DETECTION OF MACROLIDE RESISTANT GENES OF *Streptococcus pneumoniae* ISOLATED FROM PATIENTS IN HUSM AND ITS CLINICAL OUTCOMES

By:

Dr Wan Norliyana binti Wan Mahmud

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ABBREVIATIONS

ANSORP	Asian Network for Surveillance of Resistant Pathogens
BAL	Bronchio-alveolar lavage
CAP	Community-acquired pneumonia
CLSI	Clinical and Laboratory Standard Institute
CSF	Cerebrospinal fluid
ETT	Endotracheal tube secretion
HUSM	Hospital Universiti sains Malaysia
IPD	Invasive pneumococcal disease
MDR	Multidrug resistance
MIC	Minimal inhibitory concentrations
PCR	Polymerase chain reaction
Vitek MS MALDI-TOF	Vitek MS Matrix-Assisted Laser Desorption Ionization- Time of Flight Mass Spectrometry System
WHO	World Health Organization

ABSTRACT

A STUDY ON DETECTION OF MACROLIDE RESISTANCE GENES OF *Streptococcus pneumonia* ISOLATED FROM PATIENTS IN HOSPITAL USM AND ITS CLINICAL OUTCOMES

Introduction

Streptococcus pneumoniae is one of the leading pathogen causing pneumonia, meningitis, bacteremia and bacterial otitis media worldwide. It cause more serious disease in young patients less than 5 years old, elderly aged more than 65 years and in patients with underlying medical condition such as malignancy, chronic obstructive airway disease, chronic liver disease, chronic renal disease, diabetes mellitus and smokers. Global increase in antibiotic resistance in *S.pneumoniae* remains a serious concern worldwide. In global multi-country study of antimicrobial susceptibility in *S. pneumoniae*, revealed that regional rates of antibiotic resistance were consistently the highest in Asia. The dramatic increase in in-vitro resistance of *S.pneumoniae*, particularly beta-lactams and macrolide antibiotic raised the questions on clinical impact of antimicrobial resistance on clinical outcomes.

The aim of this study is to describe the clinical characteristics and outcome of *S. pneumoniae* infection, antibiotic susceptibility pattern, distribution of macrolide-resistance determinants and its relationship with macrolides susceptibility pattern and clinical outcomes among patients in Hospital Universiti Sains Malaysia, Kelantan.

Methodology

This is a descriptive cross sectional study conducted in Hospital Universiti Sains Malaysia. *S.pneumoniae,* in which non-duplicate isolates were collected from various clinical specimens from June 2014 to December 2015. Susceptibility to six antibiotics i.e penicillin, erythromycin, azithromycin, vancomycin, trimetophrim-sulfamethoxazole and amoxycillin-clavulanic acid were determined using E-test strips (BioMerieux SA, France). The results were interpreted according to CLSI guidelines. All isolates were subjected to polymerase chain reaction (PCR) analysis to detect macrolide-resistance determinants. Patients' clinical data including demographic, clinical diagnosis, risk factors, and outcomes were obtained from clinical notes.

Results

A total of 113 patients with positive growth of *S. pneumoniae* from clinical samples were included in the study. Community-acquired pneumonia is the predominant presentation of pneumococcal infection. Penicillin resistance rate was 7.1%, with MIC ranging between 0.012 – >32 µg/ml and MIC_{90} of 1µg/ml. Approximately 26.5% of the isolates resistant to erythromycin with MIC range of 0.03 – >256 1µg/ml and MIC_{90} of 32 µg/ml. Among the erythromycin-resistant isolates, majority were found to have *mef*(A) gene (50.4%), *erm*(B) gene (20%), 16.7% with combination of *mef*(A) and *erm*(B) and 13.3% with none of the two genes. There were no significant association between presence of macrolide resistance determinants with mortality ($p = 0.837$) or complications ($p > 0.999$ for empyema and cardiac complication; $p = 0.135$ for subdural abscess)

Conclusion

Overall, the isolates showed good susceptibility towards all antibiotics tested except for azithromycin. The outcome and complications of pneumococcal diseases were not significantly different between macrolide-resistance than those with macrolide-susceptible groups and were not affected by the presence of macrolide resistance determinants in the pneumococcal isolates.

ABSTRAK

KAJIAN PENGESANAN GEN RINTANGAN MACROLIDE DALAM *Streptococcus pneumoniae* YANG DI ISOLAT DARI PESAKIT DI HOSPITAL USM DAN KAITAN DENGAN KESAN KLINIKAL

Pengenalan

Streptococcus pneumoniae adalah salah satu patogen utama yang menyebabkan jangkitan paru-paru, jangkitan selaput otak, jangkitan dalam darah dan jangkitan pada telinga tengah di seluruh dunia. Jangkitan adalah lebih serius dalam kalangan pesakit muda berumur kurang dari 5 tahun, warga tua yang berumur lebih daripada 65 tahun dan juga pesakit yang mempunyai masalah perubatan yang sedia ada seperti kanser, penyakit saluran pernafasan yang kronik, penyakit hati kronik, penyakit buah pinggang kronik, kencing manis dan perokok. Peningkatan secara global kerintangan antibiotik dalam kalangan *S. pneumoniae* menjadi kebimbangan yang serius di seluruh dunia. Satu kajian yang melibatkan pelbagai negara melaporkan bahawa kadar rintangan antibiotik dalam kalangan *S. pneumoniae* secara konsisten adalah paling tinggi di rantau Asia. Peningkatan dramatik rintangan *S. pneumoniae in-vitro*, terutamanya terhadap antibiotik beta-lactams dan macrolide membangkitkan persoalan mengenai kesan klinikal rintangan antibiotik terhadap hasil klinikal.

Tujuan kajian ini adalah untuk menggambarkan ciri-ciri klinikal dan kesan klinikal jangkitan *S. pneumoniae*, corak sensitiviti antibiotik, dan menentukan hubungkait antara

gen rintangan - macrolide dengan corak sensitiviti terhadap macrolides dan kesan klinikal dalam kalangan pesakit di Hospital Universiti Sains Malaysia, Kelantan.

Tatacara

Kajian irisan lintang deskriptif ini dijalankan di Hospital Universiti Sains Malaysia. Isolat *S. pneumoniae*, bukan pendua dari pelbagai spesimen klinikal dikumpul dari Jun 2014 hingga Disember 2015. Sensitiviti terhadap enam antibiotik iaitu penisilin, erythromycin, azithromycin, vancomycin, trimetophrim-sulfamethoxazole dan amoxycillin-clavulanic acid telah dijalankan menggunakan ujian E-test (bioMérieux SA, Perancis). Keputusan telah ditafsirkan mengikut garis panduan CLSI. Ujian tindakbalas rantai polimer (PCR) telah dijalankan untuk mengesan penentu rintangan macrolide. Data klinikal pesakit termasuk data demografi, diagnosa klinikal, faktor-faktor risiko, dan kesan klinikal diperolehi daripada nota klinikal.

Keputusan

Seramai 113 pesakit dengan kultur dari sampel klinikal positif untuk *S. pneumoniae* telah dimasukkan dalam kajian ini. Klinikal manifestasi jangkitan pneumokokal yang utama adalah jangkitan pada paru-paru. Kadar kerintangan terhadap penicillin adalah 7.1%, dengan MIC antara 0.012 - > 32 μg / ml dan MIC_{90} ialah 1μg / ml. 26.5% isolat rintang terhadap eryhromycin dengan MIC antara 0.03 - > 256 1μg / ml dan nilai MIC_{90} daripada 32 μg / ml. Majoriti (50.4%) isolat yang rintang terhadap erythromycin mempunyai gen *mef*(A). Isolat yang mempunyai hanya gen *erm*(B) dan kombinasi kedua-dua *mef*(A) dan *erm*(B) adalah masing-masing 20% dan 16.7%. Manakala isolat yang tidak mempunyai

kedua-dua gen adalah 13.3%. Didapati tidak ada hubungkait yang signifikan antara kehadiran gen rintangan macrolide dengan kematian (p = 0.837) atau komplikasi (p > 0.999 untuk empyema dan komplikasi jantung; p = 0.135 untuk subdural abscess)

Kesimpulan

Secara keseluruhan, isolat menunjukkan tahap sensitiviti yang baik terhadap semua antibiotik yang diuji kecuali azithromycin. Kesan klinikal dan komplikasi penyakit pneumokokal tidak berbeza secara signifikan antara pesakit yang sensitif terhadap macrolide daripada mereka yang rintang terhadap macrolide, dan tidak terjejas dengan kehadiran gen penentu rintang macrolide di dalam isolat pneumokokal.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Streptococcus pneumoniae (pneumococcus) is one of the leading causes of the mortality and morbidity worldwide. In 2005, World Health Organization (WHO) estimated that 1.6 million deaths were caused by this agent annually, where 0.7- 1 million are children aged less than 5 years. *S. pneumoniae* is a Gram positive bacteria, spread by airborne droplets and is a primary causes of bacterial pneumonia, acute otitis media, sinusitis and bacteraemia and meningitis worldwide. It causes more serious infection in infants less than 3 years old and in adults older than 65 years old (Wardlaw *et al.*, 2006). Conditions that predispose to pneumoccoccal diseases include underlying medical condition such as malignancy, asthma, chronic obstructive airway disease, diabetes mellitus and smoking. Approximately 10% of all patients with invasive pneumococcal disease die due to their illness but case-fatality rates are higher for the elderly and patient with underlying medical conditions.

WHO estimates that in the Asia Pasific region, 49 out of 98 cases of pneumonia deaths in children are due to pneumococcal pneumonia. In Malaysia, 4% of the 7000 deaths among children less than 5 years old were estimated due to pneumococci (incidence of overall pneumonia deaths of 10.2 out of 10,000 children aged under 5 years (Rohani *et al.*, 1999b). Several studies done in Malaysia found that pneumonia is the most common pneumococcal disease in Malaysia (Rzeszutek et al., 2004; Lim et al., 2007; Rohani et al., 2011).

For pneumococcal infections, antibiotics are one of the choices of treatment. However, several studies have shown that in recent years, there has been dramatic increased in the prevalence of antibiotic resistance in *S. pneumoniae* particularly against those of the first line antibiotics such as penicillin, as well as to macrolide antibiotics such as erythromycin, that makes pneumoccocal infections difficult to treat especially in children and elderly (Song *et al.,* 2004a). Asian Network for Surveillance of Resistant Pathogens (ANSORP) study 2004 previously has reported an alarming increase of erythromycin resistant S. *pneumoniae*. In some Asian countries, the reported prevalence of penicillin and macrolide resistance was the highest in the world (Song *et al.*, 2004b).

Macrolides act by binding to the 23S subunit of the 50S ribosome, by attaching at domain V of the peptidyl transferase loop, thereby blocking protein synthesis (Mandell et al., 2014). There are two commonly described mechanisms for macrolide resistance. The first one is target site modification in the 23S rRNA of the large 50S ribosomal subunit by a ribosomal methylase encoded by the *erm (B)* (erythromycin ribosome methylation) genes on plasmids or transposons on chromosomes that are self-transferable, resulting in decreased binding of the antibiotics to targets on the ribosome. The second mechanism is by drug efflux system encoded by the *mef (A)* gene (macrolide efflux), a transposable element, and consists of transmembrane domains across the cytoplasmic membrane. This efflux pump confer resistance by excludes macrolides from the bacterium.

The disease burden of pneumococcal infections has increased due to widespread emergence of antimicrobial resistance in many countries during the past few decades. The increased resistance rate to penicillin and macrolide caused the treatment for

pneumococcal diseases complicated and difficult. Previous surveillance studies showed that more than 60% of pneumococcal isolates from Asian countries were resistant to erythromycin and 22.7% were penicillin-resistant (Kim *et al.*, 2012; Song *et al.*, 1999).

Infectious Diseases Society of America/American Thoracic Society recommended macrolides as a drug of choice for empiric therapy of community-acquired pneumonia (CAP) for outpatient treatment in previously healthy and low-risk patient, and in patients with comorbidities or use of antimicrobials within the previous 3 months, beta-lactam plus a macrolide was strongly recommended. For patients requiring hospitalization, a parenteral beta-lactam (with or without a macrolide) was advocated (Mandell *et al.*, 2007). Data confirm that macrolide resistance in pneumococci is a serious problem in many Asian countries including in Malaysia during the past few decades. This complicated the treatment for pneumococcal diseases and increased the disease burden. However, data specifically evaluate the impact of macrolide resistance on clinical outcomes are scarce.

An Asian Network for Surveillance of Resistant Pathogens (ANSORP) Study, Song *et al.* (2004b), found out that bacteremia and mechanical ventilation were significant risk factors for death, but any kind of antibiotic resistance was not associated with increased mortality due to pneumococcal pneumonia. However, in this study, the number of patients from each participating country was relatively small, and the quality of clinical care were different in each participating center, therefore the findings were not represent the overall status of the clinical characteristics or the final outcome in each country.

1.2 Rationale of the study

Because of the frequency and importance of pneumococcal infections in our population, and with increasing prevalence of macrolide-resistant S. *pneumoniae*, the clinical impact of resistant strain should be further clarified specifically in our country. To our knowledge, there is no study yet that describe the association between the macrolide resistance determinants with the clinical outcomes. Therefore, the aim of this study is to determine the clinical characteristic and clinical outcomes of pneumococcal infections caused by macrolide-resistant S. *pneumoniae*, specifically in relation to the presence of macrolide- resistance determinant genes, as well as to determine the proportion of macrolide resistant genes and current antibiotic susceptibility pattern of common antibiotic used against S. *pneumoniae* in our population that can be served as surveillance data in our country.

1.3 Literature Review

1.3.1 History and taxonomy of *Streptococcus pneumoniae (S. pneumoniae*/ pneumococcus)

Streptococcus pneumoniae was first described independently by two microbiologist, Louis Pasteur in France and George M. Sternberg from United States in 1881 and was named as *Microbe septicemique du salive* by Pasteur and *Micrococcus pasteuri* by Sternberg (Watson *et al.*, 1993). By 1886, the organism was called as *Pneumococcus* because of its ability to cause pulmonary disease, then was renamed as *Diplococcus pneumoniae* in 1920 by Winslow *et al.* (1920) because of its characteristic appearance in Gram stain and later on given its present name as *Streptococcus pneumoniae* in 1974 based on its characteristic growth as chains of cocci in liquid medium (Edward N.Janoff, 2015).

S. pneumoniae is classified under the order of Lactobacillales, family of Streptococcaceae, genus of S*treptococcus* and species of *S. pneumoniae*. There are currently 97 serotypes of *S.pneumoniae* known, distinguished by the polysaccharide capsular structure (Geno *et al.*, 2015).

1.3.2 Epidemiology

Streptococcus pneumoniae is one of the leading pathogen worldwide causing pneumonia, meningitis, bacteremia and bacterial otitis media. Pneumonia is the leading cause of death of children, accounting for 1 in 5 deaths, and *S. pneumoniae* is the leading cause of bacterial childhood pneumonia (Bennett *et al.*, 2015). Pneumococcal

diseases including pneumonia, sepsis, and meningitis are the leading cause of bacterial deaths in young children, causing up to one million deaths per year in those younger than 5 years (O'Brien *et al.*, 2009).

Invasive pneumococcal disease (IPD), defined as isolation of *S. pneumoniae* from a normally sterile site, examples blood, cerebrospinal fluid (CSF), surgical aspirate; pleural, pericardial, peritoneal, bone, or joint fluid. IPD commonly affects young children aged less than two years and adult aged ≥ 65 years and case fatality rate higher in immunocompromised individuals, elderly and patients with certain underlying diseases (CDC, 2010).

In Malaysia, a 5 year retrospective study at Universiti Malaya Medical Centre done by Nur *et al.* (2008) examining patients in all age groups identified *S. pneumoniae* as the major causative agent in bacterial meningitis (23.4%), and other study also reported that *S. pneumoniae* is one of the leading pathogens (8 out of 58; 13.8%) in childhood meningitis following *H. influenzae* type B accounting for 48.3% (Hussain *et al.*, 1998). In another study done in Kuala Lumpur, the percentage of *S. pneumoniae* isolated from adult patients with community-acquired pneumonia was 13.2% (Liam, 2005). In a study that investigated childhood IPD, pneumonia was the most common type of IPD in children younger than 14 years of age (Lim *et al.*, 2007).

1.3.3 *Streptococcus pneumoniae* colonization

Children are the main carriers of *Streptococcus pneumoniae* (Bogaert *et al.*, 2004; Hussain *et al.*, 2005). The organism begins to colonize the nasopharynx as early as first week of life. The prevalence of colonization increases from less than 10% to a peak at

70% to 100% at age one year, persists through the third year of life, and decreases thereafter to adult rates of approximately 10% depending on the sampling location, methods, and culture (Benin *et al.*, 2003).

In one cohort study that investigated the age-dependent carriage rate in healthy children and adolescents aged 1–19 years, the peak incidence of pneumococcal colonisation was at the age of 3 years (55%) then steadily decline and stable at rate of 8% after the age of 10 years old (Bogaert *et al.*, 2004).

In the United States, a prospective study has shown that generally pneumococci colonize an infant at around 6 months of age and can be detected for a mean of about 4 months (Gray and Dillon, 1988). The individual serotype can persist from one week to 6 months, but in adults, the serotype persists for shorter periods, usually two to 4 weeks (Ekdahl *et al.,* 1997), but can be longer up to several months (Heffron R., 1979).

The risk factors for nasopharyngeal carriage in young children included day care center attendance and having young siblings (Regev-Yochay *et al.*, 2004), while for adolescent and adults the risk factors include acute upper respiratory tract infection, cigarette smoking and asthma (Cardozo *et al.*, 2008). Ethnicity, crowding, environmental, and socioeconomic factors such as income also contributed to the risk of nasopharyngeal carriage of *S. pneumoniae.* (Bogaert *et al.*, 2004).

1.3.4 Risk factors for pneumococcal infection

Factors that predispose to pneumococcal infection include conditions that leads to defective antibody formation, whether primary (congenital) such as

agammaglobulinaemia, common variable agammaglobulinaemia or secondary (acquired), example multiple myeloma, chronic myeloid leukaemia and HIV infection (Bennett *et al.*, 2015). Defective complement pathway and insufficient number or defective function of polymorphonuclear leukocyte as seen in patient with alcoholism, liver cirrhosis, diabetes mellitus, renal disease and glucocorticoid treatment were also associated with increased risk of getting pneumococcal disease.

Other risk factors for invasive pneumococcal disease include asplenia, age more than 65 years, underlying chronic lung diseases and prior respiratory viral infection (van der Poll and Opal, 2009). Nosocomial pneumonia caused by *Streptococcus pneumoniae* following any cause of hospitalization also had been reported. Other factors include crowding, alcoholism, cigarette smoking, poverty and recent use of antibiotic may contribute to pneumococcal disease (Kim *et al.*, 1996).

The risk of invasive pneumococcal disease in closed populations and in the community may be reduced by the widespread use of pneumococcal vaccine (Albrich *et al.*, 2007; Valenzuela *et al.*, 2007).

1.3.5 Virulence factors and pathogenesis

Nasopharyngeal colonization of *S. pneumoniae* is required for transmission of bacteria and development of invasive disease (Weinberger *et al.*, 2008). Pneumococci bind to mucosal epithelial cells of the nasopharynx and caused disease by contiguous spread to the sinuses or middle ear causing sinusitis or otitis media, aspiration into the lung causing pneumonia, or invasion of the bloodstream causing septicaemia (Bogaert *et al.*, 2004; Tuomanen *et al.*, 1995)

The pathogenesis of pneumococcal infection is a complex interplay between pneumococcal virulence determinants and the host immune response. This virulence determinants and the corresponding immune responses produce four key effects that involved in the pathogenesis of pneumococcal diseases; adhesion, invasion, inflammation and shock (Gillespie and Balakrishnan, 2000).

Pneumococcal capsule is the most important virulence factor for pneumococcal and play a crucial role during colonisation, invasion, and dissemination from the respiratory tract (Nelson *et al.*, 2007). Capsular polysaccharide avoid ingestion and killing by host phagocytic cells (Edward N.Janoff, 2015; Musher, 1992), inhibits complement and immunoglobulin-binding to host receptors (van der Poll and Opal, 2009), and also enhances pneumococcal colonization by limiting mucus-mediated clearance (Nelson *et al.*, 2007).

Virtually all *S. pneumoniae* expressed pneumolysin (Kadioglu *et al.*, 2008) which is cytotoxic to host phagocytic and respiratory epithelial cells and have the ability to inhibit ciliary action of epithelial cells. (Zhang *et al.*, 2007).

1.3.6 Clinical significance

S.pneumoniae can directly spread from the nasopharyngeal site of colonization and causes infection of the middle ear, lung, bronchi, and sinuses. The organism may spread hematogenously to the heart, bones and joint. Infection to the pleural, peritoneal cavity and central nervous system may occur by direct extension or hematogenous route (Bennett *et al.*, 2015).

S. pneumoniae is a major cause of community-acquired pneumonia (CAP) accounting up to 36% of adult community-acquired pneumonia. In the United States, estimated that as many as 400,000 hospitalizations occur annually are due to pneumococcal pneumonia (CDC, 2015b). The risk for serious disease is higher in older adults because they usually have underlying medical conditions such as chronic obstructive pulmonary disease, malignancy, heart disease, liver disease and diabetes (Edward N.Janoff, 2015; Laurichesse *et al.*, 2001).

Complications of pneumococcal pneumonia include pleural effusion, empyema, lung abscess, and less common are pericarditis and endocarditis (Musher, 1992). In Malaysia, CAP also the main clinical manifestation of pneumococcal infections, followed by sepsis, otitis media, and conjunctivitis. Most of the patients aged 50 years or more had preexisting chronic conditions such as current or treated pulmonary tuberculosis, chronic obstructive airway diseases, asthma or lung cancer (Rohani *et al.*, 1999b).

S. pneumoniae is also a common cause of bacteremia and meningitis in children and adults (Winn and Koneman, 2006). Among elderly patients, case-fatality rate for pneumococcal bacteremia range from 20% to as high as 60% among elderly patients. (CDC, 2015b).

S. pneumoniae also a leading cause of acute otitis media in children (Block, 1997; Bluestone *et al.*, 1992). Complications of acute pneumococcal otitis media include mastoiditis, bacteremia and tympanic membrane perforation (Kouppari *et al.*, 2000).

Treatment failures for acute otitis media due to multidrug-resistant *S pneumoniae* have been reported (Pichichero and Casey, 2007).

1.3.7 Antibiotic resistance

In global multi-country studies of antimicrobial susceptibility in *S. pneumoniae*, an Asian Network for Surveillance of Resistant Pathogens (ANSORP) study by Kim *et al.* (2012) revealed that regional rates of antibiotic resistance were consistently the highest in Asia. Isolates that had the highest resistance rates generally are from China, Hong Kong, South Korea, Taiwan, Thailand and Vietnam (up to 92%).

Macrolides are one of the choice of treatments for the infection caused by *S. pneumoniae*, especially for empirical therapy for patient with CAP in outpatient setting (Mandell et al.,2007; MOH., 2014) . However in recent years, there is an alarming increased in resistant rate of *S.pneumoniae* towards macrolide antibiotics in the world including Malaysia were reported in the literature. In China, erythromycin resistance rates for *S. pneumoniae* increased from 35-53% in 1996-1999, to over 75% by 2001 and South Korea showed that resistant rate to erythromycin remained high over period of 1996-2001 (75-85%). While in Malaysia, 54 out of 165 isolates (32%) were resistant to erythromycin in year 2012 (Kim *et al.*, 2012).

Resistance to macrolides antibiotic occur by several mechanisms in which two major mechanisms are by modification of the target site (ribosomal), mediated by *erm*(B) gene usually carried on conjugative transposons, which may facilitate rapid dissemination of erythromycin resistance. The second major mechanism is active (proton-dependent) efflux, which encoded by *mef*(A) genes and can be transferred by

conjugation (Lynch and Martinez, 2002). *Erm*(B) gene caused methylation at the macrolide-binding site of the 23S rRNA results in high-level resistance (≥ 64 μg/mL) compared to efflux pump encoded by the *mef*(A) that usually confer low level resistance (≤16 μg/mL) (Edward N.Janoff, 2015).

A study by Song *et al.* (2004a) reported that ribosomal methylation encoded by *erm*(B) was the most common mechanism of erythromycin resistance in China, Taiwan,Sri Lanka and Korea, while in Hong Kong, Singapore, Thailand and Malaysia, efflux pump encoded by *mef*(A) gene was more common. *Erm*(B) was found in more than 50% of pneumococcal isolates either alone or in combination with *mef*(A) in most Asian countries except Hong Kong, Malaysia and Singapore.

A previous local study reported that among 71 isolates, 25 (35.2%) isolates were erythromycin susceptible, 3 (4.2%) were intermediate and 42 (60.6%) were resistant. *Mef*(A) alone was found in 53 isolates (74.7%), followed by combination of both *mef*(A) and *erm*(B) gene in 15 (21.1%). Three (4.2%) isolates have none of the two genes. Isolates with both *mef*(A) and *erm*(B) showed very high MICs to erythromycin (≥ 256 μg/mL) (Nathan *et al.*, 2013).

The rapid increase in the prevalence of macrolide resistance worldwide including Asian countries are due to widespread use of macrolides in clinical practice (Felmingham *et al.*, 2007; Kim *et al.*, 2012) and because of clonal spread of macrolide-resistant strains (Kim *et al.*, 2012).

Penicillin resistance in *S. pneumoniae* is caused by structural changes in penicillin-binding proteins (PBP) results in reduced affinity for penicillin and other beta-lactam

antibiotics (Linares *et al.*, 1992). Six PBPs have been identified in *S. pneumoniae* including PBPs 1a, 1b, 2a, 2b, 2x and 3, where the majority of the resistance are caused by PBP 1a, 2x and 2b (Lynch and Zhanel, 2005). ANSORP study in 2012 reported a decrease in the prevalence of penicillin resistance in non-meningeal pneumococci whereby only 4.6% were non-susceptible to penicillin and fully resistance only found in China (2.2%) and South Korea (0.3%). All 142 non-meningeal pneumococci isolated from Malaysia were sensitive to penicillin. Whilst for the meningeal isolates, 23.1% were resistant (Kim *et al.*, 2012). From local data, incidence of penicillin resistance was increase from two per cent in 1985 (Cheong *et al.*, 1988), to 10.9% in 1997 (Rohani *et al.*, 1999a), and 31.78% in 2009 (Rohani *et al.*, 2011). A more recent study that investigated 255 clinical isolates collected from 2005-2010 reported a higher resistant rate which was 50.2% (Arushothy *et al.*, 2016).

The ANSORP group also reported a high rate of resistance to trimethoprim-sulfamethoxazole (50.2%) among *S. pneumoniae* in Asian countries *(Kim et al., 2012)*. Resistance occur due to mutations in the dihydrofolate reductase (*dhfr*) gene (Lynch and Zhanel, 2009a). In Malaysia, resistance to trimetophrim-sulfamethoxazole was reported to range from 22% to 44% (Kim *et al.*, 2012; Nathan *et al.*, 2013; Rohani *et al.*, 2011).

Vancomycin-resistant *S. pneumoniae* have not been reported (Kim *et al.*, 2012; Zhanel *et al.*, 2003). However, the emergence of vancomycin tolerance (the ability of bacteria to survive but not proliferate in the presence of a bactericidal antibiotic) in *S. pneumoniae* have been described in the previous studies (McCullers *et al.*, 2000; Novak

et al., 1999). In Malaysia, none of the *S. pneumoniae* isolates investigated in previous studies showed resistance to vancomycin (Rohani *et al.*, 2011).

Prior antibiotic exposure is the major risk factor for amplification of resistance, and clonal spread facilitates dissemination of drug-resistant strains (Lynch and Martinez, 2002). Macrolide use was the single most important factor for the emergence of macrolide resistance in vivo (Malhotra-Kumar *et al.*, 2007)

Although antibiotic resistance among *S. pneumoniae* was noted increasing in trend worldwide, the clinical significance of in vitro resistance is controversial (Lynch and Martinez, 2002; Lynch and Zhanel, 2005). In a matched case-control study of patients with bacteremic pneumococcal infection, a breakthrough bacteremia during macrolide therapy was observed among patients infected with an erythromycin-resistant pneumococcus, thus indicate that in vitro macrolide resistance is clinically relevant (Lonks *et al.*, 2002). Several studies reported a similar mortality rates of invasive pneumococcal diseases due to macrolide-resistant or macrolide-susceptible strains (Moreno *et al.*, 1995).

1.3.8 Laboratory identification of *Streptococcus pneumoniae*

1.3.8.1 Microscopy

S. pneumoniae is a Gram-positive cocci occuring in pairs (diplococci) or short chain. The cocci are about 1 µm in diameter, ovoid in shape with their distal end narrowed (lancet-shaped). They are non-motile and non-sporing (Edward N.Janoff, 2015).

1.3.8.2 Culture

S. pneumoniae is an aerobic and facultative anaerobic organism, grows best in the air with 5-10% carbon dioxide (CO_2), at an optimum temperature of $37^{O}C$. The organism grows well on media with 5-10% serum, blood or heated blood which supplies the nutrients, pH buffer and catalase (Ross, 1996). On blood agar, the colonies are small, smooth, transparent and surrounded by greenish zone of α-haemolysis. Initially the colonies are tiny, convex then become flat or depressed centrally called 'draughtsman colonies' after 24-48 hour of incubation (Reller *et al.*, 2008). Some strain may produce very large capsule and form larger mucoid colony (Ross, 1996).

Figure 1: *Streptococcus pneumoniae* growth on sheep blood agar showed "draughtsman" colonies with Optochin disc showed zone of inhibition

(Adapted from Reller *et al.* (2008))

The mainstay of diagnostic tests are Gram stains and microbiological cultures (Lynch and Zhanel, 2009b). Examination of sputum samples before antibiotics were administered and performance of culture within 24 hour of antibiotic therapy yielded

the correct diagnosis in more than 80% of pneumococcal pneumonia cases (Musher *et al.*, 2004)

1.3.8.3 Biochemical test

S. pneumoniae produce catalase negative and oxidase negative reaction. They can be identified in the laboratory by two reactions: susceptibility to optochin (ethyl hydrocuprein hydrochloride), and solubility in bile salts (sodium deoxycholate).

Optochin susceptibility test

Susceptibility to Optochin provides a reliable and simple test to differentiate *S.pneumoniae* from viridans streptococci. Five µg of optochin disc is placed on a blood agar or MHBA plate that has been confluently spread with a broth suspension of suspected *S.pneumoniae* colonies, incubate at $37^{O}C$ in a 5-10% CO_2 incubator (Ross, 1996). A zone of inhibition of 14mm or more around a 6-mm disc indicates susceptibility to optochin and the organism can presumptively identifies as *S. pneumoniae*. If the zone of inhibition is smaller than 14mm, other alternative identification test (e.g., bile solubility) should be performed because some of the viridans streptococci may show small zone of inhibition (Winn and Koneman, 2006). Optochin susceptibility test was reported to have sensitivity of 100% and specificity of 98-100% (Chandler et al.,2000., JA Kellogg et al.,2001). Optochin-resistant *S.pneumoniae* have been reported but rarely encountered (Fenoll *et al.*, 1990).

Bile solubility test

Bile salts (Sodium deoxycholate) is capable to lyse *S.pneumoniae* selectively when added to actively growing bacteria in agar or broth media. Bile salts activates the autolytic enzymes (pneumolysin) of *S.pneumoniae* and accelerate their natural lytic reaction observed with culture (Winn and Koneman, 2006). Clearing of broth suspension containing *S.pneumoniae* colonies with addition of sodium deoxycholate indicate that the organism is bile-soluble.

Bile solubility is very sensitive and specific for identification of *S. pneumoniae,* 100% and 99% respectively (Kellogg *et al.*, 2001). *S. pneumoniae* can be differentiated from other viridans streptococci by optochin susceptibility and bile solubility, whereby *S. pneumoniae* isolates are typically susceptible to optochin and are bile soluble, whereas streptococci viridans are typically resistant to optochin and are bile insoluble (Reller *et al.*, 2008)

1.3.4.4 Other method

Rapid test

Pneumococcal antigen detection such as latex agglutination test is available for rapid detection of *S.pneumoniae*. It is widely used for the diagnosis of pneumococcal meningitis (Samra *et al.*, 2003) and rapid detection of *S. pneumoniae* in blood culture (Altun *et al.*, 2016). This method is easy to perform with sensitivity of 99.6–100 % and specificity of 64 - 98 %, respectively (Altun *et al.*, 2016). Rapid immunochromatographic test (ICT) that detects the polysaccharide cell wall antigen of *S. pneumoniae* (NOW *S. pneumonia*e urinary antigen test; Binax) is also available. It has a sensitivity of 70%–80% and a specificity of more than 90% if applied to urine samples (Briones *et al.*, 2006; Rosón *et al.*, 2004).

Semi-automated/ automated system method

Several semi-automated and automated systems are commercially available for identification of *S. pneumoniae,* and reported to produce acceptable identification results (Brigante *et al.*, 2006). Semi-automated systems include Vitek 2 system (bioMérieux Vitek, USA), Phoenix system (Becton Dickinson Diagnostic Systems, Sparks, MD) and MicroScan WalkAway-96 System (Dade Behring). In one study, Vitek 2 system correctly identified 96.9% of *S. pneumoniae* and one of the accurate and acceptable means for performing identification and antibiotic susceptibility tests for medically relevant Gram-positive cocci (Ligozzi *et al.*, 2002).

Example of automated system is Vitek MS Matrix-Assisted Laser Desorption Ionization- Time of Flight Mass Spectrometry System (Vitek MS MALDI-TOF). The sensitivity and specificity of the Vitek MS MALDI-TOF for the identification of *S. pneumoniae* are 99.1% and 100% respectively and the performance is similar to that of the optochin susceptibility test for routine identification of pneumococcal isolates (Dubois *et al.*, 2013).

Nucleic acid amplification test (NAAT)

Nucleic acid amplification test such as polymerase chain reaction (PCR) have been used to detect *S.pneumoniae* DNA in blood, sputum and other body fluids such as bronchio-alveolar lavage and cerebrospinal fluid. In a patient with a pneumococcal pneumonia, PCR using blood specimen has a wide range of sensitivity ranging from 29% to 100%. Whilst when using sputum samples, PCR was reported to have higher positivity rate ranging from 68% to 100% (Murdoch, 2004). Pneumolysin (*ply*), *lytA* and *psa(A)* gene

were used as specific targets for detection of *S. pneumoniae* DNA. *Ply* gene is also present in some viridans streptococci. Thus, the use of this gene can lead to false detection of pneumococci. *LytA* and *psaA* genes were the most specific for the detection *S. pneumoniae* (Maria da Gloria *et al.*, 2007).

1.3.9 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing is very important as it can confirm susceptibility to the antimicrobial agent chosen and guide the clinician to the appropriate empirical treatment, as well as to detect the resistance in bacterial isolates (Reller *et al.*, 2009). There are several methods of antimicrobial susceptibility test for bacteria available, which include dilution method that based on minimum inhibitory concentration (MIC) and disk diffusion method that measure zone of inhibition. MIC is the minimal concentration of an antimicrobial agent (in microgram/milliliter) required to inhibit or kill the microorganism (Jorgensen and Turnidge, 2015). It is the least amount of antimicrobial that will inhibit visible growth of an organism after overnight incubation (Miles and Amyes, 1996). Automated commercial methods also available such as Vitek 2 (bioMerieux, Durham, NC), Phoenix (BD,Sparks, MD) and MicroScan (Siemens Healthcare Diagnostics, Deerfil, IL).

The broth macrodilution, broth microdilution, agar dilution and disk diffusion methods are the reference methods based on Clinical and Laboratory Standard Institute (CLSI). The results of susceptibility test are interpreted according to CLSI interpretive criteria that categorized into susceptible, intermediate and resistant. Susceptible category is defined as when the bacterial isolates are inhibited by the antimicrobial agent when

recommended dosage is used. Isolates with MIC in intermediate category show lower response rate compare to susceptible isolates but shows clinical efficacy when higher than normal dosage of drug are used. The bacterial isolates are considered resistant when they are not inhibited by the usual dosage of drug scheduled.

1.3.9.1 Disk Diffusion method

In this method, agar plate is uniformly inoculated with the standardized inoculum of test organism. A paper disk impregnated with a fixed concentration of antibiotic then is placed on the agar surface and incubated under suitable conditions. The antibiotic then diffuses into the agar and inhibits the growth of the organism producing zone of inhibition that is measured to determine the sensitivity.

This test is simple, does not required special equipment, the result is easy for interpretation, and able to test large number of microorganism and antimicrobial agent (Balouiri *et al.*, 2016), and the least costly of all susceptibility methods (Reller *et al.*, 2009). However, the disadvantages are it inability to distinguish between bactericidal and bacteriostatic effect because inhibition of the bacterial growth does not necessarily mean bacterial death (Balouiri *et al.*, 2016) and lack of automation of the test (Reller *et al.*, 2009). The method need to be standardized as zone of inhibition is largely affected by inoculum size, quality of medium or agar plate used, incubation condition and technical staff performance. Antimicrobial agents such as vancomycin, polymyxin, and macrolides such as clarithromycin have higher molecular weights and therefore diffuse poorly in an agar plate (Schwalbe *et al.*, 2007). These poor diffusion combine with

poorly resolved concentration gradient around these disks can cause inaccurate reading of the results.

1.3.9.2 Antimicrobial gradient method (E-test)

This method determines the susceptibility of the antimicrobial agents by establishment of an antimicrobial concentration gradient in an agar medium. Etest® (bioMe´rieux AB Biodisk) is one of the commercialized test consists of a predefined gradient of antibiotic concentrations on a plastic strip used to determine the MIC of antimicrobial agent. In this method, the strip that is impregnated with increasing concentration gradient of an antimicrobial agents is placed on surface of agar plate that has been inoculated with tested microorganism.

After an overnight incubation, the MIC is determined by reading a value at the intersection of the strip and the growth inhibition ellipse. The advantages of the test is it simple and easy to be done and have flexibility of testing the drug that chosen by the laboratory. However one of the limitations of the Etest is it's high cost. This method is expensive if many drugs are to be tested (Reller *et al.*, 2009). This method is best used if only need to test one or two drugs or when testing a fastidious organism that require a special incubation conditions, example is when testing sensitivity of *S.pneumoniae* to penicillin (Jorgensen *et al.*, 1994).

1.3.9.3 Broth dilution method

Broth macrodilution method is one of the earliest method for antimicrobial susceptibility testing (Reller *et al.*, 2009). The twofold dilutions of antibiotic are prepared, and an adjusted bacterial inoculum is added to each tube containing the antimicrobial agent. The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the tubes as detected by the unaided eye (Coyle, 2005). The advantage of this method is the generation of a quantitative result. The major disadvantages are the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test (Reller *et al.*, 2009).

Broth microdilution MIC testing is performed in a polystyrene panel make a broth dilution technique more practical and popular (Reller *et al.*, 2009). The standard tray contains 96 wells and contains 7–8 dilutions of 12 different antimicrobial agents (Coyle, 2005). The advantages of this method include the generation of MICs, the reproducibility and convenience of having preprepared panels, and the economy of reagents and space that occurs due to the miniaturization of the test. The main disadvantage is because of some inflexibility of drug selections available in standard commercial panels (Reller *et al.*, 2009).

1.3.10 Treatment and prevention

Infectious Diseases Society of America suggested that for management of community-acquired pneumonia in adults in outpatient setting, a macrolide (azithromycin, clarithromycin, or erythromycin) are recommended for previously healthy and no risk

factors for drug-resistant *S. pneumoniae* (DRSP) infection. A b-lactam plus a macrolide are preferred for patients with comorbidities, such as chronic heart, lung, liver, or renal disease, diabetes mellitus, alcoholism, malignancies or other risks for infection caused by DRSP. Whilst for patients that required hospitalization, a respiratory fluoroquinolone (moxifloxacin, gemifloxacin, or levofloxacin) or a b-lactam plus a macrolide are strongly recommended (Mandell *et al.*, 2007).

Pneumococcal strains from patients requiring hospitalization should undergo antibiotic susceptibility test because of increasing antibiotic resistance worldwide and susceptibilities to standard agents are no longer assured (van der Poll and Opal, 2009). Non-meningeal *S pneumoniae* strains with intermediate or high-level resistance to penicillin can still be treated with high-dose, β-lactam antibiotics (penicillins, or second or third generation cephalosporins (Woodhead *et al.*, 2005). Whilst for the meningeal strain with even intermediate resistance, use of other agents are necessary to assure a successful outcome (Mandell *et al.*, 2007).

There are two types of pneumococcal vaccine currently available in Malaysia, includes polysaccharide vaccine (PPV) (Pneumo 23 Polyvalent Vaccine from Sanofi Pasteur and Pneumovax 23 Vaccine from Merck Sharp & Dohme) and conjugate vaccine (PCV) (Prevenar-13 from Pfizer) (Victor K E Lim, 2014). Pneumococcal polysaccharide vaccine consists of the 23 most common capsular serotypes that cause invasive pneumococcal disease (Bogaert *et al.*, 2004), poorly immunogenic in children less than two years old, compared to PCV that highly immunogenic in young children (Millar *et*

al., 2008). The incidence of pneumococcal diseases in all age group markedly reduced by the widespread use of conjugate vaccine (CDC, 2015a).