UNIVERSITI PUTRA MALAYSIA

GENOTYPIC AND GENOMIC CHARACTERIZATION OF PILI-CARRYING CLINICAL ISOLATES OF Streptococcus pneumoniae FROM TWO MAJOR TERTIARY HOSPITALS IN MALAYSIA

NURUL DIANA BINTI DZARALY

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By

NURUL DIANA BINTI DZARALY

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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March 2022

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Pili are detected in a small proportion of the pneumococcal population but the discovery of non-flagellar pili has recently been associated with pneumococcal pathogenesis in humans. The information regarding piliated isolates remains scarce, especially in terms of genomic studies undertaken in Malaysia. Therefore, this study aimed to characterize a collection of pili-carrying and none pili-carrying pneumococcal isolates of clinical origin for serotypes, antibiotic resistance, genotyping and comparative whole-genome. In total, 142 clinical isolates were collected from Hospital Sultanah Nur Zahirah (HSNZ), Terengganu and Hospital Sungai Buloh (HSB), Selangor between August 2017 and December 2019. Those isolates were subjected to serotyping and antimicrobial susceptibility tests, detection of pneumococcal virulence and pilus genes. Multilocus sequence typing (MLST) and phylogenetic analysis were performed only for the piliated isolates, while selected invasive piliated isolates were subjected to whole-genome sequence analysis. Demographic analysis showed that pneumococcal infection was higher in males (57.7%) than females (42.3%), while most isolation sites were from sputum (35.2%), followed by blood (34.5%), eye (10.6%), tracheal aspirate (8.5%) and pus (5.6%). The most frequent were serotypes 6A/B (18.3%), 19F (16.2%), 14 (12.7%), 19A (12.0%), and 23F (9.2%), which were vaccine serotypes. Serotype 15B/C was the predominant non-vaccine serotype among the pneumococcal collections. Most isolates were resistant to erythromycin (44%), tetracycline (41%) and trimethoprim-sulfamethoxazole (30%). Piliated isolates occurred in a proportion of 23.2%; 51.5% of them were multidrug-resistant (MDR) and the majority had serotype 19F. This study revealed that ST236 and ST320 were the predominant sequence types (ST) among the piliated isolates and were genetically related to the PMEN clones Taiwan^{19F}-14 and CC271. ST236 was the most prevalent ST in HSNZ, while ST320 was the most common ST in HSB. Interestingly, three novels, ST15604, ST16430 and ST16499, were found among the piliated isolates. In the phylogenetic analysis, the piliated isolates were grouped into three major clades, supported by 100% bootstrap values. Seven invasive piliated pneumococcal isolates from HSNZ and HSB were subjected to whole-genome

analysis. The genomic content of all the piliated isolates was diverse, with the presence of various mobile genetic elements (MGEs) such as phage and insertion sequence, as well as virulence factors and resistance determinants. *In-silico* MLST, five different STs were reported; ST236, ST320, ST386, ST671 and ST695. Overall, a BLAST search identified two major variants of the PI-1 and PI-2 genes, which were conserved with minor mutations within the variant's groups. Core genome analysis of all the representative piliated isolates and another 35 global references formed three major clades. An interesting observation was that the piliated isolates; TSP95, SSP45 and SSP46 were closely related to the South Korea strains, indicating their long persistence over more than a decade. They may have evolved and be descended from the South Korea international clones. TSP106 from clade III was grouped into a strain with the same geographical origin as the Malaysian strains. Thus, this analysis provided insights into the characteristics of piliated isolates in the Malaysian context and showed that they are related to resistance determinants and ST. Therefore, continuing the surveillance, prevention and control of *S. pneumoniae* in this region can be regarded as important.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN GENOTIP DAN GENOM TERHADAP ISOLAT KLINIKAL PEMBAWA PILI *Streptococcus pneumoniae* DARI DUA HOSPITAL TERTIARI UTAMA DI MALAYSIA

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Pili dikesan dalam sebahagian kecil populasi pneumokokus tetapi mutakhir ini penemuan pili bukan flagelum telah dikaitkan dengan patogenesis pneumokokus pada manusia. Maklumat tentang isolat berpili masih terhad, terutamanya dalam konteks kajian genomik di Malaysia. Oleh yang demikian, kajian ini bertujuan untuk mencirikan koleksi isolat pneumokokus pembawa pili dan bukan pembawa pili daripada sampel klinikal dari segi serotip, rintangan antibiotik, pencirian genotip, dan perbandingan jujukan penuh genom. Secara keseluruhan, 142 isolat klinikal telah dikumpulkan dari Hospital Sultanah Nur Zahirah (HSNZ), Terengganu dan Hospital Sungai Buloh (HSB), Selangor dari Ogos 2017 sehingga Disember 2019. Ujian serotip dan kerentanan antimikrob serta pengesanan virulens pneumokokus termasuk gen pilus telah dijalankan terhadap kesemua isolat tersebut. Multilocus sequence typing (MLST) dan analisis filogenetik hanya dilakukan terhadap isolat berpili manakala analisis jujukan genom penuh dijalankan terhadap isolat berpili invasif yang terpilih. Analisis demografi menunjukkan bahawa jangkitan pneumokokus adalah lebih tinggi dalam kalangan lelaki (57.7%) berbanding wanita (42.3%), manakala kebanyakan tapak pengasingan isolasi adalah daripada kahak (35.2%) diikuti oleh darah (34.5%), mata (10.6%), aspirat trakea (8.6%), dan nanah (5.6%). Serotip 6A/B (18.3%) merupakan serotip yang paling kerap dijumpai, diikuti oleh serotip 19F (16.2%), 14 (12.7%), 19A (12.0%), dan 23F (9.2%), yang merupakan serotip vaksin. Serotip 15B/C ialah serotip bukan vaksin utama dalam koleksi pneumokokus tersebut. Kebanyakan isolat menunjukkan rintangan terhadap eritromisin (44%), tetrasiklin (41%), dan trimetoprim-sulfametoksazol (30%). Peratusan isolat berpili adalah pada kadar 23.2%; 51.5% daripadanya menunjukkan rintangan terhadap pelbagai ubat (MDR) dan kebanyakannya mempunyai serotip 19F. Kajian ini mendapati bahawa ST236 dan ST320 merupakan jenis jujukan (sequence type atau ST) utama isolat berpili dan secara genetik berkait rapat dengan klon PMEN Taiwan19F-14 dan CC271. ST236 ialah ST yang paling lazim di HSNZ, manakala ST320 ialah ST yang paling biasa ditemui di HSB. Menariknya, tiga novel iaitu ST15604, ST16430, dan ST16499 dijumpai dalam isolat berpili. Dalam analisis filogenetik, isolat berpili dikategorikan

kepada tiga klad utama, disokong oleh nilai bootstrap 100%. Tujuh isolat pneumokokus berpili yang invasif dari HSNZ dan HSB telah dipilih untuk analisis jujukan genom penuh. Kesemua isolat berpili tersebut mempunyai kandungan genom yang berbeza dengan kewujudan pelbagai unsur genetik bergerak (MGEs) seperti phage, insertion sequence, faktor virulens, dan penentu rintangan. Dalam in-silico MLST, lima ST yang berbeza telah dilaporkan: ST236, ST320, ST386, ST671, dan ST695. Secara keseluruhan, carian BLAST mengenal pasti dua varian utama iaitu gen PI-1 dan PI-2 dengan mutasi kecil dalam kumpulan varian tersebut. Analisis genom teras terhadap tujuh isolat berpili dan 35 wakil pencilan rujukan global lain membentuk tiga klad utama. Pemerhatian yang menarik ialah isolat berpili TSP95, SSP45, dan SSP46 berkait rapat dengan isolat dari Korea Selatan yang menunjukkan kewujudan berterusan selama lebih satu dekad. Isolat ini mungkin berkembang dan berasal daripada klon antarabangsa Korea Selatan. TSP106 daripada klad III mempunyai ciri-ciri geografi yang sama dengan isolat Malaysia. Secara keseluruhannya, analisis ini memberikan gambaran tentang ciri-ciri isolat berpili dalam konteks Malaysia dan menunjukkan bahawa ia mempunyai kaitan dengan penentu rintangan dan ST. Oleh itu, pengawasan, pencegahan, dan kawalan *S. pneumoniae* secara berterusan di rantau ini adalah penting.

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
ANI	Average Nucleotide Identity
AST	Antibiotic Susceptibility Test
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
CAP	Community-acquired pneumonia
CARD	Comprehensive Antimicrobial Resistance Database
cbpA	Choline binding protein A
CC	Clonal Complexes
CDS	Coding sequence
CLSI	Clinical and Laboratory Standards Institutes
CTAB	Cetyl trimethylammonium bromide
DHPS	Dihydropteroate synthetase
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
HGT	Horizontal gene transfer
HSB	Hospital Sungai Buloh
HSNZ	Hospital Sultanah Nur Zahirah
IPD	Invasive pneumococcal disease
LRTIs	Lower respiratory tract infections
lytA	Pneumococcal autolysin A
Mbp	Megabase pairs
MDR	Multidrug-resistant
MGE	Mobile genetic element
MIC	Minimal inhibitory concentrations

MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules'
NGS	Next Generation Sequencing
NMRR	National Medical Research Registration
NT	Non-typeable
pavA	Pneumococcal adherence and virulence factor A
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PCV	Pneumococcal Conjugate Vaccine
PCV10	10-Valent Conjugate Vaccine
PCV13	13-Valent Conjugate Vaccine
PCV7	7-Valent Conjugate Vaccine
PFGE	Pulsed-Field Gel Electrophoresis
PHASTER	Phage search tool enhanced released
ply	Pneumococcal autolysin A
PMEN	Pneumococcal Molecular Epidemiology Network
PPV	Pneumococcal polysaccharide vaccine
pspA	Pneumococcal surface protein A
RGI	Resistance Gene Identifier
ROS	Reactive oxygen species
S. pneumoniae	*Streptococcus pneumoniae*
ST	Sequence Type
TNF	Tumour necrosis factor
URT	Upper respiratory tract
VFDB	Virulence Factors Database
WBC	White blood cells
WGS	Whole-genome sequencing
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Streptococcus pneumoniae (pneumococcus) is a Gram-positive bacterium that frequently colonizes the human nasopharynx and becomes normal flora in the upper human respiratory tract. Under favourable conditions, pneumococci migrate to other sites within the body, causing both invasive pneumococcal diseases (IPD), including meningitis and bacteremia, and non-invasive diseases, such as pneumoniae, otitis media and sinusitis (Engholm et al., 2017; Örtqvist et al., 2005). Worldwide, pneumococcal infection is a major cause of mortality and morbidity which frequently affects children under two years old, the elderly and patients with comorbid diseases. According to the World Health Organization (WHO), about 808,694 children under five years old die due to pneumonia, accounting for 15% of total death cases in 2017 (WHO, 2019). Malaysia, a rapidly developing country in Southeast Asia, is undergoing substantial industrialization and urbanization, raising concerns about the health of its population, in which respiratory diseases, particularly pneumonia, are among the leading causes of hospitalization and death. According to the Department of Statistics, pneumonia is the third most common cause of death in Malaysia among children under five, with an estimated 3.8 per 100,000 cases per year; *S. pneumoniae* is one of the causative agents (Department of Statistics Malaysia, 2017; Maimaiti et al., 2015).

The polysaccharide capsule in *S. pneumoniae* is considered to be the most important virulence factor, with 100 serotypes (Ganaie et al., 2020). The 7-valent conjugate vaccine (PCV7) was first implemented in the United States (US) in 2000 and in Europe in 2001, while the 10-valent conjugate vaccine (PCV10) and the 13-valent conjugate vaccine (PCV13) were introduced in 2008 and 2009, respectively, to reduce the burden of pneumococcal diseases. Additionally, pneumococci are equipped with a wide virulence regiment, such as pneumolysin, choline-binding proteins, neuraminidase, hyaluronate lyase, autolysin and many others. Recently, the discovery of a long, filamentous, pilus-like structure in Gram-positive bacteria, which are uncommonly characterized specifically in *S. pneumoniae*, has added another element to the pneumococcal virulence regiment. The role of pili is to enhance the ability of pneumococci to adhere to epithelial cells; at the same time, the piliated strain was found to be significantly more virulent in a murine model of invasive diseases (Barocchi et al., 2006). This suggests that pneumococcal pili provide an additional advantage to initiate colonization, leading to the downstream infection and pathogenesis process (Bagnoli et al., 2008; Kreikemeyer et al., 2011; Nelson et al., 2007). To date, two pili islets have been detected in pneumococci, namely PI-1 (Barocchi et al., 2006) and PI-2 (Bagnoli et al., 2008).

S. pneumoniae typing is important for providing epidemiological data and facilitating pneumococcal disease treatment. Typing methods for studying the epidemiology of *S. pneumoniae* were initially based on phenotypic markers, such as the optochin test, bile

solubility and Gram-staining. However, this conventional phenotyping remains unsatisfactory and lacking in discriminatory strength. Later, genotyping methods based on chromosomal DNA were used, such as gene amplification and sequencing analysis. The molecular typing method provides an optimal combination of technical feasibility, discriminatory power, interpretability and portability in strain comparison, due to the specific nature of DNA sequences. Various epidemiological typing methods have been developed based on DNA sequence platforms, such as multilocus sequence typing (MLST) and 16S rRNA (El Aila et al., 2010; Enright & Spratt, 1998)

MLST analysis was developed to construct a phylogenetic study of *S. pneumoniae* that was based on a nucleotide sequence of seven housekeeping genes distributed over the bacterial chromosome and conserved during evolution (Enright & Spratt, 1998). This technique enables the determination of both the sequence type (ST) and the clonal complex (CC) to which the typed isolates belong. MLST became a widely accepted tool for molecular and evolutionary studies of the pathogen at the molecular level (Dingle et al., 2001). Currently, the whole-genome sequence (WGS) is regarded as a highly attractive tool for epidemiological studies (Ben Zakour et al., 2012). This approach is utilized to evaluate the genetic relatedness of bacterial isolates based on a sequence analysis of the whole-genome. Moreover, numerous phenotypic features can be inferred by WGS, including the virulence and antibiotic resistance of a particular pathogen. WGS also allows the determination of genetic markers, such as the presence or absence of a gene that might be linked to the prevalence, severity and virulence of a disease (Al-Obaidi et al., 2018).

1.2 Problem Statements

Pili has recently been associated with pneumococcal pathogenesis in humans. The information regarding piliated isolates in Malaysia is scarce. Moreover, few studies on the genome sequence of *S. pneumoniae* have been performed, with one study reporting the draft genome sequence of specific Malaysian strains (Jindal et al., 2018). In addition, most studies focused mainly on demographics, the antimicrobial susceptibility pattern and serotyping (Arushothy et al., 2019; Subramaniam et al., 2018). Less academic coverage has been devoted to molecular genotyping and the detailed genomic make-up of the strains, especially in piliated pneumococcal isolates.

To understand the genetic pattern of pneumococci, especially in relation to pili and their potential pattern changes, as well as the factors associated with infection, studies representing different geographical areas of Peninsular Malaysia are warranted. This would enable any potential variations or similarities in relevant matters to be further addressed. The main scope of this study is the analysis approach for piliated isolates, which employed MLST and WGS analysis, and the combination of all the isolates to ascertain their demographic and phenotypic features in connection with the genetic background of molecular epidemiology. Given the potentially life-threatening effects that pili might contribute in terms of disease and transmission activity, it is important to characterize piliated isolates to address the outstanding questions relating to its epidemiology, population and evolution. The emergence of a subpopulation carrying a

pilus trait that is not common in Gram-positive bacteria should not be underestimated. Therefore, a collection of clinical pneumococcal isolates from two major tertiary hospitals; Hospital Sultanah Nur Zahirah (HSNZ), Terengganu, on the east coast and Hospital Sungai Buloh (HSB), Selangor, on the west coast of Peninsular Malaysia were characterized for their demographic, antibiotic susceptibility and genotypic properties. These covered serotypes, virulence factors, including pilus genes, MLST and WGS, specifically on piliated isolates.

1.3 General Objective

The general objective of this study is to characterize a collection of pili-carrying and none pili-carrying pneumococcal isolates of clinical origin for serotypes, antibiotic resistance, genotyping, and the whole-genome.

1.4 Specific Objectives

1. To determine the antimicrobial susceptibility patterns of the isolate collection.
2. To determine the serotype distribution of clinical *S. pneumoniae* isolates.
3. To determine the presence and distribution of important pneumococcal virulence genes including pilus genes in clinical *S. pneumoniae* isolates.
4. To determine the sequence type (ST) and clonal complex (CC) in piliated isolates based on MLST
5. To describe the whole-genome and comparative genomics of representative invasive-piliated isolates, against those in the GenBank database.

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Conference

Nurul Diana Dzaraly , Cheah Yun Shuan, Niazlin Mohd Taib, Siti Norbaya Masri, Nor Iza A. Rahman, Mohd Nasir Mohd Desa. The Occurrence of Pili Carrying Pneumococci of Clinical Origin Exhibiting Disease-Associated Serotypes and Multidrug-Resistance. 2nd International Conference on Biomedical & Health Sciences Research 2018. The Everly Hotel, Putrajaya Malaysia, 2018

Nurul Diana Dzaraly, Mohd Nasir Mohd Desa, AbdulRahman Muthanna, Siti Norbaya Masri, Niazlin Mohd Taib, Zarizal Suhaili, Nurshahira Sulaiman, Nurul Hana Zainal Baharin, Nor Iza A. Rahman, Tuan Suhaila Tuan Soh, Fatimah Haslina Abdullah. Antimicrobial Susceptibility and Serotype Distribution of Pneumococcal Isolates at A Major Tertiary Hospital in Klang Valley, Malaysia. International Congress of the Malaysian Society for Microbiology 2021 (ICMSM2021), 2021

Achievements

1. Winner of a Travel Grant for the best abstract entitled “The Occurrence of Pili Carrying Pneumococci of Clinical Origin Exhibiting Disease-Associated Serotypes and Multidrug-Resistance” at the 2nd International Conference on Biomedical & Health Sciences Research (ICBHSR), October, 2018, Putrajaya, Selangor.
2. 3rd Prize in the Virtual 3 Minutes Thesis (3MT) Competition, April, 2021, at faculty level; Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM).
3. Finalist of the Virtual UPM 3MT Finals Competition, as a representative of the Faculty of Medicine and Health Sciences, April, 2021.