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

Jayakayatri Jeevajothi Nathan*, Niazlin Mohd Taib*, Mohd Nasir Mohd Desa, Siti Norbaya Masri*, Rohani Md Yasin***, Farida Jamal*, Sreenivasa Rao Sagineedu****, Arunkumar Karunanidhi***

*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 regiment 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 (Varaldo *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

This article was accepted: 9 January 2013
Corresponding Author: Niazlin Mohd Taib, Jayakayatri Jeevajothi Nathan, Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia
Email: niazlin@medic.upm.edu.my / jjayakayatri@yahoo.com

Table I: *Streptococcus pneumoniae* isolates from 71 patients

Sites of source	No. of isolates (adults/children)	% of strains by serotype (adults/children)		% of strains by erythromycin determinant		
		1	19F	*mef*(A) (1/19F)	*erm*(B) + *mef*(A) (1/19F)	none (1/19F)
Invasive	17/14	15.5/4.2	8.4/15.5	15.5/15.5	0/7.04	4.2/0
Respiratory	17/8	0/0	23.9/11.3	0/29.6	0/7.04	0/0
Others	8/7	2.8/0	11.3/9.9	2.8/11.3	0/7.04	0/0
Total	42/29	15.5/4.2	43.6/36.7	18.3/ 56.4	0/21.1	4.2/0

Invasive- blood, CSF, pleural fluid and synovial fluid; Respiratory- sputum, NPA, tracheal aspirate, throat swab and nasal swab; Others- ear, eye, HVS and pus

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

Antibiotics	MIC_{50} µg/mL	MIC_{90} µg/mL	MIC range µg/mL	% Susceptibility		
				Susceptible	Intermediate	Resistant
Penicillin	0.50	1.5	0.002 - 32	95.8	1.4	2.8
Amoxicillin-clavulanic acid	0.38	3	0.016 - 256	85.9	14.1	0
Cefotaxime	0.25	1.5	0.002 - 32	81.7	12.7	5.6
Ceftriaxone	0.38	1.5	0.016 - 256	78.9	21.1	0
Trimethoprim-sulfamethoxazole	1	8	0.002 - 32	42.3	23.9	33.8
Erythromycin	2	256	0.016 - 256	35.2	4.2	60.6

$MIC_{50/90}$ = MIC at which 50% or 90% of the isolates are inhibited

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% CO_2 at 37°C. All isolates were stored in brain heart infusion broth (Oxoid Hampshire, U.K.) supplemented with 10% glycerol at -80°C 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 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 manufacturer's recommendations included in the packaging inserts. Standard quality control strain *S. pneumoniae* ATCC 49619 (GenBank: Z74778 *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% CO_2 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′-CGTACCTGGATATCACCG-3′ and 5′-GTAACAGTTGACGATACTGG-3′, while that for the internal region of *mef*(A) are 5′-CTGTATGAGCTACTGTCTGG-3′ and 5′- CCAGCTTAGTATACGTAC-3′ (Nagai *et al,* 2001). 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 (dH_2O). *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 AlphaImager® 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).

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%), HVS (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 higher in invasive sites, whereas serotype 19F 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 MIC_{50}/MIC_{90} 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

Table III: Distribution of MIC, MIC_{50}, MIC_{90} (µg/mL) for 71 isolates of *S. pneumoniae* (MIC_{50} and MIC_{90} values are reported in BOLD and UNDERLINED numbers, respectively).

	.002	.003	.004	.006	.008	.012	.016	.023	.032	.047	.064	.094	.125	.19	.25	.38	.50	.75	1	1.5	2	3	4	6	8	12	16	24	32	48	64	96	128	192	256
PG	1			1	1	5	4	3	2	1			1	1	2	9	**8**	11	6	8	6		1												
XL							23	1				2	2	3	2	**4**	7	9	4	4		10													
CT				1		4	8	4	2	4		6	1		**6**	5	10	6	4	3	3		1	1	1			1						
TX							24		1				3	1	1	**11**	8	5	2	15														
TS						1					1		4	3	8	3	7	3	**6**	3	3	1	5	13	3	1	1		5						
EM							6			1	5	7	5	1			1	1	1	5	**8**	7	9	1										13

Ab - antibiotics; MIC - minimum inhibitory concentrations; PG - penicillin; XL - amoxicillin-clavulanic acid; CT - cefotaxime; TX - ceftriaxone; TS - trimethoprim-sulfamethoxazole; EM - erythromycin.

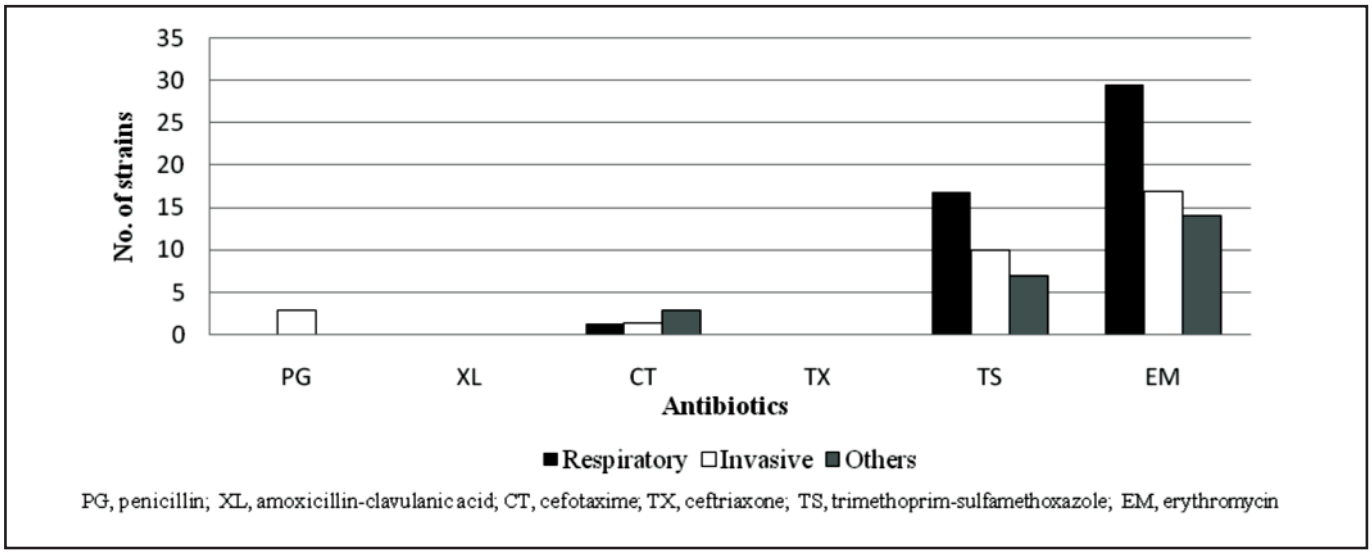

Fig. 1 : Distribution of antibiotic resistance patterns in relation to the sources of isolates. Numbers on the Y-axis represent the frequency of strains with the respective antibiogram patterns at the corresponding sources of isolates.

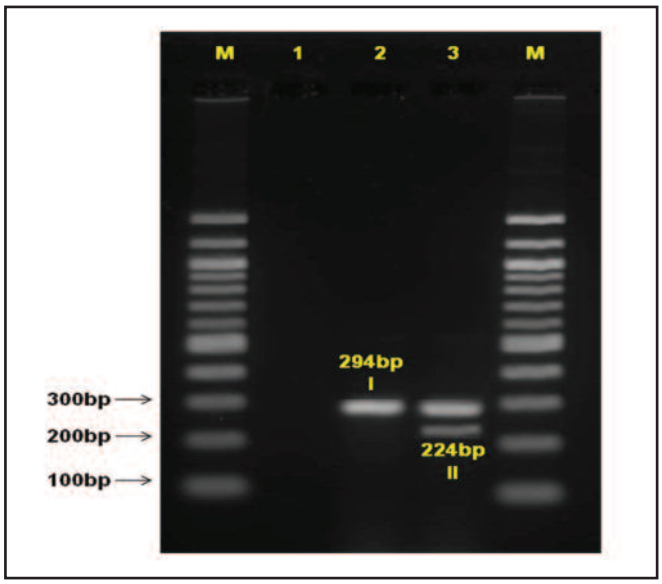

Fig. 2 : Scanned image of gel showing 3 different patterns of erythromycin resistant determinant genes, *mef*(A), *mef*(A) + *erm*(B) combination and none of the two genes. M, DNA molecular mass size marker (100bp DNA ladder). Lane 1 to 3 are PCR amplicons of *mef*(A) and *erm*(B). Lane 1 has no band representing absence of the two genes.

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 *mef*(A) and *erm*(B). *mef*(A) alone or in combination with *erm*(B) were detected in all 43 erythromycin resistant strains with MICs ≥1 µg/mL. Among the 28 erythromycin susceptible isolates, *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, *mef*(A) was detected in 29 (67.4%) isolates and *erm*(B) + *mef*(A) in 14 (32.6%) isolates. The prevalence of *erm*(B) and *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 *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% in 1998 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 *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,

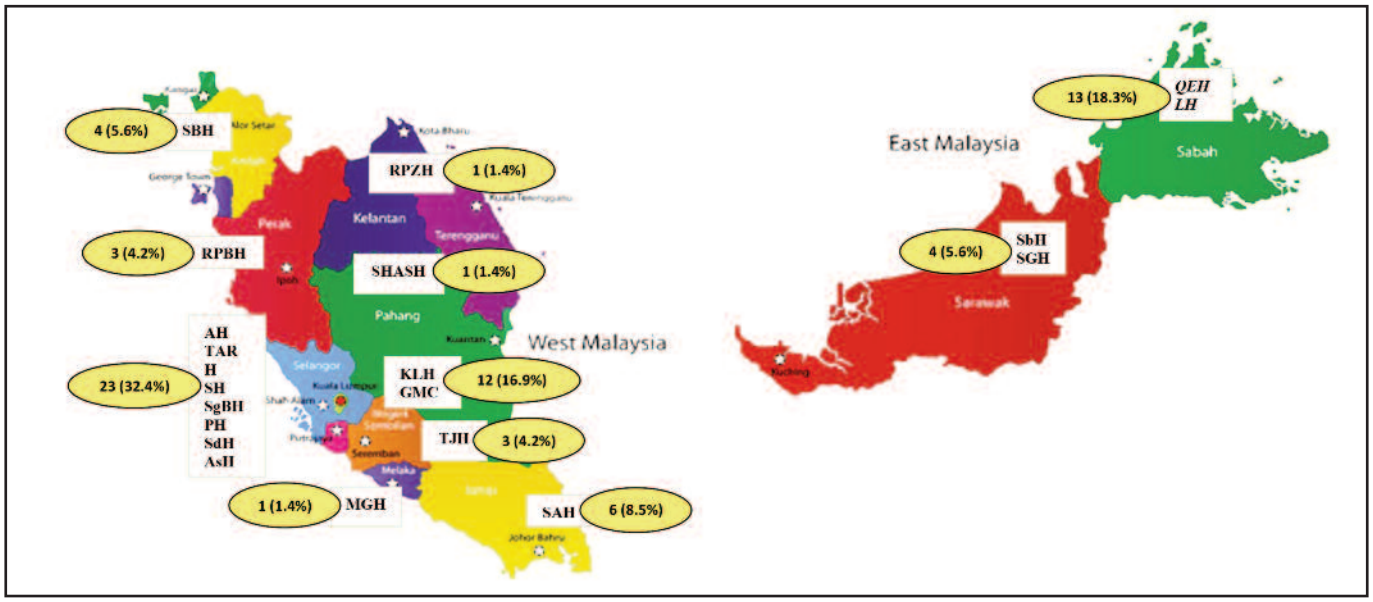

Fig. 3 : Pneumococcal isolates collected from different hospitals in different regions of Malaysia. Malaysia map was adopted from this website http://www.smn2012.com/culture.php. SBH - Sultanah Bahiyah Hospital; RPBH - Raja Permaisuri Bainun Hospital; KLH - Kuala Lumpur Hospital; GMC - Gleneagles Medical 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 *mef*(A) and *erm*(B) showed high MICs ranging from 0.5-256 µg/mL as compared to strains with *mef*(A) alone with MICs ranging from 0.016-256 µg/mL. Isolates with none of the two genes had MIC levels ranging from of 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 *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 *S. pneumoniae* containing *mef*(A) alone. However, among *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 Kaltoft, 2002; Soh *et al.*, 2000; Rohani *et al.*, 1999; Moreno *et al.*, 1995) and thus the emergence of ERSP among these two serotypes is epidemiologically relevant . The geographical incidence of *S. pneumoniae* was not discussed because the isolates were pre-selected for only two serotypes. Nevertheless, the current *in vitro* susceptibilities of the *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

1. Azoulay-Dupuis E, Rieux V, Muffat-Joly M, Bedos JP, Vallee E, Rivier C, Isturiz R, Carbon C, Moine P. Relationship between capsular type, penicillin susceptibility, and virulence of human *Streptococcus pneumoniae* isolates in mice. Antimicrob Agents Chemother 2000; 44(6): 1575-7.
2. Chen J, Liu L, Wang G, Chen Y, Luo Z, Huang Y, Fu Z, Liu E. Correlation between usage of macrolide antibiotic and resistance of *Streptococcus pneumoniae* clinic isolates from Chongqing children Hospital. Paediatr Pulmonol 2009; 44(9): 917-21.
3. Clarke R, Ross S, Walker T, Woods D. Rational use of antibiotics in respiratory tract infections. George Street, Dunedin. bpacnz better medicine, 2006.
4. Clinical and Laboratory Standards Institute®. Performance standards for antimicrobial susceptibility testing; 19th Informational Supplement M100-S19. Wayne, PA: CLSI 2009.
5. Desa MNM, Thong KL, Parasakthi N. A correlation between the genes responsible for penicillin and erythromycin resistance in *Streptococcus pneumoniae* and the minimum inhibitory concentration (MIC) values: A potential approach for molecular detection of susceptibility. Malaysian J Biochem Mol Biol 2005; 12: 55-7.
6. Dias R, Louro D, the Antimicrobial Resistance Surveillance Program in Portugal, Canica M. Antimicrobial Susceptibility of Invasive *Streptococcus pneumoniae* isolates in Portugal over an 11-year period. Antimicrob Agents Chemother 2006; 50(6): 2098-105.
7. Hsueh PR. Taiwan struggles against antibiotic resistance. APUA Newslett 2004; 21: 5.
8. Hsueh PR. Decreasing rates of resistance to penicillin, but not erythromycin, in *Streptococcus pneumoniae* after introduction of a policy to restrict antibiotic usage in Taiwan. Clin Microbiol Infect 2005; 11(11): 925-7.
9. Konradsen HB, Kaltoft MS. Invasive Pneumococcal Infections in Denmark from 1995 to 1999: Epidemiology, Serotypes, and Resistance. Clin Vaccine Immunol 2002; 9(2): 358-65.
10. Le CF, Palanisamy NK, Mohd Yusof MY, Sekaran SD. Capsular serotype and antibiotic resistance of *Streptococcus pneumoniae* isolates in Malaysia. PLoS ONE 2011; 6(5): e19547.
11. Lee NY, Song JH, Kim S, Peck KR, Ahn KM, Lee SI, Yang Y, Li J, Chongthaleong A, Tiengrim S, Aswapokee N, Lin TY, Wu JL, Chiu CH, Lalitha MK, Thomas K, Cherian T, Perera J, Yee TT, Jamal F, Warsa UC, Van PH, Carlos CC, Shibl AM, Jacobs MR, Appelbaum PC. Carriage of antibiotic-resistant pneumococci among Asian children: a multinational surveillance by the Asian Network for Surveillance of Resistant Pathogens (ANSORP). Clin Infect Dis 2001; 32(10): 1463-9.
12. McGee L. The coming of age of niche vaccines? Effect of vaccines on resistance profiles in *Streptococcus pneumoniae*. Curr Opin Microbiol 2007; 10(5): 473-8.
13. Moreno S, Garcia-Leoni ME, Cercenado E, Diaz MD, Bernaldo de Quiros JC, Bouza E. Infections caused by erythromycin-resistant *Streptococcus pneumoniae*: Incidence, risk factors, and response to therapy in a prospective study. Clin Infect Dis 1995; 20(5): 1195-200.
14. Nagai K, Shibasaki Y, Hasegawa K, Davies TA, Jacobs MR, Ubukata K, Appelbaum PC. Evaluation of PCR primers to screen for *Streptococcus pneumoniae* isolates and beta-lactam resistance, and to detect common macrolide resistance determinants. J Antimicrob Chemother 2001; 48(6): 915-8.
15. Rohani MY, Raudzah A, Ng AJ, Ng PP, Zaidatul AR, Asmah I, Murtaza M, Parasakthy N, Mohd Yasmin MY, Cheong YM. Epidemiology of *Streptococcus pneumoniae* infection in Malaysia. Epidemiol Infect 1999; 122(1): 77-82.
16. Soh S.W, Poh CL, Lin RV. Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* isolates from pediatric patients in Singapore. Antimicrob Agents Chemother 2000; 44(8): 2193-2196.
17. Song JH, Lee NY, Ichiyama S, Yoshida R, Hirakata Y, Fu W, Chongthaleong A, Aswapokee N, Chiu CH, Lalitha MK, Thomas K, Perera J, Yee TT, Jamal F, Warsa UC, Vinh BX, Jacobs MR, Appelbaum PC, Pai CH. Spread of drug-resistant *Streptococcus pneumoniae* in Asian countries: Asian Network for Surveillance of Resistant Pathogens (ANSORP) study. Clin Infect Dis 1999; 28(6): 1206-11.
18. Song JH, Chang HH, Suh JY, Ko KS, Jung SI, Oh WS, Peck KR, Lee NY, Yang Y, Chongthaleong A, Aswapokee N, Chiu CH, Lalitha MK, Perera J, Yee TT, Kumarasinghe, G, Jamal F, Kamarulzaman A, Parasakthi N, Van PH, So T, Ng TK; on behalf of the ANSORP Study Group. Macrolide resistance and genotypic characterization of *Streptococcus pneumoniae* in Asian countries: a study of the Asian Network for Surveillance of Resistant Pathogens (ANSORP). J Antimicrob Chemother 2004a; 53: 457-63.
19. Song JH, Jung SI, Ko KS, Kim NY, Son JS, Chang HH, Ki HK, Oh WS, Suh JY, Peck KR, Lee NY, Lu Q, Chongthaleong A, Chiu CH, Lalitha MK, Perera J, Yee TT, Kumarasinghe G, Jamal F, Kamarulzaman A, Parasakthi N, Van PH, Carlos C, So T, Ng TK, Shibi A. High prevalence of antimicrobial resistance among clinical *Streptococcus pneumoniae* isolates in Asia (an ANSORP Study). Antimicrob Agents Chemother 2004b; 48(6): 2101-7.
20. Sutcliffe J, Tait-Kamradt A, Wondrack L. *Streptococcus pneumoniae* and Streptococcus pyogenes resistant to macrolides but sensitive to clindamycin: a common resistance pattern mediated by an efflux system. Antimicrob Agents Chemother 1996; 40(8): 1817-24.
21. Tait-Kamradt A, Davies T, Appelbaum PC, Depardieu F, Courvalin P, Petitpas J, Wondrack L, Walker A, Jacobs MR, Sutcliffe J. Two new mechanisms of macrolide resistance in clinical strains of *Streptococcus pneumoniae* from Eastern Europe and North America. Antimicrob Agents Chemother 2000; 44(12): 3395-401.
22. Woodhead M, Blasi F, Ewig S, Huchon G, Ieven M, Ortqvist A, Schaberg T, Torres A, van der Heijden G, Verheij TJM. Guidelines for the management of adult lower respiratory tract infections. Eur Respir J 2005; 26: 1138-80.
23. Varaldo PE, Montanari MP, Giovanetti E. Genetic elements responsible for erythromycin resistance in Streptococci. Antimicrob Agents Chemother 2009; 53(2): 343-53.