CAPSULAR SEROTYPE, ANTIBIOTICS SUSCEPTIBILITY AND RESISTANCE GENE PROFILE OF *Streptococcus pneumoniae* FROM NASAL CARRIAGE OF HEALTHY CHILDREN IN SELANGOR, MALAYSIA

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the Degree of Master of Science

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Research on pneumococcal carriage epidemiology are very limited in Malaysia and some other developed countries. This is because most study always focus on symptomatic diseases for prevention and control, meanwhile high level of asymptomatic carriage rate is the starting point of pneumococcal infections. This factors interest us to research on pneumococcal carriage rate, its virulence genes, serotypes distribution, antimicrobial susceptibility pattern and macrolide resistance determinant genes. Three hundred and ten nasal swab samples were collected from healthy children of both ≤5years old and >5years old with the consent of their parents and guardians. The samples were collected from three selected child care centre; kindergarten, orphanage home and refugee school in Selangor and Kuala Lumpur. Sixty isolates of *Streptococcus pneumoniae* were recovered from all nasal swabs making the carriage rate of 26.3% for ≤5years old and 10.4% for >5years old children respectively. All isolates were carrying the virulence genes i.e 63.3% (38) of *ply* and *lytA* gene whereas 36.7% (22) carrying a single gene of either *ply* or *lytA*. Among the 22 single gene carrying isolates, we selected 12 *lytA* gene isolates for 16s rRNA sequence analysis for further confirmation of *Streptococcus pneumoniae* strain and only 7 isolates were of *Streptococcus pneumoniae* strain, making the total carriage isolates 17.7% (55). We successfully serotyped the 55 isolates by multiplex PCR techniques with the following descending prevalence sequence result: 7A/7F, 23F, 19F, 11A/D and 15B/15C. The vaccine serotype was accounted for 71% and the non-vaccine serotype was 29%. Antibiotic susceptibility testing showed cefotaxime and ceftriaxone were the most susceptible antibiotics to *Streptococcus pneumoniae* with 50.9% and 49% respectively, erythromycin susceptible *Streptococcus pneumoniae* has 38.2%, penicillin on the other hand has 20% susceptibility rate. Despite penicillin having the highest rate of non-susceptibility (80%), erythromycin has the highest rate of resistance with 43.6%. Further study on macrolide resistance determinant genes revealed *mefA* gene has the higher rate of 67.2% (37) while *ermB* gene has 25.5% (14) and *mefA+ermB* has 7.3% (4). This proved that macrolide resistance gene that highly triggers the high resistance level of erythromycin is *mefA*. Pneumococcal carriage

rate has decreased over time from previous studies. Serotype analysis has shown the importance of vaccinating the children to reduce the carriage rate and infection. Antibiotics of interest on the other hand should always be tested before prescription, so as to avoid misuse, overuse and unsuitable antibiotics. Continuous study is needed to keep track of pneumococcal carriage rate with serotype distribution, and to understand the antibiotics susceptibility pattern. If the resistant rate is higher, it is crucial to analyse the resistance determinant gene with further molecular study for comprehensive knowledge of *Streptococcus pneumoniae* in Malaysia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

SEROTYPE CAPSULAR, ANTIBIOTIK KENDERAAN DAN RINTANGAN PROFIL GENE *Streptococcus pneumoniae* DARIPADA PENGANGKUTAN NASAL KANAK-KANAK SIHAT DI SELANGOR, MALAYSIA

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Penyelidikan tentang epidemiologi pembawa pneumokokus sangat terhad di Malaysia dan beberapa negara maju yang lain. Ini kerana kebanyakan kajian memfokuskan kepada penyakit bergejala untuk pencegahan dan kawalan, manakala kadar pembawa tanpa gejala yang tinggi adalah titik permulaan jangkitan pneumokokus. Faktor ini menarik minat kami untuk menyelidik tentang kadar pembawa pneumokokus, gen virulensinya, taburan serotaip, corak kerentanan antimikrob dan gen penentu rintangan makrolid. Tiga ratus sepuluh sampel swab hidung telah dikumpul daripada kanak-kanak yang sihat berumur ≤5 tahun dan >5 tahun dengan persetujuan ibu bapa dan penjaga mereka. Sampel dikumpul dari tiga pusat jagaan kanak-kanak terpilih; tadika, rumah anak yatim dan sekolah pelarian di Selangor dan Kuala Lumpur. Enam puluh isolat *S.pneumoniae* telah dikenalpasti daripada kesatan hidung kanak2 berdasarkan pengamatan pertumbuhan isolat dan ujian biokimia menjadikan kadar pembawa 26.3% untuk ≤5tahun dan 10.4% untuk kanak-kanak berumur >5tahun. Semua isolat membawa gen virulensi iaitu 63.3% (38) *ply* dan gen *lytA* manakala 36.7% (22) membawa gen tunggal sama ada *ply* atau *lytA.* Antara 22 gen tunggal yang membawa isolat, kami memilih 12 isolat gen *lytA* untuk analisis jujukan rRNA 16s bagi pengesahan lanjut strain *Streptococcus pneumoniae.* Daripada 12 isolat, hanya 7 disahkan sebagai strain *S.pneumoniae*, menjadikan kadar pembawa adalah 17.7%(55) di kalangan kanak-kanak. Ujian PCR multipleks terhadap 55 strain *S.pneumoniae* memberikan hasil jujukan prevalen serotaip secara menurun secara berikut : 7A/7F, 23F, 19F, 11A/D dan 15B/15C. Pecahan jenis serotaip yang terkandung dalam vaksin adalah 71% manakala 29% merupakan serotaip yang tidak terkandung dalam vaksin konjugat. Ujian kerentanan terhadap empat antibiotik menunjukkan cefotaxime dan ceftriaxone adalah antibiotik yang paling rentan kepada Streptococcus pneumoniae dengan 50.9% dan 49% masing-masing, manakala kadar kerentanan erythromycin adalah 38.2% dan penicillin hanya 20%. Walaupun penicillin mempunyai kadar ketidakrentanan (non-susceptible) tertinggi (80%), erythromycin menunjukkan kadar rintangan tertinggi iaitu 43.6%. Kajian lanjut mengenai gen rintangan maklolid menunjukkan gen *mefA* yang tinggi iaitu 67.2% (37)

manakala gen *ermB* adalah 25.5% (14) dan *mefA+ermB* 7.3% (4). Ini menunjukkan *mefA* adalah gen yang mencetuskan tahap rintangan tinggi terhadap erythromycin. Kajian ini menunjukkan kadar pembawa bakteria pneumokokus telah menurun berbanding kajian lepas. Analisis serotaip juga menunjukkan kepentingan vaksin pneumokokus di kalangan kanak-kanak untuk mengurangkan kadar pembawa dan jangkitan penyakit. Antibiotik sebaliknya hendaklah sentiasa diuji sebelum preskripsi, untuk mengelakkan penyalahgunaan, penggunaan berlebihan dan antibiotik yang tidak sesuai. Kajian berterusan diperlukan untuk menjejaki kadar pembawa pneumokokus berserta taburan serotaip, dan memahami taburan kerentanan antibiotik. Jika kadar rintangan lebih tinggi, adalah penting untuk menganalisis gen penentu rintangan seterusnya membuat kajian lanjutan secara terperinci. untuk pengetahuan menyeluruh tentang *Streptococcus pneumoniae* di Malaysia.

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LIST OF ABBREVIATIONS

^{0}C	Degree Celcius
%	Percentage
</≤	Less than
>/≥	Greater than
µg	Microgram
µl	Microlitre
µm	Micrometre
AIDS	Acquire Immunodeficiency Syndrome
ANSORP	Asian Network for Surveillance of Resistant Pathogens
AMR	Antimicrobial Resistance
ART	Antiretroviral Therapy
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
Bp	Base point
CAP	Community Acquire Pneumonia
cbpA	Choline binding protein A
CC	Clonal Complex
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institutes
CO_2	Carbon dioxide
CRO	Ceftriaxone
CTX	Cefotaxime
DNA	Deoxyribonucleic acid

DRSP	Drug Resistance *Streptococcus pneumoniae*
E-test	Epsilometer test
ENSSP	Erythromycin Non-susceptible *Streptococcus pneumoniae*
ERSP	Erythromycin Resistance *Streptococcus pneumoniae*
ermB	Erythromycin Ribosomal Methylase B
ERY	Erythromycin
ESSP	Erythromycin Susceptible *Streptococcus pneumoniae*
HGT	Horizontal gene transfer
HIV	Human Immunodeficiency Virus
I	Intermediate
ICT	Information Communication Technology
IPD	Invasive Pneumococcal Diseases
JKEUPM	Ethic Committee for Research involving Human Subject
KL	Kuala Lumpur
lytA	Pneumococcal autolysin A
MDR	Multidrug resistant
MDRSP	Multidrug resistant *Streptococcus pneumoniae*
mefA	Macrolide efflux protein
MIC	Minimum Inhibitory Concentration
MLST	Multi-Locus Sequence Typing
Mm	Millimetre
NC	Negative control
NCBI	National Center for Biotechnology Information
NESp	Non-encapsulated *Streptococcus pneumoniae*
NIP	National Immunization Programme
NT	Non-typeable
NTP	Non-typeable pneumococcus

NVT	Non-vaccine serotypes
PAF	Platelet-activating factor
PC	Positive control
PCR	Polymerase Chain Reaction
PCV	Pneumococcal conjugate vaccine
PCV7	7-valent conjugate vaccine
PCV10	10-valent conjugate vaccine
PCV13	13-valent conjugate vaccine
PEN	Penicillin
PFGE	Pulsed-Field Gel Electrophoresis
PH	Potential of Hydrogen
PICU	Pediaetric Intensive Care Unit
Ply	Pneumolysin
PPV	Pneumococcal Polysaccharide Vaccine
PRSP	Penicillin resistant *Streptococcus pneumoniae*
psaA	Pneumococcal surface adhesin A
pspA	Pneumococcal surface protein A
pspC	Pneumococcal surface protein C
R	Resistant
S	Susceptible
S.pneumoniae	*Streptococcus pneumoniae*
Sp.	Specie
Spp.	Species
ST	Sequence type
UK	United Kingdom
UPM	University Putra Malaysia
URT	Upper Respiratory Tract

USA	United State of America
UV	Ultraviolet radiation
VT	Vaccine serotype
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Being a pathogenic bacterium, numerous research has been conducted in developing vaccines and antibiotics for *Streptococcus pneumoniae* prevention and treatment. However, *Streptococcus pneumoniae* is known to develop resistant gene for many of the proposed medications. Due to this, contemporary researches, including this study, are investigating how *Streptococcus pneumoniae* is becoming resistant to antibiotics and what determinant genes of macrolide triggers their prevalence (Schroeder and Stephens, 2016). It is one of the most gravitating infectious disease organism that happens to range from 75.4% (75,400) out of 100,000 hospitalized patients among Ministry of Health hospitals in Malaysia in the year 2010 (Le *et al.*, 2012) as it ranked 25th among 99 pathogenic bacteria and pneumonia happens to be the 4th leading cause of death (Song, Moon H Nahm and Moseley, 2013). In another study, report analyzed that majority of <2years old hospitalized children and >50 years old adults in Malaysia are infected with *Streptococcus pneumoniae* (Le *et al.*, 2012) and this makes pneumococci serve as one of the major cause of hospitalization in Malaysia.

Also known as pneumococcal, *Streptococcus pneumoniae* is a bacterium that colonizes the human nasopharynx and cause invasive and non-invasive diseases in adults, young adults and children of less than five years of age (Sohail *et al.*, 2018), majorly affected are the infant whose immune system have not be immunized. Nasal cavity is the uppermost part of the respiratory system that is divided into respiratory and olfactory segment and serves as the habitat for microflora and pathogenic organisms that lives on the nasal cavity as colonizers of which isolation frequently takes place as carriage isolates. *Streptococcus pneumoniae* is one the nasal cavity colonizer that brings in disease manifestation; some other related colonizers are *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella cattarrhalis* and *Neisseria meningitides.* Pneumococci on its own end enables the colonization by its virulence factor activity that enhance its ability to evade the early components of the human immune response leading to pneumonia, otitis media, sinusitis, meningitis and septicemia diseases (Kadioglu *et al.*, 2008). All of which propagate to high rate of mortality and morbidity (Izurieta *et al.*, 2018).

Streptococcus pneumoniae are gram positive bacteria that belongs to *Firmicutes phylum* with a cell wall that plays an important key roles in it shape, growth, cell division and interactions with the component of human host. Pneumococci has it means of being differentiated from other *Streptococcus spp* with it alpha hemolysis pattern on sheep blood agar. It is also an encapsulated and non-capsulated diplococcus bacterial with close to 98 serotypes which serves as it main virulence factor in their capsular polysaccharide (Arushothy *et al.*, 2019). All these serotypes does not cause severe pneumococcal diseases but differ in invasiveness and non-invasiveness, age, geographical distribution,

antimicrobial resistance, and time. With its over 90 serotypes varying geographically and increase in resistance (Arushothy *et al.*, 2019), research and investigation on serotypes distribution severity and antimicrobial resistance of pneumococcus in Malaysia keep increasing rapidly (Le *et al.*, 2011). Moreover, Pneumococcal surface protein A(PspA), Pneumococcal surface protein C (PspC), Pneumococcal surface adhesin A(PsaA), Choline binding protein A(CbpA), pneumolysin (*ply*), autolysin (*LytA*), hyaluronidase, neuraminidase are the pneumococcal proteins and enzymes that precipitate the prevalence of pneumococcal diseases as virulence factors (Song, Moon H. Nahm and Moseley, 2013).

1.2 Problem Statement

Regardless of the availability of antimicrobial therapy and vaccination program, pneumococcal infection keeps prevailing (Cornick and Bentley, 2012) and based on clinical failure, macrolide are associated with the treatment of pneumococcal infections (Schroeder and Stephens, 2016). Pneumococcal serotypes also differ in geographic areas, age, time and period of distribution (Henriques-normark and Tuomanen, 2013).Thus, this increases the rate of macrolide resistance as the bacteriostatics antibiotics became widely use and variation in serotype which have impact on the vaccination. We approach this research for proper and further studies on the serotype distributions and trends of antibiotics susceptibility pattern as well as distribution of the resistance gene on macrolide antibiotics of *Streptococcus pneumoniae*.

1.3 Research Hypothesis

H1. The carriage differs according to age, time and location.

H2. *Streptococcus pneumoniae* possess the virulence properties of autolysin (*lytA*) and pneumolysin (*ply*).

H3. The capsular serotype differs in antibiotics susceptibility and resistance gene profile.

H4. Antibiotics susceptibility pattern and macrolide resistance genes of *Streptococcus pneumoniae* differs in rate.

1.4 Objectives

1.4.1 General Objective

To characterize the capsular serotype, antibiotics susceptibility pattern and the genetic analysis of macrolide resistance to *Streptococcus pneumoniae* in children carriage.

1.4.2 Specific Objectives

a) To identify the proportion of pneumococcal nasal carriage among health children from some selected nursery homes in Malaysia.

b) To determine the difference in serotype distribution of the nasal carriage isolates of *Streptococcus pneumoniae* by multiplex PCR

c) To determine the minimum inhibitory concentration (MIC) profile of antibiotics against *Streptococcus pneumoniae* isolates using E-test method.

d) To determine the macrolide-resistance determinant genes of *Streptococcus pneumoniae* isolates by PCR.

e) To relate macrolide-resistance genes and erythromycin susceptibility profile of *Streptococcus pneumoniae* isolates.

1.5 Thesis Organization

This thesis is organized as follows:

- **Chapter 1** is an introductory into the domain of the research where the aim, objectives, hypothesis and problem statements are presented.

- **Chapter 2** provides a comprehensive review on the previous and current state of *Streptococcus pneumoniae* regarding its carriage rate, serotype distribution, antibiotics susceptibility and macrolide resistance genes.

- **Chapter 3** presents the methodology of the research defining suitable techniques of microbiological experimentation and molecular methods.

- **Chapter 4** describes the resulting outcome of the method used on the research.

- **Chapter 5** discusses the out of the research methodology and results in relation with previously related research.

- **Chapter 6** concludes the research with the summarization of the research achievements, conclusions, potential enhancements and future recommended investigation.

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