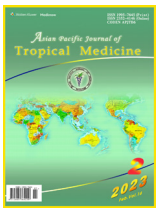

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Molecular epidemiology of piliated pneumococcal isolates at a major tertiary hospital in the Klang Valley, Malaysia

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ABSTRACT

Objective: To characterise a collection of pili-carrying and none pili-carrying pneumococcal isolates of clinical origin for serotypes, antibiotic resistance and genotype.

Methods: In total, 42 clinical isolates were collected between October 2017 and December 2019. Those isolates were analysed for antimicrobial susceptibility, serotype distribution, detection of pneumococcal virulence and pilus genes. Multilocus sequence typing was performed only for piliated isolates, followed by phylogenetic analysis.

Results: The common isolation sites among the pneumococcal isolates were tracheal aspirate (28.6%), blood (26.2%), and sputum (23.8%). Fifty percent isolates were resistant to erythromycin, tetracycline (50.0%) and trimethoprim-sulfamethoxazole (43.0%). The most frequent were serotypes 19F (28.6%), 6A/B (23.8%) and 19A (14.3%). Piliated isolates were detected in a small proportion (33.3%); 64.3% were multidrug-resistant. ST320 was the prevalent sequence type among the piliated isolates and genetically related to the Pneumococcal Molecular Epidemiology Network clones Taiwan^{19F}-14 (CC271). In the phylogenetic analysis, some piliated isolates showed a close association having similar ST320, carrying serotype 19A and both pilus genes indicating their clonal spread.

Conclusions: Pneumococcal lineages of piliated isolates have been globally disseminated and pili could have played a role in the spread of antibiotic resistant clones.

KEYWORDS: Pneumococci; Pili; Genotype; Malaysia

1. Introduction

Streptococcus (S.) pneumoniae is a Gram-positive bacterial pathogen that may asymptomatically colonise the upper human respiratory tract and it has the potential to cause conjunctivitis, otitis media,

Significance

This study showed the persistence of ST320, being the most common sequence type among piliated isolates and genetically related to the global antibiotic-resistant Pneumococcal Molecular Epidemiology Network clones Taiwan^{19F}-14 (CC271). The phylogenetic analysis also indicated a close genetic lineage among piliated isolates indicating their successful spread locally and globally.

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lower respiratory tract infection, bacteremia, and meningitis[1,2]. According to a World Health Organization (WHO) report, *S. pneumoniae* is the leading cause of mortality and morbidity, killing half-a-million children under the age of five each year, with most of the deaths having been reported particularly in developing countries, including Africa and Asia[3,4]. The incidence of pneumococcal diseases varies considerably, depending on the age of patients, particularly young children (less than 5 years old) and the elderly (over 60 years old)[4].

The polysaccharide capsule in *S. pneumoniae* is considered to be the most important virulence factor, with 100 serotypes having been identified thus far[5]. A 7-valent conjugate vaccine (PCV7) that offers protection against seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F), was introduced and made available for children in 2000 in the United States, while the 10-valent (PCV10; 1, 5 and 7F) and 13-valent (PCV13; 3, 6A and 19A) were introduced in 2008 and 2009, respectively, resulting in a greater impact in reducing hospitalisations for pneumonia, particularly in young children[6]. Recently, the discovery of a long, filamentous, pilus-like structure in Gram-positive bacteria, which are uncommonly characterised specifically in *S. pneumoniae*, has added another element to the pneumococcal virulence regiment. To date, two pili islets have been detected in pneumococci, namely PI-1 and PI-2[7,8].

Pili have recently been associated with pneumococcal pathogenesis in humans. The information regarding piliated isolates in Malaysia is scarce. In addition, most studies focused mainly on demographics, the antimicrobial susceptibility pattern and serotype distribution[9,10]. There were also fewer studies on the molecular genotyping and virulence gene profiles, particularly pili gene, especially in piliated pneumococcal isolates. Multilocus sequence typing (MLST) which serves as a portable approach for tracing global dissemination patterns would address the evolutionary genetic lineage of the piliated strains. Given the potentially life-threatening effects that pili might contribute in terms of disease development, it is important to characterise piliated isolates to address the outstanding questions related to its epidemiology, population and evolution. Therefore, a collection of clinical pneumococcal isolates from a major tertiary hospital in the densely populated Klang Valley of Malaysia was characterised for their demographic, antibiotic susceptibility, serotypes, virulence factors including pilus genes, and MLST on piliated isolates.

2. Subjects and methods

2.1. Ethical considerations

Ethical approval for this descriptive study was granted by the Medical Research and Ethics Committee of the Malaysian Ministry of Health, National Medical Research Register (approval no. NMRR 17-1025-35696).

2.2. Bacterial isolates

Clinical isolates of *S. pneumoniae* were obtained from the Microbiology Laboratory of the Department of Pathology at Sungai Buloh Hospital (HSB), Selangor. This is a major tertiary hospital with over 600 licensed beds and located in Klang Valley, Selangor, west coast of Peninsular Malaysia. A total of 42 clinical isolates were collected between October 2017 and December 2019. Phenotypic and genotypic analyses were performed on the isolates in October 2019 to February 2021. Each clinical isolate was acquired from a different individual, from both invasive and non-invasive sites. *S. pneumoniae* ATCC 49619 was used as a positive control for the standard confirmatory test. The pneumococcal isolates were confirmed as *S. pneumoniae* by the following characteristics; α-haemolysis, bile solubility and susceptibility to ethylhydrocupreine disc (optochin).

2.3. Antimicrobial susceptibility test

The antibiotic susceptibility testing of all isolates was performed by disk diffusion method for erythromycin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin (Oxoid, USA). Meanwhile, the E-test method (BioMérieux, France) was performed to determine the minimal inhibitory concentration (MIC) of penicillin, ceftriaxone, and cefotaxime. Both methods and interpretation of the results were performed according to the manufacturer's instruction and the Clinical and Laboratory Standard Institute (CLSI) for meningitis and non-meningitis[11]. Multidrug-resistance (MDR) was defined as resistant to three or more antimicrobial agents of different classes[12]. Quality control analysis was performed using *S. pneumoniae* ATCC 49619.

2.4. Genomic DNA extraction

Genomic DNA was extracted by the GeneAll Exgene kit (GeneAll Biotechnology Co. Ltd, Korea) as per the manufacturer's instructions.

2.5. Detection of S. pneumoniae virulence and pilus genes

Commonly virulence-associated genes of *S. pneumoniae* (*ply*, *lytA*, *cbpA*, *pavA* and *pspA*) and pilus genes (*rlrA*, *rrgA*, *rrgC* and *sipA*) were screened using PCR with primers and running conditions, as previously described[13]. PCR reactions were performed in BioRadMyCycler™ Thermal Cycler (BioRad, USA).

2.6. Determination of capsular types

Pneumococcal capsular types of pneumococcal isolates were performed by sequential multiplex PCR using published primers recommended by the Center for Disease Control and Prevention[14]. Primers were sorted into six multiplex sets named A, B, C, D, E and F, as previously described[15,16]. Four different serotypes and a pair of primers targeting a conserved region of the pneumococcal capsular polysaccharide synthesis gene (*cpsA*) to serve as an internal positive control were included in each set of primer pairs.

2.7. Multilocus sequence typing (MLST)

MLST was subjected only to pili-carrying pneumococcal isolates in this study. The following seven housekeeping genes, namely, *aroE, gdh, gki, recP, spi, xpt*, and *ddl*, were amplified by PCR, sequenced and analysed as previously described[17]. Allele numbers and sequence type (ST) of piliated isolates were assigned according to the PubMLST database (https://pubmlst.org/organisms/streptococcus-pneumoniae). Sequence and STs that could not be found in the database were submitted to the curator as new novel STs. The PHYLOViZ software was aligned for assigning the isolates with clonal complexes (CC), defined as cluster sharing at least five out of seven common alleles. The STs of piliated isolates in our study were compared to Pneumococcal Molecular Epidemiology Network (PMEN) clones in the PMEN database (https://www.pneumogen.net/gps/pmen.html).

2.8. Phylogenetic analysis

Analysis of the sequenced DNA of MLST genes was also performed using MEGA software version 7[18], which is available on the www.megasoftware.net website. The sequences were trimmed and full contiguous sequences were generated from the forward and reverse primer sequences. The nucleotide sequences were aligned using MUSCLE, which is available through the same software. The available reference 49347, 13506, 38581, 8663, 12544, 7083, 126508 and 38687 for the respective STs were retrieved from the MLST database and included in the analysis as the control. The phylogenetic tree was constructed by MEGA7 using the concatenated sequences (all seven genes were combined) of the products and with the reference sequences included. The phylogenetic tree was constructed using the maximum-likelihood technique based on the Tamura-Nei model, while the reliability of the tree was estimated through bootstrap analysis with 1 000 replicates.

2.9. Statistical analysis

Chi-square was used to compare the demographic characteristics of the patients with the phenotypic and genotypic variables of the pneumococcal isolates; these included their ages, sex, the sites of isolation, vaccine/non-vaccine serotypes, and the piliated isolates. Statistical significance was indicated by $P<0.05$.

3. Results

3.1. Demographic data

Among the 42 pneumococcal isolates collected within the study period, the most frequent site of isolation was tracheal aspirate (n=12; 28.6%) followed by blood (n=11; 26.2%), sputum (n=10; 23.8%) and pus (n=4; 9.5%). The remaining isolates were from nasopharynx (n=3; 7.1%), bronchial aspirate (n=1; 2.4%), and cerebrospinal fluid (n=1; 2.4%). The ages of patients ranged from one month to 90 years old. Demographic analysis showed the isolation frequency of pneumococcal isolates was slightly higher in male (n=28; 66.7%) compared to female subjects (n=14; 33.3%). The common pattern for age group distribution of pneumococcal isolates was 13-50 years (n=19; 45.2%); followed by >50 years (n=12; 28.6%); and ≤5 years (n=11, 26.2%). Table 1 depicts the association of isolation sites; invasive and non-invasive, which showed no significant association in relation to sex, age, vaccine serotype, multidrug resistance and piliated isolates.

Table 1. Isolation sites of invasive and non-invasive source among pneumococcal isolates in relation to age, sex, vaccine serotypes, multidrug-resistant (MDR) pattern and pilus genes.

Site of isolation	[a]Invasive (n=12)	[b]Non-invasive (n=30)	P-value
Age			
≤ 12 years (n=11)	4	7	0.443
>12 years (n=31)	8	23	
Sex			
Male (n=28)	8	20	0.501
Female (n=14)	4	10	
Vaccine serotypes			
Vaccine serotype (n=32)	9	23	0.13
Non-vaccine serotype (n=10)	3	7	
MDR pattern			
[c]MDR (n=11)	2	9	0.37
[d]Non-MDR (n=31)	10	21	
Pilus genes			
Piliated strains (n=14)	3	11	0.468
Non-piliated strains (n=28)	9	19	

[a]Invasive site (blood, cerebrospinal fluid); [b]Non-invasive site (tracheal aspirate, sputum, pus, bronchial aspirate, nasopharyngeal); [c]MDR= resistance to ≥ 3 antibiotics; [d]Non-MDR=resistance to < 3 antibiotics.

3.2. Antimicrobial susceptibility profile

Meningitis and non-meningitis susceptibilities of beta lactam antibiotics breakpoint were used for penicillin, ceftriaxone, and cefotaxime. Only one isolate from cerebrospinal fluid was associated with meningitis, while other isolates were assumed as non-meningitis in this study. Among the 42 pneumococcal isolates, the majority were resistant to erythromycin (*n*=21; 50%), tetracycline (*n*=21; 50%), and trimethoprim-sulfamethoxazole (*n*=18; 43%). Meanwhile, 26.2% (11/42) of the total collection was classified as MDR in this study, with only two invasive isolates had MDR phenotype (*n*=2; 18.2%). None of the pneumococcal isolates were resistant to penicillin. All isolates were also susceptible to vancomycin, ceftriaxone and cefotaxime. The distribution of antimicrobial susceptibilities for all isolates in this study is shown in Table 2.

Table 2. Antimicrobial susceptibility pattern among the collection of 42 isolates.

	Susceptible	Intermediate	Resistant
Penicillin	100%	-	-
Ceftriaxone	100%	-	-
Cefotaxime	100%	-	-
Erythromycin	45%	5%	50%
Tetracycline	43%	7%	50%
Trimethoprim-sulfamethoxazole	52%	5%	43%
Vancomycin	100%	-	-

Susceptibility test by E-test (minimal inhibitory concentration determination) for penicillin, ceftriaxone and cefotaxime. Susceptibility test by disk diffusion for tetracycline, erythromycin, trimethoprim-sulfamethoxazole and vancomycin.

3.3. Distribution of serotypes

Among the 42 pneumococcal isolates, 39 were successfully serotyped, while the other three were classified as non-typeable as they were not amplified for any of the molecular targets, including *cpsA* which served as the internal positive control. There were ten different serotypes with six different serogroups; the most prevalent was serotype 19F (*n*=12; 28.6%), followed by serotypes 6A/B (*n*=10; 23.8%), 19A (*n*=6; 14.3%), 15B/C (*n*=3; 7.1%), 7A/F (*n*=2; 4.8%), 23A (*n*=2; 4.8%), 4 (*n*=1; 2.4%), 14 (*n*=1; 2.4%), 23F (*n*=1; 2.4%) and 11A/D (*n*=1; 2.4%). Meanwhile, serotype 19F was the most dominant among invasive isolates (*n*=3, 25.0%).

For immunisation vaccine coverage, the proportion of isolates expressed the serotypes in PCV10 and PCV13 were 64.3% (27/42) and 78.6% (33/42), respectively. Serotype 15B/C was predominant among non-vaccine serotypes with three isolates (7.1%). Serotype distributions varied according to patients' age group (Table 3). Serotype 6A/B (*n*=6; 31.6%) showed a high percentage in subjects of 13-50 years, while among subjects in the >50 years group, the most common serotypes was 19F (*n*=6; 50.0%).

Table 3. Serotype distribution of pneumococcal isolates in relation to age group.

Serotype	≤ 5 years (*n*=11)	6-12 years (*n*=0)	13-50 years (*n*=19)	>50 years (*n*=12)	Total (*n*=42)
4	-	-	1	-	1
6A/B	2	-	6	2	10
7A/F	-	-	2	-	2
11A/D	1	-	-	-	1
14	-	-	1	-	1
15B/C	1	-	2	-	3
19A	3	-	1	2	6
19F	3	-	3	6	12
23A	-	-	2	-	2
23F	-	-	1	-	1
Non-typeable	1	-	-	2	3

3.4. Occurrence of virulence and pilus genes

Ply, *lytA*, *cpbA*, *pavA*, and *pspA* were detected in all 42 pneumococcal isolates. However, only 14 isolates (33.3%) carried at least one of the pilus genes. Of these, six isolates (42.9%) were detected for PI-1 alone, one isolate (7.1%) for PI-2 alone, and seven isolates (50.0%) presented both genes (PI-1+ PI-2). The isolation sites among the piliated isolates were from both tracheal aspirate and sputum at 28.6% (4/14) and 28.6% (4/14), respectively. The majority of PI-1 alone and both PI-1+PI-2 isolates belonged to serotype 19F (*n*=5; 35.7%), followed by serotype 19A (*n*=4; 28.6%), and 6A/B (*n*=3; 21.4%). PI-2 alone isolate was observed only in serotype 7A/F.

The frequency of piliated pneumococcal isolates targeted by PCV10 and PCV13 was 64.3% (9/14) and 92.3% (13/14), respectively. In relation to antibiotic susceptibility pattern, isolates with PI-1 alone and both PI-1+PI-2 were frequently resistant to erythromycin (*n*=11; 79%), tetracycline (*n*=10; 71%), and trimethoprim-sulfamethoxazole (*n*=11; 79%). None of the piliated isolates were resistant to penicillin, ceftriaxone, cefotaxime and vancomycin. The only PI-2 alone-isolate was susceptible to all antibiotics except for trimethoprim-sulfamethoxazole. Of this PI-1 alone and both PI-1+PI-2 isolates, nine (64.3%) were MDR. Meanwhile, our study found only three piliated isolates from the invasive site out of fourteen isolates carrying pilus genes. The three isolates presented both PI-1+PI-2 isolates and had MDR phenotype (Table 4).

3.5. Multilocus sequence typing (MLST) analysis

MLST was subjected only to pneumococcal isolates carrying pilus genes (*n*=14). All the respective amplicons from the isolates matching the expected DNA band size were successfully sequenced yielding distinct nine STs. The most frequently represented genotype was ST320 (*n*=6; 42.9%), followed by ST271 (*n*=1; 7.1%), ST90 (*n*=1; 7.1%), ST3544 (*n*=1; 7.1%), ST1161 (*n*=1; 7.1%), ST2040 (*n*=1; 7.1%), ST695 (*n*=1; 7.1%). As shown in Table 4, the three piliated isolates from the invasive site had ST320 (*n*=2), followed by

Table 4. Genotypic characteristics of the piliated pneumococcal isolates in relation to serotypes, isolation sites, Pneumococcal Molecular Epidemiology Network clones, antibiotic susceptibilities and pilus genes.

ST	CC	Serotype	Invasive site	Non-invasive	PMEN clones	Number of isolates resistant to antibiotics							MDR	PI-1	PI-2	PI-1+
						PEN[a]	CRO[a]	CTX[a]	TET[b]	ERY[b]	SXT[b]	VAN[b]	(*n*)	alone	alone	PI-2
	CC271	19F (*n*=2)	(*n*=1)	(*n*=1)		S (2)	S (2)	S (2)	R (2)	R (2)	R (2)	S (2)	Yes (2)	-	-	2
ST320	CC271	19A (*n*=3)		(*n*=3)	DLV of Taiwan ^{19F}-14	S (3)	S (3)	S (3)	R (3)	R (3)	R (2), S (1)	S (3)	Yes (2), No (1)	-	-	3
	CC271	NT (*n*=1)	(*n*= 1)			S (1)	S (1)	S (1)	R (1)	R (1)	R (1)	S (1)	Yes	-	-	1
ST271	CC271	19F (*n*=1)		(*n*=1)	SLV of Taiwan^{19F}-14	S (1)	S (1)	S (1)	R (1)	R (1)	R (1)	S (1)	Yes	1	-	-
ST16499	CC271	19F (*n*=1)		(*n*=1)	DLV of Taiwan^{19F}-14	S (1)	S (1)	S (1)	R (1)	R (1)	R (1)	S (1)	Yes	1	-	-
ST90	CC90	6A/B (*n*=1)		(*n*=1)	Spain^{6B}-2	S (1)	S (1)	S (1)	S (1)	R (1)	S (1)	S (1)	No	1	-	-
ST695	CC695	19A (*n*=1)	(*n*=1)		-	S (1)	S (1)	S (1)	S (1)	S (1)	S (1)	S (1)	No	1	-	-
ST3544	-	7A/F (*n*=1)		(*n*=1)	SLV Denmark^{12F}-34	S (1)	S (1)	S (1)	S (1)	S (1)	R (1)	S (1)	No	-	1	-
ST2040	-	6A/B (*n*=1)		(*n*=1)	SLV Poland^{6B}_20	S (1)	S (1)	S (1)	R (1)	R (1)	R (1)	S (1)	Yes	1	-	-
ST1161	-	6A/B (*n*=1)		(*n*=1)	SLV England^{14}_9	S (1)	S (1)	S (1)	R (1)	R (1)	R (1)	S (1)	Yes	1	-	-
ST16430	-	19F (*n*=1)		(*n*=1)	-	S (1)	S (1)	S (1)	I (1)	S (1)	R (1)	S (1)	No	-	-	1

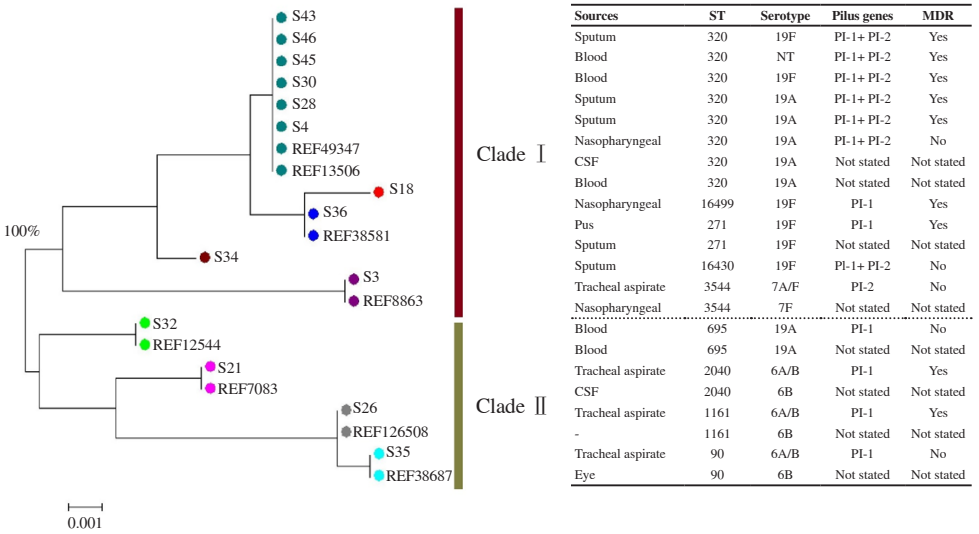
ST: sequence type; CC: clonal complex; NT: non-typeable; isolation sites: invasive and non-invasive; PMEN: Pneumococcal Molecular Epidemiology Network; SLV: single-locus variant; DLV: double-locus variant; PEN: penicillin; TET: tetracycline; ERY: erythromycin; CRO: ceftriaxone; CTX: cefotaxime; SXT: trimethoprim-sulfamethoxazole; VAN: vancomycin.; MDR: multidrug resistance; [a]susceptibility test by E-test (MIC determination) for penicillin, ceftriaxone and cefotaxime; [b]susceptibility test by disk diffusion for tetracycline, erythromycin, trimethoprim-sulfamethoxazole and vancomycin.

ST695 (*n*=1), respectively (Table 4). Two new novel sequence types ST16499 and ST16430 were identified in this study; both two from nasopharynx and sputum, resistant to erythromycin, tetracycline and trimethoprim-sulfamethoxazole, carrying serotype 19F with PI-1 only and PI-1+PI-2 respectively.

Meanwhile, a total of eight piliated isolates belonged to CC271 which was also the predominant CC, accounting for 80%. One piliated isolate belonged to CC90 and one piliated isolate belonged to CC695. Piliated isolates presented STs similar to six of the 43 clones recognised by the PMEN, sharing at least five MLST alleles. One isolate was related to Spain^{6B}-2, four were single-locus locus variant (SLV) of Taiwan^{19F}-14, Poland^{6B}-20, Denmark^{12F}-34 and England^{14}-9 respectively, and seven were double-locus variant (DLV) of Taiwan^{19F}-14. A comparison of these piliated isolates with PMEN clones showed that 85.7% (*n*=12) of all isolates were grouped into the international antibiotic resistant clones, while the predominant PMEN clones were Taiwan^{19F}-14 (Table 4).

3.6. Phylogenetic analysis

All nucleotide sequences of the seven housekeeping genes were aligned in the specified order and phylogenetic analysis was performed among all the 14 piliated isolates (labelled with S followed by numbers) and 8 reference sequences from the MLST database (labelled with REF followed by identity number). The tree formed two obvious clades Ⅰ and Ⅱ with 100% bootstrap confidence interval values at all branching (Figure 1). The largest clade Ⅰ consists of ten isolates while clade Ⅱ with four isolates. The clades also showed a clear segregation among STs where clade Ⅰ predominantly harbours ST320 frequently carrying both pili genes,



Sources	ST	Serotype	Pilus genes	MDR
Sputum	320	19F	PI-1+ PI-2	Yes
Blood	320	NT	PI-1+ PI-2	Yes
Blood	320	19F	PI-1+ PI-2	Yes
Sputum	320	19A	PI-1+ PI-2	Yes
Sputum	320	19A	PI-1+ PI-2	Yes
Nasopharyngeal	320	19A	PI-1+ PI-2	No
CSF	320	19A	Not stated	Not stated
Blood	320	19A	Not stated	Not stated
Nasopharyngeal	16499	19F	PI-1	Yes
Pus	271	19F	PI-1	Yes
Sputum	271	19F	Not stated	Not stated
Sputum	16430	19F	PI-1+ PI-2	No
Tracheal aspirate	3544	7A/F	PI-2	No
Nasopharyngeal	3544	7F	Not stated	Not stated
Blood	695	19A	PI-1	No
Blood	695	19A	Not stated	Not stated
Tracheal aspirate	2040	6A/B	PI-1	Yes
CSF	2040	6B	Not stated	Not stated
Tracheal aspirate	1161	6A/B	PI-1	Yes
-	1161	6B	Not stated	Not stated
Tracheal aspirate	90	6A/B	PI-1	No
Eye	90	6B	Not stated	Not stated

Figure 1. Phylogenetic analysis formed two major clades among the piliated pneumococcal isolates with clade I being predominant exhibiting mostly serotype 19A of ST320, and all the both PI-1+PI-2 and some PI-1 alone. Bootstrap sampling at 1000 replicates showed 100% at all branching. The distribution of sources of isolation, sequence type (ST), serotype, pilus genes and multidrug resistance for respective isolates are shown in the right columns. The reference sequences were retrieved from the MLST database comprising identity (ID) numbers 49347 (Vietnam), 13506 (Malaysia), 38581 (China), 8663 (Saudi Arabia), 12544 (China), 7083 (Egypt), 126508 (China), 38687 (China); ID number was preceded by REF indicating reference sequence, and ID labelled with S (Selangor) and numbers represent the isolates in this study. S18 and S34 are a newly assigned ST (REF sequence is not available). CSF: cerebrospinal fluid.

followed by ST271, ST3544, ST16430 and ST16499; while clade][for ST695, ST2040, ST1161 and ST90. All reference sequences of the different STs were grouped according to their respective comparable ST of the isolates in this analysis.

4. Discussion

S. pneumoniae is a well-known opportunistic pathogen carrying various virulence factors, including pili. Molecular investigations of genetic lineages representing particularly piliated isolates from Malaysia are still limited. Hence, *S. pneumoniae* typing is important for providing epidemiological data and facilitating pneumococcal disease management. Sungai Buloh Hospital is one of the major tertiary hospitals apart from a few other major hospitals in Selangor within the Klang Valley, which is the most developed region in Malaysia. Based on the 2018 Malaysia economic report, Selangor tops the list and highest contributor to the national gross domestic product (GDP). Selangor has always been Malaysia's economic powerhouse and sustaining a major contribution to the most impressive economic performance sector[19]. Thus, the genetic background of pneumococcal isolates from this area is of interest to observe the dynamics of pneumococcal infection and also dissemination.

For the site of isolation, the current study showed that tracheal aspirate (28.6%) was the most common source with certain serotype distribution. Meanwhile, a male (66.7%) predominance was noted in the study population, which was similar to a recent study in Japan[20]. The rates varied substantially with age from a new born of one month to adults of 90 years. Our study also revealed the variety of age groups of pneumococcal infections, where the highest incidence was observed among subjects aged 13-50 years. This is an interesting observation as young children and the elderly are commonly known as the high-risk group. Nevertheless, the isolation sites were not significantly related to the discrete variables.

None of the pneumococcal isolates reported resistance to penicillin in our study. A previous study from Malaysia reported a low prevalence of penicillin resistance at only 5.6%[10]. While resistance was observed for erythromycin, tetracycline and trimethoprim-sulfamethoxazole: 50%, 50% and 43% respectively, with an MDR rate of 26.2%. A similar finding was documented in Malaysia with resistance to both erythromycin and tetracycline at 42.9%[9]. The pattern of antimicrobial susceptibility profile in the present study also revealed 100% sensitivity to ceftriaxone, cefotaxime and vancomycin.

The analysis of the serotype distribution showed serotype 19F at the largest proportion (28.6%) followed by serotype 6A/B and 19A. This is in concordance with previous studies that showed serotype 19F as the common serotype among isolates from different sites of isolation in various Malaysian hospitals[21,22]. In Malaysia, the pneumococcal conjugate vaccine (PCV) has been implemented as part of the national immunisation program since December 2020 to the toddler which includes serotype 19F. Meanwhile, about 7.1% of non-typeable serotypes among pneumococcal isolates were identified in our collection. Those isolates were not detected for any of the targeted serotypes including the internal positive control gene (*cpsA*). This isolate could be of other serotypes, whereby the associated primers were not included since the primers were limited only to 48 serotypes available based on the Malaysian setting.

Pili have been detected in a small proportion of the pneumococcal population in humans. Pili are part of a virulence factor regiment in *S. pneumoniae* and play a significant role in pathogenesis by promoting adhesion, colonisation and cellular invasion of the host tissue to provide an extra advantage to the isolates[8]. PI-1 has been reported to be present in 16.6% to 35%[23,24]; whereas the second type of pili, PI-2 was found to occur in approximately 16% to 21% of pneumococcal isolates[25,26]. The prevalence of piliated isolates in this study was within that range with only 33.3% of isolates possessing at least one of the pilus genes. The latest local Malaysian finding reported 19% of a pneumococcal collection at a major tertiary hospital in the east coast of peninsular Malaysia were piliated isolates[13]. The piliated isolates in this current study were predominantly collected from sputum and tracheal aspirate. This was in concordance with a previous study in Japan that reported that sputum was the common source of piliated isolates[27].

In addition, isolates carrying PI-1 alone and both PI-1+PI-2 showed a similar frequency of resistance to erythromycin (79%), tetracycline (71%), and trimethoprim-sulfamethoxazole (79%), while about 64.3% of piliated isolates in our study were also MDR. Earlier evidence showed that MDR was strongly associated with piliated isolates[28]. Serotype 19F was also predominant and linked to the presence of PI-1 alone and both PI-1+PI-2, followed by serotype 19A and 6A/B. Similarly, a recent study in China and Indonesia found that serotype 19F was the most frequently associated with PI-1 and PI-2 genes[29,30]. Thus, it can be proposed serotype 19F is a predominant serotype among piliated isolates. This was also consistent with our recent report involving isolates from east coast of peninsular Malaysia[13].

Our study showed a diverse genetic lineage among the piliated isolates with nine different STs including two newly assigned STs. PI-1 and PI-2 are frequently found in pneumococcal lineages that are related to international PMEN clones. ST320 was the predominant among piliated isolates in our collection and also belonged to CC271 (Taiwan^{19F}-14) while the majority of them were 19A and MDR. In contrast, our recent findings revealed ST236 as the most prominent ST among piliated isolates on the east coast of peninsular Malaysia[13]. According to the recent data, ST320 is derived from the lineage of Taiwan^{19F}-14 clone (ST236) and has become prevalent

and spread throughout Asia including China[31]. The emergence of ST320 was the combined result of vaccine selective pressure, antimicrobial pressure, and the propensity of pneumococci to undergo recombination[32].

This was similar to a previous study that mentioned ST320 was the predominant ST among piliated isolates in Russia[33]. The previous study mentioned that CC271 was more common in multidrug-resistant pneumococcal clones (Taiwan^{19F}-14) and at the same time carried piliated elements[29]. Apart from ST320, other STs: ST90, ST2040, and ST1161 were also related to the widely disseminating PMEN clones including Spain^{6B}-2, England^{14}_9 and Poland^{6B}, respectively. Meanwhile, only one PI-2 isolate in this present study was found in ST3544 and related to SLV Denmark^{12F}-34. Based on these observations, it can be suggested that pili genes are often associated with successful pneumococcal lineages and antimicrobial resistance pattern[34].

Two major phylogenetic clades were observed among piliated isolates in this study indicating the clonal diversity of the isolates. The phylogenetic tree indicated a close genetic lineage among piliated isolates, notably those expressing both pilus genes. In clade Ⅰ, ST320 was the most prominent ST among vaccine serotype (19A) and multidrug resistance supporting the close association of the sub-group. Interestingly, the only isolate with PI-2 alone in clade Ⅰ had serotype 7A/F and non-MDR phenotype, belonging to ST3544. Serotype 7F is included in PCV13; a study conducted in Portugal showed a strong association of serotype 7F with PI-2 alone[35]. Meanwhile, the two novel STs ST16499 and ST16430 are also represented in clade Ⅰ carrying PI-1 alone and both PI-1+PI-2; both STs showed a prominent serotype 19F, which was vaccine serotype and one of them was MDR. Clade Ⅱ was the second major clade that represented the diversity of STs including ST90, ST695, ST2040, and ST116. Interestingly, this clade consisted of only PI-I alone isolates; most of them were vaccine type (serotype 6A/B and 19A).

Overall, the piliated isolates were diverse but linked with certain common features. The phylogenetic analysis revealed that piliated pneumococcal isolates in this study commonly shared close correlation due to being clustered together with some having similar serotypes and STs, carrying both pilus genes. Therefore, it is likely that they could have clonally originated and were then dispersed across the population.

This study has a limitation, with a low represented number of isolates throughout a 2.6 years collection period and the fact that these isolates were collected back in 2017 till 2019. The lower number of isolates in HSB is probably due to the presence of several tertiary hospitals available in the Klang Valley whereby the admission with potential pneumococcal infection is segregated in the different hospitals. A previous study accumulated 95 strains of pneumococcal collection in the Klang Valley setting[22]. Despite the limited collection, we managed to collect 42 pneumococcal isolates and 14 piliated strains were identified with a total accumulation of 33.3% in this study. The prevalent serotype of piliated isolates was associated with clonal spread involving serotypes of 19F, 19A and 6A/B, which are mostly covered by PCVs and have been associated with a high frequency of MDR. The predominant ST of piliated isolates at this setting was ST320 and linked with Taiwan^{19}-14 (CC271). Based on these findings, it can be suggested that global pneumococcal lineages of piliated isolates have been disseminated in this area and pili could have played a role in the spread of antibiotic resistant clones.

Conflict of interest statement

The authors report no conflict of interest.

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Authors' contributions

MNMD and NDD contributed to the study conception and design. MNMD, NDD, AM and NHZB implemented the study. NDD, NS, NAAR, SO analysed and contributed to the interpretation of the results. MNMD, NM, NM, ZS, NKP, NIAR, TSTO and FHA revised the work critically. All authors read and approved the final manuscript for publishing.

References

[1] Henriques-Normark B, Tuomanen EI. The pneumococcus: Epidemiology, eicrobiology and pathogenesis. *Cold Spring Harb Perspect Med* 2013; **3**(7): a010215.

[2] Engholm DH, Kilian M, Goodsell DS, Andersen ES, Kjærgaard RS. A visual review of the human pathogen *Streptococcus pneumoniae*. *FEMS Microbiol Rev* 2017; **41**(6): 854-879.

[3] Wahl B, O'Brien KL, Greenbaum A, Majumder A, Liu L, Chu Y, et al. Burden of *Streptococcus pneumoniae* and *Haemophilus influenzae* type b disease in children in the era of conjugate vaccines: Global, regional, and national estimates for 2000-15. *Lancet Glob Health* 2018; **6**(7): e744-e757.

[4] World Health Organization. *Pneumonia in children*. [Online]. Available from: https://www.who.int/news-room/fact-sheets/detail/pneumonia. [Accessed on 22 November 2022].

[5] Ganaie F, Saad JS, Mcgee L, Van Tonder AJ, Bentley SD, Lo SW, et al. A new pneumococcal capsule type, 10d, is the 100th serotype and has a large cps fragment from an oral *Streptococcus*. *mBio* 2020; **11**(3): e00937-20.

[6] Alicino C, Paganino C, Orsi A, Astengo M, Trucchi C, Icardi G, et al. The impact of 10-valent and 13-valent pneumococcal conjugate vaccines on hospitalization for pneumonia in children: A systematic review and meta-analysis. *Vaccine* 2017; **35**(43): 5776-5785.

[7] Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, et al. A pneumococcal pilus influences virulence and host inflammatory responses. *Proc Natl Acad Sci* 2006; **103**(8): 2857-2862.

[8] Bagnoli F, Moschioni M, Donati C, Dimitrovska V, Ferlenghi I, Facciotti C, et al. A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells. *J Bacteriol* 2008; **190**(15): 5480-5492.

[9] Arushothy R, Ahmad N, Amran F, Hashim R, Samsudin N, Azih CRC. Pneumococcal serotype distribution and antibiotic susceptibility in Malaysia: A four-year study (2014-2017) on invasive paediatric isolates. *Int J Infect Dis* 2019; **80**: 129-133.

[10]Subramaniam P, Jabar KA, Kee BP, Chong CW, Nathan AM, de Bruyne J, et al. Serotypes & penicillin susceptibility of *Streptococcus pneumoniae* isolated from children admitted to a tertiary teaching hospital in Malaysia. *Indian J Med Res* 2018; **148**(2): 225-231.

[11]Clinical and Laboratory Standards Insititute. *M100 performance standards for antimicrobial susceptibility testing*. 30th edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.

[12]Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**(3): 268-281.

[13]Dzaraly ND, Mohd Desa MN, Muthanna AR, Masri SN, Taib NM, Suhaili Z, et al. Antimicrobial susceptibility, serotype distribution, virulence profile and molecular typing of piliated clinical isolates of pneumococci from east coast, Peninsular Malaysia. *Sci Rep* 2021; **11**(1): 8220.

[14]Centers for Disease Control and Prevention. *Resources and protocols–Multiplex conventional PCR schemes for pneumococcal serotype deduction*. [Online]. Available from: https://www.cdc.gov/streplab/pneumococcus/resources.html. [Accessed on 24 January 2020].

[15]Shakrin NNSM, Masri SN, Taib NM, Nordin SA, Jamal F, Desa MNM. Genotypic characterization of Malaysian human isolates of *Streptococcus pneumoniae* from carriage and clinical sources. *Comp Immunol Microbiol Infect Dis* 2014; **37**(5-6): 347-354.

[16]Shakrin NNSM, Balasubramaniam SD, Yusof HA, Mastuki MF, Masri SN, Taib NM, et al. Evaluation of PCR-based approach for serotype determination of *Streptococcus pneumoniae*. *Trop Biomed* 2013; **30**(2): 338-344.

[17]Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: Identification of clones associated with serious invasive disease. *Microbiology* 1998; **144**(11): 3049-3060.

[18]Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016; **33**(7): 1870-1874.

[19]Department of Statistics Malaysia. *Press Release State Socioeconomic Report 2018. Socioeconomic performance by state*. [Online]. Available from: https://www.dosm.gov.my/v1/index.php?r=column/pdfPrev&id=a0c3UGM3MzRHK1N1WGU5T3pQNTB3Zz09. [Accessed on 24 July 2019].

[20]Yanagihara K, Kosai K, Mikamo H, Mukae H, Takesue Y, Abe M, et al. Serotype distribution and antimicrobial susceptibility of *Streptococcus pneumoniae* associated with invasive pneumococcal disease among adults in Japan. *Int J Infect Dis* 2021; **102**: 260-268.

[21]Nathan JJ, Mohd Desa MN, Thong KL, Clarke SC, Masri SN, Md Yasin R, et al. Genotypic characterization of *Streptococcus pneumoniae* serotype 19F in Malaysia. *Infect Genet Evol* 2014; **21**: 391-394.

[22]Goh SL, Kee BP, Abdul Jabar K, Chua KH, Nathan AM, Bruyne J, et al. Molecular detection and genotypic characterisation of *Streptococcus pneumoniae* isolated from children in Malaysia. *Pathog Glob Health* 2020; **114**(1): 46-54.

[23]Knupp-Pereira PA, Marques NTC, Teixeira LM, Póvoa HCC, Neves FPG. Prevalence of PspA families and pilus islets among *Streptococcus pneumoniae* colonizing children before and after universal use of pneumococcal conjugate vaccines in Brazil. *Braz J Microbiol* 2020; **51**(2): 419-425.

[24]Kawaguchiya M, Urushibara N, Aung MS, Ito M, Takahashi A, Habadera S, et al. High prevalence of antimicrobial resistance in non-vaccine serotypes of non-invasive/colonization isolates of *Streptococcus pneumoniae*: A cross-sectional study eight years after the licensure of conjugate vaccine in Japan. *J Infect Public Health* 2020; **13**(8): 1094-1100.

[25]Hjálmarsdóttir M, Pétursdóttir B, Erlendsdóttir H, Haraldsson G, Kristinsson KG. Prevalence of pilus genes in pneumococci isolated from healthy preschool children in Iceland: Association with vaccine serotypes and antibiotic resistance. *J Antimicrob Chemother* 2015; **70**(8): 2203-2208.

[26]Zähner D, Gudlavalleti A, Stephens DS. Increase in pilus islet 2-encoded pili among *Streptococcus pneumoniae* isolates, Atlanta, Georgia, USA. *Emerg Infect Dis* 2010; **16**(6): 955-962.

[27]Miyazaki H, Shibuya R, Chang B, Inukai T, Miyazaki Y, Ubukata K, et al. Genetic characteristics of piliated *Streptococcus pneumoniae* serotype 35B, increased after introduction of pneumococcal vaccines in Japan. *J Infect Chemother* 2020; **26**(11): 1198-1204.

[28]Moreno BQ, Araque M. Molecular characterisation of multidrug-resistant pneumococcal clones colonising healthy children in Mérida, Venezuela. *J Glob Antimicrob Resist* 2018; **14**: 45-50.

[29]Fu J, Li L, Liang Z, Xu S, Lin N, Qin P, et al. Etiology of acute otitis media and phenotypic-molecular characterization of *Streptococcus pneumoniae* isolated from children in Liuzhou, China. *BMC Infect Dis* 2019; **19**(1): 168.

[30]Safari D, Valentiya F, Salsabila K, Paramaiswari WT, Tafroji W, Hammerschmidt S, et al. The prevalence of pilus islets in *Streptococcus pneumoniae* isolates from healthy children in Indonesia. *Access Microbiol* 2021; **3**(1): acmi000184.

[31]Fu J, Yi R, Jiang Y, Xu S, Qin P, Liang Z, et al. Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* causing invasive diseases in China: A meta-analysis. *BMC Pediatr* 2019; **19**(1): 424.

[32]Golden AR, Adam HJ, Karlowsky JA, Baxter M, Nichol KA, Martin I, et al. Molecular characterization of predominant *Streptococcus pneumoniae* serotypes causing invasive infections in Canada: The SAVE study, 2011-15. *J Antimicrob Chemother* 2018; **73**(7): vii20-vii 31.

[33]Protasova IN, Wan TW, Bakhareva NV, Hung WC, Higuchi W, Iwao Y, et al. Molecular characterization of *Streptococcus pneumoniae*, particularly serotype19A/ST320, which emerged in Krasnoyarsk, Russia. *Microbiol Immunol* 2017; **61**(9): 359-370.

[34]Dzaraly ND, Muthanna AR, Mohd Desa MN, Taib NM, Masri SN, Rahman NIA, et al. Pilus islets and the clonal spread of piliated *Streptococcus pneumoniae*: A review. *Int J Med Microbiol* 2020; **310**(7): 151449.

[35]Horácio AN, Silva-costa C, Diamantino-miranda J, Lopes JP, Ramirez M, Melo-cristino J, et al. Population structure of *Streptococcus pneumoniae* causing invasive disease in adults in Portugal before PCV13 availability for adults: 2008-2011. *PLoS One* 2016; **11**(5): e0153602.

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