CHAPTER V

RESULTS

1. BACTERIAL STRAINS

A total of 385 *Streptococcus pneumoniae* were isolated from patients in the King Chulalongkorn Memorial Hospital (Bangkok, Thailand), between January 2003 and December 2007. Each isolate was from different patient. Most *S. peumoniae* were isolates from respiratory tract. *S. pneumoniae* were collected from invasive site ; blood (11.43%, n=44) and cerebrospinal fluid (CSF) (1.81%, n=7) and non-invasive site ; sputum (61.29%, n=236), nasal swab (7.28%, n=28), endotracheal (6.75%, n=26), eye swab (5.71%, n=22), nasopharynx (2.86%, n=11), ear swab (1.29%, n=5), throat swab (0.78%, n=3), and pus (0.78%, n=3). All isolates were from male (63.38%, n=244) and female (36.62%, n=141). The ranges of age were 1 day to 95 years old. The age distribution of the patients was <1-2 years (15.80%, n=61), 3-14 years (8.10%, n=31), >14-64 years (47.30%, n=182) and >64 years (28.80%, n=111). (The results are shown in Table 7).

All isolates were identified as *S. pneumoniae* based on colonial morphology, gram stain, cell morphology and biochemical tests. Colonies on sheep blood agar were small and graynish, with a greenish zone of alpha-hemolysis surrounding them (Figure 18). Gram stain of *S. pneumoniae* showed gram-positive diplococci (Figure 19). Isolates were identified as *S. pneumoniae* by their susceptibility to optochin, solubility in bile.

Table 7 Pateint demographics and culture source of 385 isolates of *S. pneumoniae*

Parameter	Group	No. of isolates	Percentage of total (%)
Age	<1 -2	61	15.80
(years)	3-14	31	8.10
	>14-64	182	47.30
	>64	111	28.80
Gender	male	244	63.38
	female	141	36.62
Source			
invasive site			
	blood	44	11.43
	CSF	7	1.81
non-invasive site			
	sputum	236	61.29
	nasal swab	28	7.28
	endotracheal	26	6.75
	eye swab	22	5.71
	nasopharynx	11	2.86
	ear swab	5	1.29
	Throat swab	3	0.78
	pus	3	0.78

Figure 18 *S. pneumoniae* on the blood agar plate.

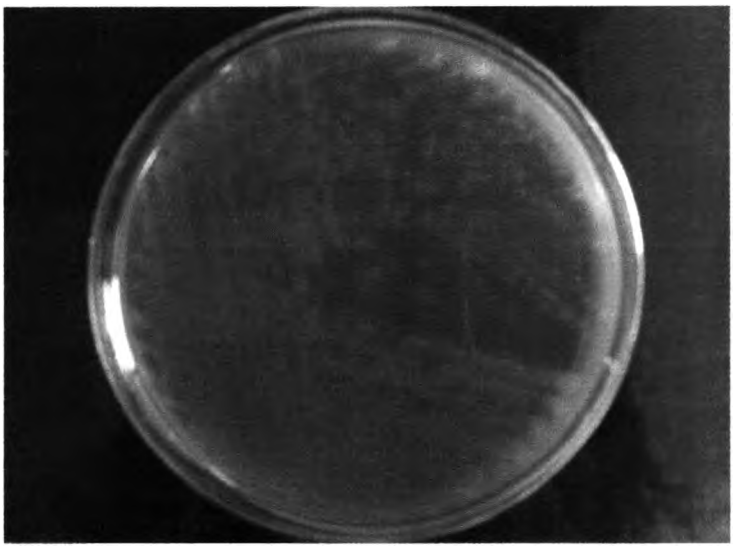
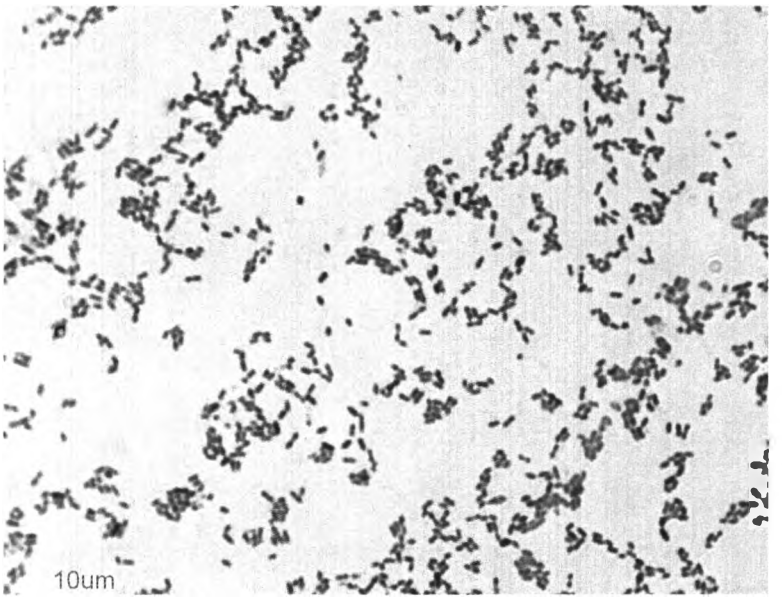


Figure 19 Gram stain of *S. pneumoniae* (100X)



2. DETERMINATION OF ANTIBIOTIC SUSCEPTIBILITY OF *S. PNEUMONIAE*

Susceptibility of *S. pneumoniae* to erythromycin, clarithromycin and clindamycin were determined by agar dilution method. The MIC is the lowest concentration of antimicrobial agent required to inhibit the growth of a microorganism *in vitro*. The MIC_{50} and MIC_{90} are the lowest concentration of anitimicrobial agents required to inhibit 50% and 90% of isolate tested, respectively. MIC breakpoints of erythromycin, clarithromycin and clindamycin were ≥ 1 μg/ml. Antimicrobial susceptibility and resistance rates of 385 *S. pneumoniae* isolates are shown in Table 8 and appendix IV.

Prevalence of erythromycin resistance was 54.02% (208/385). The MIC ranged from 0.03 to >512 μg/ml. MIC_{50} and MIC_{90} were 8 μg/ml and >512 μg/ml, respectively. Distribution of the MICs for erythromycin are shown in Figure 20. It was demonstrated that MICs of erythromycin-susceptible *S. pneumoniae* isolates ranged from 0.03 to 0.25 μg/ml. Most of erythromycin-susceptible isolates (69.49%) had erythromycin MIC of 0.125 μg/ml. Of the 208 erythromycin-resistant isolates, 53.85% had erythromycin MIC range of 1 to 16 μg/ml and 46.15% had high-level erythromycin resistance with the MIC of >512 μg/ml.

Similar to erythromycin susceptibility, the MIC ranged from 0.03 to >512 μg/ml. MIC_{50} and MIC_{90} were 8 μg/ml and >512 μg/ml, respectively. Distribution of the MICs for clarithromycin are shown in Figure 21. It was demonstrated that MICs of clarithromycin-susceptible in *S. pneumoniae* isolates ranged from 0.03 to 0.25 μg/ml. Most of clarithromycin-susceptible isolates (98.87%) had clarithromycin MIC of 0.03 to 0.125 μg/ml. Of the 207 clarithromycin-resistant isolates, 53.62% had clarithromycin MIC range of 1 to 8 μg/ml and 44.93% had high level resistance with the MIC of ≥256 μg/ml. All clarithromycin-resistant isolates were resistant to erythromycin. One of the 385 *S. pneumoniae* isolates was resistant to erythromycin (MIC 8 μg/ml) but was susceptible to clarithromycin (MIC 0.125 μg/ml).

Prevalence rates of clindamycin resistance was 25.20% (97/385). The MIC ranged from 0.03 to >512 μg/ml. MIC_{50} and MIC_{90} were 0.125 μg/ml and >512 μg/ml, respectively. Distribution of the MICs for clindamycin are shown in Figure 22. It was demonstrated that MICs of clindamycin-susceptible in *S. pneumoniae* isolates ranged from 0.03 to 0.25 μg/ml. Most of clindamycin-susceptible isolates (62.5%) had clindamycin MIC of 0.125 μg/ml. Most of clindamycin-resistant isolates (97.94%) had clindamycin MIC of ≥128 μg/ml.

Table 8 Macrolides and clindamycin MICs and resistance rates of 385 *S. pneumoniae* isolates

Antimicrobial agents	MICs (μg/ml)			Resistance rate (%)
	Range	MIC_{50}	MIC_{90}	
Erythromycin	0.03->512	2	>512	54.02
Clarithromycin	0.03->512	2	>512	53.76
Clindamycin	0.03->512	0.125	>512	25.20

Figure 20 MIC distribution for erythromycin against 385 *S. pneumoniae*

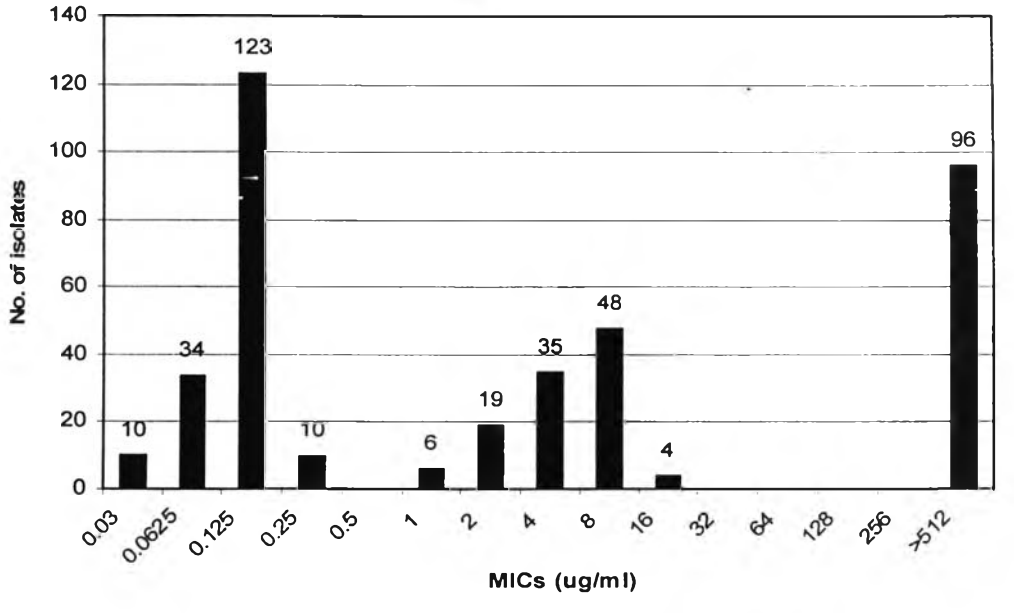


Figure 21 MIC distribution for clarithromycin against 385 *S. pneumoniae*

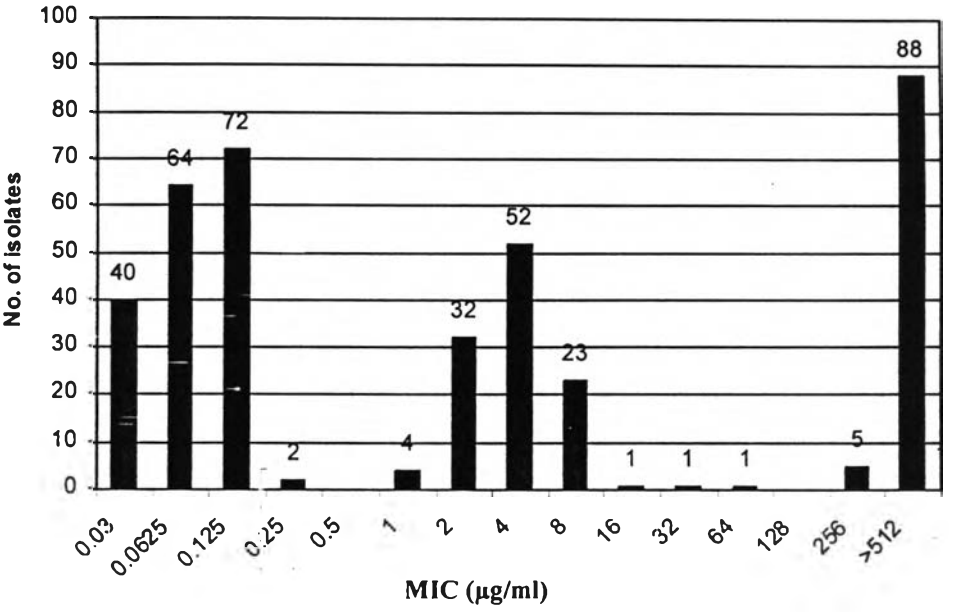


Figure 22 MIC distribution for clindamycin against 385 *S. pneumoniae*

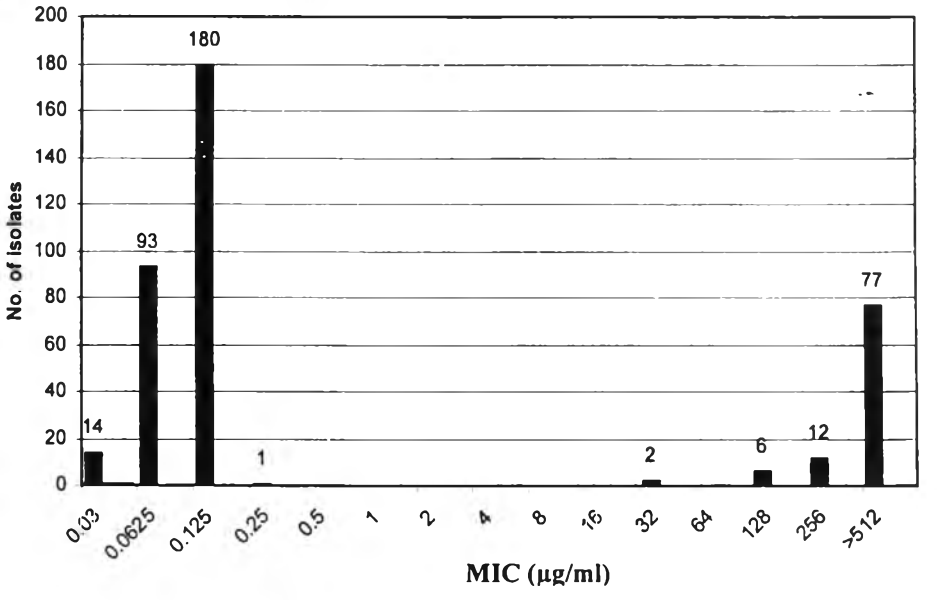


Table 9 Antibiotic susceptibility of the erythromycin, clarithromycin and clindamycin among *S. pneumoniae* isolates collected during 2003-2007

Antibiotic	Year				
	2003 (n=80)	2004 (n=84)	2005 (n=76)	2006 (n=68)	2007 (n=77)
Erythromycin					
MIC_{50} (μg/ml)	4	1	0.125	4	0.25
MIC_{90} (μg/ml)	>512	>512	>512	>512	>512
MIC range (μg/ml)	0.03->512	0.03->512	0.03->512	0.03->512	0.03->512
%Resistance	67.50	51.19	47.37	57.35	48.05
Clarithromycin					
MIC_{50} (μg/ml)	4	0.125	0.125	2	0.125
MIC_{90} (μg/ml)	>512	>512	>512	>512	>512
MIC range (μg/ml)	0.03->512	0.03->512	0.03->512	0.03->512	0.03->512
%Resistance	67.50	50.00	47.37	57.35	46.75
Clindamycin					
MIC_{50} (μg/ml)	0.125	0.125	0.125	0.125	0.125
MIC_{90} (μg/ml)	>512	>512	>512	>512	>512
MIC range (μg/ml)	0.03->512	0.03->512	0.03->512	0.03->512	0.03->512
%Resistance	35	26.20	21.05	29.41	14.29

INCIDENCE OF ANTIBIOTIC SUSCEPTIBILITY OVER THE 5 YEAR STUDY PERIOD (2003-2007)

A total of 385 isolates of *S. pneumoniae* were collected over the 2003-2007 : year 2003, 80 (20.80%) isolates ; year 2004, 84 (21.82%) isolates ; year 2005, 76 (19.74%) isolates ; year 2006 , 68 isolates (17.66%) and year 2008, 77 (20%) isolates. The rate of erythromycin resistance in this study was decreased from Year 2003 to 2005 (Year 2003, 67.5% ; Year 2004, 51.19% ; Year 2005, 47.37%), increased in Year 2006 (57.35%) and decreased again in Year 2007. MIC90 of erythromycin and clarithromycin were 512 μg/ml in Year 2003 to 2005.

Clarithromycin susceptibility showed similarlity trends regarding susceptibility to erythromycin. The rate of clindamycin-resistance was decreased from Year 2003 to 2005 (Year 2003, 35% ; Year 2004, 26.20% and Year 2005, 21.05%), increased in Year 2006 (29.42%) and decreased in Year 2007 (14.29%). Clindamycin MIC_{50} was 0.125 µg/ml and MIC_{90} was >512 µg/ml in Year 2003 to 2007. The results are shown in Table 9.

3. PHENOTYPE DETECTION OF MACROLIDE RESISTANCE MECHANISM

For all *S. pneumoniae* (n=385) isolates, macrolide resistance phenotypes were identified by a double disc test using erythromycin and clindamycin. Of the 208 erythromycin-resistant isolates, 46.15% (n=96) were $cMLS_B$ phenotype, 53.85% (n=112) were M phenotype (Figure 23). The $cMLS_B$ phenotype isolates were resistant to both erythromycin and clindamycin whereas M phenotype isolates were resistant to erythromycin but remained susceptible to clindamycin. The $iMLS_B$ phenotype was not detected. The $iMLS_B$ phenotype isolates were resistant to erythromycin and blunting around of the clindamycin disc (Figure 24).

The relationship between MIC and phenotype of macrolide resistance showed that isolates with erythromycin MICs of 1 to16 µg/ml had M phenotype while isolates with MIC of >512 µg/ml exhibited MLS_B phenotype.

Figure 23 Macrolide resistance phenotypes in 208 erythromycin-resistant *S. pneumoniae* isolates

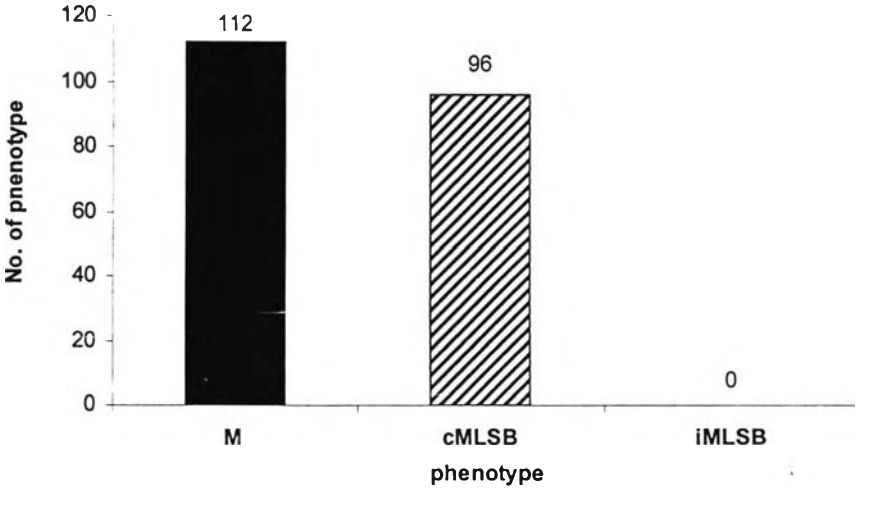
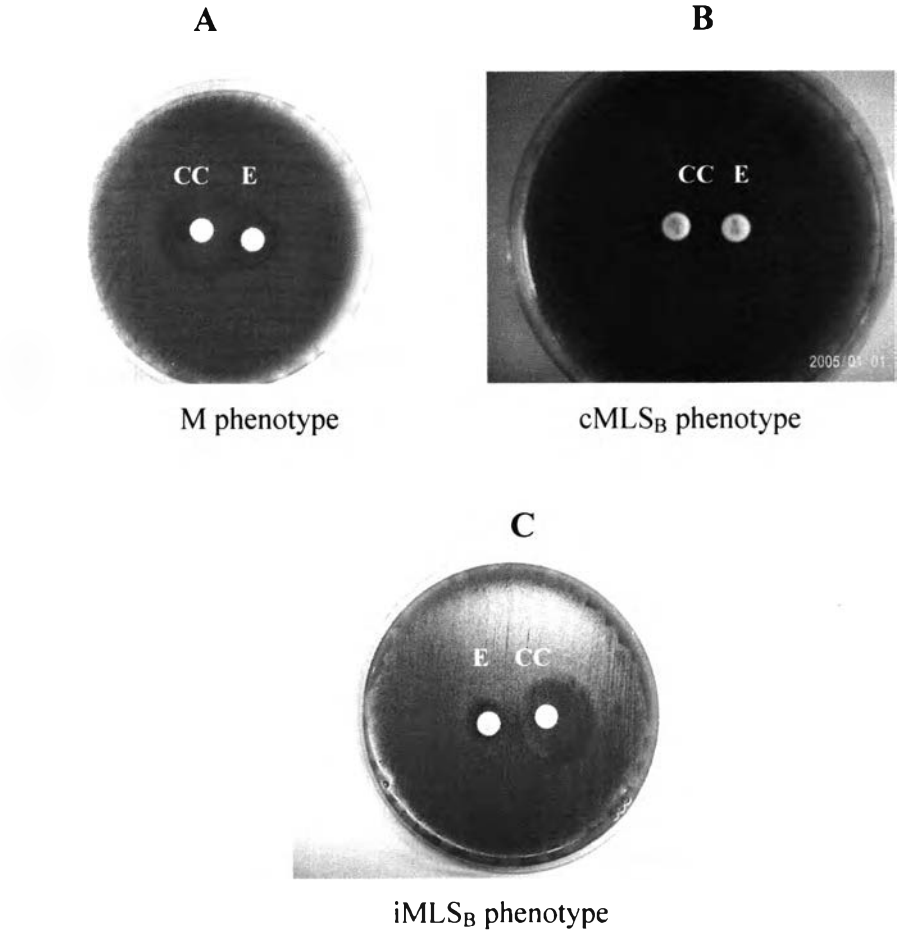


Figure 24 Double disc diffusion test for macrolide resistance phenotypes (E ; erythromycin (15 μg), CC ; clindamycin (2 μg), A ; M phenotype, B ; $cMLS_B$ phenotype and C ; $iMLS_B$ phenotype).



4. SCREENING FOR *MEF* AND *ERM* (B) GENES

The presence of *mef* gene and *erm* (B) gene in 385 *S. pneumoniae* were screened by multiplex PCR (Figure 25). The PCR products were 346 bp for *mef* gene and 639 bp for *erm* (B) gene. The *erm* (B) gene was found in 95 isolates (45.67%) and the *mef* gene was found in 112 isolates (53.85%). One isolate (0.48%) carried both *erm* (B) and *mef* genes.

The correlation between the erythromycin susceptibility and macrolide resistance genes in *S. pneumoniae* are shown in Figure 26. Isolates carrying either *mef* or *erm* (B) genes or both were resistant to macrolides. Erythromycin MICs of isolates containing *erm* (B) alone or both *erm* (B) and *mef* genes were >512 µg/ml. In contrast, isolates harboring *mef* genes had erythromycin MICs of 1-16 µg/ml. One of erythromycin-resistant isolates that was susceptible to clarithromycin carried the *mef* gene and showed M phenotype. The results demonstrated that isolates containing *erm* (B) alone or *erm* (B) in combination with *mef* genes exhibited high level MICs. On the other hand, isolates carring only *mef* gene exhibited low level MICs.

Isolates with $cMLS_B$ phenotype were genotypically confirmed by the presence of the *erm* (B) gene. Similarily, isolates with the M phenotype contained the *mef* gene. One (0.48%) of the 96 $cMLS_B$ phenotype strains harbored both *erm* (B) and *mef* genes. There was a perfect correlation between phenotype and genotype (Table 10).

Screening for the presence of *mel* gene. The presence of *mel* gene in the 112 *S. pneumoniae* isolates carring the *mef* gene was determined by PCR. The results demonstrated that all 112 isolates harbored the *mel* gene.

Figure 25 Electrophoresis of *erm* (B) and *mef* PCR products by multiplex PCR. M = 100 bp marker, lane1-2 : macrolide-susceptible strains, lane3-4 : macrolide-resistant strains carrying *erm* (B) gene, lane5-6 : macrolide-resistant strains carrying *mef* gene, lane 7 : macrolide-resistant strain carrying both *erm*(B) and *mef* genes, lane 8-9 : negative control (D.D.W).

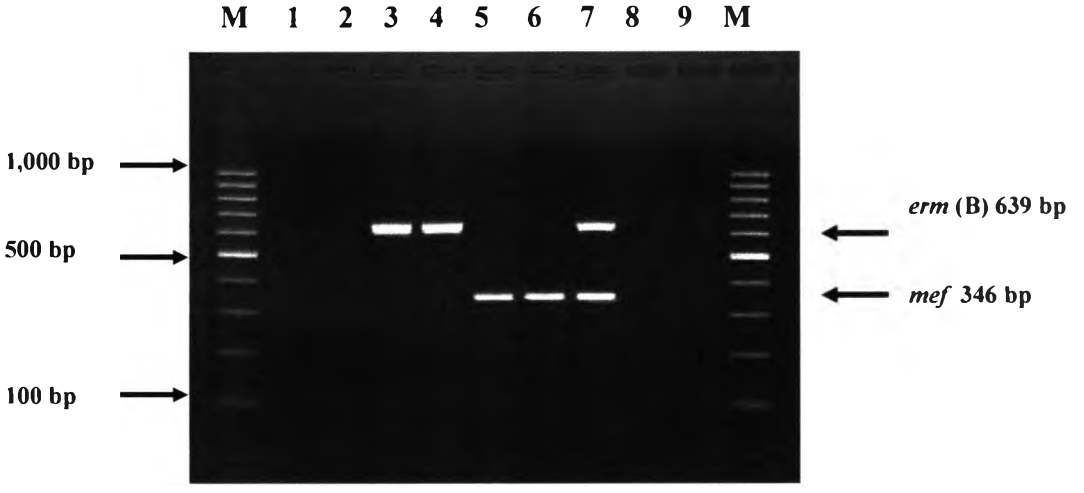


Figure 26 Relationship between erythromycin susceptibility and macrolide resistance genes.

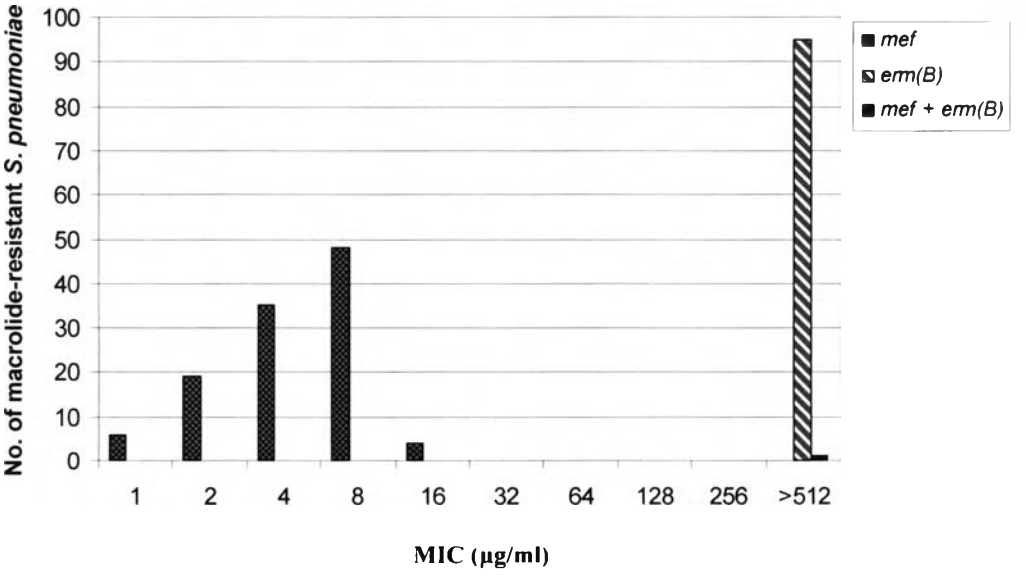


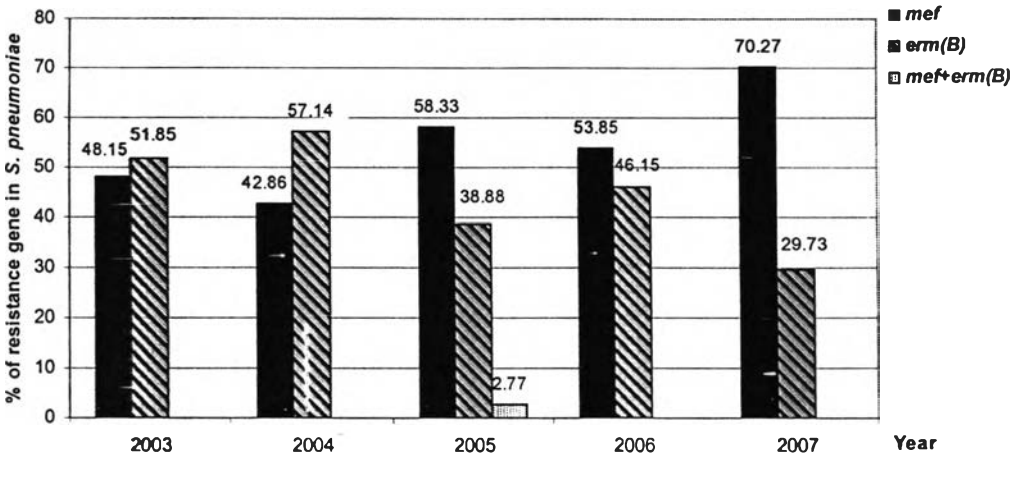
Table 10 Correlation between the resistance phenotypes and genotypes of erythromycin-resistant *S. pneumoniae* isolates.

Phenotypes	No. of isolates	Genotypes		
		erm (B)	*mef*	*erm*(B)+*mef*
$cMLS_B$	96	95	0	1
$iMLS_B$	0	0	0	0
M	112	0	112	0

INCIDENCE OF MACROLIDE RESISTANCE MECHANISMS OVER THE 5 YEAR STUDY PERIOD (2003-2007)

The distribution of macrolide resistance mechanisms among erythromycin-resistant isolates of *S. pneumoniae* collected during years 2003 to 2007 of the study is shown in Figure 27. The incidence of *mef*-positive *S. pneumoniae* isolates changed from 48.15% (2003) to 70.27% (2007). However, data from Year 2005-2007 show that *mef*-positive was consistently the most expressed genotype whereas the incidence of *erm* (B)-positive *S. pneumoniae* variable changed from 2003 to 2007 were 51.85%, 57.14%, 38.88%, 46.15% and 29.73%, respectively. *S. pneumoniae* strains expressing *erm* (B) gene exhibited a high level resistance to erythromycin (MIC >512 μg/ml) whereas *S. pneumoniae* isolates expressing *mef* genotype exhibited a low level resistance to erythromycin (MIC range 1-16 μg/ml). Only one isolates (2.77%) from 2005 carried dual *erm* (B) and *mef* mechanisms of macrolide resistance, displayed high level resistance to erythromycin and resistance to clarithromycin (MIC >512 μg/ml)

Figure 27 Prevalence of erythromycin resistance mechanism in *S. pneumoniae* isolated from 2003 to 2007. Black shading represents isolates with a *mef* genotype, dark pattern represents isolates with an *erm* (B) genotype and light grey shading represents isolates with both *erm* (B) and *mef* genes.



5. DETERMINATION OF *MEF* GENE TYPE BY PCR-RFLP

Determination of *mef* gene type was performed by PCR-RFLP in 112 *S. pneumoniae* isolates carrying *mef* genes. *Bam*HI and *Dra*II were used to restrict *mef* amplicons (346 bp). The *mef* (A) amplicon contains one *Bam*HI site, so restriction generates two fragments of 64 and 282 bp whereas the *mef* (E) amplicon contains no *Bam*HI restriction site (Figure 28). RFLP analysis with *Bam*HI revealed that all 112 *mef* gene amplicons were not cut by *Bam*HI. Therefore, all isolates tested carried *mef* (E) genes. As *mef* (E) amplicon contains one *Dra*II, restriction analysis with *Dra*II confirmed the results by generating 2 fragments of 112 and 234 bp. (Figure 29).

Figure 28 Agarose gel electrophoresis of *mef* amplicon restricted with restriction enzyme *Bam*HI. M : 100 bp marker, lane1-3 : macrolide-resistant strains carrying *mef* (A) gene (pre-cut), lane4-6 : macrolide-resistant strain carrying *mef* (A) gene cut by *Bam*HI.

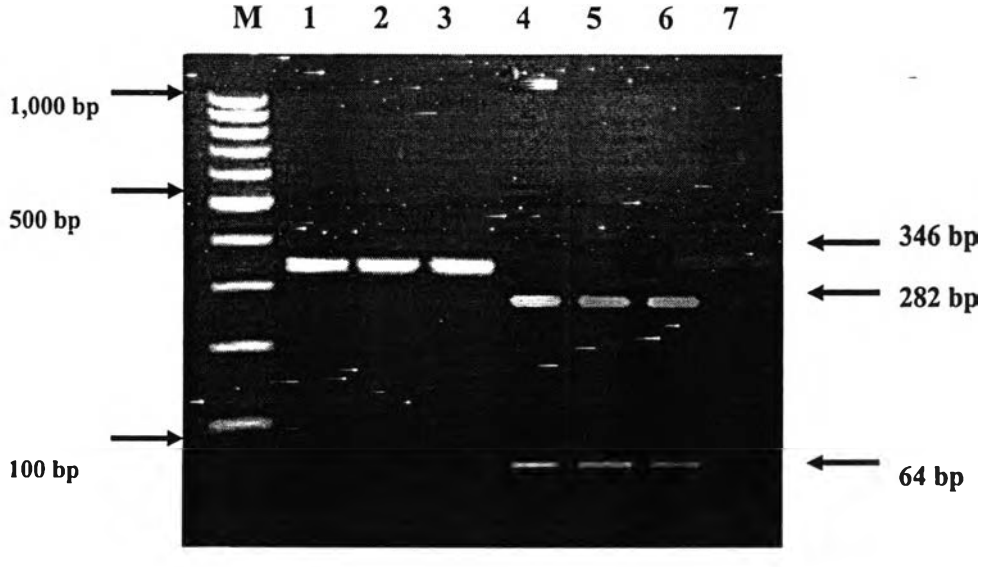
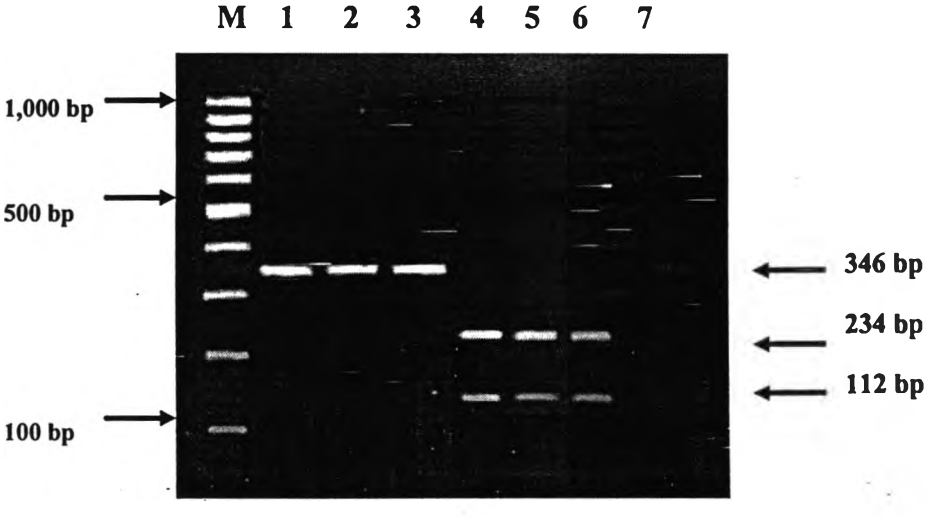


Figure 29 Restriction analysis of *mef* amplicon by *Dra*II. M : 100 bp marker, lane1-3 : *mef* (E) amplicon gene (pre-cut), lane4-6 : *mef* (E) amplicon cut by *Dra*II, one *Dra*II site, generating two fragments of 112 and 234 bp.



6. DETERMINATION OF MACROLIDE EFFLUX IN *S. PNEUMONIAE*

The activity of efflux pumps was tested by erythromycin agar dilution in the presence or absence of CCCP. A four-fold dimination MIC in the presence of CCCP was considered positive for macrolide efflux pump.

There were 112 *mef* (E)-carrying *S. pneumoniae* isolates with erythromycin MIC range of 1 to 16 μg/ml. CCCP could reduce MIC of erythromycin-resistant *S. pneumoniae* for all strains when compared with the MIC when CCCP was absent erythromycin. The CCCP did not affect the MIC of erythromycin in the isolate carring either both *mef* and *erm* (B) genes or *erm* (B) alone and erythromycin-susceptible strains. The results are shown in Table 11. Erythromycin MIC was decreased 6-9 fold in the presence of CCCP in all 112 mef-carrying *S. pneumoniae* isolates. The data indicate that *mef* (E)-carrying *S. pneumoniae* had macrolide efflux pump. The effect of CCCP on 112 erythromycin-resistant *S. pneumoniae* isolates are shown in Appendix V.

Table 11 The effect of macrolide efflux inhibitor (CCCP) on 112 erythromycin-resistant strains carrying *mef* gene.

Antibiotic	No.of fold Decreased	% (n)	MIC range (μg/ml)	
			without CCCP	with CCCP
Erythromycin	6	8.92(10)	1-4	0.015-0.0625
	7	29.46(33)	2-8	0.015-0.0625
	8	32.16(36)	4-8	0.015-0.03125
	9	29.46(33)	2-16	0.015-0.03125

7. ANALYSIS OF ENTIRE *MEF* (E) AND ENTIRE *MEL* GENES BY PCR AND SEQUENCING

Ten M-phenotype *S. pneumoniae* isolates containing *mef* (E) gene (SP47-22, SP 47-27, SP48-58, SP49-2, SP49-25, SP49-54, SP49-68, SP50-19, SP50-24 and SP50-30), with different MIC level (MIC range 1-16 µg/ml) were randomly selected for sequencing analysis of entire genes. The entire *mef* (E) and entire *mel* PCR products were 1,646 bp and 1,955 bp, respectively (Figure 30). DNA sequences were analyzed by the software available over the internet at the National Center for Biotechnology Information (http;//www.ncbi.nlm.nih.gov/BLAST) and ExPASy (www.expasy.org/), Multiple sequence alignment of sequences were analyzed by Multalin (http://bioinfo.genopoletoulouse.prd.fr/multalin/multalin.htm) and Bioedit program.

DNA sequence analysis of a 1,646-bp fragment revealed an open reading frame of 1,218 bp, encoding 405 amino acid proteins. There were no nucleotide changes in the entire *mef* (E) in any 10 M-phenotype isolates (Figure 31-32). They shared 100% nucleotide and amino acid sequences identity with the published sequence of *mef* gene in *S. pneumoniae* (accession no. AF274302, U83667 and AF376746). Furthermore, our result showed that they were 100% nucleotide and amino acid sequences identity with those of *S. salivarius* (accession no. AJ318993). *S. aureus* (accession no. AY064721), *S. intermedius* (accession no. AY064722), *S. agalactiae* (accession no. DQ445273) viridans streptococcus (accession no. EF042094). The *mef* (E) gene shared 99% nucleotide and amino acid sequences to those of *N. gonorrhoeae* (accession no. AY319932) and 90% nucleotide sequences identity and 88% amino acid sequences with those of *S. pyogenes*. (accession no. AY445042, AY657002 and AF227521).

DNA sequence analysis of a 1,955-bp fragment revealed an open reading frame of 1,464 bp, encoding a 487 amino acid proteins. There were no nucleotide changes in the entire *mel* gene in any 10 M-phenotype isolates (Figure 33-34). They shared 100% nucleotide and amino acid sequences identity with the published sequence of *mel* gene in *S. pneumoniae* (accession no. AF274302 and AF376746).

S. salivarius (accession no. AJ318993) and viridans streptococcus (EF042094). Moreover, *mel* sequence are 99% nucleotide and amino acid sequences identity with *mel* in Tn*2010* in *S. pneumoniae* (accession no. AB426626) and 97% nucleotide and amino acid sequences with *mel* in *S. pyogenes* (accession no. AF227521, AY657002, AY445042). A 119 bp *mef-mel* intergenic region was found to be 100% nucleotide and amino acid sequences identity to those of *S. pneumoniae* (accession no. AF274302), *S. pneumoniae* (accession no. AF376746) and *S. salivarius* (accession no. AJ318993). In this study, we did not find any isolates with the 99-bp deletion in *mef* (E) and *mel* intergenic region. The results are showed in Figure 35-36.

A 630 bp upstream region of *mef* (E) was compared with the published sequences of mega (accession no. AF274302). There were 23 nucleotide changes in upstream region of *mef* (E), a single T to C substitution at position -31, T to G substitution at position -54, T deletion at position -63, A to T substitution at position -78, T to G substitution at position -81, A to G substitution at position -82, a 16 bp deletion at position -155 and T to A substitution at position -345 (Figure 37). M-phenotype *S. pneumoniae* of the 10 isolates, all 22 nucleotide changes but four isolates had an additional T to A substitution at position -345 (MIC range 2-16 μg/ml). The results are showed in Table 12.

Figure 30 Agarose gel electrophoresis of upstream region of *mef* (E) (630 bp), entire *mef* (E) gene (1,646 bp) and entire *mel* gene (1,955 bp). M : 100 bp plus marker, lane1-2 : *mef*(E)-upsream, lane 3-4 : entire *mef*(E) gene, lane 5-6 : entire *mel* gene, lane 7: negative control (D.D.W).

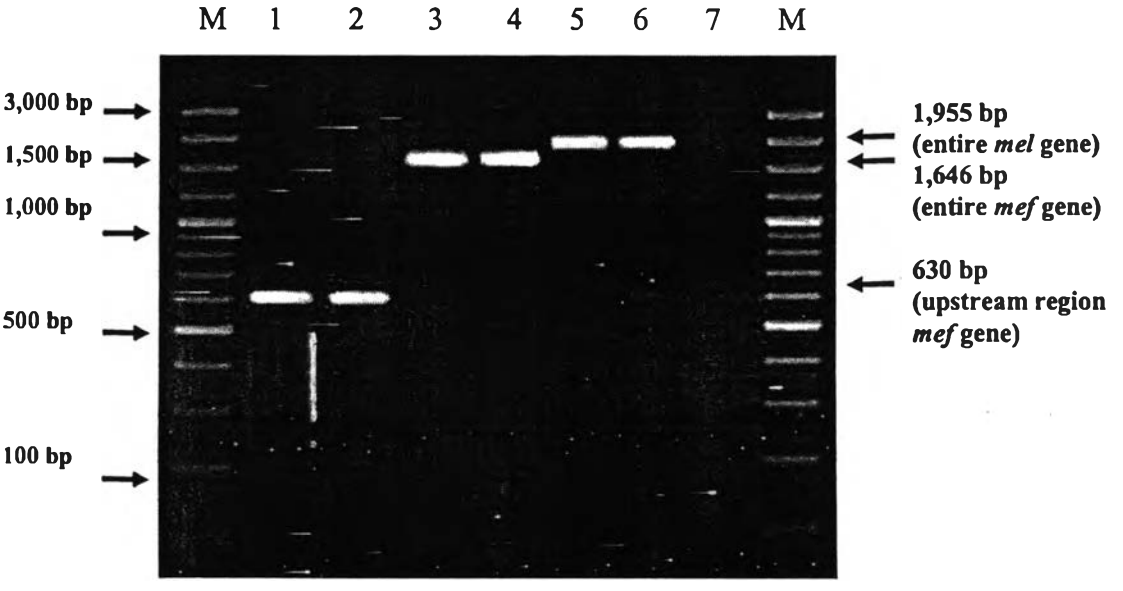


Figure 31 Multiple amino acid sequence alignment of entire Mef protein from 10 *mef*-positive *S. pneumoniae* isolates with those from *S. pneumoniae* (SP.AF274302) in GenBank.

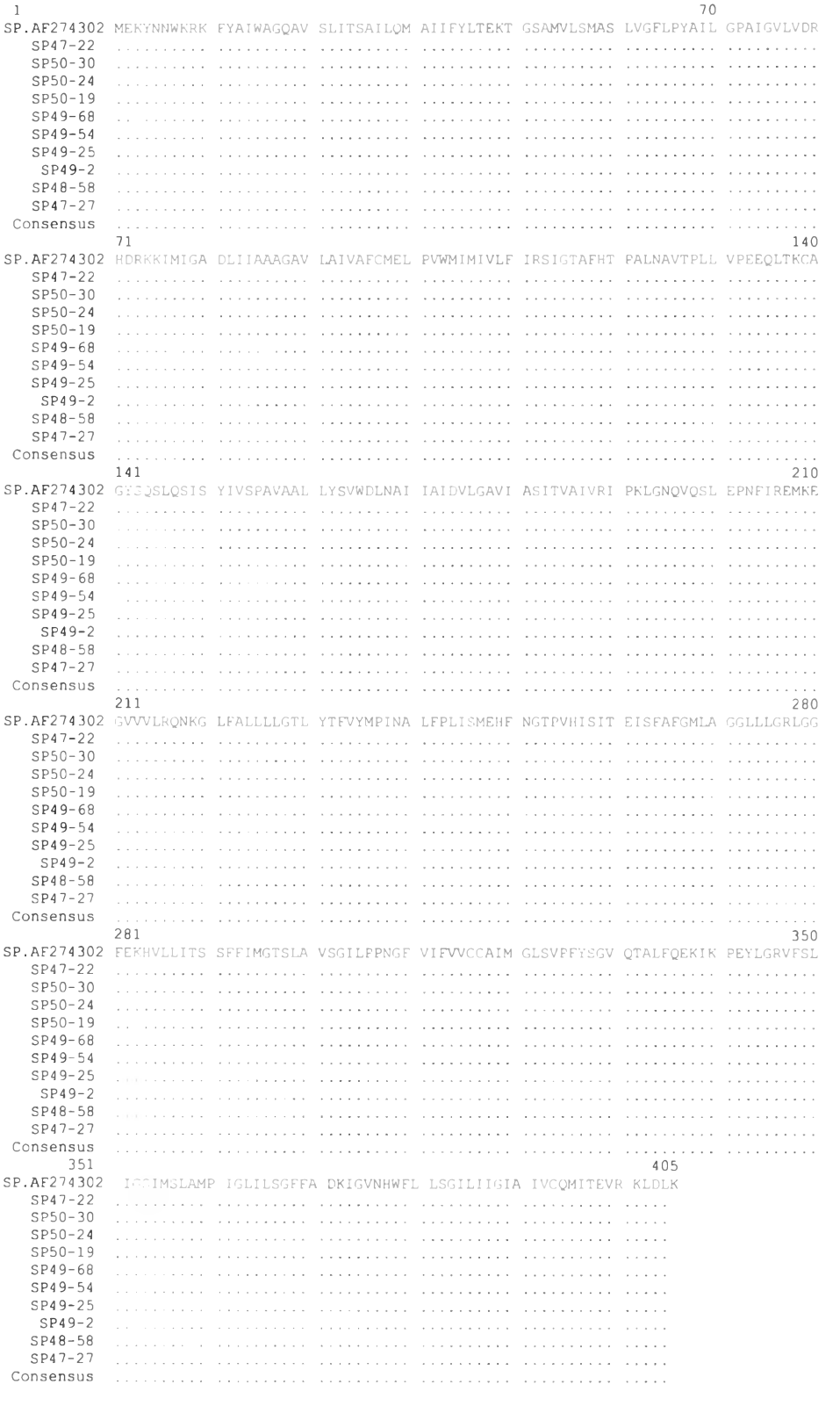


Figure 32 Multiple amino acid sequence alignment of Mef protein from *S. pneumoniae* (SP.AF274302) and those from *S. pyogenes* (SY.AF445042, SY.AY657002 and SY.AB227521), *S. salivarius* (SS.AJ318993), viridans streptococcus (SV.EF042094) and *S. pneumoniae* (SP.AF376746).

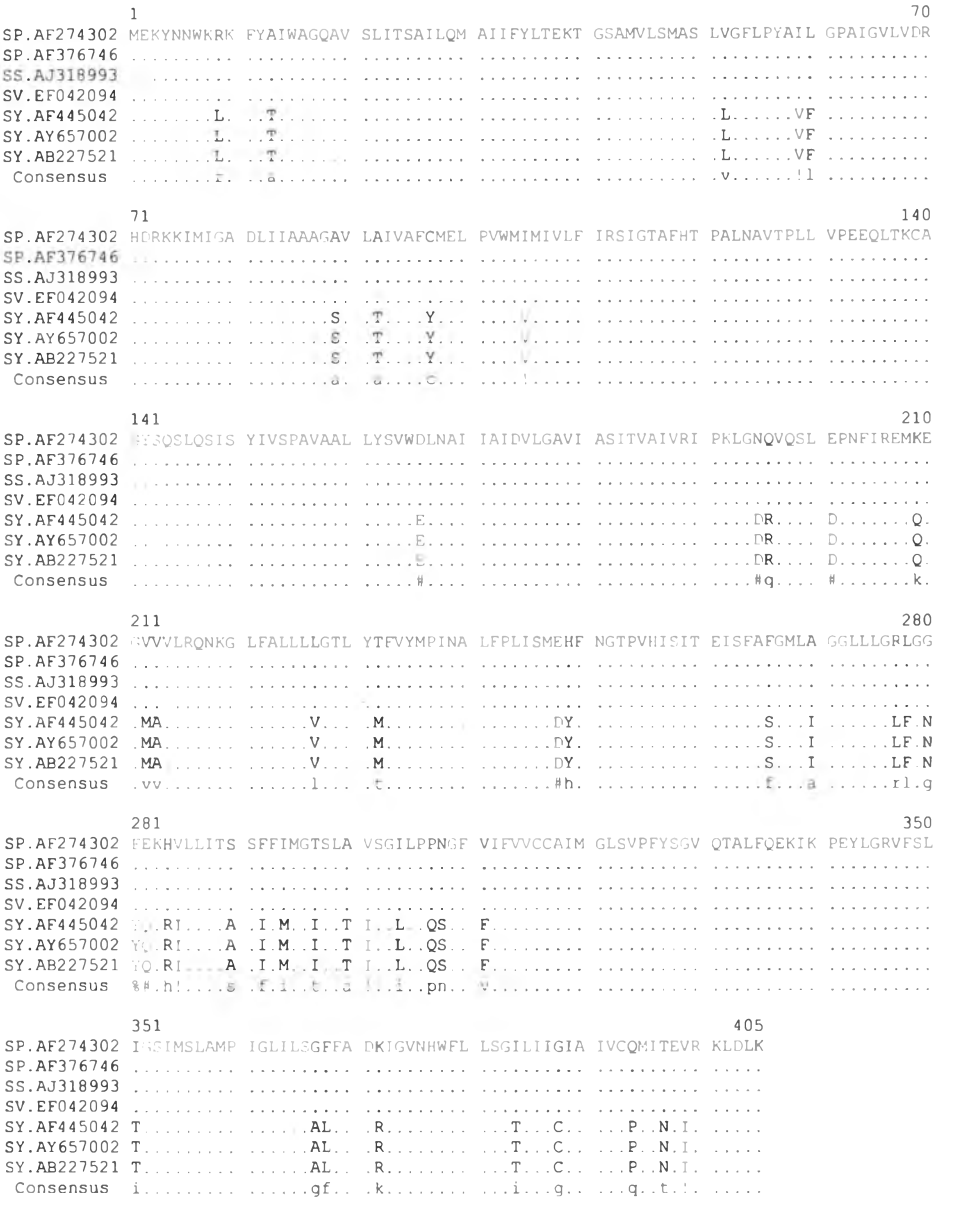
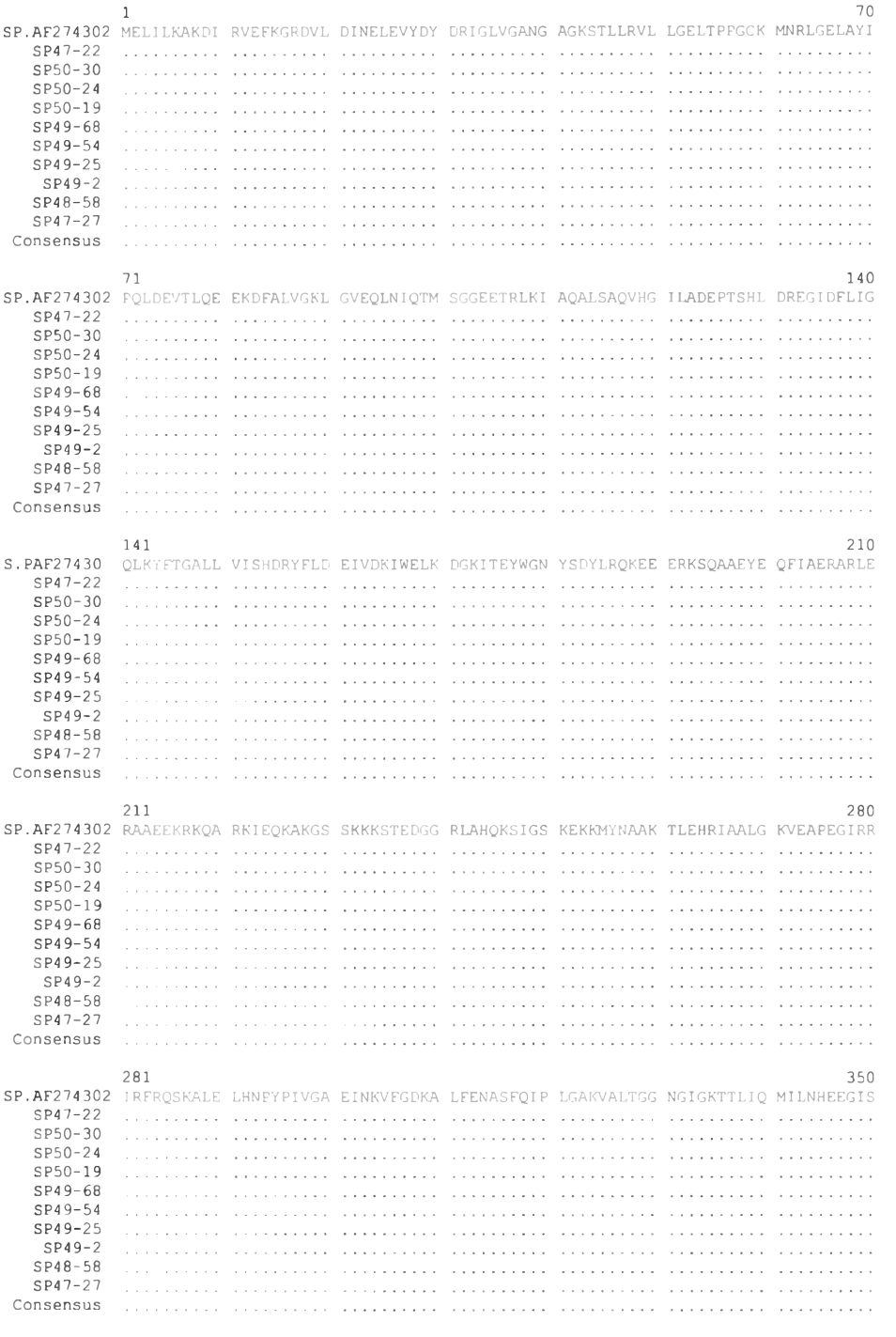


Figure 33 Multiple amino acid sequence alignment of entire Mel protein from 10 *mel*-positive *S. pneumoniae* isolates with those from *S. pneumoniae* (SP.AF274302) in GenBank.



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                351                                                                 420
SP.AF274302 ISPKAKIGYF AQNGYKYNSN QNVMEFMQKD CDYNISEIRS VLASMGFKQN DIGKSLSVLS GGEIIKLLLA
   SP47-22  .......... .......... .......... .......... .......... .......... ..........
   SP50-30  .......... .......... .......... .......... .......... .......... ..........
   SP50-24  .......... .......... .......... .......... .......... .......... ..........
   SP50-19  .......... .......... .......... .......... .......... .......... ..........
   SP49-68  .......... .......... .......... .......... .......... .......... ..........
   SP49-54  .......... .......... .......... .......... .......... .......... ..........
   SP49-25  .......... .......... .......... .......... .......... .......... ..........
    SP49-2  .......... .......... .......... .......... .......... .......... ..........
   SP48-58  .......... .......... .......... .......... .......... .......... ..........
   SP47-27  .......... .......... .......... .......... .......... .......... ..........
 Consensus  .......... .......... .......... .......... .......... .......... ..........

                421                                                                 487
SP.AF274302 KMLMGRYNIL IMDEPSNFLD IPSLEALEIL MKEYTGTIVF ITHDKRLLEN VADVVYEIRD KKINLKH
   SP47-22  .......... .......... .......... .......... .......... .......... .......
   SP50-30  .......... .......... .......... .......... .......... .......... .......
   SP50-24  .......... .......... .......... .......... .......... .......... .......
   SP50-19  .......... .......... .......... .......... .......... .......... .......
   SP49-68  .......... .......... .......... .......... .......... .......... .......
   SP49-54  .......... .......... .......... .......... .......... .......... .......
   SP49-25  .......... .......... .......... .......... .......... .......... .......
    SP49-2  .......... .......... .......... .......... .......... .......... .......
   SP48-58  .......... .......... .......... .......... .......... .......... .......
   SP47-27  .......... .......... .......... .......... .......... .......... .......
 Consensus  .......... .......... .......... .......... .......... .......... .......
```

Figure 34 Multiple amino acid sequence alignment of Mel protein from *S. pneumoniae* (SP.AF274302) and those from *S. pyogenes* (SY.AF445042, SY.AY657002 and SY.AB227521), *S. salivarius* (SS.AJ318993), viridans streptococcus (SV.EF042094) and those of *S. pneumoniae* (SP.AF376746 and SP.AB426626).

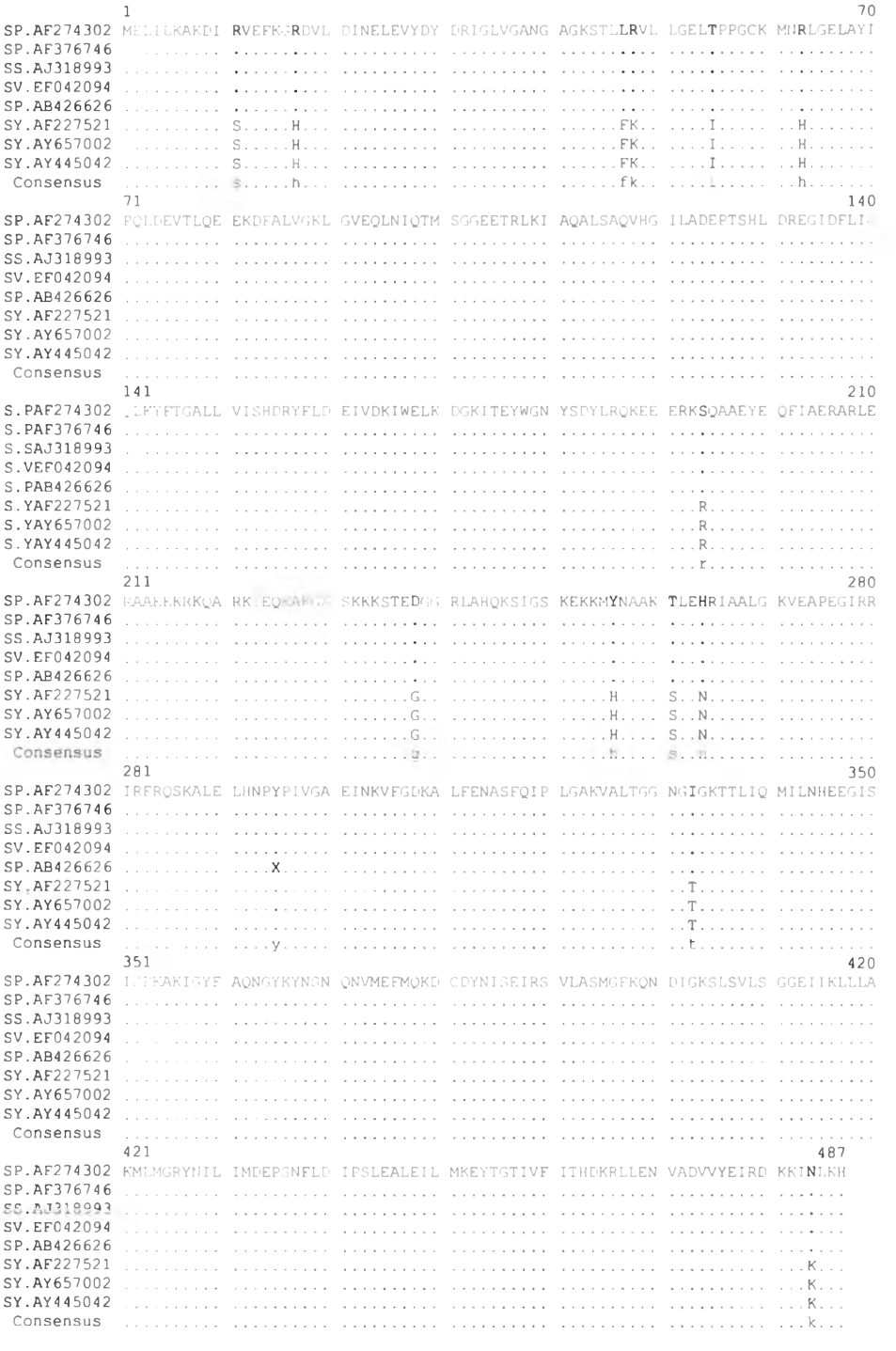


Figure 35 Multiple nucleotide sequence alignment of *mef-mel* intergenic region of 10 *S. pneumoniae* isolates with those from *S. pneumoniae* (SP.AF274302) in GenBank.

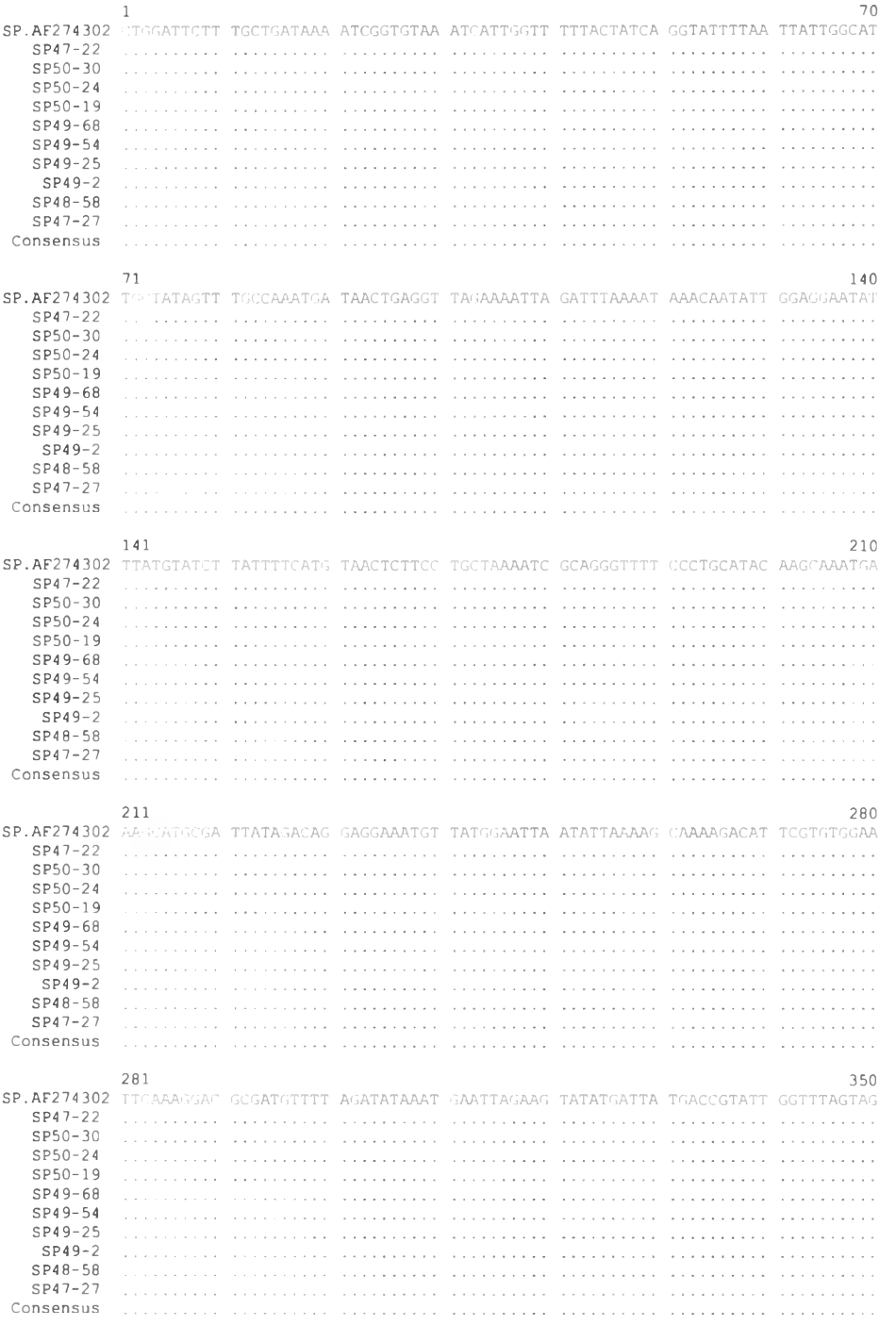


Figure 36 Multiple nucleotide sequence alignment of *mef-mel* intergenic region from *S. pneumoniae* (SP.AF274302) with those from *S. salivarius* (SS.AJ318993), viridans streptococcus (SV.EF042094) and *S. pneumoniae* (SP.AF376746). (letters in red ; *mef* gene, letters in violet ; *mel* gene, letters in blue ; *mef-mel* intergenic region and - ; 99 bp deletion).

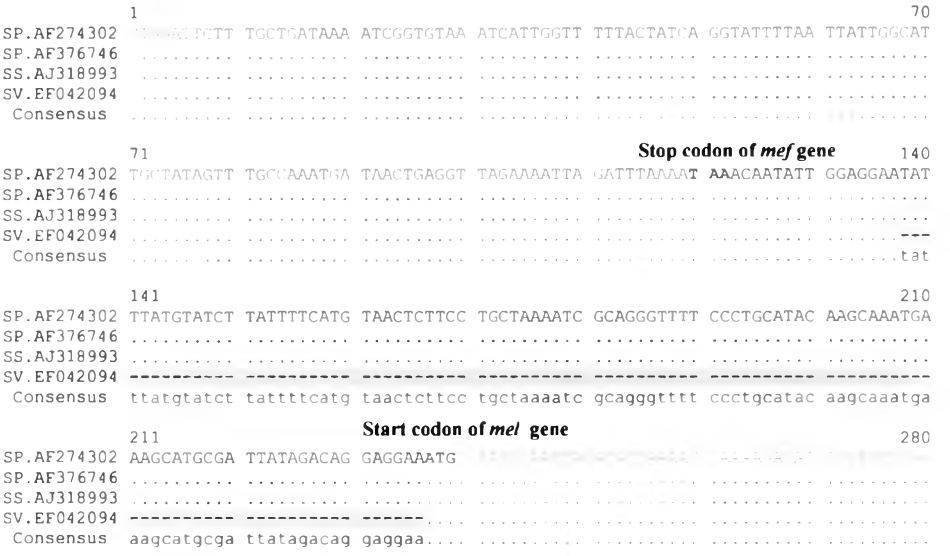
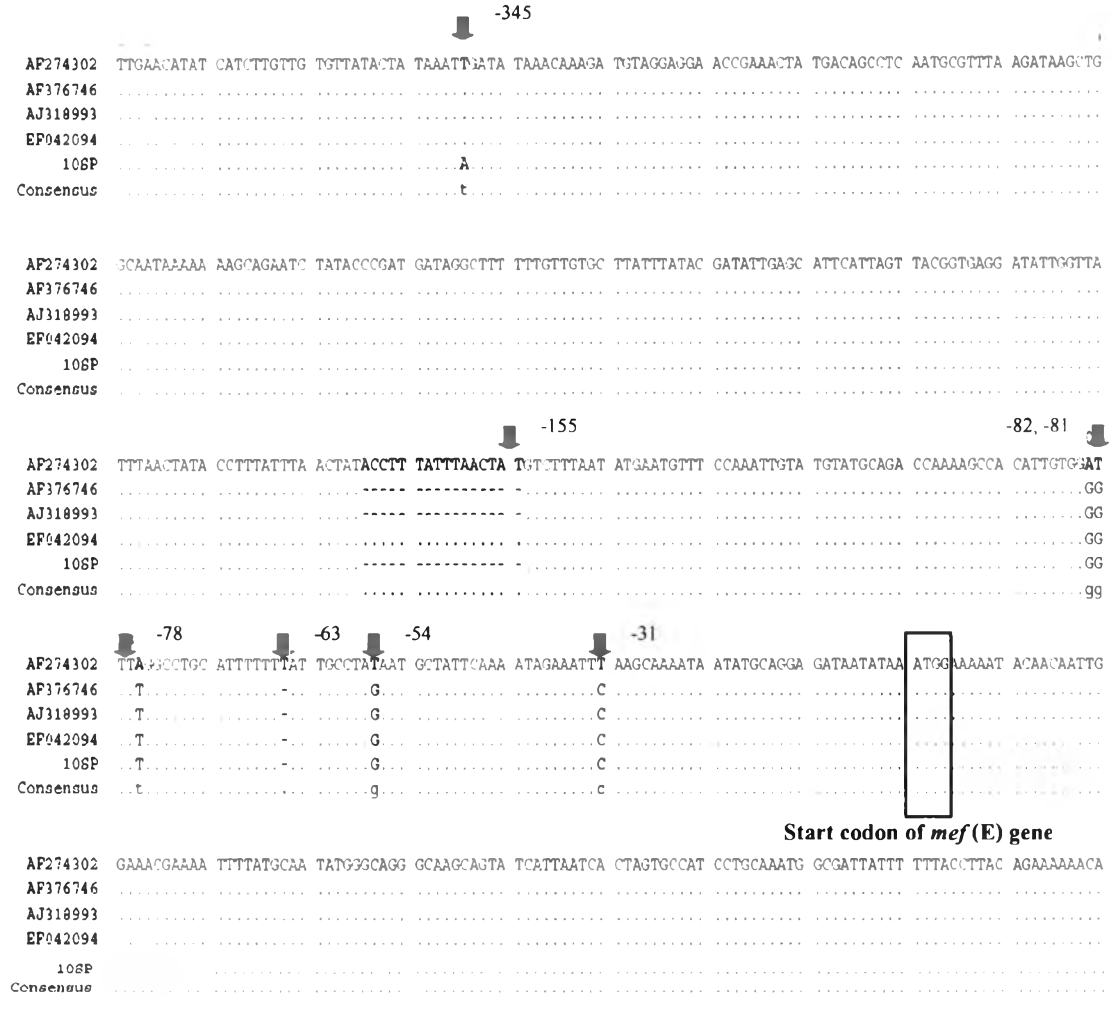


Table 12 Nucleotide sequence changes in upstream of *mef* gene in macrolide-resistant *S. pneumoniae* isolates.

Strains	MICs µg/ml	**Nucleotide sequence change at position :							
		-31	-54	-63	-78	-81	-82	-155	-345
Reference strain									
S. pneumoniae	4	T	T	T	A	T	A	ACCTTTAT	T
*(accession no.AF274302)								TTAACTAT(16bp)	
SP47-22	16	C	G	-	T	G	G	-	A
SP47-27	16	C	G	-	T	G	G	-	A
SP49-68	2	C	G	-	T	G	G	-	A
SP49-2	8	C	G	-	T	G	G	-	A
SP48-58	4	C	G	-	T	G	G	-	T
SP49-25	2	C	G	-	T	G	G	-	T
SP50-30	2	C	G	-	T	G	G	-	T
SP50-24	1	C	G	-	T	G	G	-	T
SP50-19	4	C	G	-	T	G	G	-	T
SP49-54	2	C	G	-	T	G	G	-	T

* ; GeneBank accession no.AF274302, SP ; *S. pneumoniae* , - ; deletion, ** ; Position relative to the start site of *mef* (E) gene.

Figure 37 Multiple nucleotide sequence alignment of upstream of *mef* gene from 10 *mef*-positive *S. pneumoniae* isolates with those of *S. pneumoniae* (SP.AF274302 and SP.AF376746), *S. salivarius* (SS.AJ318993) and viridans streptococcus (SV.EF042094).



Abbreviations : A, adenine ; C, cytosine ; G, guanine ; T, thymine ; 10SP, 10 M-phenotype *S. pneumoniae*.