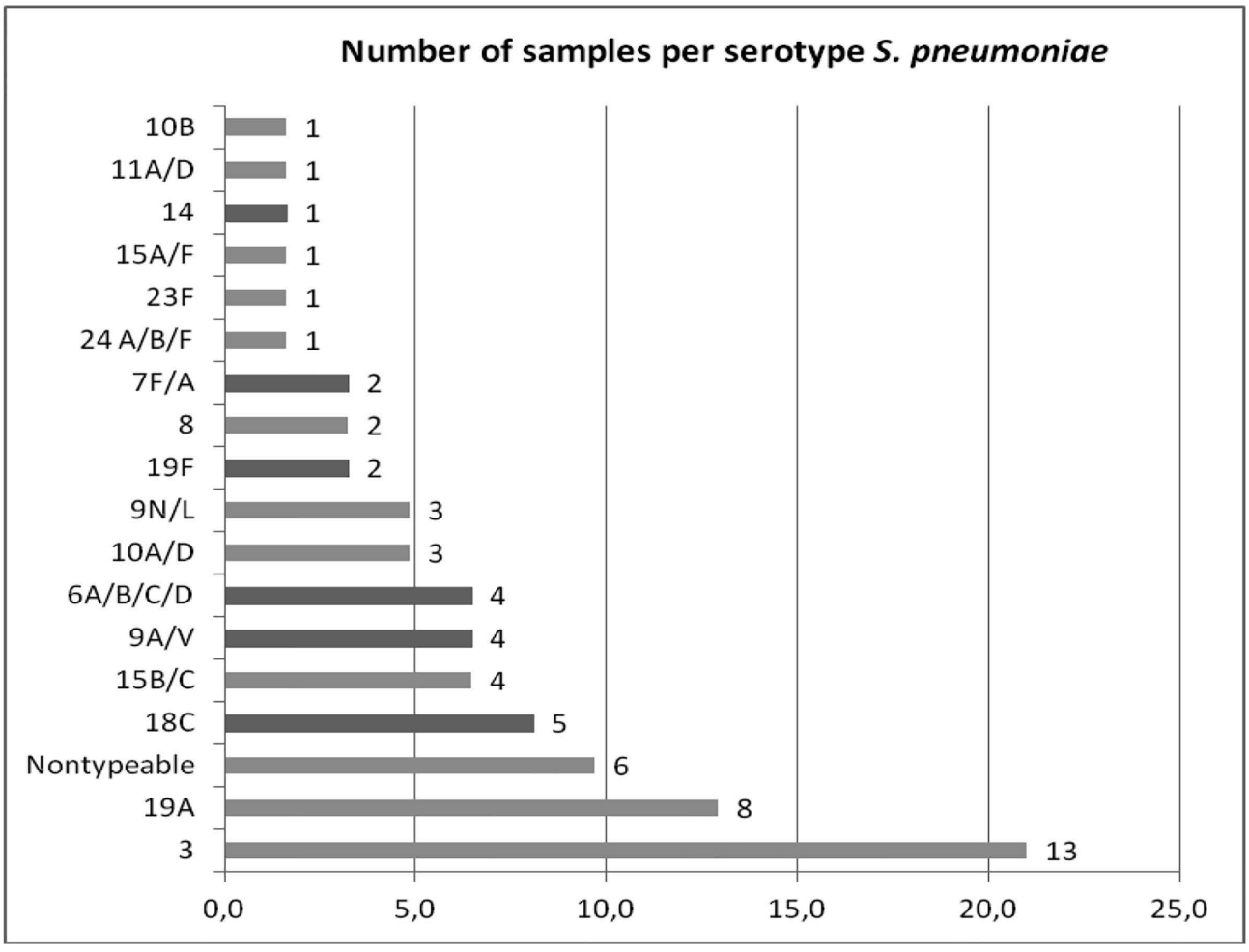

Figure 3. Number of samples per serotype/serogroup of *S. pneumoniae* from 62 positive cases of bacterial meningitis.

The obtained results provide important data for the molecular epidemiology of circulating *S. pneumoniae* strains in Bulgaria. The frequency of serotype 3 differs significantly from serotype 14 ($p < 0.05$). The pneumococcal conjugate vaccine currently used in the immunisation calendar of Bulgaria is PCV10, which does not cover *S. pneumoniae* serotypes showing the highest frequency according to this study.

Serotype 3 is related with high mortality rates in different regions of the world (18, 19, 20). The high frequency of this serotype – 21.0% of positive samples, and its association with greater mortality requires continuous monitoring of its

incidence after the introduction of PCV10. In order to provide protection against serotype 3, PCV10 may be considered to be replaced by PCV13. In the present study, all cases of meningitis caused by *S. pneumoniae* vaccine serotypes (6A/B/C/D, 7F/A, 9A/V, 14, 18C, 19F, 23F) occurred in patients that were not vaccinated.

Vaccine prophylaxis with pneumococcal conjugate vaccines significantly reduces the incidence of invasive pneumococcal infections caused by serotypes included in PCV10.

REFERENCES

1. Abdullahi O, et al. *The prevalence and risk factors for pneumococcal colonization of the nasopharynx among children in Kilifi District, Kenya*. PLoS ONE. *2012;* 7: e30787.
2. Yahiaoui RY, et al. *Prevalence and antibiotic resistance of commensal Streptococcus pneumoniae in nine European countries*. Future Microbiol. 2016; 11:737–744.
3. Bogaert D, De Groot R, Hermans PW. *Streptococcus pneumoniae colonisation: the key to pneumococcal disease*. Lancet Infect. Dis. 2004; 4:144–154.
4. World Health Organization. *Immunization, vaccines and biologicals. Pneumococcal vaccines*. Available at: http:// archives.who.int/vaccines/en/pneumococcus.shtml External Links icon. Accessed October 20, 2014.
5. World Health Organization. *23-valent pneumococcal polysaccharide vaccine: WHO position paper.* Wkly Epidemiol Rec. 2008; 83(42):373-384.
6. Kalchev Y, Kirina V, Mircheva M, Tsolova M, Setchanova L, Levterova V, Simeonovski I, Stoycheva M, Kantardjiev T, Murdjeva M. *Etiology and epidemiology of non-viral meningitis in the Plovdiv region.* General Medicine.2018; 20(2): 9-15.
7. Hsiao HJ, Wu CT, Huang JL, et al. *Clinical features and outcomes of invasive pneumococcal disease in a pediatric intensive care unit*. BMC Pediatr. 2015; 15:85.
8. Weinberger DM, Trzcinski K, Lu YJ, Bogaert D, Brandes A, Galagan J, et al. *Pneumococcal capsular polysaccharide structure predicts serotype prevalence.* PLoS pathogens. 2009; 5(6):e1000476.
9. Blumental S, Granger-Farbos A, Moïsi JC, et al. *Virulence Factors of Streptococcus pneumoniae. Comparison between African and French Invasive Isolates and Implication for Future Vaccines*. PLoS One. 2015; 10(7):e0133885.
10. Browall S, Norman M, Tångrot J, et al. *Intraclonal Variations among Streptococcus pneumoniae isolates influence the likelihood of invasive disease in children*. J Infect Dis. 2014; 209:377–388.
11. Calix JJ, Nahm MH. *A New Pneumococcal Serotype, 11E, Has a Variably Inactivated wcjE Gene*. J Infect Dis. 2010; 202(1):29-38.
12. Calix JJ, Porambo RJ, Brady AM, Larson TR, Yother J, Abeygunwardana C, Nahm MH. *Biochemical, Genetic, and Serological Characterization of Two Capsule Subtypes among Streptococcus pneumoniae Serotype 20 Strains: Discovery Of A New Pneumococcal Serotype*. J Biol Chem. 2012; 287(33):27885-27894.
13. Kay EJ, Yates LE, Terra VS, Cuccui J, Wren BW. *Recombinant expression of Streptococcus pneumoniae capsular polysaccharides in Escherichia coli*. Open Biol. 2016; 6(4):150243.
14. Ko KS, Baek JY, Song JH. *Capsular Gene Sequences and Genotypes of "Serotype 6E" Streptococcus pneumoniae Isolates*. J Clin Microbiol. 2013; 51(10):3395-3399.
15. Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MCC, Nahm MH. *Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae*. J Clin Microbiol. 2007; 45(4):1225-1233.
16. 16. Streptococcus Laboratory , June 15, 2018, Available at: *https://www.cdc.gov/streplab/pneumococcus/resources.html*
17. Oliver MB, van der Linden MPG, Kuntzel SA, Saad JS, Nahm MH. *Discovery of Streptococcus pneumoniae serotype 6 variants with glycosyltransferases synthesizing two differing repeating units*. J Biol Chem. 2015; 290(44):26474-26475.
18. Brueggemann AB, Peto TEA, Crook DW, Butler JC, Kristinsson KG, et al. *Temporal and geographic stability of the serogroup specific invasive disease potential of Streptococcus pneumoniae in children*. J Infect Dis. 2004; 190:1203–1211.
19. Harboe ZB, Thomsen RW, Riis A, Valentiner-Branth P, Christensen JJ, et al. *Pneumococcal serotypes and mortality following invasive pneumococcal disease: a population-based cohort study*. PloS Medicine. 2009; 6:1–13.
20. Jansen AGSC, Rodenurg GR, van der Ende A, van Alphen L, Veenhoven RH, et al. *Invasive pneumococcal disease among adults: association among serotypes, disease characteristics and outcome*. Clin Infect Dis. 2009; 49: e23–29.