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Pneumococcal carriage among children aged 4 – 12 years in Angola 4 years after the introduction of a pneumococcal conjugate vaccine

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ABSTRACT

Children in Angola are affected by a high burden of disease caused by pneumococcal infections. The 13-valent pneumococcal conjugate vaccine (PCV13) was introduced in the childhood immunization programme in 2013 but the serotype distribution of *Streptococcus pneumoniae* and antimicrobial susceptibility patterns are unknown. We did a cross-sectional nasopharyngeal carriage study in Luanda and Saurimo, Angola (PCV13 3rd dose coverage 67% and 84%, respectively) during November to December 2017 comprising 940 children aged 4–12 years. The main objective was to assess vaccine serotype coverage and antimicrobial susceptibility rates for *S. pneumoniae*. Our secondary aim was to characterize colonizinig strains of *Haemophilus influenzae* and *Moraxella catarrhalis*. Pneumococcal colonization was found in 35% (95% CI 32–39%) of children (n = 332), with 41% of serotypes covered by PCV13. The most common serotypes were 3 (8%), 18C (6%), 23F (6%), 11A (6%), 34 (6%), 19F (5%) and 16 (5%). Carriage of *H. influenzae* and *M. catarrhalis* was detected in 13% (95% CI 11–15%) and 15% (95% CI 13–17%) of children, respectively. Non-susceptibility to penicillin was common among pneumococci (40%), particularly among PCV13-included serotypes (50% vs. 33%; p = 0.003), although the median minimal inhibitory concentration was low (0.19 μg/mL, IQR 0.13–0.25 μg/mL). Most pneumococci and *H. influenzae* were susceptible to amoxicillin (99% and 88%, respectively). Furthermore, resistance to trimethoprim-sulfamethoxazole was>70% among all three species. Multidrug-resistant pneumococci (non-susceptible to ≥ 3 antibiotics; 7% [n = 24]) were further studied with whole genome sequencing to investigate clonality as an underlying cause for this phenotype. No clearly dominating clone(s) were, however, detected. The results indicate that continued use of PCV13 may have positive direct and herd effects on pneumococcal infections in Angola as carriage of vaccine serotypes was common in the non-vaccinated age group. Finally, amoxicillin is assessed to be a feasible empirical treatment of respiratory tract infections in Angola.

1. Introduction

Streptococcus pneumoniae cause substantial mortality and morbidity among children in low- and middle-income countries due to community acquired pneumonia (CAP), invasive pneumococcal disease (IPD) and acute otitis media (AOM) [1,2]. The bacterium frequently colonizes the nasopharynx of children, a niche it shares with *Haemophilus influenzae* and *Moraxella catarrhalis*, which also are important causes of AOM, and in the case of *H. influenzae* CAP, bacteraemia and meningitis [3,4]. General infant immunization with pneumococcal conjugate vaccines (PCV) has resulted in decreasing incidences of pneumococcal infections among children, with some herd effects in adults through reduced transmission of

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vaccine type (VT) pneumococci [2,5]. However, serotype replacement with non-vaccine serotypes (NVT) has in some cases caused emergence of invasive and/or antibiotic resistant NVTs and partially diminished herd effects [5–6]. Studies of pneumococcal carriage are of great importance for evaluation of PCV implementation [7].

In Angola, pneumococcal infections are a major health issue with a mortality rate of 216 per 100,000 children aged 1 to 59 months in 2015, predominantly related to CAP and IPD [2]. Furthermore, the high incidence of AOM among children represents an important public health concern leading to extensive morbidity due to complications such as chronic suppurative otitis media (CSOM) and hearing loss [8,9]. PCV13 was introduced in the Angolan childhood immunization programme in 2013 (3 + 0 schedule, administered at 2, 4 and 6 months) and the WHO-UNICEF estimate of national immunization coverage (WUENIC) for the 3rd PCV dose was 45–59% during 2014 to 2018 [10]. The *H. influenzae* type b (Hib) conjugate vaccine was introduced in Angola in 2006 (3 + 0 schedule) with a WUENIC between 43% and 59% since 2007 [10]. We have previously studied the bacterial spectrum associated with CSOM in Angola and detected a small number of pneumococci (n = 34), of which 35% were covered by PCV13 and 53% were penicillin non-susceptible (PNSP) [11]. Furthermore, recent studies of bacterial meningitis at a paediatric hospital in Luanda have shown that *S. pneumoniae* is the currently dominating etiological agent, and that 48% (10/21) of serotyped pneumococci were VT in 2016–2017 [12,13]. In addition to these reports, the current pneumococcal serotype distribution and antimicrobial susceptibility rates are unknown. To enable future evaluation of the immunization programme, more data on the present situation when vaccinations commenced are urgently needed.

With the main objective to characterize the spectrum of *S. pneumoniae* circulating in the Angolan population 4 years after the introduction of PCV13, we conducted a cross-sectional nasopharyngeal carriage study in children aged 4 to 12 years in Angola during November to December 2017. The study individuals belonged to an age group untargeted by routine PCV immunization and the detected serotype distribution was therefore thought to be largely unaffected by direct vaccine effects. Associations between background factors of the studied individuals and pneumococcal carriage were also investigated. A secondary aim was to determine carriage rates and antimicrobial susceptibility of *H. influenzae* and *M. catarrhalis*. The current study was part of a project initiated by the Department of Otorhinolaryngology at Hospital Josina Machel, Luanda and the Faculty of Medicine, Agostinho Neto University, Luanda to investigate etiology, epidemiology and treatment strategies of otitis media in Angola.

2. Materials and methods

2.1. Study setting

Enrolment of study participants was conducted during November to December 2017 in Luanda, the capital of Angola, and in villages surrounding Saurimo, the province capital of Lunda Sul in north-eastern Angola. Participating children, accompanied by their parent(s) or guardian(s), were recruited at sampling stations set up outdoors after church gatherings, at local community centers and schools. A small proportion (13%; n = 126) of the samples were collected from children visiting outpatient departments at Hospital Josina Machel and Hospital Pediátrico David Bernardino, Luanda. These children were recruited before seeing a physician and the diagnosis leading to their visit could therefore not be recorded. Enrolment of participants to the study was organized and implemented by personnel of the Department of Otorhinolaryngology at Hospital Josina Machel, Luanda. Upon inclusion, the parent(s) or guardian(s) of the children provided oral consent for the child to participate in the study. According to data obtained from the Angolan Ministry of Health (personal communication, MF) coverages of the 3rd dose of PCV13 (67% and 84%, respectively) and the Hib vaccine (65% and 83%, respectively) were slightly lower in Luanda as compared to Lunda Sul in 2017, but do not indicate any substantial difference in immunization patterns. These numbers are official administrative coverage estimates, which may explain the fact that they differ from the national WUENIC [10].

2.2. Collection of nasopharyngeal samples

Trans-nasal nasopharyngeal sampling was performed with flexible mini-tip flocked swabs (FLOQSwabs®; Copan Italia, Brescia, Italy) and collected in skimmed milk-tryptone-glucose-glycerine (STGG) medium [7]. The samples were transported in cooler-bags with ice-blocks and stored at −80 °C at the National Institute for Health Research, Luanda for a maximum of 1 month until transportation to Sweden (in cooler-bags with ice-blocks). Upon arrival at Clinical Microbiology, Department of Translational Medicine, Lund University (Malmö, Sweden) samples were frozen at −80 °C until further processing.

2.3. Collection of participant background information

In connection with the microbiological sampling, background information of the included children was collected through an interview of their parent or guardian. No medical/vaccination records were available and the data was based on the history provided by the parent or guardian. Questions concerned age and sex of the child, ongoing chronic or acute medical conditions, recent antibiotic therapy and vaccination status (*i.e.*, whether the child received routine vaccines or not), number of children in the household, presence of any tobacco smoker in the household, and access to electricity/water as well as cooking method in the household. In response to questions regarding ongoing medical conditions various replies were given, comprised of both unspecific symptoms (*e.g.* "fever", "headache", "otalgia") as well as diagnoses (*e.g.* CSOM, malaria). Replies that were sufficiently specific were used to assess if children had signs of ongoing infections or any chronic ear or respiratory tract condition. Unspecific answers (*e.g.* "yes") were excluded from the analyses and treated as missing answers. Height and weight of the children was recorded and BMI calculated.

2.4. Culture conditions and species identification

All samples were cultured on blood agar and chocolate blood agar at 35 °C in 5% CO_2 for approximately 48 h. An optochin disk was included on the blood agar to facilitate identification of *S. pneumoniae*. Single colonies of suspected *S. pneumoniae,* of the most common colony morphology if multiple were present, were selected and re-cultured on blood agar for 16–24 h and tested for optochin susceptibility to confirm species. Suspect colonies of *H. influenzae* and *M. catarrhalis* were re-cultured on chocolate blood agar and species identification of these isolates was confirmed with Matrix-assisted laser desorption/ionization - time of mass spectrometry (MALDI-TOF MS). Confirmed isolates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were stored at −80 °C in glycerol-supplemented horse serum.

2.5. Antimicrobial susceptibility testing

Isolates were screened for resistance to clinically relevant antibiotics using disk diffusion tests (Oxoid, Basingstoke, UK). Benzylpenicillin and amoxicillin minimum inhibitory concentrations

(MICs) of pneumococci were determined with gradient tests (Etest; bioMérieux, Marcy-l'Étoile, France). Pneumococcal isolates that were non-susceptible to ≥ 3 of antibiotics (benzylpenicillin, erythromycin, clindamycin, tetracycline, trimethoprim-sulfamethoxazole or norfloxacin) during screening were regarded as possible multidrug-resistant (MDR) pneumococci and were further studied with broth microdilution to determine MDR phenotype (Sensititre; Thermo Fisher Scientific, Waltham, MA). All results from susceptibility testing were interpreted according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST) 2020 clinical breakpoint tables [14].

2.6. Serotyping of pneumococci

All pneumococcal isolates, except 4 that were lost due to contamination, were serotyped (n = 328) according to a sequential 6-reaction multiplex polymerase chain reaction (PCR) protocol combined with latex agglutination and the Quellung reaction, as previously described [11]. Briefly, crude DNA extracts were produced by heating overnight growth from blood agar plates in sterile water to 99 °C for 10 min followed by centrifugation to remove cellular debris. PCR was performed as specified by da Gloria Carvalho *et al.* [15] with primers published by the CDC [16] distributed in 6 sequential reactions. When required, the serotype of isolates positive in any PCR reaction was further specified through the Quellung reaction with Neufeldt antisera (SSI Diagnostica, Denmark). Isolates that were negative in all PCR reactions twice were serotyped with the ImmuLex Pneumotest kit (SSI Diagnostica) and Neufeldt antisera. Isolates that were negative for the *cpsA* gene (included in all PCR reactions) twice or whose serotype could not be confirmed through latex agglutination, were regarded nontypeable (NT).

2.7. Capsule typing of H. Influenzae

H. influenzae capsule types were determined by MALDI-TOF MS [17,18]. All results were confirmed by PCR as previously described [19–21]. In cases of discrepancies between the two methods the results from PCR typing are reported.

2.8. Whole genome sequencing of antibiotic non-susceptible pneumococci

Whole genome sequencing (WGS) described by Kavalari *et al.* [22], was performed on possible MDR pneumococcal isolates to investigate whether these phenotypes could be explained by clonality and to determine resistance mechanisms. Briefly, genomic DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and fragment libraries were constructed using a Nextera XT Kit (Illumina, Little Chesterford, UK) followed by 250-bp paired-end sequencing (MiSeqTM; Illumina) according to the manufacturer's instructions. The paired-end Illumina data were *de novo* assembled using SKESA assembler [23]. Bioinformatic analyses were done using NCBI Genome Workbench v 3.3.1 (NCBI, Bethesda, MD) if not otherwise specified. Identification of the 16S rRNA position 203 was performed as previously described by Scholz *et al.* [24] to confirm pneumococcal species based on the presence of cytosine at this position. When needed, multilocus sequence analysis (MLSA) was used to assign species [25]. Capsular loci were analysed to determine capsular genotype using the PneumoCaT tool [26]. To assign multilocus sequence type (MLST) [27] and global pneumococcal sequence cluster (GPSC) [28] of isolates, and to identify penicillin binding protein (PBP) profiles [29] and additional resistance genes, assembled genomes were uploaded to Pathogenwatch [30]. The genomic sequence data for the 26 isolates are deposited in the Genbank (https://www.ebi.ac.uk/ena) (ENA accession no. is: PRJEB39312).

2.9. Statistical analyses

All statistical analyses were performed in SPSS Statistics version 26 (IBM, Armonk, NY). Background information on included individuals, colonization rates and antimicrobial susceptibility patterns are presented descriptively. The Chi-square test or Fisher's exact test (when appropriate) and the Mann-Whitney U test were used to compare proportions and medians between groups, respectively. Two-tailed p-values < 0.05 were considered statistically significant. If a significant difference was indicated in a contingency table with more than two rows or columns, *post-hoc* testing was done with two-by-two Chi-square tests with an adjusted significance level depending on the number of analyses performed (Bonferroni correction). Univariate and multivariate logistic regression was performed to investigate associations between background variables and pneumococcal carriage rate, calculating odds ratios (OR) with 95% confidence intervals (95% CI). In these analyses BMI and the number of siblings in the household were converted to binary variables with cut-offs set as BMI < 5th percentile (age and sex specific) as defined by the WHO [31] and ≥ 5 children (*i.e.,* > the overall median), respectively.

2.10. Ethical considerations

Accompanying parent(s) or guardian(s) of the included children gave their informed consent for participation after receiving information about the study. Authorization for the study was obtained from the Ethical committee at the Medical National Council of Angola, the Scientific Council of the Faculty of Medicine of Agostinho Neto University and the General Director of Hospital Josina Machel.

3. Results

3.1. Study population

Nasopharyngeal culture samples were collected from a total of 940 children aged 4 to 12 years (median 8 years) in community settings in Luanda (n = 654; 70%) and Saurimo, Lunda Sul (n = 160; 17%), and at 2 hospitals in Luanda (n = 126; 13%). Background characteristics of the included individuals are listed in Table 1.

3.2. Carriage of S. pneumoniae occurs in one third of children

S. pneumoniae, *H. influenzae*, and *M. catarrhalis* were isolated from 35% (95% CI 32–39%; n = 332), 13% (95% CI 11–15%; n = 121), and 15% (95% CI 13–17%; n = 139) of samples, respectively. Simultaneous carriage of pneumococci and *H. influenzae* was detected in 6% of samples, pneumococci and *M. catarrhalis* in 5%, *H. influenzae* and *M. catarrhalis* in 1%, and all 3 bacterial species in 2%. Median age of carriers and non-carriers differed only regarding *M. catarrhalis* (7 [IQR 6–9] and 9 [IQR 7–11] years, respectively; p < 0.001). Colonization rates at the different study sites are presented in Fig. 1. Univariate and multivariate regression analysis was performed to investigate association of demographic and clinical variables with pneumococcal carriage (Table 2).

3.3. Nearly half of isolated pneumococci consists of vaccine serotypes

In total 328 pneumococcal isolates were serotyped, and the overall and site-specific serotype distributions are presented in

Table 1
Demographical and clinical background characteristics of sampled children at the different inclusion sites. Values are presented as *n* (%) for categorical variables and median (IQR) for continuous variables.

Characteristic[a]	Missing data (*n*)	Total	Luanda (community)	Luanda (hospital)	Saurimo (community)
Number (*n*)		940	654	126	160
Age (years)	0	8 (7–10)	9 (7–10)	8 (6–10)	9 (7–11)
Female sex	0	444 (47)	323 (49)	44 (35)	69 (43)
BMI	1	15.6 (14.4–16.8)	15.6 (14.5–16.8)	15.4 (14.2–17.2)	15.7 (14.5–16.7)
Chronic ear/auditory or respiratory tract symptoms	167	49 (6)	12 (2)	21 (19)	16 (11)
Ongoing infectious symptoms	134	59 (7)	8 (1)	39 (66)	12 (8)
Antibiotic treatment last month	142	62 (8)	19 (4)	43 (35)	0
Vaccinated according to schedule	144	642 (81)	403 (79)	114 (93)	125 (78)
Number of children in the household	4	4 (3–5)	4 (3–5)	3 (3–5)	4 (3–5)
Smoker in the household	2	122 (13)	64 (10)	14 (11)	44 (28)
Access to grid electricity	21	696 (76)	579 (92)	117 (93)	160 (100)
Access to running water	490	76 (17)	40 (13)	30 (73)	6 (6)
Cooking method in household	166				
- Coal		2 (0.3)	2 (0.3)	0	0
- Gas		771 (99.6)	643 (99.5)	124 (100)	4 (100)
- Electricity		1 (90.1)	1 (0.2)	0	0

[a] All data except BMI is based on the history provided by the children's parent or guardian.

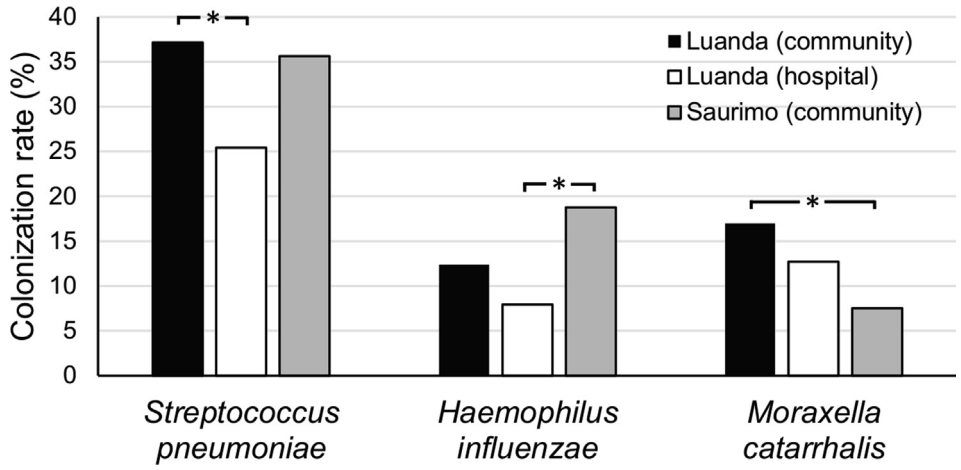

Fig. 1. Nasopharyngeal colonization of *S. pneumoniae, H. influenzae* and *M. catarrhalis* among Angolan children aged 4–12 years in community settings in Luanda and Saurimo, Lunda Sul, and 2 hospitals in Luanda. A statistically significant difference (with Bonferroni correction; $p < 0.017$) between any two collection sites is indicated with an asterisk.

Table 2
Logistic regression analysis assessing association of clinical and demographical variables with pneumococcal carriage. ORs with 95% CIs indicating statistical significance are shown in bold. BMI, body mass index.

Variable	Missing data (*n*)	Frequency of variable (carriers/total)	Univariate regression (OR [95% CI])	Multivariate regression (OR [95% CI])[a]
Age[b]	0		0.95 (0.89–1.01)	0.95 (0.88–1.02)
Female sex	0	158/444	1.02 (0.78–1.34)	1.06 (0.77–1.46)
BMI < 5th percentile	1	47/113	1.35 (0.91–2.02)	1.10 (0.69–1.76)
Site of inclusion	0			
- Luanda (community)		243/654	= reference	= reference
- Luanda (hospital)		32/126	**0.58 (0.37–0.89)**	0.43 (0.17–1.07)
- Saurimo (community)		57/160	0.94 (0.65–1.34)	0.61 (0.30–1.24)
Chronic ear/auditory or respiratory tract symptoms	167	18/49	0.91 (0.50–1.65)	1.59 (0.77–3.29)
Ongoing infectious symptoms	134	18/59	0.70 (0.40–1.25)	1.14 (0.48–2.72)
Antibiotic treatment last month	142	8/62	**0.22 (0.10–0.47)**	**0.27 (0.09–0.83)**
Vaccinated according to schedule	144	244/642	0.99 (0.69–1.42)	1.09 (0.73–1.61)
$\geq$ 5 children in the household	4	139/381	1.09 (0.83–1.43)	0.90 (0.65–1.24)
Smoker in the household	2	44/122	1.03 (0.70–1.54)	1.55 (0.96–2.51)
Access to grid electricity	21	243/696	0.87 (0.64–1.19)	0.97 (0.52–1.83)
Access to running water	490	28/76	0.95 (0.57–1.59)	–
H. influenzae colonization	0	70/121	**2.92 (1.98–4.31)**	**2.37 (1.51–3.72)**
M. catarrhalis colonization	0	67/139	**1.88 (1.31–2.71)**	1.43 (0.92–2.20)

[a] The total number of cases included in the multivariate model was 688 (73%). All variables except "access to running water", which was excluded due to the large proportion of missing data, were included in the multivariate model.
[b] Continuous variable.

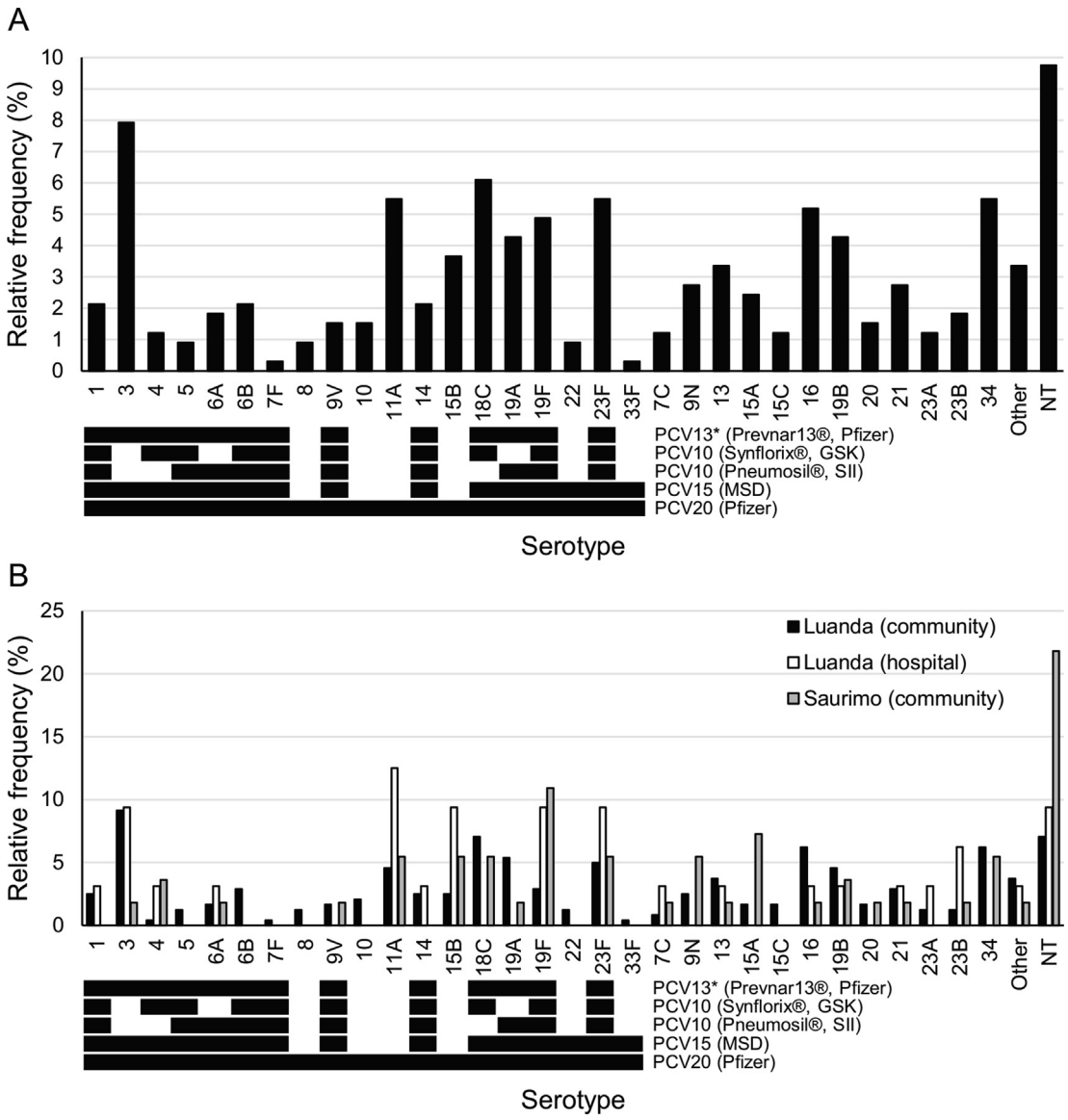

Fig. 2. Serotype distribution of *S. pneumoniae* detected in nasopharyngeal samples from Angolan children aged 4–12 years in community settings in Luanda and Saurimo, Lunda Sul, and 2 hospitals in Luanda. The overall distribution of pneumococcal serotypes (A) and their relative frequency at each study site (B) are presented. Serotypes included in different currently marketed or upcoming PCVs are grouped to the left on the x-axis indicated by the black bars below. The proportion of serotyped pneumococci covered was 41% for the PCV13 (Prevnar13® [Pfizer, New York City, NY]; indicated by asterisk) currently used in Angola compared to 27% ($p < 0.001$) for Synflorix® (GlaxoSmithKline, Brentford, UK), 26% ($p < 0.001$) for Pneumosil® (Serum Institute of India, Pune, IN), both PCV10, and 42% ($p = 0.8$) for PCV15 (Merck Sharp & Dohme, Kenilworth, NJ) and, finally, 54% ($p = 0.001$) for PCV20 (Pfizer). NVTs representing < 1% of all isolates are pooled as "Other". SII, Serum Institute of India; GSK, GlaxoSmithKline; MSD, Merck Sharp & Dohme.

Fig. 2. A large proportion of analyzed serotypes were included in PCV13 (n = 134; 41%), representing 5 of the 10 most common serotypes (serotypes 3 [8%], 18C [6%], 23F [6%], 19F [5%] and 19A [4%]), and we conclude a VT carriage rate of 14%. The most common NVTs were 11A (6%), 34 (6%), 16 (5%), 19B (4%) and 15B (4%). The proportions of serotypes covered by other current or proposed PCVs are indicated in Fig. 2. Most *H. influenzae* were determined to be NTHi (n = 109; 90%). The 12 (10%) encapsulated strains comprised 7 Hif, 2 Hie, and 1 isolate each of Hib, Hic and Hid. The results from MALDI-TOF MS typing were consistent with those from PCR typing except for a few cases of NTHi (n = 4), which were misclassified as Hie by MALDI-TOF MS typing [17,18].

3.4. Non-susceptibility to penicillin is common among pneumococci

Antimicrobial susceptibility rates of the isolated bacteria are presented in Table 3. Penicillin non-susceptibility was common among *S. pneumoniae* (40%), but all PNSP isolates exhibited only intermediate resistance with a median MIC of 0.19 μg/mL (IQR 0.13–0.25 μg/mL), and no differences were noted between the study sites. PCV13-included serotypes were significantly associated with penicillin non-susceptibility (50% vs. 33%; p = 0.003). Prevalence of PNSP within specific serotypes is outlined in Fig. 3. Twenty-four (7%) MDR *S. pneumoniae* isolates, *i.e.*, non-susceptible to ≥ 3 antibiotics, were detected and further characterized with WGS and broth microdilution (Table 4).

Table 3
Antimicrobial susceptibility patterns of isolated bacteria. Figures are presented as *n* (%).

	S. pneumoniae	*H. influenzae*	*M. catarrhalis*
Benzylpenicillin[a]			
- S	195 (60)	103 (85)[b]	3 (2)[b]
- I	130 (40)		
- R		18 (15)	136 (98)
Amoxicillin			
- S	322 (99)	106 (88)	
- I	1 (0.3)		
- R	2 (0.6)	15 (12)	
Erythromycin			
- S	322 (99)		
- I	1 (0.3)		
- R	2 (0.6)		
Clindamycin			
- S	232 (99)		
- R	2 (0.6)		
Tetracycline			
- S	257 (79)	105 (87)	135 (97)
- I	12 (4)		
- R	56 (17)	16 (13)	4 (3)
Trimethoprim-sulfamethoxazole			
- S	57 (18)	19 (16)	41 (29)
- I	6 (2)		
- R	262 (81)	102 (84)	98 (71)
Norfloxacin			
- S	324 (99.7)		
- R	1 (0.3)		

[a] Intravenous non-meningitis EUCAST breakpoints for *S. pneumoniae*; 0.06 < I ≤ 2 μg/mL.
[b] Screening substance for betalactamase-production.

3.5. Several global pneumococcal sequence clusters (GPSC) occur among MDR pneumococci but no dominating cluster was found

To further investigate molecular epidemiology and resistance mechanisms of pneumococcal MDR isolates, these were subjected to WGS. Identified GPSCs, MLST sequence types (STs), capsular genotypes, and resistance genes are listed in Table 4. The two most common GPSCs found were 94 (*n* = 7) and 10 (*n* = 5), predominantly comprising serogroup 23 and serotype 3, respectively. Two isolates (*S. pneumoniae* #110 and #152) shared 6/7 alleles with Pneumococcal Molecular Epidemiology Network (PMEN; https://www.pneumogen.net/pmen/) clones Spain^{9V}-3 ST156 and Taiwan^{19F}-14 ST236, respectively. Importantly, identification of cytosine at the 203 position of 16S rRNA sequence confirmed *S. pneumoniae* species in 25/26 analysed isolates, while the remaining isolate (#123) was revealed to be *Streptococcus pseudopneumoniae* through MLSA. Results corresponding to this isolate have been omitted from previous sections but are included in Table 4 for the sake of completeness.

4. Discussion

Previous studies on the microbiology of pneumococci in Angola are scarce, a fact that limits the possibilities to forecast vaccine effects and to make future evaluations. We have conducted a nasopharyngeal carriage study in children that have not been targeted by PCV immunization (aged 4–12 years in 2017). Importantly, our results reveal that 41% of colonizing pneumococci were covered by PCV13 4 years after the introduction of the vaccine and that these serotypes were more frequently PNSP (50% vs. 33%).

An overall pneumococcal carriage rate of 35% was detected, slightly lower than the 43% reported among 5 to 15-year-old children in a systematic review of pneumococcal carriage in sub-Saharan Africa, but the rates varied considerably in the included studies that were performed prior to introduction of PCV [32]. In a study including unvaccinated 5 to 15-year-old children after introduction of PCV in Malawi, pneumococcal carriage was detected in 37% of 89 individuals [33]. Colonization by *H. influenzae* and recent antibiotic use were the only two studied variables that were significantly associated with pneumococcal carriage in a multivariate logistic regression (Table 2). A positive but non-significant trend (OR 1.43) was also seen in the association between *M. catarrhalis* and pneumococci. The positive association between colonization with the three studied species has been described earlier [34]. This finding may both be explained by inter-species "cooperation" or be dependent on other host or environmental factors [4]. Unfortunately, many answers recorded regarding medical conditions and symptoms of the studied individuals were inconclusive which is a limitation for these analyses.

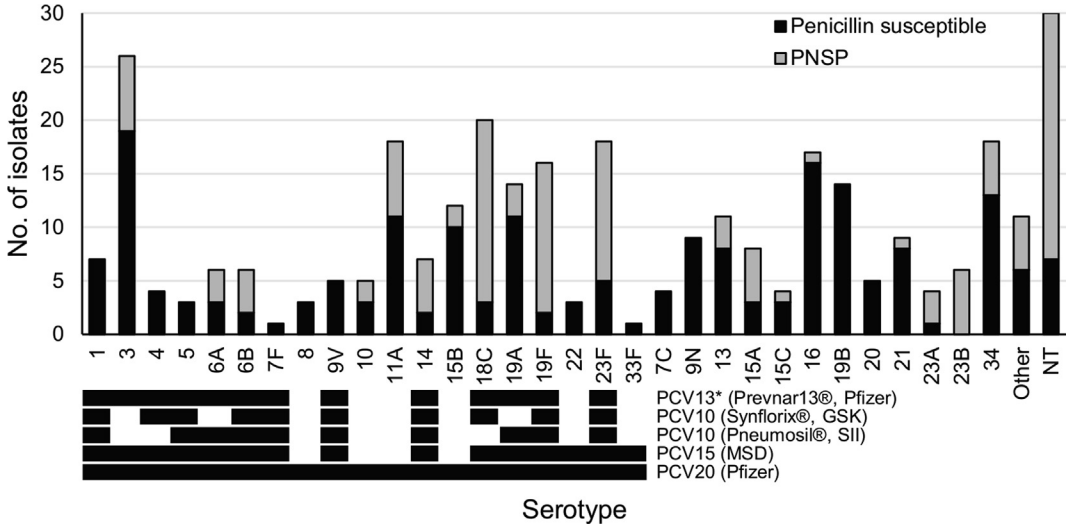

Fig. 3. Prevalence of PNSP among detected pneumococcal serotypes. Serotypes included in different currently marketed or upcoming PCVs are grouped to the left on the x-axis indicated by the black bars below. Penicillin non-susceptibility was more frequent among serotypes included in the PCV13 currently used in Angola (indicated by asterisk) than non-PCV13 serotypes (50% vs. 33%; $p = 0.003$). Penicillin non-susceptibility is defined as a minimal inhibitory concentration > 0.06 mg/L. NVTs representing < 1% of all isolates are pooled as "Other". SII, Serum Institute of India; GSK, GlaxoSmithKline; MSD, Merck Sharp & Dohme.

Table 4
Findings regarding species and serotype determination, molecular epidemiology, resistance patterns and resistance mechanisms of pneumococcal MDR isolates analysed with WGS. Twenty-four (7%) isolates exhibited MDR phenotype according to EUCAST 2020 clinical breakpoints and were analysed with WGS and broth microdilution. Two additional isolates were included in the analyses, but were revealed to be a *Streptococcus pseudopneumoniae* (#123) and a non-MDR *S. pneumoniae* (#795), respectively. GPSC, global pneumococcal sequence cluster; MLST, multi-locus sequence type; PcG, benzylpenicillin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; TMP-SMX, trimethoprim-sulfamethoxazole; MOX, moxifloxacin; NR, not relevant.

No.	Serotype	Capsular genes	16S rRNA position 203	GPSC	MLST[a]	PcG[b]	ERY	CLI	TET	TMP-SMX	MOX	*ermB*	*mefA/E*	*tetM*	PBP1a	PBP2b	PBP2x	Inferred PcG MIC
16	10	10F	C	Novel	Novel1 (2–57-62–3-6–88-309)	I (0.12)	I (0.5)	S	R (>8)	R (4)	S	–	+	+ (Tn916)	2	183	242	0.12
31	6B	6E	C	129	3207	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn1545)	Novel	Novel	Novel	0.12
110	15C	15C	C	6	166	I (2.0)[e]	R (>2)	R (>1)	S	R (>4)	S	+	–	NR	15	12	7	2
123[c]	NT	–	A	–		I (0.12)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	–	–	–	–
152	19F	19F	C	1	271	I (2.0)[e]	R (>2)	R (>1)	R (>8)	R (>4)	S	+	+	+ (Tn5251)	13	49	8	4
153	38	38	C	117	6103	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	2	Novel	Novel	0.25
186	NT	NT	C	Novel	Novel2 (1–9-novel-154–15-1–262)	I (0.5)	S	S	R (>8)	R (>4)	R (2)	NR	NR	+ (Tn916)	Novel	176	Novel	0.25
214	11A	11A	C	Novel	Novel3 (1–16-54–1-9–1-824)	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	Novel	Novel	Novel	0.25
229	38	38	C	117	6103	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	2	Novel	Novel	0.25
312	23A	10A/23A	C	Novel	Novel4 (2–5-54–63-?-6–8)	I (0.25)	S	S	R (>8)	R (4)	S	NR	NR	+ (Tn1545)	150	1	242	0.25
329	23F	23F	C	94	Novel5 (7–16-8–8-6–60-14)	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	19	Novel	Novel	0.25
505	3	3	C	10	700	I (0.5)	S	S	R (>8)	R (4)	S	NR	NR	+ (Tn1545)	17	15	22	0.5
535	3	3	C	10	700	I (0.5)	S	S	R (>8)	R (4)	S	NR	NR	+ (Tn1545)	17	15	22	0.5
543	6A	6A	C	62	912	I (0.5)	S	S	R (8)	R (>4)	S	NR	NR	+ (Tn916)	192	Novel	Novel	0.5
561	23F	23F	C	94	Novel6 (7–16-8–8-6–60-17)	I (0.12)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	12	Novel	Novel	0.25
570	3	3	C	10	700	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn1545)	17	15	22	0.5
585	19F	19F	C	Novel	9716	I (0.12)	S	S	R (>8)	I (2)	S	NR	NR	+	19	Novel	Novel	0.25
595	23F	23F	C	94	Novel5 (7–16-8–8-6–60-14)	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	12	Novel	104	0.12
631	23F	23F	C	94	Novel6 (7–16-8–8-6–60-17)	I (0.12)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	12	Novel	Novel	0.25
632	10	10A	C	10	3135	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn1545)	150	1	22	0.25
689	3	3	C	10	700	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn1545)	17	15	Novel	0.5
705	19F	19F	C	94	Novel7 (7–16-8–8-6–142-9)	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	19	Novel	Novel	0.25
706	15A	15A	C	Novel	Novel8 (1–8-73–47-36–1-6)	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+	Novel	Novel	Novel	0.25

Table 4 (*continued*)

No.	Serotype	Capsular genes	16S rRNA position 203	GPSC	MLST[a]	PcG[b]	ERY	CLI	TET	TMP-SMX	MOX	*ermB*	*mefA/E*	*tetM*	PBP1a	PBP2b	PBP2x	Inferred PcG MIC
795[d]	23F	23F	C	94	Novel5 (7–16-8–8-6–60-14)	I (0.25)	S	S	R (>8)	S	S	NR	NR	+ (Tn916)	12	Novel	104	0.12
879	23F	23F	C	94	Novel9 (7–16-novel-8–6-60–14)	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	12	Novel	104	0.12
886	NT	NT	C	Novel	Novel10 (7–9-1–38-13–1-74)	I (0.12)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn1545)	Novel	0	Novel	0.12

[a] Allele signatures of novel MLSTs are presented as (*aroE-gdh-gki-recP-spi-xpt-ddl*).
[b] Intravenous non-meningitis EUCAST breakpoints; 0.06 < I ≤ 2 μg/mL
[c] Identified as *S. pseudopneumoniae* using MLSA.
[d] Isolate *S. pneumoniae* #795 was initially classified as MDR but was revealed to be susceptible to trimethoprim-sulfamethoxazole and is therefore not classified as MDR.

In the present study, it is unknown to which extent the detected serotype distribution has been indirectly affected by the infant immunization programme. Nevertheless, nasopharyngeal carriage of VT pneumococci was confirmed to still exist in children (14%) at our study sites, where the vaccine coverage of PCV13 (3 dosages) was 52–84% in 2017 (according to WHO/UNICEF and official Angolan estimates). In recent studies performed in Malawi, Ghana and Tanzania after PCV-introduction it has been noted that VT carriage persists in children even if a decrease is observed [35–37]. Several factors may contribute to these findings, including incomplete vaccine coverage, lack of a booster dose during the second year of life resulting in waning serotype-specific immunity, and transmission of VT pneumococci from parents and older siblings [35,37,38]. Taken together, it is crucial that the vaccine coverage of PCV in Angolan children is increased, but an alternate vaccination schedule (2 + 1 or 3 + 1) is likely also to be of importance to eliminate VT transmission [39].

The two most common serotypes found, VTs 3 (8%) and 18C (6%), have been identified in Malawi [33], Ghana [35], Tanzania [36], The Gambia [38] and, Uganda [40], but generally less frequently than in our study. Both serotypes are common causes of IPD [41]. However, the effect of PCV13 on serotype 3 carriage is likely lower than for other included serotypes which may contribute to its continued transmission [42]. The NVTs presented in Fig. 2 generally have low invasive disease potential but do cause substantial numbers of IPD worldwide as they become more prevalent in carriage [41]. Specifically, this applies to serotypes 9N, 10A, 11A, 15A/B/C, 23A and 23B [6]. Future higher-valency PCVs may become impotant to prevent emerging NVTs [43]. Among currently marketed and proposed PCVs, Pfizer's PCV20 candidate exhibited a higher coverage (54%) of the detected serotype distribution than the currently used PCV13 (41%) (Fig. 2).

Penicillin non-susceptibility was more common among VT strains (50% vs. 33%), explained by high rates among serotypes 6A, 6B, 14, 18C, 19F, and 23F. Further use of PCV may therefore result in decreasing rates of PNSP in Angola which has been observed in Ghana [35]. However, some NVTs were also frequently PNSP, specifically serotypes 15A, 23A, and 23B which has been previously reported and risk to attenuate vaccine effects on total PNSP prevalence [6,44]. Despite the high frequency of PNSP, virtually all pneumococci (99%), as well as the majority of *H. influenzae* (88%), were susceptible to amoxicillin. Most bacteria were non-susceptible against trimethoprim-sulfamethoxazole (83%) which was similarly reported recently from Tanzania and may be related to high use of the substance as prophylactic treatment in HIV-positive individuals [36]. Based on the results, amoxicillin is a feasible empirical treatment of respiratory tract infections and AOM in outpatient settings in Angola while tetracycline and, in particular, trimethoprim-sulfamethoxazole are unfavourable choices. Broth microdilution was only used to determine MICs of MDR isolates which is a limitation of the study as gradient tests may underestimate the MICs of penicillin for *S. pneumoniae* 1–2 dilution steps [45]. However, even though this may affect the presented median penicillin MIC, it is unlikely that the SIR classifications were affected as most MICs of penicillin detected were in the low-intermediate range.

Studies of the molecular epidemiology of *S. pneumoniae*, in addition to serotyping, in relation to PCV implementation are of importance as virulence of specific strains may be as dependent on genetic lineage as serotype, and the latter may switch within a lineage [28]. We performed a limited, explorative investigation with WGS of molecular epidemiology and resistance genes among MDR pneumococci. All serotype 3 and serogroup 23 isolates belonged to GPSCs 10 and 94, respectively. According to the Global Pneumococcal Sequencing Project database [46] GPSC10 has been identified globally and exhibit several serotypes, but all serotype 3 isolates have been reported from sub-Saharan Africa and, similar to our results, exhibit ST700. All GPSC94 isolates previously reported have been found in sub-Saharan Africa and mostly exhibit serotypes 19A or 23F. These findings suggest that clonality may explain multi-resistant phenotypes among *S. pneumoniae* in Angola. Many different GPSCs and STs were seen, however, and too few isolates were studied to draw any conclusions. Further studies on the molecular epidemiology of pneumococci, including both carriage and disease-associated isolates, are needed to clarify which genetic lineages may be of significant clinical importance in Angola.

Whole genome sequencing revealed that one isolate identified as *S. pneumoniae* belonged to the closely related species *S. pseudopneumoniae*. The incorrect classification of this isolate exemplifies

the difficulty to discriminate *S. pneumoniae* from other mitis group streptococci [47]. The fact that no additional, but labour intense, methods were used to confirm pneumococcal species is a limitation of this study. It is possible that the high number of NT pneumococci observed may include additional incorrectly identified *S. pseudopneumoniae*.

Capsule typing of *H. influenzae* revealed that the majority of colonizing strains were indeed NTHi (90%). The high agreement in typing results for MALDI-TOF MS and PCR underlines the strength of MALDI-TOF MS as a rapid method for capsule typing of *H. influenzae*. A few NTHi were misclassified as Hie by MALDI-TOF MS. This is a known limitation of the MALDI-TOF MS typing method and isolates classified as Hie should be confirmed by PCR typing [18]. Only a single isolate of Hib was detected, a finding that is consistent with a Kenyan study of Hib carriage detecting carriage in only 2 of 1408 children aged > 5 years, after the introduction of the conjugated Hib vaccine [48]. Even though the estimated coverage of Hib-immunization, alike PCV-coverage, has been suboptimal since its introduction in 2006 [10], the low carriage prevalence and previously reported decline of meningitis and empyema caused by *H. influenzae* may most likely be related to a vaccine effect [13,49].

5. Conclusions

The current study has provided a first description of the spectrum of pneumococci circulating among unvaccinated children in Angola four years after the introduction of PCV13 in the national childhood immunization programme. Enduring nasopharyngeal carriage of VT pneumococci, which were more often PNSP, indicate that continued, and more comprehensive administration of PCV13 may have positive effects on pneumococcal disease in the country. Additionaly, a vaccine schedule including a booster dose may reduce transmission of VT pneumococci further and future higher-valency vaccines may have a broader impact. Further investigations of phenotypic and genetic characteristics of colonizing and infecting pneumococci in Angola are of great importance for assessing effects of the vaccination programme and for formulating future effective treatment and prevention strategies.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: FU, HCS and KR participate in projects supported by Pfizer. TP has participated in projects supported by Sanofi Pasteur. Remaining authors declare no conflicts of interest.

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