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Characteristics of serogroup 20 *S.pneumoniae* isolates from Brazil

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Abstract

Background: Although serogroup 20 is not part of any conjugate pneumococcal vaccine, its serotype 20A, but not 20B, belongs to the polysaccharide 23-valent formula. Little is known about its clinical, laboratorial and epidemiological characteristics.

Methods: The purpose of this study was to evaluate the bacterial genotypes (by PFGE and MLST), clinical characteristics of patients (from review of medical records) and antimicrobial susceptibility of serogroup 20 isolates which were recovered from patients with invasive pneumococcal disease (IPD) from 2007 to 2012. Subtyping to determine 20A and 20B types was also performed by sequencing the genes of the *cps* locus.

Results: Sixteen isolates were genotyped and were highly related. All pneumococci were resistant to tetracycline and 31 % were non-susceptible to trimethoprim/sulfamethoxazole. Penicillin MIC ranged from 0.004 to 1 μg/mL and non-susceptibility (MIC ≥ 0.12 μg/mL) was observed in 5/16 isolates (31 %). All isolates belonged to subtype 20B. Most patients were male with a median age of 62 years and presented at least one underlying disease (mostly respiratory conditions). All isolates belonged to ST8889 and to a unique PFGE clone.

Conclusions: A high clonal occurrence of serotype 20B pneumococci recovered from patients with IPD in Brazil was observed. As a non-PCV10 serotype, selective pressure may be responsible for this unusual occurrence of serogroup 20. However, temporal variation effect should not be underestimated; therefore it is an issue that warrants continued monitoring.

Keywords: *Streptococcus pneumoniae*, Invasive pneumococcal disease, Serogroup 20, Molecular epidemiology

Abbreviations: GHC, Grupo Hospitalar Conceição; HCPA, Hospital de Clínicas de Porto Alegre; HMD, Hospital Mãe de Deus; ICU, Intensive care units; IPD, Invasive pneumococcal diseases; MIC, Minimal inhibitory concentration; MLS, Multilocus sequence typing; PCR, Polymerase chain reaction; PCV, Pneumococcal conjugate vaccine; PFGE, Pulsed-field gel electrophoresis; PPV23, Pneumococcal polyssacharyde vaccine; ST, Sequence type.

Background

To reduce the burden of pneumococcal diseases, especially invasive cases, different vaccine formulations have been introduced worldwide [1–6].

Serogroup 20 is part of the 23-valent polysaccharide pneumococcal vaccine (PPV23), but it is not included in any of the available conjugate formulations. Albeit in a low proportion, this serotype has been found in the nasopharynx of children [7–12] and has also been reported as a cause of invasive disease [13–20]. Although little is known about specific characteristics related to its virulence, this serogroup has been associated with increased disease severity, invasiveness and mortality [15, 21]. In addition, serogroup 20 has also been linked with some clinically relevant resistance phenotypes, such as levofloxacin resistance [22].

In Brazil, serogroup 20 has recently been recognized among the more prevalent serotypes in the post-vaccine period [23]. Analysis of these isolates by multilocus sequence typing (MLST) identified them as belonging to the same (and newly described) sequence type (ST) 8889. Cálix and co-workers (2012) [24] subtyped isolates

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belonging to serogroup 20 based on differences in the *cps* locus and designated these variants as 20A and 20B. They showed distinguishable antigenicity when human sera from patients who were vaccinated with the PPV23 were used. The authors concluded that this vaccine contains serotype 20A polysaccharide, but not 20B.

Compared with other serotypes, there is a restricted number of strains of serogroup 20 described in the MLST database (http://pubmlst.org/spneumoniae/) and therefore additional molecular epidemiology analyses remain to be explored. The aim of this work was to characterize isolates of serogroup 20 recovered from patients with IPD in Porto Alegre, South Brazil.

Methods

Study setting and bacterial isolates

S. pneumoniae isolates from patients with IPD have been systematically collected as part of our surveillance studies. From January 2007 to December 2012, 358 pneumococci were recovered from patients attending three hospitals in Porto Alegre, Brazil: Hospital Mãe de Deus (HMD), Grupo Hospitalar Conceição (GHC) and Hospital de Clínicas de Porto Alegre (HCPA). One isolate per patient was considered. Identification was confirmed by optochin susceptibility and bile solubility tests [25]. Serotyping was performed for 336 isolates by multiplex PCR [26] and/or Quellung reaction. Among all *S. pneumoniae*, 4.8 % (16/336) were identified as serogroup 20 and included in the present study. Isolates were stored in Skim Milk® (Difco) with 5 % glycerol at -80 °C.

Antimicrobial susceptibility tests

Minimal inhibitory concentration (MIC) to the following antimicrobials was determined by broth microdilution, as recommended by CLSI, 2013 [27]: penicillin, ceftriaxone, erythromycin, tetracycline, trimethoprim/sulfamethoxazole, levofloxacin, chloramphenicol and vancomycin. The reference strain *S. pneumoniae* ATCC 49619 was used for quality control.

Genetic characterization of serogroup 20 cps locus

Regions of the capsular genes that previously demonstrated genetic variability between serotypes 20A and 20B [28] were sequenced: *wcjE*, *wchA* and *whaF*. DNA extraction was performed using a 5 % suspension of Chelex® 100 resin (Biorad). The oligonucleotide sequences used were designed based on the nucleotide sequence of the published serogroup 20 *cps* locus (GenBank™ accession number CR931679.1) and are shown in Table 1. PCR reaction included 1.2 μM of each primer, 2.5U of *Taq* DNA polymerase and 3.5 mM of $MgCl_2$. Reaction conditions included an initial denaturation at 94 °C for 4 min, 30 cycles of denaturation (94 °C for 45 s), annealing (56 °C for 45 s) and extension (72 °C for 2.5 min) and a final extension at 72 °C for 5 min. PCR products were purified with ExoSAP-IT (Affymetrix USB, Santa Clara, CA) and cycle sequenced using the BigDye Terminator V3.1 chemistry (Life Technologies, Carlsbad, CA). Sequencing reactions were analyzed on an ABI 3130xl genetic analyzer (Applied Biosystems, Carlsbad, California, USA).

Molecular typing

Pulsed-field gel electrophoresis (PFGE) and MLST techniques were used for molecular typing. PFGE was performed according to McEllistrem et al. (2000) [28] and Pinto et al. (2013) [29]. PFGE patterns were clustered by UPGMA. A dendrogram was generated from a similarity matrix calculated using the Dice similarity coefficient with an optimization of 0.5 % and a tolerance of 1.5 %. A non-invasive serogroup 20 isolate (079-12), recovered during the same period from a sputum sample, was included for comparative purposes. Clonal relationship among isolates was defined according to parameters published by Tenover and co-workers (1995) [30].

MLST was previously performed by our group [23], according to Enright & Spratt (1998) [31], using modified primers described at CDC's Streptococcus Laboratory website (http://www.cdc.gov/streplab/alt-mlst-primers.html).

Analysis of medical records

Patient's records were evaluated to obtain information such as age, gender, intensive care unit (ICU) admission, outcome and occurrence of the following underlying conditions: diabetes, hypertension, HIV infection,

Table 1 Primers sequences used for PCR and sequencing

Region	Sequence	Amplicon (bp)	Reference
wcjE	5'-AGCCTTACTATCCGATCAACG-3'	1334	Calix et al, 2012 [24]
	5'-CTTGTTATGACGCGCTTACC-3'		Calix et al, 2012 [24]
wchA	5'-CCTGTTACTTGCGAACGATG-3'	374	Calix et al, 2012 [24]
	5'-GACCAACGATAGCTCCACAAA-3'		This article
whaF	5'- TGAATTTGAAGAGATAAGGGAAA-3'	457	This article
	5'-CCCGTGTTACATAAGGTGTTG-3'		This article

liver diseases, chronic kidney failures, asplenia, chronic obstructive pulmonary disease, autoimmune disease, transplantation, neoplasia, smoking and alcoholism.

Ethical considerations

This retrospective study was approved by the Research Ethical Committee of Grupo Hospitalar Conceição (Project number 11-205). Patient records and information were anonymized and de-identified prior to analysis.

Results and discussion

From 2007 to 2012, 16 out of 336 pneumococci recovered from patients with IPD belonged to serogroup 20. Yildirim and co-workers (2012) [17] evaluated prevalence of serotypes causing invasive diseases in two periods: directly after PCV7 implementation in USA (2000-2002) and a few years later (2009-10) and also observed an increase in the proportion of invasive disease caused by serogroup 20. However, other studies have reported a stable participation of this serogroup in invasive disease over the years [15].

Yearly, the proportion of serogroup 20 increased from 2007 (1/43, 2 % were serotype 20) to 2011 (with 11/125, 9 % of all pneumococci serotyped as 20) and decreased after that (1/76, 1 % in 2012). Indeed, most of the cases of invasive disease caused by serogroup 20 were detected after the implementation of PCV10 (69 %; 11/16), mainly in the year 2011. However, such effect of the vaccine would be unlikely only one year after the beginning of the vaccination program, especially among adults, and in addition only one serogroup 20 case was observed in 2012. It is well known that temporal variations in the distribution of pneumococcal serotypes are expected, independent of selective pressure due to antibiotic use or vaccination and is a more likely explanation for the changes in prevalence seen in this study than is vaccine-related serotype replacement [32].

Most patients were male (56 %; 9/16). For two patients, no gender data were available. The age of patients varied from 37 to 85 years old and the average and median ages were 61.6 and 62 years old, respectively. Eight (50 %) patients were ≥65 years old. Most isolates were recovered from patients admitted at GHC (81 %; 13/16). Isolates from HCPA and HMD represented 12 % (2/16) and 6 % (1/16), respectively.

The majority of isolates (87 %; 14/16) were from blood and the remainder from cerebrospinal fluid (CSF). Although some authors have reported both invasive [13, 33] and non-invasive [20] infections caused by pneumococci from serogroup 20 in adults, studies consistently describe this serogroup in invasive disease among children [15, 17, 19, 36]. Most studies report serogroup 20 associated with bacteremia and/or meningitis [21, 34]. Also, serogroup 20 appears to be found in a very small proportion of pneumococci in the nasopharynx of children [7–12].

Medical records were available for 12 patients (Table 2). All of them presented at least one underlying condition.

Table 2 Clinical manifestations of patients presenting with invasive disease caused by serogroup 20 *S. pneumoniae* isolates

ID#	Date[a]	Origin	Source	Age	DM	SAH	HIV	Liver disease	COPD	Neoplasia	Smoking	Alcoholism	ICU	Death
009–07	pre	GHC	blood	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
044–09	pre	GHC	blood	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
045–09	pre	GHC	blood	85–89	no	yes	no	no	no	no	no	no	no	no
079–09	pre	GHC	CSF	55–59	no	yes	no	no	yes	no	yes	yes	no	no
014–11	pre	GHC	blood	70–74	yes	no	no	no	no	yes	no	no	no	no
056–11	post	GHC	blood	50–54	no	no	yes	no	no	no	no	no	no	no
089–11	post	HCPA	blood	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
103–11	post	HCPA	CSF	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
114–11	post	GHC	blood	70–74	yes	yes	no	no	yes	no	yes	no	no	no
121–11	post	GHC	blood	65–69	no	yes	no	no	yes	no	No	no	no	no
124–11	post	GHC	blood	65–69	no	no	no	yes	yes	no	yes	yes	yes	yes
130–11	post	GHC	blood	35–39	no	no	no	no	no	no	yes	yes	yes	no
136–11	post	GHC	blood	65–69	no	no	no	no	no	yes	yes	no	no	no
142–11	post	GHC	blood	75–79	no	no	no	no	no	yes	no	no	no	yes
161–11	post	HMD	blood	50–54	no	no	no	no	no	no	no	no	no	no
031–12	post	GHC	blood	80–84	yes	yes	no	no	no	no	no	no	no	no

[a]Date is referred to periods pre-vaccination (from 2007 to 2010) and post-vaccination (from 2010 to 2012)

GHC Grupo Hospitalar Conceição, *HCPA* Hospital de Clínicas de Porto Alegre, *HMD* Hospital Mãe de Deus, *NA* not available, *DM* diabetes mellitus, *SAH* systemic arterial hypertension, *COPD* chronic obstructive pulmonary disease, *ICU* admission to intensive care unit

The most common underlying conditions were chronic obstructive pulmonary disease (COPD) (4/12; 33 %), alcoholism (4/12; 33 %), systemic arterial hypertension (5/12; 42 %) and smoking (5/12; 42 %). Indeed, it is well-established that pneumococcal diseases (considering all serotypes) are facilitated by abnormal conditions of the respiratory tract or underlying conditions [6]. Three patients (25 %) needed admission to ICU.

Five of the 12 (42 %) patients with known outcome died. However, it is difficult to established mortality attributable to pneumococcal infection as underlying conditions were a common feature. Despite these confounders, some studies have demonstrated an increased risk of invasive disease and/or poor outcome related to serogroup 20. In this context, Grabenstein and co-workers (2014) [33] performed a systematic review to characterize differences in serious outcome between pneumococcal serotypes; among seven adult studies evaluating meningitis, serogroup 20 was among the group with elevated risk. Also, Jansen and co-workers (2009) [13] demonstrated that serogroup 20 was among the serotypes that caused meningitis and bacteremia without focus in a relatively high proportion compared to other serotypes. Other studies focusing on analysis of invasiveness demonstrate that this serogroup, along with others, was found to have an enhanced propensity to cause invasive disease [21]. Despite this important characteristic of invasiveness, serogroup 20 was associated with low rates of case-fatality [13]. In contrast, eight deaths among 569 IPD cases reported by Hsu and co-workers (2010) [15] were associated with a group of non-PCV7 isolates, including serogroup 20.

All pneumococci were resistant to tetracycline and 31 % (5/16) were non-susceptible to trimethoprim/sulfamethoxazole (2 with intermediate resistance and 3 fully resistant). Interestingly, we had previously characterized the antimicrobial susceptibility profile of 159 pneumococci recovered from invasive disease in our region [35] and resistance to tetracycline was observed in 22 % of the isolates. Penicillin MICs ranged from 0.004 to 1 μg/mL and resistance (MIC ≥ 0.12 μg/mL) was observed in 31 % (5/16) MIC_{50} for penicillin was very low (<0.03 μg/mL); MIC_{90} was 0.5 μg/mL and one isolate had an MIC of 1.0 μg/mL. Among the limited number of available studies describing antimicrobial susceptibility profiles of serogroup 20 isolates, non-susceptibility to penicillin was not observed by Dunais et al (2011) [7], while one paper reported MICs higher than 0.12 μg/mL [36]. Our isolates were susceptible to all other antimicrobials tested. Indeed, as described in literature, resistance to other antimicrobials appears to be low. Rudolph and co-workers (2013) [37] found a very small proportion of isolates of serogroup 20 non-susceptible to erythromycin among invasive pneumococci recovered from Alaska (1986–2010). Recently, Guo and co-workers (2014) [22] reported two pneumococci belonging to serotype 20B presenting resistance to quinolones in China.

All but one isolate (121–11) were previously submitted to the MLST website; all isolates represented a unique and newly described sequence type: ST8889 [23]. Rudolph and co-workers (2013) [37] typed an erythromycin non-susceptible serogroup 20 isolate and found it to be ST1030, which is one of the 95 serogroup 20 sequence types listed at the MLST website. These STs are distributed in 12 groups (with 59 STs) and 35 singletons. ST 8889 is part of the major clonal complex (CC235) and is a single locus variant of the ancestor, ST235 (Fig. 1).

PFGE grouped all the 16 invasive isolates in a single clone (similarity of 80 % or more in the band pattern). Most isolates (87 %; 14/16) had an identical band profile, and the remaining two pneumococci (044–99 and 045–99) presented a very similar band pattern (Fig. 2). The two isolates recovered from CSF were included in the major PFGE band profile and the ones recovered from patients attending different hospitals (HMD and HCPA) also presented the major band profile. Interestingly, the non-invasive isolate (079–12) included for comparison purposes presented the most distinct band pattern, as shown in Fig. 1, and it was not included in the same clone.

According to Calix and co-workers (2012) [24], serotype 20B (GenBank™ accession number JQ653093.1) is identical to the reference strain of serogroup 20 (GenBank™ accession number CR931679.1), except for the occurrence of a silent mutation in *wcjE* (936 c::t). On the other hand, serotype 20A (GenBank™ accession number JQ653094.1) presents three alterations in genes of the *cps* locus: mutation in *wchA* (659 t::g), mutation in *whaF* (898 a::c) and an adenosine insertion within a polyadenosine tract in *whaF* (position 881). None of the 16 pneumococci had these alterations. The *wchaA* gene of our isolates was identical to the strains CR931679.1 and JQ653093.1 (20B), as well as for *whaF*. The *wcjE* gene of isolates included in this study did not have the 936 c::t mutation observed in 20B (JQ653093.1). However, as this is a silent mutation, we can deduce that the protein constitution of our isolates is the same as the previously described 20B. Serotype 20B seems to be the more common subtype identified amongst serogroup 20 isolates, at least among the restricted number of serogroup 20 isolates subtyped so far. Our subtyping results correlated with Calix et al (2012) [24] who identified serotype 20B amongst their isolates, which were also all from IPD.PPV23 includes

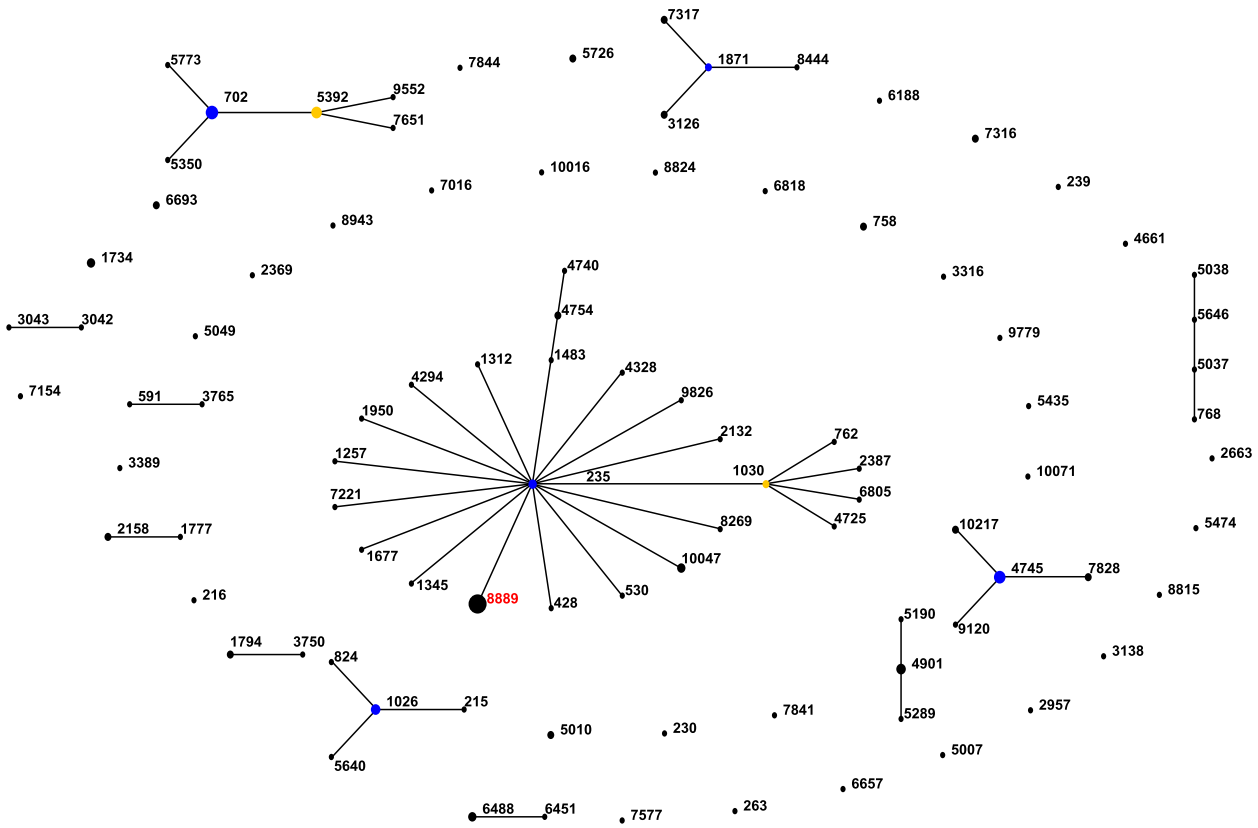

Fig. 1 Population snapshot of 151 serogroup 20 isolates in the *S.pneumoniae* MLST database (accessed in April 2015) based on eBURST analysis. Each ST encountered is indicated by a circle, with their diameters being proportional to the numbers of isolates. ST described by our group [23] is highlighted in red

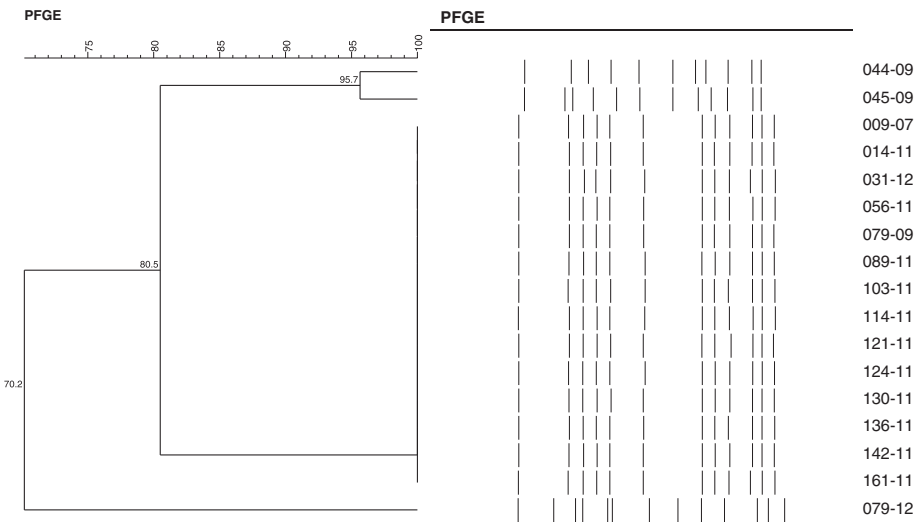

Fig. 2 Representative dendogram of the invasive serogroup 20 pneumococci generated by PFGE. A non-invasive isolate belonging to serogroup 20 (079-12) was included for molecular epidemiology comparison, only

serotype 20A and although Calix and co-workers [24] infer there might be effective cross-protection against 20B, they suggest epidemiological analyses are warranted to confirm these data.

Conclusions

Serogroup 20 is an infrequent non-PCV serotype and little is known about its molecular epidemiology and clinical disease presentation. As changes in the pneumococcal population are expected due to temporal variation and/or selective pressure of vaccination/antimicrobial use, it is important to generate data to better understand the evolution of serotypes not included in available conjugate vaccine formulations. As far as we know, this is the first study devoted to this serotype in Latin America. We focused on the occurrence of a specific ST of serotype 20B, a serotype not included neither in the conjugate nor polysaccharide vaccines. This observation leads to the need of further surveillance for this non-vaccine serotype.

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Avalilability of data and materials

Not applicable.

Authors' contribution

JC: Performed MLST, MLST analysis, wrote the manuscripts. FHS: MLST analysis, reviewed manuscript. PH: Performed MLST. GRdC: identified pneumococci, performed serotyping. MM: identified pneumococci, performed serotyping. PAd'A, CD: MLST analysis, wrote and reviewed manuscript. LM: reviewed manuscript. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

Consent for publication

All authors consent to publish the manuscript as it was presented here.

Ethics approval and consent to participate

This retrospective study was approved by the Research Ethical Committee of Grupo Hospitalar Conceição (Project number 11-205).

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