Vaccine 29 (2011) 1770–1776

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Clonal and clinical profile of *Streptococcus pneumoniae* serotype 19A causing pediatric invasive infections: A 2-year (2007–2009) laboratory-based surveillance in Madrid

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ARTICLE INFO

Article history:
Received 10 November 2010
Received in revised form
22 December 2010
Accepted 22 December 2010
Available online 7 January 2011

Keywords:
S. pneumoniae
Serotype 19A
Invasive disease
Children
Susceptibility

ABSTRACT

Studies of clonality and clinical profile of serotype 19A invasive pneumococcal disease in children (IPD-19A) are worthy after PCV7 introduction. A prospective, hospital-based surveillance of IPD-19A, culture and/or PCR confirmed, was performed in 2007–2009 in Madrid (all 22 hospitals with pediatric departments). Sixty-two cases were found: 90.3% in children <5 years, 87.1% in <36 months, and 74.2% in ≤18 months. Clinical presentations: meningitis (22.6%), primary bacteremia (19.4%), secondary bacteremia to otic foci (SBOF; 17.7%), bacteremic pneumonia (17.7%), pediatric parapneumonic empyema (PPE; 17.7%) and others (4.8%). Presentations by age: meningitis (35.7%), SBOF (28.6%) and primary bacteremia (21.4%) in children <12 months, bacteremic pneumonia, PPE and primary bacteremia (26.3% each) in 12–23 months, and bacteremic pneumonia (33.3%) and PPE (26.6%) in ≥24 months. Sequence types ST276 and ST320 represented 83.0% isolates, all oral-penicillin/erythromycin non-susceptible. In nonmeningeal isolates, non-susceptibility to parenteral penicillin/cefotaxime was 0%/17.6% (ST276) and 93.8%/75.0% (ST320). Non-susceptibility in ST276 and ST320, prevalence of these STs among 19A isolates, and serotype 19A prevalence among IPDs, indicate the importance of 19A inclusion in PCV13 for IPD-19A prevention and for reducing 19A nasopharyngeal carriage, thus preventing 19A otitis (one-third of 19A bacteremia in this study were from otic origin).

1. Introduction

Geographic- and age-related differences in the incidence of certain *Streptococcus pneumoniae* serotypes have lead to the proposal that each serotype can be considered a different pathogen from the epidemiological perspective [1]. However this perspective may not reflect the real potential of a serotype to cause disease because serotypes with low disease potential, but to which children are frequently exposed, may cause a large proportion of invasive pneumococcal disease (IPD) while serotypes that are highly invasive may be low prevalent in invasive disease if children are rarely exposed to them [2]. Although serotype 19A has been classified as having low invasive disease potential [2–4], its incidence greatly increased in the 2000 in Spain (with oral penicillin and erythromycin non-susceptibility prevalence as in the pre-vaccination era) [5] and other countries [6]. The reason for this may be that serotype 19A, which was one of the most prevalent serotypes (together with the group included in the 7-valent conjugate vaccine (PCV7)) prior to the introduction of PCV7, in both invasive isolates [5,7] and colonising strains [8], appears equally capable of causing nasopharyngeal colonisation, acute otitis media and invasive disease [9,10]. In addition, the macrolide and penicillin non-susceptibility prevalence in this serotype made it selectable by antibiotic use [5]. For all these facts, serotype 19A could fill the ecologic niche left by the reduction of the number of PCV7 types after vaccine introduction [5,11,12].

For prevalent serotypes, age and tropism for the infection site should be studied on a geographical basis since it has been suggested that the serotype is the most important determinant for causing serious infections [2]. The question of clonality as disease

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doi:10.1016/j.vaccine.2010.12.114

determinant remains opened; stated another way, does genotype within 19A serotype contribute to define IPD?

The aim of this study was to describe the clonal and clinical profile of IPD caused by serotype 19A (serotyped by Quellung and/or PCR) in children in the post-vaccination era (2007–2009) in Madrid, the single Spanish region (with approximately 6 million inhabitants) where PCV7 is included in the childhood vaccination calendar since October 2006 (PCV7 was introduced in Spain in June 2001 but only in the private market).

2. Patients and methods

A prospective, 2-year (May 2007–April 2009), hospital-based surveillance of IPD was carried out in all hospitals (20 centres from May 2007 to April 2008, adding two new centres from May 2008 to April 2009) with pediatric departments located in the Autonomous Region of Madrid, Spain. All hospitalised children (<15 years old) with IPD confirmed by culture and/or PCR at local laboratories were considered. IPD was defined as the presence of *S. pneumoniae* in sterile fluids. Local Research Ethics Committees approved the study protocol. All pneumococcal isolates were sent to a single reference laboratory (*Microbiology Department of the Universitary Clinic Hospital in Madrid*) for serotyping by Quellung reaction. Pleural and cerebrospinal fluids not yielding positive culture were also sent to the reference laboratory for being analysed by pneumolysin (*ply*) and autolysin (*lyt*) genes PCR [13,14]. Pneumococci confirmed by PCR were serotyped using a real-time PCR assay [15]. Only IPD cases caused by serotype 19A (by Quellung and/or PCR) were considered for the present study. Basic demographic data (age, gender and PCV7 vaccination status), length of hospital stay, admission in ICU and outcome were recorded.

An automated rep-PCR DNA fingerprinting [16], with modifications, was used for bacterial genotyping by rep-PCR (bioMérieux, Marcy-L'Etoile, France). DNA was extracted from several colonies of an overnight subculture. PCRs utilized 25 ng of purified DNA and reagents in the *Enterococcus* DiversiLab DNA fingerprinting kit (Bacterial Barcodes). For the best discrimination of *S. pneumoniae*, this kit was recommended by the manufacturer. Cycling conditions for PCR included an initial denaturation of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 70 °C for 90 s, with a final extension at 70 °C for 3 min. PCR products were separated in a microfluidics DNA chip (Bacterial Barcodes) in the Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA) according to the protocol provided by Bacterial Barcodes. Rep-PCR fingerprint profiles were compared by using the Pearson correlation coefficient with DiversiLab v3.1 software (Bacterial Barcodes) that assesses both band position and band intensity. Isolates were characterized as being "similar" or subtypes if they had ≥95% similarity and ≤3 band different.

A subset of strains from representative patterns by rep-PCR was studied by the multilocus sequence typing (MLST) as previously described [17]. Briefly, internal fragments of the aroE, gdh, gki, recP, spi, xpt, and ddl genes were amplified by PCR from chromosomal DNA using the primer pairs described by Enright and Spratt [17]. The amplified fragments were directly sequenced in each direction using the same primers of the initial amplification. The sequences at each of the seven loci were compared with the sequences of all of the known alleles at those loci. Sequences 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://www.mlst.net). The alleles at each of the seven loci defined the allelic profile (in the order aroE, gdh, gki, recP, spi, xpt, and ddl) of each isolate as well as their sequence type. The relatedness between the isolates was represented as a dendrogram, constructed by the unweighted pair-group method with arithmetic averages, from the matrix of pairwise differences in the allelic profiles.

Susceptibility to penicillin, cefotaxime, erythromycin and levofloxacin was determined by microdilution following CLSI recommendations [18]. Current CLSI breakpoints [19] were considered for non-susceptibility (intermediate plus resistance) interpretation: oral penicillin and parenteral penicillin for meningitis (≥0.12 μg/ml), parenteral penicillin for non meningitis (≥4 μg/ml), cefotaxime for non meningitis (≥2 μg/ml), cefotaxime for meningitis (≥1 μg/ml), erythromycin and clindamycin (≥0.5 μg/ml) and levofloxacin (≥4 μg/ml).

The macrolide resistance phenotypes were determined by the double disc diffusion method with erythromycin (15 μg) and clindamycin (2 μg) discs on Mueller-Hinton agar supplemented with 5% sheep blood. The plates were incubated overnight in 5% CO_2 atmosphere at 35 °C. The *erm*(B) and *mef*(A/E) genes were detected by PCR [20], with a subsequent PCR to differentiate between *mef*(A) and *mef*(E) genes [21].

3. Results

During the study period 330 cases of IPD were identified (114 bacteremic pneumonia, 100 pediatric parapneumonic empyema – PPE, 45 meningitis, 44 primary bacteremia and 27 cases corresponding to other IPDs). Of the 330 IPDs, the 62 (18.8%) cases caused by serotype 19A were considered in this study: 53 (85.5%) cases confirmed by culture yielding growth of *S. pneumoniae* and 9 (14.5%; 8 pleural fluids and 1 cerebrospinal fluid) exclusively by PCR identification (with amplification for both *lyt* and *ply* genes). A total of 36 (58.1%) children were male. Twenty-eight (45.2%) children were <12 months old, 19 (30.6%) were 12–23 months and 15 (24.2%) were ≥24 months. Fig. 1 shows cumulative distribution of cases by age in months. The distribution showed that 90.3% cases occurred in children younger than 5 years, 87.1% in children younger than 36 months, and 74.2% in children aged ≤18 months. Of the 62 children included, 8 (12.9%) had not received any PCV7 dose and 54 (87.1%) had received at least one PCV7 dose (9.3% four doses, 70.4% three doses, 16.7% two doses, 1.8% one dose and 1.8% unknown number of doses). The distribution of cases by clinical presentation was as follows: meningitis ($n = 14$; 22.6%), primary bacteremia ($n = 12$; 19.4%), secondary bacteremia to otic foci (3 acute otitis media and 8 mastoiditis as primary diagnoses) (SBOF; $n = 11$; 17.7%), bacteremic pneumonia ($n = 11$; 17.7%), PPE ($n = 11$; 17.7%), and others ($n = 3$; 4.8%).

Fig. 2 shows clinical presentations of serotype 19A IPD by age group, with three different clinical profiles. In children younger than 12 months, meningitis (35.7%) was the most frequent clinical presentation followed by SBOF (28.6%) and primary bacteremia (21.4%). In children aged 12–23 months equal rates of bacteremic pneumonia, PPE and primary bacteremia (with 26.3% each) were found, while in children of 24 months or older bacteremic pneumonia (33.3%) and PPE (26.6%) were the most frequent presentations.

Table 1 shows demographic and clinical data for the total population and by clinical presentation of IPD. Children presenting meningitis were younger (median 7.5 months) than those presenting bacteremic pneumonia or PPE (median 20.0 and 15.0 months, respectively), with median age of approximately 11 months for children with primary bacteremia or SBOF. Median days of hospitalisation and percentages of ICU admission were higher in children with meningitis (16.5 days and 78.6%, respectively) and PPE (20.0 days and 63.6%, respectively) than in those presenting bacteremia: bacteremic pneumonia (9.0 days and 18.2%, respectively), primary