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-invasivo	e 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.

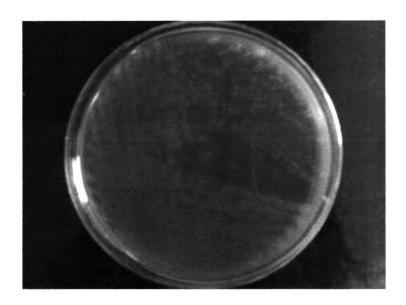
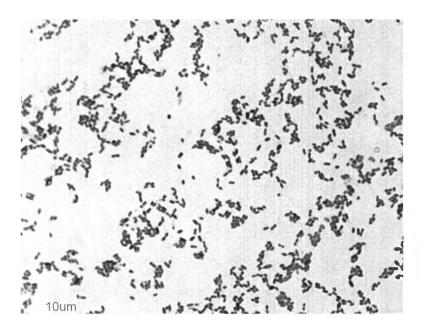


Figure 19 Gram stain of S. pneumoniae (100X)



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 MIC₅₀ and MIC₉₀ 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 $\geq 1 \mu g/ml$. 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 μ g/ml. MIC₅₀ and MIC₉₀ were 8 μ g/ml and >512 μ g/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 μ g/ml. Most of erythromycin-susceptible isolates (69.49%) had erythromycin MIC of 0.125 μ g/ml. Of the 208 erythromycin-resistant isolates, 53.85% had erythromycin MIC range of 1 to 16 μ g/ml and 46.15% had high-level erythromycin resistance with the MIC of >512 μ g/ml.

Similar to erythromycin susceptibility, the MIC ranged from 0.03 to >512 μ g/ml. MIC₅₀ and MIC₉₀ were 8 μ g/ml and >512 μ g/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 μ g/ml. Most of clarithromycin-susceptible isolates (98.87%) had clarithromycin MIC of 0.03 to 0.125 μ g/ml. Of the 207 clarithromycin-resistant isolates, 53.62% had clarithromycin MIC range of 1 to 8 μ g/ml and 44.93% had high level resistance with the MIC of \geq 256 μ g/ml. All clarithromycin-resistant isolates were resistant to erythromycin. One of the 385 *S. pneumoniae* isolates was resistant to erythromycin (MIC 8 μ g/ml) but was susceptible to clarithromycin (MIC 0.125 μ g/ml).

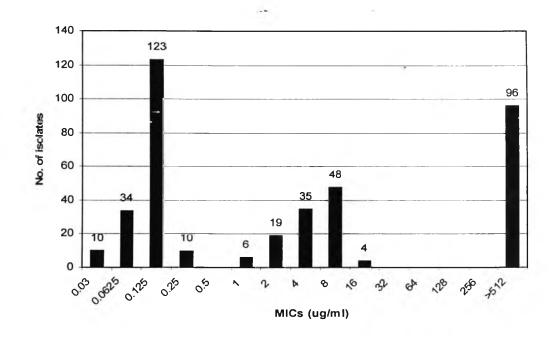
Prevalence rates of clindamycin resistance was 25.20% (97/385). The MIC ranged from 0.03 to >512 μ g/ml. MIC₅₀ and MIC₉₀ were 0.125 μ g/ml and >512 μ g/ml, respectively. Distribution of the MICs for clindamycin are shown in Figure 22. It was demonstrated that MICs of clindamycin-susceptible in *S. pneumoniae* isolates ranged from 0.03 to 0.25 μ g/ml. Most of clindamycin-susceptible isolates (62.5%) had clindamycin MIC of 0.125 μ g/ml. Most of clindamycin-resistant isolates (97.94%) had clindamycin MIC of ≥128 μ g/ml.

Table 8 Macrolides and clindamycin MICs and resistance rates of 385

S. pneumoniae isolates

Antimicrobial	N	⁄IICs (μg/ml)		Resistance rate (%)
agents	Range	MIC ₅₀	MIC ₉₀	7
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





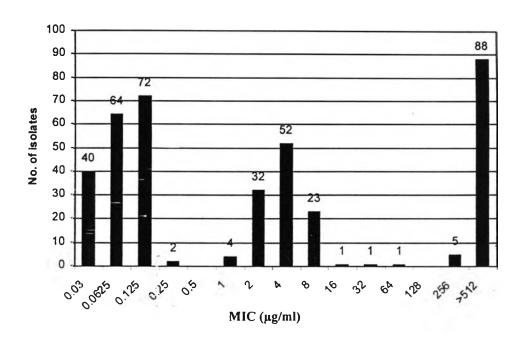


Figure 22 MIC distribution for clindamycin against 385 S. pneumoniae

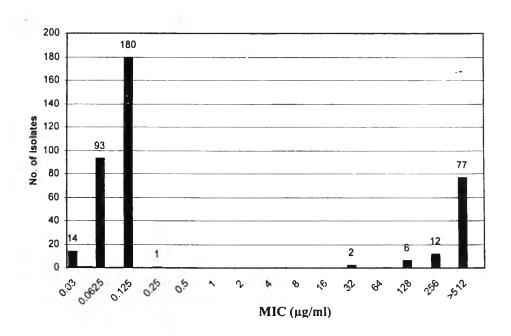




Table 9 Antibiotic susceptibility of the erythromycin, clarithromycin and clindamycin among *S. pneumoniae* isolates collected during 2003-2007

Antibiotic			Year		
	2003	2004	2005	2006	2007
	(n=80)	(n=84)	(n=76)	(n=68)	(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)

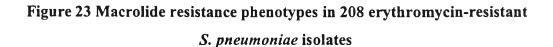
A total of 385 isolates of *S. pneumoniae* were collected over the 2003-2007: year 2003, 80 (20.80%) isolates; year 2004, 84 (21.82%) isolates; year 2005, 76 (19.74%) isolates; year 2006, 68 isolates (17.66%) and year 2008, 77 (20%) isolates. The rate of erythromycin resistance in this study was decreased 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 μ g/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 μ g/ml and MIC_{90} was >512 μ g/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 to 16 μ g/ml had M phenotype while isolates with MIC of >512 μ g/ml exhibited MLS_B phenotype.



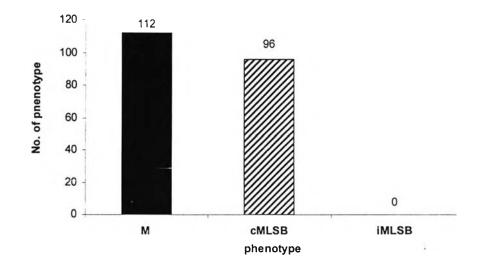
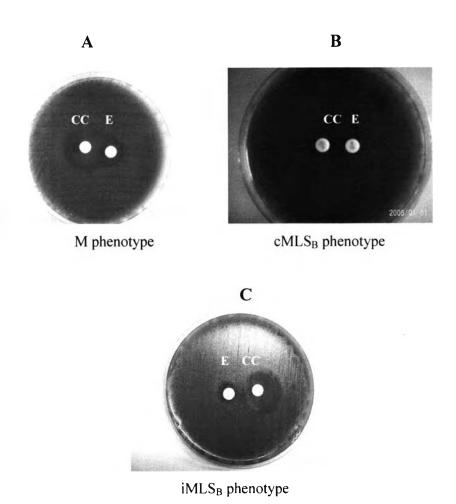


Figure 24 Double disc diffusion test for macrolide resistance phenotypes (E; erythromycin (15 μ g), CC; clindamycin (2 μ g), A; M phenotype, B; cMLS_B phenotype and C; iMLS_B phenotype).



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. Similarly, 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).

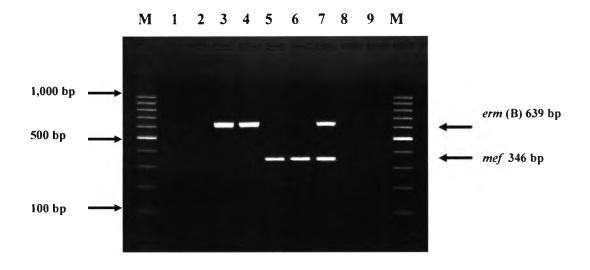


Figure 26 Relationship between erythromycin susceptibility and macrolide resistance genes.

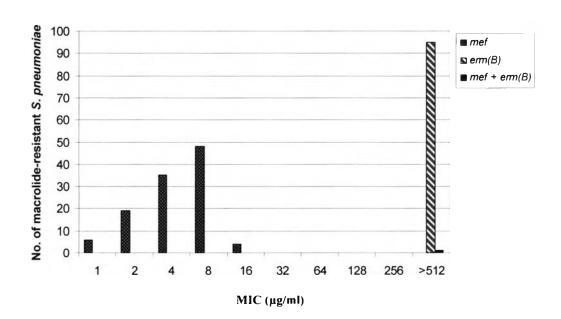


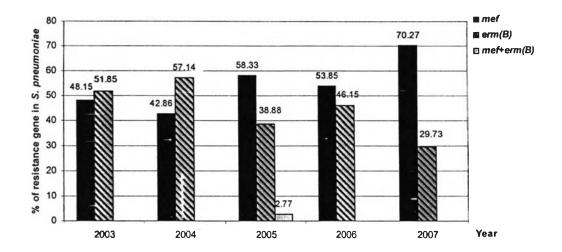
Table 10 Correlation between the resistance phenotypes and genotypes of erythromycin-resistant S. pneumoniae isolates.

Phenotypes	No. of isolates	-	Genotyp	es
		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. BamHI and DraII were used to restrict mef amplicons (346 bp). The mef (A) amplicon contains one BamHI site, so restriction generates two fragments of 64 and 282 bp whereas the mef (E) amplicon contains no BamHI restriction site (Figure 28). RFLP analysis with BamHI revealed that all 112 mef gene amplicons were not cut by BamHI. Therefore, all isolates tested carried mef (E) genes. As mef (E) amplicon contains one DraII, restriction analysis with DraII 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 *BamHI*. 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 *BamHI*.

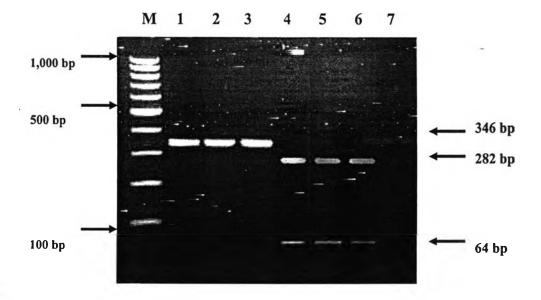
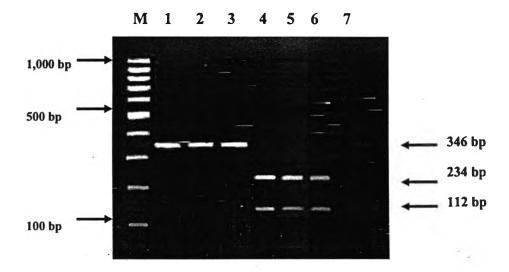


Figure 29 Restriction analysis of *mef* amplicon by *DraII*. M: 100 bp marker, lane1-3: *mef* (E) amplicon gene (pre-cut), lane4-6: *mef* (E) amplicon cut by *DraII*, one *DraII* site, generating two fragments of 112 and 234 bp.



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 erythromycinresistant strains carrying *mef* gene.

Antibiotic	No.of fold	% (n)	MIC range	e (μg/ml)
	Decreased	,	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 Tn2010 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).

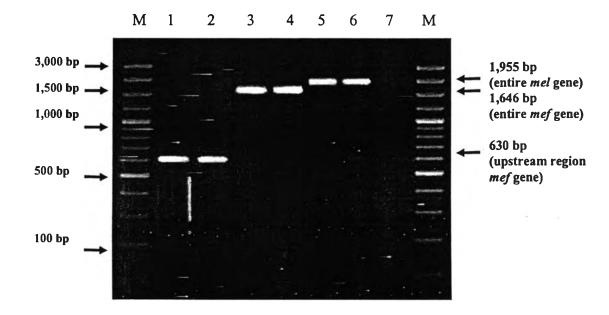


Figure 31 Multiple amino acid sequence alignment of entire Mef protein from 10 *mef*-positive *S. pneumoniae* isolates with those from *S. pneumoniae* (SP.AF274302) in GenBank.

1						7.0	
SP.AF274302	MEKYNNWKRK	FYAIWAGQAV	SLITSAILOM	AIIFYLTEKT	GSAMVLSMAS		GPAIGVLVDR
SP47-22							
SP50-30							
SP50-24 SP50-19							
SP49-68							
SP49-54							
SP49-25							
SP49-2 SP48-58							
SP47-27							
Consensus							
SP.AF274302	71 HDRKKIMIGA	DITIANACAN	LAIVAECMEL	DUDDMINIVE	IRSIGTAFHT	DALMAVEDII	140 VPEEOLTKCA
SP47-22	HURKKIMIGA	DLITAAAGAV	LAIVAFCMEL	PVWMIMIVLE	IKSIGIAFHI	FALNAVIPLL	VPEEQLINCA
SP50-30							
SP50-24							
SP50-19							
SP49-68 SP49-54							
SP49-25							
SP49-2							
SP48-58 SP47-27							
Consensus							
00110011040	141						210
SP.AF274302	GYGQSLQSIS	YIVSPAVAAL	LYSVWDLNAI	IAIDVLGAVI	ASITVAIVRI	PKLGNQVQSL	EPNFIREMKE
SP47-22 SP50-30							
SP50-24							
SP50-19							
SP49-68							
SP49-54 SP49-25							
SP49-2							
SP48-58							
SP47-27							
Consensus	211						280
SP.AF274302		LFALLLLGTL	YTFVYMPINA	LFPLISMEHF	NGTPVHISIT	EISFAFGMLA	
SP47-22							
SP50-30 SP50-24							
SP50-19							
SP49-68							
SP49-68 SP49-54							
SP49-68 SP49-54 SP49-25							
SP49-68 SP49-54							
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27							
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58							
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus	281	SEFIMGTSLA	VSGILPPNGF			OTALFOEKIK	350
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22		SFFIMGTSLA	VSGILPPNGF	VIFVVCCAIM		QTALFQEKIK	350 PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30				VIFVVCCAIM	GLSVPFYSGV	QTALFQEKIK	
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24				VIFVVCCAIM	GLSVPFYSGV	QTALFQEKIK	
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30	FEKHVLLITS			VIFVVCCAIM	GLSVPFYSGV		PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54	FEKHVLLITS			VIFVVCCAIM	GLSVPFYSGV		PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP. AF274302 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-55	FEKHVLLITS			VIFVVCCAIM	GLSVPFYSGV		PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-2	FEKHVLLITS			VIFVVCCAIM	GLSVPFYEGV		PEYLGRVFSL
SP49-68 SP49-24 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP. AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-25 SP49-25	FEKHVLLITS			VIFVVCCAIM	GLSVPFYSGV		PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-27 Consensus	FEKHVLLITS			VIFVVCCAIM	GLSVPFTEGV		PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus	FEKHVLLITS			VIFVVCCAIM	GLSVPFYEGV	405	PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-27 Consensus	FEKHVLLITS	· IGLILSGFFA	\ DKIGVNHWFI	VIFVVCCAIM	GLSVPFYSGV	405 KLDLK	PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-25 SP49-27 Consensus 351 SP.AF274302 SP47-27 Consensus	FEKHVLLITS) IGLILSGFF#	\ DKIGVNHWFI	VIFVVCCAIM	GLSVPFYEGV	405 KLDLK	PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP50-20 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus 351 SP.AF274302 SP57-22 SP50-30 SP50-24	FEKHVLLITS	> IGLILSGFF#	V DKIGVNHWFI	VIFVVCCAIM	GLSVPFYSGV	405 R KLDLK	PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-25 SP49-27 Consensus 351 SP.AF274302 SP47-27 Consensus	IGSIMSLAME	· IGLILSGFFA	A DKIGVNHWFI	VIFVVCCAIM	GLSVPFYEGV	405 KLDLK	PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-25 SP49-25 SP49-25 SP49-27 Consensus 351 SP.AF274302 SP47-27 Consensus 351 SP.AF274302 SP47-27 SP49-28 SP49-29 SP49-29 SP49-29 SP49-29 SP49-29 SP49-29 SP49-29	FEKHVLLITS) IGLILSGFF#	\ DKIGVNHWFI	VIFVVCCAIM	GLSVPFYEGV	405 ** KLDLK	PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP50-20 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus 351 SP.AF274302 SP50-19 SP49-68 SP49-25 SP49-25 SP49-25 SP49-25 SP49-25	FEKHVLLITS	P IGLILSGFF#	A DKIGVNHWFI	VIFVVCCAIM	GLSVPFYSGV	405 ** KLDLK	PEYLGRVFSL
SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-25 SP49-25 SP49-25 SP49-27 Consensus 351 SP.AF274302 SP47-27 Consensus 351 SP.AF274302 SP47-27 SP49-28 SP49-29 SP49-29 SP49-29 SP49-29 SP49-29 SP49-29 SP49-29	IGGIMSLAME	P IGLILSGFF#	A DKIGVNHWFI	VIFVVCCAIM	GLSVPFYEGV	405 KLDLK	PEYLGRVFSL
SP49-68 SP49-24 SP49-25 SP49-2 SP48-58 SP47-27 Consensus SP.AF274302 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-27 Consensus SP47-27 Consensus SP47-27 SP49-25 SP49-25 SP49-25 SP49-25 SP49-25 SP49-25 SP50-30 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25	IGGIMSLAME) IGLILSGFF#	A DKIGVNHWFI	VIFVVCCAIM	GLSVPFYSGV	405 **KLDLK	PEYLGRVFSL

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).

	1						70
SP AF274302	MEKYNNWKRK	FYATWAGOAV	SLITSALLOM	ALIFYLTEKT	GSAMVLSMAS	LVGFLPYAIL	
SP.AF376746							
SS.AJ318993							
SV.EF042094							
SY.AF445042							
SY.AY657002							
SY.AB227521							
Consensus						.v!1	
	71						140
CD AF274302	HORKKIMIGA	DITIANACAV	LATVACCMET	DV/WMTMTV/I F	IRSIGTATHT	PAINAVTPII	
SP.AF376746			LAIVARCHEL				
SS.AJ318993							
SV.EF042094							
SY.AF445042			.TY				
SY.AY657002			T Y				
SY.AB227521			T Y	V			
Consensus				!			
	141						210
SP.AF274302			LYSVWDLNAI				
SP.AF376746 SS.AJ318993							
SV. EF042094							
SY.AF445042			E				
SY.AY657002			E				
SY.AB227521							
Consensus			#			#g	# k .
	211						280
SP.AF274302	GVVVLRQNKG		YTFVYMPINA				GGLLLGRLGG
SP.AF376746	TVVVLRQNKG						GGLLLGRLGG
SP.AF376746 SS.AJ318993	CVVVLRQNKG						GGLLLGRLGG
SP.AF376746 SS.AJ318993 SV.EF042094	CVVVLRQNKG		**************************************				GGLLLGRLGG
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042				Y.			GGLLLGRLGG
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002				DYDY.		SI	GGLLLGRLGG
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002	MA					SI SI	GGLLLGRLGG
SP. AF376746 SS. AJ318993 SV. EF042094 SY. AF445042 SY. AY657002 SY. AB227521	MA					SI SI	GGLLLGRLGG LF.N LF.N LF.N LF.N
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus	MAMAMAVV	VVVVVVV				S. I. S. I. S. I. S. I. S. I.	GGLLLGRLGG
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AF457002 SY.AB227521 Consensus		VVVVVVV	M		GLSVPFYSGV	S. I.	GGLLLGRLGG
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AF657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746	MA	VVVVVVVV	MMMMtVSGILPPNGF		GLSVPFYSGV	S. I.	GGLLLGRLGGLF.NLF.NLF.NLF.Nrl.g
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993		VVVVVVV	.MMMMMM		GLSVPFYSGV	S. I.	GGLLLGRLGG
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094	MAMAMAVV	VVVVl	.M. .M. .M. .t. VSGILPPNGF	DY. DY. DY. DY. #h.	GLSVPFYSGV	S. I.	GGLLLGRLGGLF.NLF.NLF.Nrl.g 350 PEYLGRVFSL
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042	MAMAMAVV	VVVVl	.MMMMMM		GLSVPFYSGV	S. I.	GGLLLGRLGG
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094	MA	SFFIMGTSLA	.M. .M. .M. .t. VSGILPPNGF		GLSVPFYSGV	S. I.	GGLLLGRLGGLF.NLF.NLF.Nrl.g
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AF457002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002	MAMAVV. 281 FENHVLLITS	SFFIMGTSLA	M		GLSVPFYSGV	S. I.	LF N LF N
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AF445042 SY.AF457002 SY.AB227521	MA	SFFIMGTSLA	.MM		GLSVPFYSGV	S. I.	LF N LF N
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AP657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AF445042 SY.AF445042 SY.AF45042 SY.AF45042 SY.AF45042 SY.AF45042			M. M. M. VSGILPPNGF	DY	GLSVPFYSGV	S. I.	LF N LF N
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302	MA	SFFIMGTSLA	VSGILPPNGF I. L. QS I. L. QS I. L. QS I. L. QS I. D. QS I. D. QS II. DRIGVNHWFL		GLSVPFYSGV	OTALFQEKIK	LF N LF N
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302 SY.AB227521 SP.AF274302 SP.AF376746	MAMAMAMAVV281 FEKHVLLITS MARIA YO.RIA YO.RIA % H.h!S	SFFIMGTSLAI.M.I.T.I.M.I.T.I.M.I.T.I.M.I.T.I.M.I.T.I.T	VSGILPPNGF I.L.QS I.L.QS I.L.QS I.L.QS I.L.QS I.L.DS	DY. DY. DY. #h. VIFVVCCAIM F. F. F. LSGILIIGIA	GLSVPFYSGV	OTALFQEKIK	LF N LF N
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AY657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993	MAMAVV 281 FEKHVLLITS 10.RIA YO.RIA YO.RIA 351 INSIMSLAMP	SFFIMGTSLA I.M. I.T. I.M.	VSGILPPNGF I. L. QS. I. DRIGVNHWFL	DY. DY. DY. #h VIFVVCCAIM F. F. LSGILLIGIA	GLSVPFYSGV	S. I.	LF N LF N
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AP657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AF445042 SY.AF45042 SY.AF457002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094	MA	SFFIMGTSLA	M	PY	GLSVPFYSGV	S. I.	LF N LF N
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AP657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AP657002 SY.AB227521 Consensus SP.AF274302 SY.AF27521 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AF445042	MAMAMAVV 281 FENHVLLITS	SFFIMGTSLA	.MMM	DY. DY. DY. Hh. VIFVVCCAIM F. F. F. LSGILLIGIA	GLSVPFYSGV	S. I.	LF N LF N
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AP657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AP657002 SY.AB227521 Consensus SP.AF274302 SY.AF27521 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AF445042	MA	SFFIMGTSLA	.MMMMMMMMMM.	DY. DY. DY. Hh. VIFVVCCAIM F. F. F. LSGILLIGIA	GLSVPFYSGV	S. I.	LF N LF N
SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AP657002 SY.AB227521 Consensus SP.AF274302 SP.AF376746 SS.AJ318993 SV.EF042094 SY.AF445042 SY.AP657002 SY.AB227521 Consensus SP.AF274302 SY.AB27521 Consensus	MAMAVV 281 FEKHVLLITS 11.8 II. A 10. RI. A 10. RI. A 11.8 IMSLAMP T. T. T.	SFFIMGTSLA	.MMMMMMMMMM.	P. LSGILIIGIA LSGILIIGIA T. C. T. C.	GLSVPFYSGV IVCQMITEVR P. N. I. P. N. I.	S. I.	LF N LF N

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.

	1						70
SP.AF274302	MELILKAKDI	RVEFKGRDVL	DINELEVYDY	DRIGLVGANG	AGKSTLLRVL	LGELTPFGCK	MNRLGELAYI
SP47-22							
SP50-30							
SP50-24							
SP50-19 SP49-68							
SP49-54							
SP49-25							
SP49-2							
SP48-58							
SP47-27							
Consensus							
CD NEG34300	71	ENDERTHCK!	CUEOTALIOTTA	CCCCCCDIVI	BODI CROUNC	II A D C D T C III	140
SP.AF274302 SP47-22	PQLDEVTLQE	EKDIALVIKL	GAEGTUIĞIW	SGGELIKLKI	AQALSAQVHG	ILADEPISAL	DREGIDELIG
SP50-30							
SP50-24							
SP50-19							
SP49-68							
SP49-54							
SP49-25							
SP49-2							
SP48-58							
SP47-27 Consensus	,						
Consensus							
	141						210
S.PAF27430	QLKYFTGALL	VISHDRYFLD	EIVDKIWELK	DGKITEYWGN	YSDYLRQKEE	ERKSQAAEYE	QFIAERARLE
SP47-22							
SP50-30							
SP50-24							
SP50-19							
SP49-68 SP49-54							
SP49-25							
SP49-2							
SP48-58							
SP47-27							
Consensus							
0000040							
3311343							200
	211	BKIFOKAKGS	SKKKSTEDGG	RIAHOKSIGS	KEKKMYNAAK	TLEHRIAALG	280 KVFAPEGIRR
SP.AF274302 SP47-22	211	RKIEQKAKGS	SKKKSTEDGG	RLAHQKSIGS	KEKKMYNAAK	TLEHRIAALG	
SP.AF274302	211	RKIEQKAKGS	SKKKSTEDGG	RLAHQKSIGS	KEKKMYNAAK	TLEHRIAALG	KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24	211	RKIEQKAKGS	SKKKSTEDGG	RLAHQKSIGS		TLEHRIAALG	KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19	211	RKIEQKAKGS	SKKKSTEDGG			TLEHRIAALG	KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68	211	RKIEQKAKGS	SKKKSTEDGG			TLEHRIAALG	KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54	211	RKIEQKAKGS					KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-54	211	RKIEQKAKGS					KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54	211	RKIEQKAKGS					KVEAPEGIRR
SP. AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-2	211	RKIEQKAKGS					KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-55 SP49-2 SP48-58	211	RKIEQKAKGS					KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-25	211 RAAEEKRKQA	RKIEQKAKGS					KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-25 SP48-58 SP47-27 Consensus	211 RAAEEKRKQA						KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-27 Consensus	211 RAAEEKRKQA	LHNPYPIVGA	EINKVFGDKA	LFENASFQIP	LGAKVALTGG	NGIGKTTLIQ	KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-27 Consensus SP.AF274302 SP47-22	211 RAAEEKRKQA	LHNFYPIVGA	EINKVFGDKA	LFENASFQIP	LGAKVALTGG	NGIGKTTLIQ	KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-27 Consensus	211 RAAEEKRKQA	LHNPYPIVGA	EINKVFGDKA	LFENASFQIP	LGAKVALTGG	NGIGKTTLIQ	KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-2 SP48-58 SP47-27 Consensus	211 RAAEEKRKQA	LHNFYPIVGA	EINKVFGDKA	LFENASFQIP	LGAKVALTGG	NGIGKTTLIQ	KVEAPEGIRR 350 MILNHEEGIS
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-27 Consensus SP.AF274302 SP47-27 Consensus	211 RAAEEKRKQA	LHNPYPIVGA	EINKVFGDKA	LFENASFQIP	LGAKVALTGG	NGIGKTTLIQ	KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54	211 RAAEEKRKQA	LHNPYPIVGA	EINKVFGDKA	LFENASFQIP	LGAKVALTGG	NGIGKTTLIQ	KVEAPEGIRR 350 MILNHEEGIS
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-55 SP49-25 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-54 SP49-54 SP49-54	211 RAAEEKRKQA	LHNPYPIVGA	EINKVFGDKA	LFENASFQIP	LGAKVALTGG	NGIGKTTLIQ	KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25	211 RAAEEKRKQA	LHNPYPIVGA	EINKVFGDKA	LFENASFQIP	LGAKVALTGG	NGIGKTTLIQ	WEAPEGIRR 350 MILNHEEGIS
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP48-58 SP47-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-54 SP49-54 SP49-52 SP48-58	211 RAAEEKRKQA	LHNFYPIVGA	EINKVFGDKA	LFENASFQIP	LGAKVALTGG	NGIGKTTLIQ	KVEAPEGIRR
SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25 SP49-27 Consensus SP.AF274302 SP47-22 SP50-30 SP50-24 SP50-19 SP49-68 SP49-54 SP49-25 SP49-25	211 RAAEEKRKQA	LHNFYPIVGA	EINKVFGDKA	LFENASFQIP	LGAKVALTGG	NGIGKTTLIQ	KVEAPEGIRR 350 MILNHEEGIS

	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	MKEYTGTIVE	ITHDKRLLEN	VADVVYEIRD	KKINLKH
SP47-22							
SP50-30							
SP50-24							
SP50-19							
SP49-68							
SP49-54							
SP49-25							
SP49-2							
SP48-58							

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).

SP.AF274302	1	DUEEK : DOW	DINETEUVOV	T.D.T.C.I.V.CANC	NOVOT: I DVI	LOSI TODOCCE	70
SP.AF376746			DINELEVIDI				
SS.AJ318993							
SV.EF042094							
SP.AB426626 SY.AF227521							
SY.AY657002							н
SY.AY445042							Н
Consensus		\$h			fk		h
an 15074300	71		GVEOLNIOTM			* * * * * * * * * * * * * * * * * * * *	140
SP.AF274302 SP.AF376746	POLDEVILQE		GAEÖTNIÖLW			ILADEPTSHL	DREGIDFLI
SS.AJ318993							
SV.EF042094							
SP.AB426626							
SY.AF227521 SY.AY657002							
SY.AY445042							
Consensus							
	141						210
S.PAF274302 S.PAF376746	.LFYFTGALL		EIVDKIWELK		_	_	_
S.SAJ318993							
S.VEF042094							
S.PAB426626							
S.YAF227521							
S.YAY657002 S.YAY445042							
Consensus						r	
	211						280
SP.AF274302							
SP.AF376746 SS.AJ318993							
SV.EF042094							
SP.AB426626							
SY.AF227521			G				
SY.AY657002 SY.AY445042			G			SN	
Consensus						5	
	281						350
SP.AF274302			EINKVFGEKA				
SP.AF376746 SS.AJ318993							
SV.EF042094							
SP.AB426626		X					
SY.AF227521 SY.AY657002							
SY.AY445042						T	
Consensus		y					
an 1000.000	351						420
SP.AF274302 SP.AF376746	LIFEAKIGYE	AQNGYKYNSN	ONVMEFMOKE	0 = 11.11.11.11	VLASMGFKQN	2 1 3110 00 - 00	J-JD I I I I I I I I I
SS.AJ318993							
SV.EF042094							
SP.AB426626							
SY.AF227521 SY.AY657002						• • • • • • • • • •	
SY.AY445042							
Consensus							
CD \$5034300	421						487
SP.AF274302 SP.AF376746			IPSLEALEIL				
SS.AJ318993							
SV.EF042094							
SP.AB426626 SY.AF227521							
SY.AY657002							
SY.AY445042							
Consensus							k

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.

CD 75074300	1 HTGGATTCTT	maama nann	ATCGGTGTAA	A TO A TIME COMP	TTTACTATCA	COTATOTAL	70
SP.AF274302 SP47-22	CIGGALICII	TGCTGATAAA	AICGGIGIAA	ATCATTGGTT	ITIACIATCA	GGIAIIIIAA	TTATTGGCAT
SP50-30							
SP50-24							
SP50-19							
SP49-68							
SP49-54							
SP49-25							
SP49-2 SP48-58							
SP47-27							
Consensus							
00110011000							
	71						140
SP.AF274302	TGCTATAGTT	TGCCAAATGA	TAACTGAGGT	TAGAAAATTA			GGAGGAATAT
SP47-22							
SP50-30 SP50-24							
SP50-19							
SP49-68							
SP49-54							
SP49-25							
SP49-2							,
SP48-58							
SP47-27 Consensus							
Collsellsus							
	141						210
SP.AF274302	TTATGTATCT	TATTTTCATG	TAACTCTTCC	TGCTAAAATC	GCAGGGTTTT	CCCTGCATAC	AAGCAAATGA
SP47-22							
SP50-30							
SP50-24 SP50-19							
SP49-68							
SP49-54							
SP49-25							
SP49-2							
SP48-58							
SP47-27							
Consensus							
	211						280
SP.AF274302	AARCATGCGA	TTATAGACAG	GAGGAAATGT	TATGGAATTA	ATATTAAAAG	CAAAAGACAT	TCGTGTGGAA
SP47-22							
SP50-30							
SP50-24 SP50-19							
SP49-68							
SP49-54							
SP49-25							
SP49-2							
SP48-58							
SP47-27							
Consensus							
	281						350
SP.AF274302		GCGATGTTTT	AGATATAAAT	GAATTAGAAG	TATATGATTA	TGACCGTATT	
SP47-22							
SP50-30							
SP50-24							
SP50-19 SP49-68							
SP49-68							
SP49-25							
SP49-2							
SP48-58							
SP47-27							
Consensus							

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).

	1						70
SP.AF274302	TI - WITCTT	TGCTGATAAA	ATCGGTGTAA	ATCATTGGTT	TTTACTATCA	GGTATTTTAA	TTATTGGCAT
SP.AF376746							
SS.AJ318993							
SV.EF042094							
Consensus							
	71				Ston co	don of <i>mef</i> ger	ne 140
SP.AF274302		TOCCAAATGA	TARCTGAGGT	TAGAAAATTA		AAACAATATT	
SP.AF376746	I'N IMINGII	100000000000000000000000000000000000000	TWICTOMOOT	111011111111111111111111111111111111111	-37111171101112	70010101111111	3011031211111
SS.AJ318993							
SV.EF042094							
Consensus							
00110011000							
	141						210
SP.AF274302	141 TTATGTATCT	TATTTTCATG	TAACTCTTCC	TGCTAAAATC	GCAGGGTTTT	CCCTGCATAC	
SP.AF274302 SP.AF376746		TATTTTCATG	TAACTCTTCC		GCAGGGTTTT	CCCTGCATAC	
SP.AF376746 SS.AJ318993			TAACTCTTCC				
SP.AF376746 SS.AJ318993 SV.EF042094	TTATGTATCT						AAGCAAATGA
SP.AF376746 SS.AJ318993	TTATGTATCT						AAGCAAATGA
SP.AF376746 SS.AJ318993 SV.EF042094	TTATGTATCT			tgctaaaatc			AAGCAAATGA aagcaaatga
SP.AF376746 SS.AJ318993 SV.EF042094	TTATGTATCTttatgtatct	tattttcatg	taactettee	tgctaaaatc			AAGCAAATGA
SP.AF376746 SS.AJ318993 SV.EF042094 Consensus	TTATGTATCTttatgtatct		taactettee	tgctaaaatc			AAGCAAATGA aagcaaatga
SP.AF376746 SS.AJ318993 SV.EF042094 Consensus	TTATGTATCTttatgtatct	tattttcatg	taactettee	tgctaaaatc			AAGCAAATGA aagcaaatga
SP.AF376746 SS.AJ318993 SV.EF042094 Consensus SP.AF274302 SP.AF376746	TTATGTATCTttatgtatct	tattttcatg	taactettee	tgctaaaatc of <i>mel</i> gene	gcagggtttt		AAGCAAATGA aagcaaatga 280
SP.AF376746 SS.AJ318993 SV.EF042094 Consensus SP.AF274302 SP.AF376746 SS.AJ318993	ttatgtatct 211 AAGCATGCGA	tattttcatg	taactettee Start codon (GAGGAAATG	tgctaaaatc	gcagggtttt	ccctgcatac	AAGCAAATGA aagcaaatga 280

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 strai	in										
S. pneumoniae	4	T	T	T	A	T	A	ACCTTTAT	T		
*(accession no.A	AF274302)							TTAACTAT(16bp))		
SP47-22	16	C	G	-	T	G	G	•	A		
SP47-27	16	C	G	·X · _	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).

-345										
3.0074300	mmon han man	CAM/FINATION	TGTTATACTA	maaam#aama	Thancasaca	martin con a con	ACCCARACTA	TOROROGOTO	3 A TYC COTTET A	AGATAAGUTG
AF376746	TIGAACATAT	CATCTTGTTG	TGITATACTA	TAAATPJATA	TAAACAAAGA	TOTAGGAGGA	ACCGAAACIA	TOACAGCGTC	AATGCGTTA	AGATMAGE 1G
AF3/6/46 AJ318993										
EF042094										
106P				3						
Consensus				+						
Consensus										
AF274302	GCAATAAAAA	AAGCAGAATC	TATACCCGAT	GATAGGCTTT	TTTGTTGTGC	TTATTTATAC	GATATTGAGC	ATTCATTAGT	TACGGTGAGG	ATATTGGTTA
AF376746										
AJ318993										
EF042094										
108P										
Consensus										
	-155									
AP274302	TTTAACTATA	CCTTTATTTA	ACTATACCTT	TATTTAACTA	TGTGTTTAAT	ATGAATGTTT	CCAAATTGTA	TGTATGCAGA	CCAAAAGCCA	CATTGTGGAT
AF376746										
AJ318993										
EF042094					•					_.
108P					*					, , , , , , , , , GG
Consensus				• • • • • • • • • • • • • • • • • • • •	•					gg
	-78		-63	-54		-31		1		
AF274302	TTAGGCTGC	ATTTTTTAT	TGCCTATAAT	GCTATTCAAA	ATAGAAATTT	AAGCAAAATA	ATATGCAGGA	GATAATATAA	ATGGAAAAAT	ACAACAATTG
AF376746	T		G		C			, , . , . , .		
AJ318993	T	y	G		C					
EF042094	T		G		C				000000000000000000000000000000000000000	0.000
108P	T		G		C					
Consensus	t		g		C					
	Start codon of mef (E)									gene
AF274302	GAAACGAAAA	TTTTATGCAA	TATGGGCAGG	GCAAGCAGTA	TCATTAATCA	CTAGTGCCAT	CCTGCAAATG	GCGATTATTT	TTTACCTTAC	AGAAAAAACA
AF376746									,	
AJ318993										
EF042094										
108P										
Consensus										

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