Prevalence of macrolide resistance and in vitro activities of six antimicrobial agents against clinical isolates of Streptococcus pneumoniae from a multi-center surveillance in Malaysia


*Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia, **Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia, ***Bacteriology Division, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia, ****Department of Pharmaceutical Chemistry, School of Pharmacy, International Medical University, Bukit Jalil, 57000 Kuala Lumpur, Malaysia

SUMMARY
The in vitro activities of 6 antimicrobial agents against clinical isolates of Streptococcus pneumoniae (pneumococci) were investigated and the erythromycin minimum inhibitory concentrations (MICs) were correlated with the two major macrolide resistance determinants, mef(A) and erm(B). MICs of commonly used antibiotics as well as the presence of macrolide resistance determinant genes in all isolates were tested. Seventy one pneumococcal isolates collected at Institute for Medical Research (IMR) were included in this study. Phenotypic characterization, MIC determination using E-test strips and polymerase chain reactions for antibiotic resistance determination were included. Among the isolates, 25 (35.2%) isolates were erythromycin susceptible, 3 (4.2%) were intermediate and 42 (60.6%) were resistant. Fifty three isolates (74.7%) were found with mef(A) alone, 15 (21.1%) isolates with erm(B) + mef(A) combination and 3 (4.2%) isolates with none of the two genes. The in vitro activity of penicillin, amoxicillin clavulanic acid, ceftriaxone and cefotaxime is superior to trimethoprim-sulfamethoxazole and erythromycin. In conclusion, pneumococcal isolates in this study were highly susceptible to penicillin with very low MICs. However, a very high prevalence rate of erythromycin resistance was observed. Erythromycin resistant S. pneumoniae isolates with both mef(A) and erm(B) showed very high MICs ≥256 µg/mL.

INTRODUCTION
Streptococcus pneumoniae is one of the leading agents of respiratory diseases with high morbidity and mortality worldwide. Several surveillance studies indicate that various serotypes, in particular, serotypes 1 and 19F, are associated with the pneumococcal infections (Song et al., 2004a; Song et al., 2004b; Rohani et al., 1999). A number of antibiotic regimen are available in combating the infection. However, in recent years, there has been a dramatic increase in the prevalence of antibiotic resistance in S. pneumoniae particularly against those of the first line antibiotics such as erythromycin, as well as penicillin (Varaldero et al., 2009; Song et al., 2004a; Song et al., 2004b). Previous multinational surveillance of pneumococcal resistance by the Asian Network for Surveillance of Resistant Pathogens (ANSORP) has reported an alarming increase for erythromycin resistant S. pneumoniae (ERSP) since last two decades (Song et al., 2004a; Song et al., 2004b; Song et al., 1999).

There are two commonly described mechanisms for erythromycin resistance namely, target modification by a ribosomal methylase encoded by the erm(B) gene and drug efflux encoded by the mef(A) gene. ERSP from China, Sri Lanka, Taiwan and Korea is predominantly mediated by the erm(B) gene, which is usually associated with high-level resistance (MICs ≥128 µg/mL), whereas mef(A) that prevails among strains from Hong Kong, Malaysia, Singapore and Thailand shows low-level resistance (MICs 1-64 µg/mL) (Song et al., 2004a). However, in Malaysia, the current perspective on the prevalence of antibiotic resistant S. pneumoniae is largely unknown. Since the management of S. pneumoniae infections are challenging, understanding the characteristics of local strains is necessary for the development of new strategies for the prophylaxis of such infections. Therefore, this study was aimed to characterize the isolates from clinical sources in Malaysia with regard to the prevalence rates against the common antibiotics, level of erythromycin susceptibility and the genetic mechanism of ERSP. This will provide the latest scenario of antibiotic resistance in Malaysia and to prompt a new insight on the management of commonly used antimicrobials which includes revision of treatment guidelines.

MATERIALS AND METHODS

Bacterial Isolates
Seventy one consecutive single isolates of S. pneumoniae of serotypes 1 and 19F were collected at the Microbiology Unit at IMR, Malaysia and transported to Medical Microbiology
Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM) in an Amies transport medium (Copan, Italy) for further collaborative analysis. These isolates were previously isolated from 21 hospitals from different patients and collected at IMR which serves as the surveillance multicenter. The source of the isolates included sputum, nasal swab, throat swab, tracheal aspirate (TA), nasopharyngeal aspirate (NPA), high vaginal swab (HVS), eye swab, ear swab, pus, pleural fluid, synovial fluid, blood and cerebrospinal fluid (CSF) of clinically ill patients. The isolates were re-cultivated on 5% Columbia sheep blood agar (Oxoid, Malaysia) and incubated in 5% CO2 at 37°C. All isolates were stored in brain heart infusion broth (Oxoid, Hampshire, U.K.) for further collaborative analysis. The source of the isolates included invasive- blood, CSF, pleural fluid and synovial fluid; Respiratory- sputum , NPA , tracheal aspirate, throat swab and nasal swab; Others- ear, eye, HVS and pus without antibiotics awaiting further investigation.

Identification of pneumococci and serotyping
The strains were presumptively identified by colonial morphology, α-haemolysis on blood agar, Gram stain and confirmed by sensitivity to optochin using ethylhydrocupreine disc (AB Biodisk, Sweden), bile solubility and catalase negative reaction. Isolates were serogrouped by latex agglutination test using a Pneumotest-Latex kit (Statens Serum Institut, Copenhagen, Denmark) according to the manufacturer’s recommendations.

Antimicrobial susceptibility testing
The MICs of 6 antimicrobial agents (penicillin, amoxicillin-clavulanic acid, cefotaxime, ceftriaxone, trimethoprim-sulfamethoxazole and erythromycin) were determined using E-test strips (BioMerieux SA, France) according to the manufacturer’s instructions included in the packaging inserts. Standard quality control strain S. pneumoniae ATCC 49619 (GenBank: 274778 S. pneumoniae gene for dihydrofolate reductase) was included in each run. The system comprises a predefined antibiotic gradient ranging from 0.016 to 256 μg/mL and bacterial inoculum equivalent to a turbidity of 0.5 McFarland standard was inoculated onto Mueller-Hinton agar (MHA) plates supplemented with 5% sheep blood (Oxoid, Malaysia) by lawn culture. Appropriate E-test strips were carefully placed at the center of the MHA plates supplemented with 5% sheep blood and incubated at 37°C in 5% CO2 for 24 h in an incubator. The susceptibility of S. pneumoniae strains were evaluated by assessing the MICs as described by the Clinical and Laboratory Standards Institute guidelines (CLSI) (2009).

Detection of macrolide resistant determinants
Total chromosomal DNA was extracted from the isolates using a DNA extraction kit (Wizard® Genomic DNA Purification Kit, Promega Corporation, USA) according to the manufacturer’s instructions. The primer set for the internal region of erm(B) are 5'-CTGTATGAGCTACTGTCTGG-3' and 5'-CCAGCTTAGTATACTGG-3'. while that for the internal region of mef(A) are 5'-CGTACCTGGATACCG-3' and 5'-GTAAACGTGACTG-3'. The reaction mixture of PCR was a 25 μL total volume containing 12.5 μL of master mix (i-DNA Biotechnology (M) Sdn. Bhd., Malaysia), 1.0 μL of primers (forward 0.5 μL and reverse 0.5 μL), 2 μL of genomic DNA and 9.5 μL from distilled water (dH2O). erm(B) and mef(A) genes were amplified using an iCycler thermalcycler (Biometra Tpersonal, Gottingen, Germany) and the reaction conditions followed those as described by Desa et al. (2005); initial denaturation step of 3 min at 93°C, followed by 35 cycles of denaturation (1 min at 93°C), annealing (1 min at 52°C), and extension (1 min at 72°C). The reactions were finalized by polymerization for 5 min at 72°C. The PCR amplicons were visualized using UV light Alphalmager® Gel Documentation after electrophoresis using 1.4% agarose in 1X TBE containing 2.5 μL for GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., Hayward, California).

Table I: Streptococcus pneumoniae isolates from 71 patients

<table>
<thead>
<tr>
<th>Sites of source</th>
<th>No. of isolates (adults/children)</th>
<th>% of strains by serotype (adults/children)</th>
<th>% of strains by erythromycin determinant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive</td>
<td>17/14</td>
<td>15.5/4.2</td>
<td>8.4/15.5</td>
</tr>
<tr>
<td>Respiratory</td>
<td>17/8</td>
<td>23.9/11.3</td>
<td>2.8/11.3</td>
</tr>
<tr>
<td>Others</td>
<td>8/7</td>
<td>11.3/9.9</td>
<td>18.3/56.4</td>
</tr>
<tr>
<td>Total</td>
<td>42/29</td>
<td>43.6/36.7</td>
<td></td>
</tr>
</tbody>
</table>

Table II: Susceptibility rates of pneumococcal isolates and MIC values (E-test) for 6 antimicrobial classes

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>MIC50 μg/mL</th>
<th>MIC90 μg/mL</th>
<th>MIC range μg/mL</th>
<th>% Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>0.05</td>
<td>1.5</td>
<td>0.002 - 32</td>
<td>95.8</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>0.38</td>
<td>3</td>
<td>0.016 - 256</td>
<td>85.9</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.25</td>
<td>1.5</td>
<td>0.002 - 32</td>
<td>81.7</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.38</td>
<td>1.5</td>
<td>0.016 - 256</td>
<td>78.9</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>1</td>
<td>8</td>
<td>0.002 - 32</td>
<td>42.3</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2</td>
<td>256</td>
<td>0.016 - 256</td>
<td>35.2</td>
</tr>
</tbody>
</table>

MIC50 = MIC at which 50% or 90% of the isolates are inhibited.
RESULTS
Twelve isolates (16.9%) were collected in 2008, 20 isolates (28.2%) in 2009 and the remaining 39 isolates (54.9%) in 2010. All the 71 isolates matched the standard bacteriological characteristics of *S. pneumoniae*. Of the 71 pneumococcal isolates, 55 (77.5%) were of serotype 19F and 16 (22.5%) of serotype 1. Majority of the isolates were from blood (37%) followed by sputum (18%), ear (11%), TA (7%), NPA (6%), eye (6%), throat swab (3%), CSF (3%), pus (3%), nasal swab (1%), pleural fluid (1%) and synovial fluid (1%). However, for simplicity of analysis, the sites of isolation were reassigned as respiratory (sputum, NPA, tracheal aspirate, throat swab and nasal swab), invasive (blood, CSF, pleural fluid and synovial fluid) and others (ear, eye, HVS and pus) which accounted for 25 (35.2%), 30 (42.3%) and 16 (22.5%) isolates respectively. The isolates were obtained from clinically ill patients of varying ages from 6 days to 94 years. Thirty eight isolates (53.3%) were recovered from males and 33 isolates (46.5%) were from females. The isolates were sorted based on the patient's age, as < 13 years old accounted for 29 isolates (40.8%) and ≥ 13 years old for 42 isolates (59.2%). In both age-groups, serotype 19F was the most predominant serotype with a prevalence rate of 43.6% and 36.7%. Serotype 1 was found to be prevalent in 15.5% of adults and 4.2% in children respectively. Based on the isolation site, the prevalence of serotype 1 was found to be highly associated with respiratory sites (Table I).

The isolates used in the present study were from 11 different states of Malaysia, namely: Kedah, Perak, Selangor, Wilayah Persekutuan, Negeri Sembilan, Melaka, Johor, Pahang, Kelantan, Sabah and Sarawak. Majority of the isolates were from Selangor which comprised 23 isolates (32.4%), followed by Sabah with 13 isolates (18.3%) and Kuala Lumpur with 12 isolates (16.9%) (Figure 3).

The distribution of antibiotic resistance in relation to the isolation site and the susceptibility rates of the 6 antibiotics tested are summarized in Table III and Figure 1. The MIC50/MIC90 for the antibiotics against the 71 pneumococcal isolates are: penicillin 0.50/1.5, amoxicillin-clavulanic acid 0.38/3, cefotaxime 0.25/1.5, ceftriaxone 0.38/1.5, trimethoprim-sulfamethoxazole 1/8 and erythromycin 2/256 µg/mL. The percentage distribution of isolates susceptible/intermediate/resistant to each antibiotics are: penicillin 95.8/1.4/2.8, amoxicillin-clavulanic acid 85.9/14.1/0, cefotaxime 81.7/12.7/5.6, ceftriaxone 78.9/21.1/0, trimethoprim-sulfamethoxazole 42.3/23.9/33.8 and erythromycin 35.2/4.2/60.6 respectively (Table III). The MIC of the control strain was within the acceptable range. Notably, 5 isolates were found to be multidrug resistant *S. pneumoniae* (MDRSP) as these isolates were resistant to ≥ 3 antibiotics of different classes (cefotaxime, erythromycin and trimethoprim-sulfamethoxazole).

PCR results and the MIC distribution of all the 6 antibiotics tested are shown in Table III and IV. In this study, 43 out of 71 isolates were resistant to erythromycin. The two major macrolide resistance determinants, *erm*(B) and *mef*(A) were...
amplified as 224bp and 294bp fragments respectively (Figure 2). Erythromycin is the only macrolide tested in this study and all 43 erythromycin resistant strains were subjected to PCR for the presence of macrolide resistance determinant genes \textit{mef}(A) and \textit{erm}(B). \textit{mef}(A) alone or in combination with \textit{erm}(B) were detected in all 43 erythromycin resistant strains with MICs ≥1 µg/mL. Among the 28 erythromycin susceptible isolates, \textit{mef}(A) alone was detected in 25 (35.2%) isolates with MICs ≤0.25 µg/mL. Three isolates (1.4%) were found with none of the two genes with MICs ≤0.094 µg/mL.

Among the 43 ERSP, \textit{mef}(A) was detected in 29 (67.4%) isolates and \textit{erm}(B) + \textit{mef}(A) in 14 (32.6%) isolates. The prevalence of \textit{erm}(B) and \textit{mef}(A) together was frequently observed in strains with higher MIC values (≥256 µg/mL).

**DISCUSSION**

Penicillin has been the drug of choice for treating pneumococcal infection for so long before the introduction of erythromycin, and this has resulted in the widespread emergence of penicillin resistant \textit{S. pneumoniae} (PRSP) (Le et al., 2011). Macrolides are different group of antibiotic and provide as an alternative for treatment. Among the macrolide members, erythromycin serves as the first line anti-pneumococcal antibiotic for empirical treatment of community acquired lower respiratory tract infections and is extensively used for treating infant and adult pneumococcal infections (Clarke et al., 2006; Woodhead et al., 2005). Nevertheless, high prevalence rates of ERSP are also now being reported in many countries worldwide including Malaysia (Song et al., 2004b; Azoulay-Dupuis et al., 2000). In addition, several surveillance studies have also reported an increased prevalence of ERSP in community acquired and hospital acquired pneumococcal infections in Western Pacific regions and Asian regions (Chen et al., 2009; Dias et al., 2006; Song et al., 2004b). Similar trend has also been observed in Malaysia with a prevalence of ERSP as low as 3% in 1996 and 1997 which then increased up to 36.8% till 2001 (Song et al., 2004a; Song et al., 2004b Song et al., 1999). This is a worrying scenario that may require an update on the antibiotic prescription policies and regulation (Lee et al., 2001). As an example, Taiwan has introduced a policy to restrict erythromycin and penicillin usage in the country, particularly in view of the spread of highly antibiotic resistant \textit{S. pneumoniae} of the international clones (Hsueh, 2005; Hsueh, 2004).

This current study also reported an increased pattern whereby of the 71 pneumococcal isolates tested, 60.6% isolates were resistant to erythromycin. On the contrary,
Prevalence of macrolide resistance and in vitro activities of six antimicrobial agents

Fig. 3: Pneumococcal isolates collected from different hospitals in different regions of Malaysia. Malaysia map was adopted from this website http://www.smn2012.com/culture.php. SBH - Sultanah Bahiyah Hospital; RPBH - Raja Permaisuri Bainun Hospital; KLH - Kuala Lumpur Hospital; GMC - Gleneagles Medical Centre; AH - Ampang Hospital; TARH - Tengku Ampuan Rahimah Hospital; SH - Selayang Hospital; SgBH - Sungai Buloh Hospital; PH - Putrajaya Hospital; SDH - Serdang Hospital; ASH - Assunta Hospital; MGH - Melaka General Hospital; SAH - Sultanah Aminah Hospital; TJH - Tuanku Jaafar Hospital; SHASH - Sultan Haji Ahmad Shah Hospital; RPZH - Raja Perempuan Zainab II Hospital; QEH - Queen Elizabeth Hospital; LH - Likas Hospital; SBH - Sibu Hospital; SGH - Sarawak General Hospital.

other antibiotic shows a lower resistance rate with 33.8% for trimethoprim-sulfamethoxazole, 5.6% for cefotaxime and only 2.8% for penicillin. None of the isolates were resistant to amoxicillin-clavulanic acid and ceftriaxone, however 14.1% and 21.1% of intermediates were observed respectively for these two antibiotics. This could be the changing pattern whereby penicillin which was previously observed to be getting low in activity due to emergence of PRSP (Dias et al., 2006; Hsueh, 2005; Lee et al., 2001; Soh et al., 2000; Song et al., 1999) but now shows the otherwise with 95.8% of isolates in this study being susceptible. This could make penicillin as the drug of choice again.

As majority of the isolates in this study were found to be resistant to erythromycin, assessing the erythromycin determinant genes in association with the susceptibility level is warranted for surveillance purposes at molecular level. Among the 43 ERSP, strains harbouring both \textit{erm(A)} and \textit{erm(B)} showed high MICs ranging from 0.5-256 μg/mL as compared to strains with \textit{erm(A)} alone with MICs ranging from 0.016-256 μg/mL. Isolates with none of the two genes had MIC levels ranging from 0.047-0.094 μg/mL which is the susceptible category. The findings in the current study were in accordance with previous study reported earlier by McGee (2007) which showed the presence of \textit{erm(B)} to be the main culprit in causing resistant to erythromycin. This was also further validated by other studies that erythromycin MICs have a lower range of 1-32 μg/mL among \textit{S. pneumoniae} containing \textit{erm(A)} alone. However, among \textit{erm(B)} mutants, the MICs typically exceeded 128 μg/mL due to the multiple ribosomal mutations which exhibits a 2000-fold increases in MICs (Tait-Kamradt et al., 2000; Sutcliffe et al., 1996). This underscores that the emergence of ERSP and level of susceptibility nowadays are still associated with the distribution pattern of the two genes which corroborate the findings by Desa et al. (2005).

As a whole, the sample size of this study is only 71 isolates and limited to serotypes 1 and 19F only. Nevertheless the source of the isolates was geographically widespread representing many states of Malaysia to claim a merit to some extent. Although many serotypes are associated in pneumococcal infection, serotypes 1 and 19F are widely prevalent in many Asian and developed countries (Song et al., 2004b; Konradsen and Kaitoff, 2002; Soh et al., 2000; Rohani et al., 1999; Moreno et al., 1999) and thus the emergence of ERSP among these two serotypes is epidemiologically relevant. The geographical incidence of \textit{S. pneumoniae} was not discussed because the isolates were pre-selected for only two serotypes. Nevertheless, the current in vitro susceptibilities of the \textit{S. pneumoniae} isolates presented here would suggest that penicillin would re-gain as the effective treatment options for pneumococcal infections, while the erythromycin determinants are still consistent among the ERSP. Meanwhile, the two genes could serve as genetic markers at molecular level to infer the level of resistance. In conclusion, this study strongly underscores that continuous surveillance of antibiotic resistance with more antimicrobial agents are necessary to monitor the changing microbial drug resistant patterns as a whole.
ACKNOWLEDGEMENTS
The Authors are indebted to the Microbiology Unit at the Institute for Medical Research (IMR) for providing the isolates and Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Science, Universiti Putra Malaysia for providing research facilities. This study was supported by Fundamental Research Grant Scheme (FRGS: 03-10-10-951FR) from the Ministry of Higher Education (MOHE, Putrajaya, Malaysia).

CONFLICT OF INTEREST
No conflict of interest exists.

REFERENCES