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Rise of multidrug-resistant *Streptococcus pneumoniae* clones expressing non-vaccine serotypes among children following introduction of the 10-valent pneumococcal conjugate vaccine in Bulgaria

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ABSTRACT

Objectives: Pneumococcal conjugate vaccines (PCVs) have reduced the incidence of pneumococcal disease, but non-vaccine serotypes are of concern, particularly if antimicrobial-resistant. This study retrospectively evaluated the serotype-specific clonality of paediatric multidrug-resistant (MDR) invasive and non-invasive *Streptococcus pneumoniae* isolates collected following PCV10 introduction (2011–2017) in Bulgaria.

Methods: Capsular types, drug resistance patterns and multilocus sequence typing (MLST) of the most common MDR *S. pneumoniae* serotypes sampled from children were determined.

Results: Overall, the rate of MDR pneumococci was 44.6% (107/240). The most common serotypes among MDR strains were 19F (25.2%), 19A (19.6%), 6C (13.1%), 6A and 23A (6.5% each) and 15A (4.7%), contributing 75.7% of all MDR strains. With the exception of serotype 19F, the remaining serotypes were non-PCV10 types. Among MDR pneumococci, the most frequently found sequence types were ST320 (30.4%; 19A and 19F), ST386 (12.7%; 6C and 6A) and ST8029 (5.1%; 23A). The majority of MDR STs (74.7%) belonged to PMEN clonal complexes, of which the most common were CC320 (Taiwan^{19F}-14), CC315 (Poland^{6B}-20) and CC180 (Netherlands^{3}-31), accounting for 43.0%, 13.9% and 5.1%, respectively. In the post-vaccine period, a shift in the genetic structure of serotype 19A was found, with a significant increase of PMEN-14 (CC320) and a concurrent decrease of the major clone Denmark^{14}-32 (CC230) observed prior to PCV10 introduction in Bulgaria.

Conclusions: Clonality was found behind the wide distribution of MDR capsular types 19A, 6C, 23A and 3 following vaccine introduction, and a highly multiresistant and virulent clone Taiwan^{19F}-14/ST320 has emerged as a common pathogen in children.

1. Introduction

Despite the availability of pneumococcal conjugate vaccines (PCVs), *Streptococcus pneumoniae* continues to be an important cause of invasive pneumococcal disease (IPD), mainly in adults, as well as less serious but more frequent respiratory tract infections both in children and adults [1]. Antimicrobial-non-susceptible pneumococci pose a challenge for devising effective empirical antimicrobial treatment strategies not only in the pre-vaccine era but also following the introduction of PCVs.

The first PCV (PCV7) reduced the burden of IPD and changed the serotype distribution in countries that included the vaccine in their national child immunisation programme. As serotypes included in PCV7 were those that expressed the highest rates of antimicrobial resistance, PCV7 vaccination had a concomitant decreasing effect on the incidence of IPD and other non-invasive pneumococcal diseases (NIPDs) caused by antimicrobial-resistant *S. pneumoniae*. Vaccine serotypes have nearly disappeared and have largely been replaced by non-vaccine serotypes (NVTs) in countries with routine vaccination, including serotype 19A [2,3]. Importantly, PCV7 also targeted several serotypes belonging to

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antimicrobial-non-susceptible clones [4]. The frequency of serotype 19A is of concern as many 19A-associated clones, such as clonal complex 320 (CC320), are antimicrobial-resistant [2,4–6]. Since 2010, two broad-spectrum PCVs, the 10-valent (PCV10) and 13-valent (PCV13) vaccines, are available and both vaccines proved to be more effective than PCV7 [7]. In April 2010, the Bulgarian National Immunization Program introduced PCV10 as a first vaccine for universal vaccination of all infants. Prior to PCV10 implementation, PCV7 was not used in Bulgaria. PCV10 vaccine coverage among targeted age groups is very high (>90%) according to the national epidemiological data of the Bulgarian Ministry of Health.

After routine PCV10 immunisation began in 2010, few cases of IPD were observed among children and vaccine serotypes decreased in our population with pneumococcal infections [8], a trend that continued in 2017. However, following PCV10 introduction, some non-PCV10 serotypes such as 19A and 6C significantly increased among vaccinated children with IPD and NIPD compared with non-vaccinated patients [8]. Previously (2011–2016), we reported that the rates of antimicrobial-non-susceptible *S. pneumoniae* remained high in the post-PCV10 era because of the wide prevalence of some multidrug-resistant (MDR) NVTs.

The present study aimed to retrospectively characterise invasive and non-invasive MDR *S. pneumoniae* serotypes isolated in Bulgaria during 2011–2017 and to further evaluate serotype-specific clonality of paediatric MDR isolates using multilocus sequence typing (MLST) as well to determine whether changes in serotype 19A clones have occurred during the period when the vaccine was being introduced compared with those found before PCV10 introduction (1995–2010).

2. Materials and methods

2.1. Selection of pneumococcal isolates and serotyping

The Department of Medical Microbiology at the Medical University of Sofia (Sofia, Bulgaria) has been collecting pneumococcal isolates since 2011 from several clinical microbiological laboratories throughout Bulgaria located in the Medical Universities of Sofia, Plovdiv, Pleven and Varna. A total of 240 invasive ($n = 77$) and non-invasive ($n = 163$) pneumococci isolates collected during 2011–2017 were identified and were characterised regarding serotype and antimicrobial susceptibility. Data on serotypes and antimicrobial susceptibilities of isolates collected from May 2011 to May 2016 were presented previously [8].

The PCV status was determined on the basis of patient age at the date when the pneumococcal strain was isolated, as the coverage rate of PCV10 is high (>90%) among age-eligible children according to national epidemiological data. Children born 1 April 2010 or thereafter and those who had received at least three doses of PCV10 were defined as PCV10-vaccinated. This retrospective study included MDR *S. pneumoniae* serotypes most frequently isolated from children with IPD and NIPD in the post-vaccine period (2011–2017).

Pneumococcal strains were confirmed both by optochin susceptibility and bile solubility testing. Isolates were serotyped by the standard capsular reaction test using group and factor antisera (Statens Serum Institut, Copenhagen, Denmark). Serogroup 6 isolates were subjected to PCR serotyping as described elsewhere [9,10]. Vaccine coverage was estimated by calculating the percentage of isolates that expressed the serotypes included in PCV10, and the coverage rate did not included other cross-reactive serotypes. NVTs isolates were defined as those serotypes not included in PCV10.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the broth microdilution method on microtitre plates (Sensititre™ HPB-*Haemophilus/Streptococcus pneumoniae* MIC plates; Trek Diagnostic Systems Ltd., East Grinstead, UK). Minimum inhibitory concentrations (MICs) were interpreted using the 2017 European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [11]. Non-susceptible isolates were defined as those displaying intermediate or complete resistance to one or more of the antimicrobials tested. Isolates presenting a penicillin MIC of $\geq$0.12 mg/L were considered penicillin-non-susceptible and those presenting a penicillin MIC of <0.12 mg/L were considered penicillin-susceptible. A MDR phenotype was defined as non-susceptibility to three or more antimicrobial classes. PCR was used to detect the *ermB* and *mefA/E* macrolide resistance determinants in erythromycin-resistant pneumococci as described previously [12].

2.3. Multilocus sequence typing

For MLST analysis, 79 invasive and non-invasive pneumococcal isolates were selected as representatives of most frequently isolated MDR serotypes among children with pneumococcal diseases during PCV10 implementation (2011–2017). These strains were subjected to MLST performed by standard procedures [13] as described previously [12]. Briefly, seven housekeeping genes were sequenced and were compared with the pneumococcal MLST database (http://pubmlst.org/spneumoniae) to identify the alleles and respective sequence types (STs). eBURST was used to assign clonal complexes (CCs) [14]. STs were compared with the Pneumococcal Molecular Epidemiology Network (PMEN) clones at http://www.sph.emory.edu/PMEN. STs that shared at least five of seven allelic variants composed a CC.

2.4. Statistical analysis

Fisher's exact test or χ^2 test were used to compare differences in proportions of STs expressing serotype 19A collected before and after PCV10 introduction. A P-value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. Serotype distribution of multidrug-resistant pneumococci isolated following PCV10 introduction

Among 240 invasive and non-invasive *S. pneumoniae* isolates collected from children and adults with IPD and NIPD between 2011–2017, 107 (44.6%) were MDR. The rate of MDR isolates was higher among non-invasive isolates (middle ear fluid and respiratory) compared with invasive strains [88/163 (54.0%) vs. 19/77 (24.7%); $P < 0.001$]. The number of MDR pneumococci isolated from each type of infection was as follows: meningitis, bacteraemic pneumonia and bacteraemia ($n = 19$); otitis media ($n = 57$); conjunctivitis ($n = 4$); and non-bacteraemic lower respiratory tract infection ($n = 27$). The majority of MDR isolates were obtained from children (92/107; 86.0%), ranging in age from 15 days to 14 years, and the remaining 15 isolates were collected from adults (>18 years). Of the 92 children, 72 were born after the beginning of routine pneumococcal vaccination, thus the vaccinated population comprised 67.3% (72/107) of the entire MDR collection. Fifteen different serotypes and three non-typeable *S. pneumoniae* isolates were identified among the MDR isolates (Fig. 1). The most frequent MDR serotypes were 19F ($n = 27$; 25.2%), 19A ($n = 21$; 19.6%), 6C ($n = 14$; 13.1%), 6A and 23A ($n = 7$ each; 6.5%)