CHAPTER VI

DISCUSSIONS

Macrolides have become good therapeutic choice in the treatment of penicillin-resistant *S. pneumoniae* infections and in penicillin-allergic patients with penumococcal pneumonia. However, *S. pneumoniae* is currently an increasing problem worldwide. The prevalence of resistance varies wildely between countries. In this study, antimicrobial susceptibility was determined in 385 *S. pneumoniae* isolated from patients at King Chulalongkorn Memorial Hospital, Bangkok during January 2003 to December 2007. The prevalence of macrolide resistance was 54.02% for erythromycin and 53.76% for clarithromycin. Clindamycin resistance was found to be 25.20%. Data from Asian Network for Surveillance of Resistant Pathogens (ANSORP) revealed that 21.9% of *S. pneumoniae* were resistant to erythromycin in Thailand during 1998-2001 (185). A recent publication by Srifuengfung S. *et al.* (186) showed an increased prevalence of erythromycin-resistant *S. pneumoniae* in Thailand from 49.5% in 2002-2003 to 55.5% in 2004-2005. This was in accordance with 54.02% macrolide resistance observed in our study. Macrolide resistance was reported to be high in Asian countries. Prevalence of erythromycin resistance was very high in Veitnam (92.1%) and Taiwan (86%) (185). High prevalence of macrolide-resistant *S. pneumoniae* was also reported to be 83.1% in South Korea (187), 81.4% in Japan (188), 83.6% in China (23).

In contrast, the low prevalence of erythromycin resistance was observed in the study by Reyes J. *et al.* from Colombia that reported 3.3% erythromycin-resistant *S. pneumoniae* in 1995-2004 (189). Similarly, erythromycin resistance was found to be 11.2% in Germany (179) and 18% in Greece (150). The PROTEKT US study during 2000-2004 reported that erythromycin resistance were 29.3% in *S. pneumoniae* over the 4 year study period (34). A study by the Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA) found that macrolide-resistant *S. pneumoniae* increased from 9% in 2000 to 14% in 2005 (20). In the USA, the prevalence of erythromycin resistance in *S. pneumoniae* increased from 25.7% in 1994-1995 (123) to 31.5% in 1999-2000 (29). The careful

monitoring of macrolide resistance rates should continue, especially since reports of *S. pneumoniae* strains with high-level erythromycin resistance.

Our results demonstrated that the main macrolide resistance mechanism was mediated by *mef* (E) gene, responsible for macrolide efflux. There were 53.85% of erythromycin-resistant isolates, harboring the *mef* gene and exhibited the M phenotype. These isolates had low-level erythromycin MIC (MIC range 1-16 μg/ml) and clarithromycin MIC (MIC range 0.125-16 μg/ml). All M-phenotype isolates were susceptible to clindamycin. One isolate carrying *mef* (E) was resistant to erythromycin (MIC 8 μg/ml) but susceptible to clarithromycin (MIC 0.125 μg/ml). In contrast, 45.67% of erythromycin-resistant isolates carrying the *erm* (B) gene, encoding a 23S rRNA methylase showed high-level erythromycin, clarithromycin and clindamycin MICs (MIC >512 μg/ml). All exhibited the $cMLS_B$ phenotype. The main mechanism of macrolide resistance in this study is similar to that of the previous report from Thailand (186) that showed 55% M phenotype among erythromycin-resistant isolates, followed by 45% $cMLS_B$ phenotype, $iMLS_B$ phenotype was not detected. Similarly, the PROTEKT US study in 2000-2004 (34) suggested that M phenotype was the most common resistance phenotype 65.7%, followed by $cMLS_B$ phenotype (16.6%) and $iMLS_B$ phenotype was not detected. In addition, study by Linden M. *et al.* in 1992-2004 from Germany (179) reported that 63.5% of *S. pneumoniae* resistant to erythromycin displayed M phenotype and 35.6% displayed $cMLS_B$ phenotype. The study by Rantana M. *et al.* (37) in 2002 from Finland reported that 49.30% and 41% of erythromycin-resistant *S. pneumoniae* isolates harbored *mef* and *erm* (B), respectively. The $iMLS_B$ phenotype was prevalent in some countries. The study by Littauer P. *et al.* in 1993-2002 from Norway (36) showed that the most common macrolide resistance phenotype of *S. pneumoniae* was M phenotype (60%) followed by $cMLS_B$ phenotype (37%) and $iMLS_B$ phenotype 1.66%. Similarly, *S. pneumoniae* resistant to erythromycin displayed $iMLS_B$ phenotype was found in 5% in Tunisia (176) and 5.5% in Colombia (189) and 0.62% in Italy (190). In addition, between 1998 and 1999, reported that 0.6% $iMLS_B$ phenotype of *S. pneumoniae* were resistant to erythromycin from Spain by Perez-Trallero E. *et al.* (191)

The *erm* (B) gene is prevalent in erythromycin-resistant *S. pneumoniae* from European countries and South Africa. A study by Fitoussi F. *et al.* from France (18) reported that all 48 (100%) macrolide-resistant *S. pneumoniae* had *erm* (B) gene. Eldere J. *et al.* (19) found that 89.7% of erythromycin-resistant pneumococcal isolates from Belgium, carried the *erm* (B) gene. Marchese A. *et al.* (190) from Italy found that approximately 90% of erythromycin-resistant *S. pneumoniae* possessd *erm* (B). In South Africa, the *erm* (B) gene was the predominant macrolide resistance mechanism. A report of Klugman K.P. *et al.* (21) showed that macrolide resistance in *S. pneumoniae* was 83.33% and 16.67% due to *erm* (B) and *mef* genes, repectively. Wolter N. *et al.* (20) presented that macrolide-resistant *S. pneumoniae* isolated from invasive specimens contained 57% *erm* (B) gene and 27% *mef* gene.

In this study, the presence of isolates containing *erm* (B) in combination with *mef* (E) gene was found in one isolate (0.48%) which exhibited the $cMLS_B$ phenotype. Despite the high prevalence of macrolide resistance in *S. pneumoniae*, there is no report of dual *mef* and *erm* (B) genes on the mechanism of macrolide resistance in Thailand. The results from our study are in agreement with 2% of erythromycin-resistant *S. pneumoniae* reported from Tunisia (176) and Finland (37). Similarly, the presence of a dual mechanism *erm* (B) and *mef* genes, was reported to be 3.5% in Belgium (19), 5.8% in Canada (35). High prevalence of dual macrolide resistance mechanism, both mef and *erm* (B), was reported to be 39.1% in South Korea (187) and 38.6% in Korea. In Japan, the presence of a dual mechanism *erm* (B) and *mef* genes were increasing from 6.1% in 2006 (22) to 7.1% in 2007 (188). Farrell D. J. and Jenkins S.G. reported that the presence of a dual mechanism *erm* (B) and *mef* genes were increasing in prevalence from 3.3% in the PROTEKT US study in 1996-1997 to 12.2% in the PROTEKT US study in 1999-2000 (31) and to 18.4% in 2004 (34). Wolter N. *et al.* (20) from South Africa showed that 15% of macrolide-resistant *S. pneumoniae* isolates contained both *erm* (B) and *mef* genes. From data on increasing prevalence of *mef* and *erm* (B) dual mechanism of macrolide resistance in *S. pneumoniae*, it is interesting to monitor this trend in the future, due to high-level macrolide resistance.

Two *mef* variants, *mef* (A) and *mef* (E), are associated with different genetic elements. The *mef* (A) gene was located in Tn*1207.1* (26, 42) and *mef* (E) was

carried by MEGA element located on Tn*916* and Tn*2009* (49, 164). However, *mef* (A) and *mef* (E) genes were spreaded by horizontal transfer among *Streptococcus* spp. In this study, of all 112 *mef*-positive isolates carried *mef* (E). Similar to the study by Palavecino E.L. *et al.* (192) from Chile which showed that all erythromycin-resistant *S. pneumoniae* were *mef* (E). Wierzbowski A.K. *et al.* (193) from Canada reported that 95% of macrolide-resistant *S. pneumoniae* carried *mef* (E) and 5% carried *mef* (A). In addition, Dobay O. *et al.* (194) reported that there were 75% *mef* (E) and 25% *mef* (A) genes in macrolide-resistant *S. pneumoniae* in Hungary. A study by M. Rantala *et al.* (37) from Finland found that 88.79% *mef* (E) and 11.21% *mef* (A) was present in erythromycin-resistant *S. pneumoniae.* In contrast, Amezaga M.R. *et al.* from the United Kingdom (195) showed that all *mef*-positive *S. pneumoniae* isolates carried *mef* (A). Montanari M. P. *et al.* (196) from Italy showed that 55.6% of erythromycin-resistant pneumococci isolated carried *mef* (A) and 44.4% carried *mef* (E). The *mef* (A) and *mef* (E) genes were found to be spreaded by horizontal transfer among *Streptococcus* spp. Sangvik M. *et al.* (197) from Norway described that macrolide-resistant *S. pyogenes* carried 75% *mef* (A) and 25% *mef* (E) whereas macrolide-resistant viridans streptococci carried 10% *mef* (A) and 90% *mef* (E). Arpin C. *et al.* (198) in France showed that *S. agalactiae* resistant to macrolide contained of 50% *mef* (E) and 50% *mef* (A).

In this study, all isolates with *mef* gene also contain *mel* gene in ORF adjacent to *mef* gene. This was similar to the study by M. Del Grisso *et al.* (42) which found that all *mef*-positive isolates carried the *mel* gene, homologue the *msr*A gene in *S. aureus* which encoded a protein of the ABC transporter superfamily involved in macrolide efflux. The presence of the *mel* genes in all *mef*-carrying isolates confirms previous studies of Gay K. et al. (49) and Daly M M. (51) that showed presence of the *mel* gene downstream from the *mef* (E) gene. Both *mef* and *mel* genes were reported to be necessary for erythromycin resistance and drove the macrolide efflux. Macrolide efflux were inhibited by CCCP, a well established inhibitor of efflux pumps. In this study, CCCP could reduce erythromycin MIC in all M-phenotype *S. pneumoniae* isolates, confirming the presence of efflux pump. Similarly, Capobianco J. *et al.* (199) showed that the inactivation of the macrolide efflux pump by CCCP resulted in about 2.6-fold increase of erythromycin uptake in *S. pneumoniae*.

A 1,218 bp ORF, encoding 405 amino acid in all 10 M phenotype *S. pneumoniae* isolates with the MIC range of 1-16 µg/ml. The result showed that the entire *mef* (E) gene shared 100% nucleotide and amino acid sequences identity with those of *S. pneumoniae* (accession no. AF274302) (49), (accession no.U83667) (12) and (accession no. AF376746) (42). Furthermore, our results showed that *mef* (E) gene was 100% nucleotide and amino acid sequences identity to other Streptococci, including those of *S. salivarius* (accession no. AJ318993), *S. intermedius* (AY064722), *S. agalactiae* (DQ445273) and viridans streptococci (EF042094). A study by Cousin S. *et al.* (200) found that macrolide-resistant *N. gonorrhoeae* (AY319932) carrying *mef* (A) gene which shared 99% nucleotide and amino acid sequences identity to those of *S. pneumoniae*. The study by Banks D. J. *et al.* (201) showed that the *mef* (A) gene of *S. pyogenes* (accession no. AY445042, AY657002 and AF227521) had 90% nucleotide and 88% amino acid sequences identity with *mef* (E) gene of *S. pneumoniae*.

The *mel* gene adjacent to *mef* (E), was homology with the *msrA* gene of *S. aureus* (accession no. AY064721), encoding a protein of the ABC transporter superfamily involved in macrolide efflux. The entire *mel* gene contained 1,464-bp nucleotide sequences, encoding a 487 amino acid. The results showed that the entire *mel* gene shared 100% nucleotide and amino acid sequences identity to those of *S. pneumoniae* (accession no. AF274302 and accession no.AF376746), *S. salivarius* (accession no. AJ318993) and *viridans* streptococci (accession no. EF042094). The results demonstrated that *mel* gene shared 99% nucleotide and 99% amino acid sequences identity to *mel* in Tn*2010* in *S. pneumoniae* (accession no. AB426626) and 97% nucleotide and 97% amino acid sequences identity to *S. pyogenes* (accession no. AF227521, AY657002 and AY445042).

A 119 bp intergenic region between *mef* (E) and *mel* genes contained a consensus Shine-Dagarno sequence upstream from the predicted start codon for *mel* gene. Wierzbowski A. K. *et al.* (53) reported that 99-bp deletion between *mef* (E) and *mel* intergenic region appeared in 11 isolates of the 23 M phenotype *S. pneumoniae* isolates. Similarly, Gay K. *et al.* , 2001 (49) found that 99-bp deletion between *mef* (E) and *mel* genes appeared among macrolide-resistant

S. pneumoniae isolates. In contrast to this study, we did not find any isolates with the 99-bp deletion in this region.

Sequencing analysis of a 630 bp upstream region of *mef* (E) were analyzed. There were one nucleotide changes at T-31C substibution in a putative -10 and -35 regions of *mef* (E) carrying *S. pneumoniae* isolates. Other 22 nucleotide changes of upstream region of *mef* gene were at as T-54G, T deletion at position -63, A-78T, T-81G, A-82G, T-345A and 16 bp deletion at -155. However, the results demonstrated that mutation at position -345 in upstream region of *mef* (E) gene may be associated with increased erythromycin MIC value in M-phenotype isolates as four isolates carrying this mutation had the erythromycin MIC range of 2-16 μg/ml whereas the others had the erythromycin MIC range of 1-4 μg/ml. In addition, our results found that 23 nucleotide changes were associated with Tn*2009* in *S. pneumoniae* (accession no.AF376746), *S. salivarious* (accession no.AJ318993) and viridans streptococci (accession no.EF042094) from GenBank data.

In 2005, Weirzbowski *et al.* (53) showed that higher levels of expression of the *mef* (E) gene were associated with higher MICs of erythromycin in macrolide-resistant *S. pneumoniae*. However, quantification of mRNA levels does not explain translation levels or the mRNA levels detected may not reflect the levels of protein produced. This increase in *mef* (E) mRNA and the Mef (E) protein levels remains to be elucidated.