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Evolution of Erythromycin Resistance among *Streptococcus pneumoniae* Isolates in Malaysia from 2005 and 2010

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Abstract

Objectives: This study focuses on the antibiotic susceptibility pattern and distribution of the *erm*B and *mef*A virulence genes among the *Streptococcus pneumoniae* due to an increase in erythromycin resistance in *S. pneumoniae* worldwide. Methodology: We investigated 255 clinical isolates collected from 2005-2010 to det*erm*ine the serotype distribution and resistance to erythromycin in comparison to penicillin, clindamycin, clarithromycin, azithromycin, and trimethoprim sulfamethoxazole. Multiplex PCR was carried out to detect erythromycin resistance genes (*ermB* and *mef*A). Results: There were 146 (57.3%) isolates resistant to erythromycin. MIC_{90} for erythromycin is at >256 mg/L and MIC_{50} is at 16 mg/L. The *erm*B gene was detected in 25.3% of the erythromycin-resistant isolates and *mef*A gene was detected in 50.7% of the isolates. The four most common serotypes encountered are 19F, 19A, 23F and 14. The serotype distribution among the erythromycin resistant isolates was 19F (42.0%) followed by serotype 19A (11.3%), serotype 23F (9.2%) and serogroup 14 (7.0%). Conclusion: In conclusion, there is a significant rise in erythromycin resistance among the Malaysian pneumococcal isolates. The emergence of serotype 19A together with increasing prevalence of resistance to macrolide warrants for a more extensive surveillance study.

Keywords

***Streptococcus pneumoniae*, Pneumococcal, Erythromycin Resistance, Serotype, *mef*A, *erm*B**

1. Introduction

Streptococcus pneumoniae is one of the most common causative pathogens of severe invasive infections among

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infants and young children causing diseases ranging from otitis media, sinusitis, pneumonia, septicemia to meningitis. The morbidity and mortality rates of pneumococcal infections worldwide are high, particularly in developing countries. The World Health Organization (WHO) estimates the annual incidence of invasive pneumococcal disease ranging from 10 to 100 cases per 100,000 population in Europe and the United States. In the developing countries, the incidence of pneumococcal disease in children aged <5 years is several times higher than it is in industrialized countries [1]. A study by Kilgore P.E. *et al*. (2005) in China, South Korea, and Vietnam estimated the incidence of pneumococcal meningitis in children aged 1 to 23 months at 5.1 cases per 100,000 childbirths from 1999 to 2003 [2].

Studies by the Asian Network for Surveillance of Resistant Pathogens (ANSORP) have indicated that the rates of antimicrobial resistance among *S. pneumoniae* isolates in Asia continue to be in an increasing trend [3]. The ANSORP surveillance also documented that during 1996 and 1997, the prevalence rate of erythromycin resistance among *S. pneumoniae* clinical isolates was more than 60% in many areas including Taiwan, Korea, Japan and Vietnam. In Malaysia, we have observed a rise in penicillin-resistant *S. pneumoniae* (PRSP) from 0.8% in 1988 [4] to 7.0% in 1995 [5] and 25.54% in 2009 [6]. Another study in Malaysia [7] has reported higher resistant rate to other common oral antibiotics in comparison to another study [5] whereby resistance to erythromycin has increased from 1.1% to 33.33% and clindamycin from 0.4% to 18.08%.

To date, it has been reported that 93 pneumococcal serotypes exist which are further classified into 46 pneumococcal serogroups [8]. A study by Song *et al.* (2004) reported that the main serotypes among the erythromycin resistant isolates in Asia were 19F, 19A, 23A, 23F, 6A, 6B, 14, serogroup 4 and serogroup 18 [9]. Reports from Malaysia [7] indicated that the main serotypes were 19F, 19A, 6B, 14, 19B, 23F, 6A, serogroup 1, 23A, 19C and serogroup 15. This shows that there are some changes to the serotypes distribution in the last 5 years.

In particular, macrolide resistance among *Streptococcus pneumoniae* isolates has risen in recent years worldwide [10]. The main mechanisms of erythromycin resistance in *S. pneumoniae* are the ribosomal methylation encoded by the *erm*B gene (MLS_B phenotype) and macrolide efflux encoded by the *mef*A, *mef*E and *mef*I gene (M pneotype). Erythromycin-ribosomal methylase, which is encoded by the *erm*(B) gene is encoded by the methylation of adenine at position 2059 in the 23SrRNA blocks, the binding of macrolides (e.g., erythromycin), lincosamides (e.g., clindamycin), and streptogramin B (e.g., dalfopristin) and results in high-level resistance to these antibiotics (MLSB phenotype), with high erythromycin MICs (≥256 μg/ml) [11].

This study was conducted to determine the distribution of the *erm*B gene and *mef*A gene and its relationship with serotype distribution and macrolide dr*ug*s susceptibility. Through this study, we wish to provide the latest scenario of antibiotic resistance in Malaysia and develop an up-to-date guideline for the management of the prescribed antimicrobials.

2. Material and Methods

2.1. *S. pneumoniae* Isolates

Streptococcus pneumoniae isolates (n = 255) from clinical samples were collected from several state hospitals thro*ug*hout Malaysia between 2005 to 2010. The isolates were identified as *S. pneumoniae* based on typical colony morphology, gram staining, *α*-hemolysis, optochin (Oxoid Company, Britain) susceptibility and positive bile solubility test. The isolates were stored at −70˚C in 5% trypticase soy broth plus 20% (v/v) glycerol with beads [12] and were supplemented with three drops of rabbit serum for long term storage. The source of the specimen is listed below.

Invasive (n = 147)		Non-Invasive (n = 108)	
Blood	109	Sputum	65
CSF	9	Middle Ear Fluid	13
Tracheal Aspirates	29	Other	30
Children (0 - 18 years)	110	Children (0 - 18 years)	71
Adult	37	Adult	37

2.2. Antimicrobial Susceptibility Testing

Susceptibility to oxacillin, erythromycin, clindamycin, clarithromycin, azithromycin and trimethoprim-sulfamethoxazole (cotrimoxazole) were de*term*ined by Kirby-Bauer method disc diffusion method, by using Mueller-Hinton agar supplemented with 5% sheep blood. The results were interpreted based on the Clinical Laboratory Standards Institute (CLSI) guidelines (2010). The plates were incubated at 35˚C for 18 - 24 hours under 5% CO_2. *S. pneumoniae* ATCC 49619 was used as the quality control strain. Multidr*ug*-resistant *S. pneumoniae* (MDRSP) was defined as those which are resistant to three or more classes of antibiotics used in this study. An E-test (AB Biodisk, Sweden) method was applied to de*term*ine the Minimum Inhibitory Concentration for two dr*ug*s, in particular, Penicillin G (256 µg/ml) and Erythromycin (256 µg/ml). *S. pneumoniae* ATCC 49619 was used as the quality control strain and was included in each set of tests to ensure the accuracy of the results. MICs that fell between any two doubling dilutions were rounded up to the next higher one and the susceptibility categories were then assigned according to MIC (µg/ml) breakpoints, as in the CSLI guidelines. The interpretive breakpoints for *S. pneumoniae* isolate to define penicillin and erythromycin resistance: MICS of ≥2 µg/ml and 0.5 µ/ml respectively.

2.3. De*term*ination of Macrolide Resistance Mechanism

The macrolide resistance genes *erm*B and *mef*A were subjected to PCR analysis to detect *erm*B and *mef*A genes as described elsewhere [10].

2.4. Serotyping and PCV7 Coverage

The isolates streaked on sheep blood agar and grown for 18 hours at 35˚C in 5% CO_2, were serogroup based on the Quellung Reaction using Pneumotest kits (StatensSeruminstitut, Copenhagen, Denmark) [13]. Further serotyping was performed using type-specific antisera. The isolates that reacted negatively and could not be serotyped with the Pneumotest kit were classified as not-typeable.

3. Results

3.1. Erythromycin Resistance among *S. pneumoniae* Isolates

Among the 255 isolates, 100 (39.2%) were susceptible, 9 (3.5%) int*erm*ediate and 146 (57.3%) resistant to erythromycin. In 2009, the erythromycin resistance was the highest at (67.3%), followed by 2010 (62.2%), 2007 (61.1%), 2008 (52.4%), 2006 (51.2%) and 2005 (46.2%). These data shows that there is an increase in the erythromycin resistance from 2005 to 2010. The MIC_{90}s for erythromycin among the isolates were 256*ug*/ml or higher thro*ug*hout 2005 to 2010 (**Table 1**).

3.2. Correlation between Erythromycin Resistance and Other Drug Resistance

About 88.1% of the erythromycin resistant pneumococci are resistant to penicillin. Clarithromycin and azithromycin are both macrolide similar to erythromycin. About 94.4% of erythromycin resistant *S. pneumoniae* isolates were also resistant to clarithromycin and azithromycin. The high resistance within macrolide group might be due to the resistance conferred by the resistance genes. Approximately 90.8% of the erythromycin resistant isolates were also resistant to trimethoprim-sulfamethoxazole.

3.3. Serotype Distribution of the Erythromycin Resistant Isolates

Serotypes detected among the Malaysian isolates were 14, 17F, 18F, 19A, 19F, 23F, 6A, 6B, 7A and 9A. Isolates from serotype 19F were the most prevalent erythromycin resistant (ERSP) serotype (42.5%), followed by serotype 19A (11.0%), serotype 23F (8.9%) and serogroup 14 (6.8%). Approximately 77.0% of the *S. pneumoniae* isolates, serotypes were found in PCV10 and PCV 13. Of the 148 isolates, 50 are from children less than age 5 years of age and the pneumococcal serotypes are 19F (15.1%), 19A (4.1%), 14 (2.7%), 23F (2.7%), 18F (2.1%), 6B (1.4%), 6A (1.4%), 7A (0.7%) and 4.8% of the isolates are not-typeable. About 44.2% of the pneumococci were multidr*ug* resistant (MDRSP), the most significant serotypes being 19F (28.1%), 19A (11.0%) and 23F (8.9%). Almost 32.5% of the MDRSP isolates were isolated from children less than 5 years old (**Figure 1**).

Table 1. Relationship between MIC value, susceptibility to erythromycin and detection of resistance determinants from 2005 to 2010.

Year	No of isolates	Minimal inhibitory concentration (ug/ml)		Susceptibility to erythromycin (n) %			Resistance determinants (n) %		
		MIC_{50}	MIC_{90}	S[a] (%)	I[a] (%)	R[a] (%)	*erm* (B)	*mef* (A)	*erm* (B) + *mef* (A)
2005	39	6	>256	20 (51.3)	1(2.6)	18 (46.2)	5 (27.8)	9 (50.0)	4 (22.2)
2006	41	16	>256	18 (43.9)	2 (4.9)	21 (51.2)	9 (42.9)	11 (52.4)	1 (4.8)
2007	36	48	>256	14 (38.9)	0 (0.0)	22 (61.1)	8 (36.4)	10 (45.4)	4 (18.2)
2008	42	16	>256	18 (42.9)	2 (4.8)	22 (52.4)	4 (18.2)	15 (68.2)	3 (13.6)
2009	52	126	>256	15 (28.8)	2 (3.8)	35 (67.3)	3 (8.6%)	18 (51.4)	8 (22.9)
2010	45	>256	>256	15 (33.3)	2 (4.4)	28 (62.2)	8 (28.6)	11 (39.3)	9 (32.1)
Total	255	16	>256	100 (39.2)	9 (3.5)	146 (57.3)	37 (25.3)	74 (50.7)	29 (19.9)

a: Isolates were defined as susceptible (S ≥ 21 mm), int*erm*ediate (I: 16 - 20 mm) and resistant (R ≤ 15 mm) to erythromycin disc diffusion assay according to CLSI interpretation for *S. pneumoniaee*.

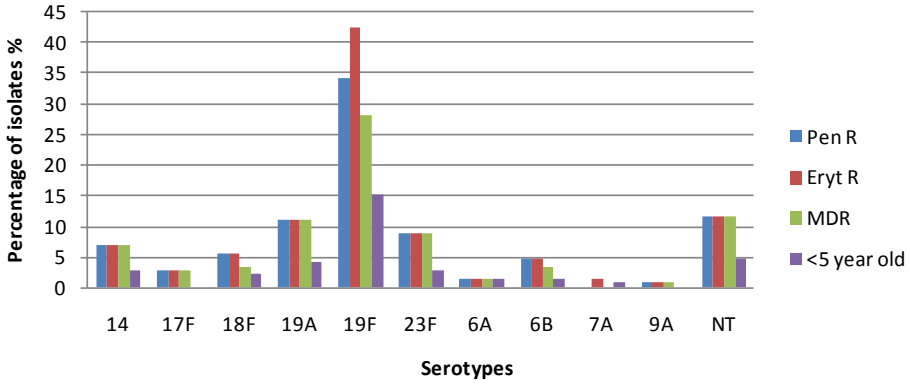

Figure 1. Distribution of *S. pneumoniae* serotype in Malaysia with relationship to penicillin, erythromycin and multidrug resistance with pediatric population.

3.4. Correlation between Macrolide-Resistant Genes and Serotype Population

The distribution of the macrolide-resistant genes can be seen as either *mef*A/*erm*B or both *erm*B and *mef*A detected together. **Table 1** shows that the *erm*B gene was detected in 25.3% of the ERSP isolates whereas *mef*A gene was detected in 50.7% of the isolates. About 19.9% of the isolates were found have to the both *erm*B and *mef*A dually. The isolates with *mef*A gene was more common than the *erm*B gene among the ERSP isolates with serotype 19F (25.4%), 19A (7.7%), 23F (4.9%) and 14 (2.8%), whereas the *erm*B gene was predominant in isolates 19F (10.6%), 23F (3.5%), 19A (2.8%) and 14 (2.1%). Interestingly, 55.0% of the ERSP isolates with both *mef*A and *erm*B gene detected dually belong to serotype 19F (**Figure 2**).

4. Discussion

An increase in macrolide resistance was observed worldwide in the past 10 years. The increment was not only documented in Malaysia but also neighboring countries [14]. In this study, the erythromycin resistance for the pneumococcal isolates has increased progressively throughout this 5 year study period. In 2005, the resistance was 46.2% and by 2010, it has increased to 62.2%. The erythromycin MIC_{50} gradually increased from 6 *ug*/ml in 2005 to >256 ug/ml in 2010. The erythromycin MIC_{90} maintained at >256 ug/ml throughout the study which reflects high resistance among the pneumococcal isolates. The increment in the MIC level, which also a clear indication of the rise in resistance shows some evidence that the increase of erythromycin consumption might be related to rise in resistance. Isolates from Hong Kong have been reported to be 80.0% resistant to erythromycin

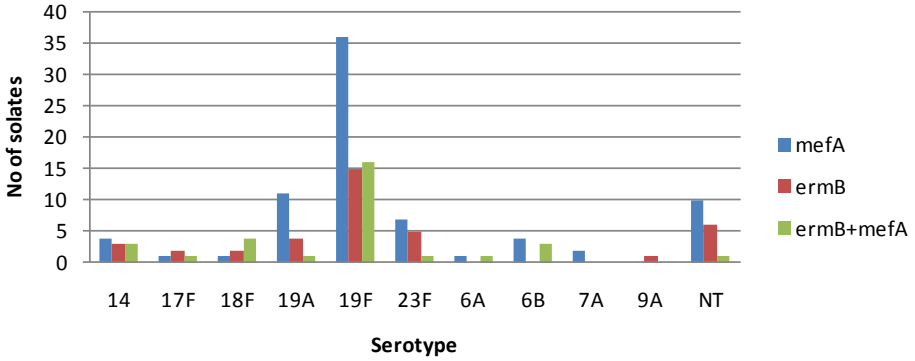

Figure 2. Relationship between ERSP serotype and distribution of macrolide-resistant genes.

[15] and 91.0% of the Taiwanese isolates were fully resistant to erythromycin [16]. According to the study by Song *et al.* (2004) [16] the prevalence of macrolide resistance is alarmingly high in Vietnam, Korea and China, where >70% of the pneumococcal isolates are fully resistant to erythromycin.

About 94.4% of erythromycin resistant isolates were also resistant to clarithromycin and azithromycin. Penicillin resistance is usually concomitant with macrolide resistance, and in our study, 88.0% of the erythromycin resistance isolates were penicillin resistant. Similar findings for the trend of penicillin resistance was also reported previously [11] [17] [18]. These data provides a relatively good representation of the local pneumococcal isolates. Penicillin resistance isolates have increased progressively from 10.9% [17], 31.0% [18] and 25.54% [11]. In our study, 50.2% of the 255 isolates were resistant to penicillin.

The rate of resistance to trimethoprim-sulfamethoxazole was 90.8% in the erythromycin resistant isolates. The extremely high level of resistance to trimethoprim-sulfamethoxazole among the pneumococci isolates in Malaysia is unexpected, as this antibioticin not frequently used for empirical treatment. A similar scenario was also reported by Soh S.W. (2000) [19] on pediatric isolates from Singapore.

Clindamycin resistance rate of 29.4% was detected among the 255 pneumococci isolates studied. Of the erythromycin resistant isolates, 51.2% were clindamycin resistant and had the macrolide-lincosamide-streptogramin B (MLS_B) phenotype.

The progressive increase in the prevalence of level of macrolide resistance among the pneumococci isolates from Malaysia draws a particular concern. Erythromycin resistance in pneumococci is due to the modification of the drug binding site which is regulated by the *erm*B gene, that is usually associated with the MLS_B phenotype and high-level resistance to erythromycin, with MIC_S of ≥64 µg/ml. Low-level erythromycin resistance, with MIC_S of 1 to 32 µg/ml, is due to the active efflux of the dr*ug* (which is regulated by the *mef*A gene [20]. Molecular characterization of the macrolide-resistant isolates in this study showed that the *erm*B gene was found in 25.3% of the pneumococcal isolates and *mef*A gene was found in 50.7% of the isolates. About 19.9% of the isolates were found have to the both *erm*B and *mef*A dually. This data s*ugg*est that almost 56% (144/255 isolates) are resistant to erythromycin and carry the resistant genes. Similar findings [21], which found more than 50% of the pneumococcal isolates from Asia carry the *erm*B and *mef*A gene. Based on these findings, it may be suggested that the use of single macrolide for the treatment of pneumococcal diseases may result in failure of antimicrobial therapy.

The four most common serotype encountered among the erythromycin pneumococcal isolates in over these 5 year study period are 19F, 19A, 23F and 14. Serotype 19F was the most prevalent erythromycin resistant serotype (42%) followed by serotype 19A (11.3%), serotype 23F (9.2%) and serogroup 14 (7.0%). Many studies, around the world, have reported the insurgence of serotype 19F, 23F and 14 among the erythromycin resistant pneumococci isolates.

The PCV7 vaccine appears to be able to confer protection against 86.1% of the local erythromycin resistant pneumococci. However, isolates from our study also show that there is an increase in the 19A serotype. The high prevalence of serotype 19A might be contributed to the reduction in disease caused by isolates from the PCV7 serotypes. Studies have shown that the proportion of serotype 19A isolates increased from 0.5% in 1995 and 0.3% during 2002-2004 to 6.93% (2008-2009) in Malaysia [11]. An insurgence of 19A serotype among the French isolates was also reported after the introduction of the PCV7 vaccine [22]. The introduction of PCV7,

PCV10 and PCV13 covered 60.3%, 66.7% and 87.8% of all isolates in China [23]. The change from PCV7 to either PCV10 or PCV13 would further improve the coverage as it has 19A serotype.

It is particularly interesting as how these serotypes are related to macrolide resistance. Other non-prevalent serotypes are also detected among the erythromycin resistant isolates. This may be due to variations in the geographical area, type of disease and age group of the patients. However, the high prevalence of erythromycin resistance in this study suggests the rapid spread of resistant clones among pneumococci in this region. Since *S. pneumoniae* has the ability to switch serotypes by horizontal transfer recombination [20] and other genetic events, it is important to monitor the frequency of the serotype exchanges in order to predict long-term efficacy of the new vaccine. Therefore continuous monitoring of antimicrobial resistance and serotype distribution of *S. pneumoniae* is important for disease management and implementation of disease control policies in Malaysia. The current study needs the support of a long-term surveillance program, epidemiological typing and also vaccination data for prevention and control of pneumococcal infections.

5. Conclusion

In conclusion, there is a significant rise in macrolide resistance among the Malaysian pneumococcal isolates. The rise of the 19A serotype is most likely linked to the introduction to PCV7. However, the emergence of serotype 19A together with increasing prevalence of resistance to macrolide warrants for a continuous surveillance of pneumococcal serotypes and a more extensive antimicrobial study.

Conflict of Interest

The authors declare that they have no conflict of interests.

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