CHAPTER IV

MATERIALS AND METHODS

Methodology Scheme

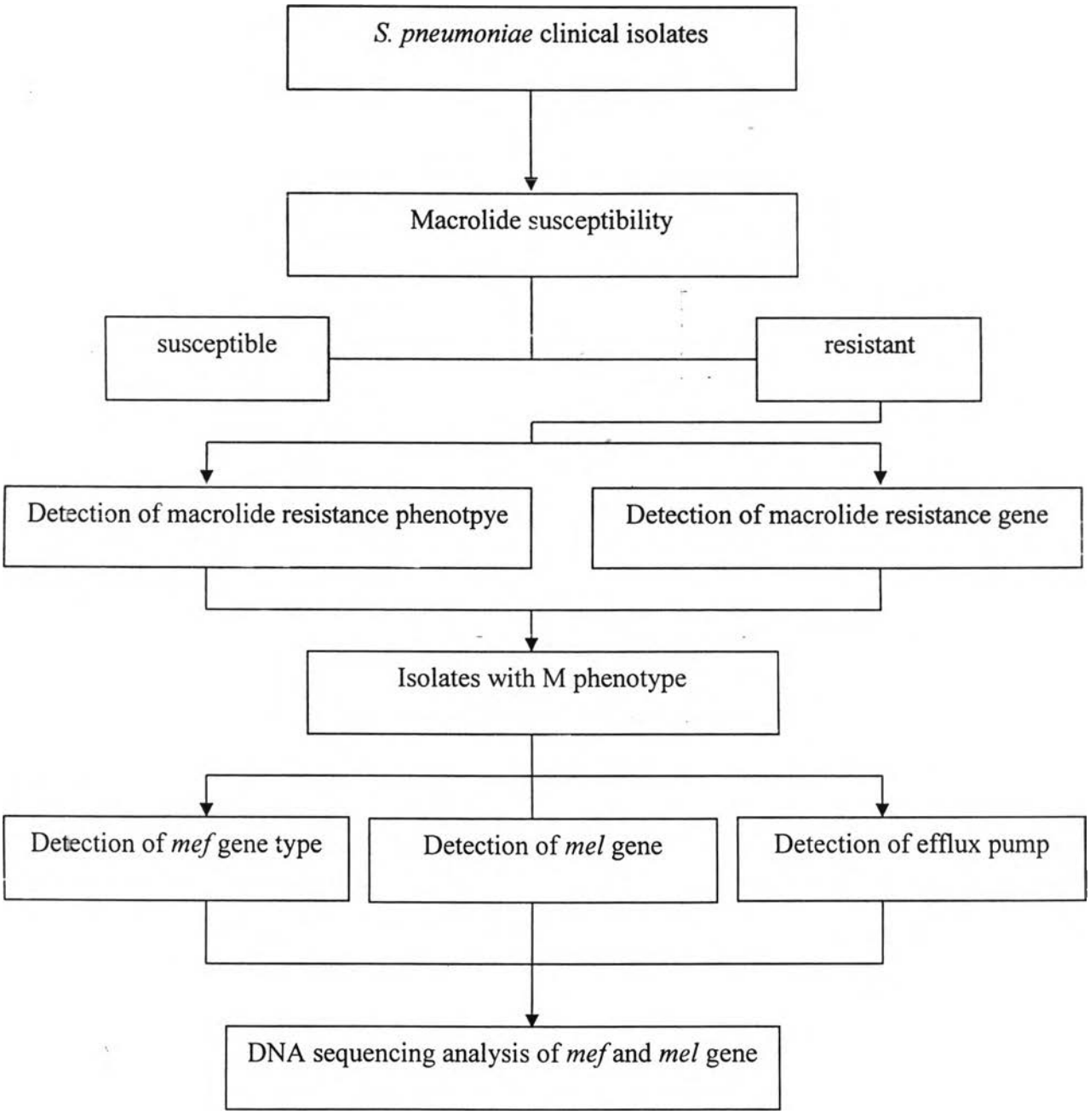

Figure 15 Methodology Scheme

PART I : CLINICAL ISOLATES AND IDENTIFICATION

1. BACTERIAL STRAINS

There were 385 *Streptococcus pneumoniae* isolates were collected from patients in the King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between January 2003 and December 2007. The specimens were collected from invasive sites (blood and CSF) and non-invasive sites (sputum, nasal swab, eye swab, nasopharynx, throat swab, endotracheal, and pus). The mean age (±standard deviation) of the patients was 42.63 ± 28.42 years (range 1 day to 95 year).

CULTURE PRESERVATION

All isolates were grown on trypticase soy agar (Oxiod, UK) supplemented with 5% (V/V) sheep blood in 5% CO_2 at 37°C for 18-24 hours. The colonies suspended in cryogenic vials containing brain heart infusion broth supplement with 50% (V/V) horse serum and were kept at -70°C until to used.

2. IDENTIFICATION OF *S. PNEUMONIAE* ISOLATES

Pneumococci on sheep blood agar form a small, round, gray, glistening colonies, α-hemolysis, first dome-shaped and later developing a central plateau with an elevated rim. Strains are enhanced by 5% CO_2. Isolates were identified to the species level by using bile solubility test, optochin susceptibility and the presence of *lytA* gene. Autolysin encoded by *lytA* gene was detected by PCR. *S. pneumoniae* ATCC49619 was used as a positive control.

2.1 BIOCHEMICAL TESTS

2.1.1 Susceptibility to optochin

S. pneumoniae is susceptible to optochin and is inhibited around a disk of optochin (viridans streptococci are not inhibited by optochin). To determine optochin susceptibility, the suspect alpha-heamolytic colony were streaked onto a blood agar plate. The optochin or "P" disk with a diameter 6 mm (BBL, Becton Dickson and Company, Coskeysville, MD) was placed on the plate. The plates were incubated in a CO_2 incubator at 37°C for 20-24 hours. *S. pneumoniae* exhibits zone of inhibition of ≥14 mm in diameter.

2.1.2 Bile solubility test

Autolysis of pneumococci is greatly enhanced by surface-active agents. Lysis of pneumococci occurs in a few minutes of 10% sodium deoxycholate (Sigma, USA). Strains were tested by take a loop of the strain from the growth on a blood agar plate. Divide the suspension into two tubes (one test and one control). The 0.5ml of 10% sodium deoxycholate was added to the test suspension and 0.5ml of 0.85% saline to the control. The tubes were gently mixed and incubated at 37°C for up to 15 minutes. Examine for evidence of clearing of turbidity in the tube marked test compared with the saline control. For positive result, suspension is clear in tube labelled test and remains turbid in control tube. For negative result, suspension remains turbid in both tubes.

2.2 Detection of *lytA* gene by polymerase chain reaction (PCR)

DNA extraction

S. pneumoniae isolates were emulsified in 200 µl of steriled nuclease-free water and boiled for 10 min. After boiling, the suspensions were centrifuged at 12,000 rpm for 10 min. The supernatant was used as DNA template.

Amplification of *lytA* gene

The reaction was performed in a final volume of 25 µl containing 1X buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 10 mM for *lytA* forward and *lytA* reverse primers previously describeded by Nagai K. *et al.*, 2001 (181) (Table 2), 0.125U of *Taq* polymerase (Fermentas, USA) and 2 µl of DNA template. The PCR mixture was amplified for 35 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension 72°C for 1 min and this was followed by a final extension at 72°C for 10 min. *S. pneumoniae* ATCC 49619 was used as a positive control.

Table 2 Oligonucleotide primers used in polymerase chain reaction.

Primer name	Sequence 5' to 3'	PCR Product (bp)	Position in *lytA* gene (AJ490805)
***lytA* forward**	5'-CAACCGTACAGAATGAAGCGC-3'	319	681-701
***lytA* reverse**	5'-TTATTCGTGCAATACTCGTGCG-3'		978-999

Analysis of amplified DNA

The PCR products were analyzed on 1.5% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). Eight microliters of PCR products were mixed with 2 µl of loading buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light transilluminator. A 100 bp DNA ladder (Promega, USA) was used as a DNA size marker. The size of PCR product was 319 bp.

PART II : MACROLIDES AND CLINDAMYCIN SUSCEPTIBILITY

Minimal inhibitory concentrations (MICs) were determined by agar-dilution technique, according to guideline from the BSAC agar dilution method (British society for antimicrobial chemotherapy) (182). The antibiotics used in this study were erythromycin, clarithromycin and clindamycin (Sigma Chemical Co., St. Louis, Mo, USA). *S. pneumoniae* isolates were grown overnight on trypticase soy agar supplemented with 5% (V/V) sheep blood.

MICs were determined on Mueller-Hinton agar (BBL, Becton Dickinson and Company, Coskeysville, MD) supplemented with 5% sheep blood. Inoculum was prepared from an overnight culture on tryptic soy agar supplemented with 5% sheep blood and the turbidity was adjusted to a 0.5 McFarland standard. Preparing dilutions of antimicrobial agent for agar dilution susceptibility test at concentrations of 0.03125 to 512 µg/ml in doubling dilutions are shown in Table 4. The suspension was inoculated on plates with two-fold dilution of antibiotics. A multipoint inoculator was used to deliver 1-2 µl of inoculum suspension to the agar dilution plates. The final inoculum was approximately 10^4 cfu/spot (Figure 16-17). The plates were incubated in 5% CO_2, at 37°C for 18 h. The MIC is defined as the lowest concentration of antimicrobial agent at which there is no visible growth.

Breakpoint criteria used were those defined by the the National Committee for Clinical Laboratory Standards, 2003; Clinical and Laboratory Standards Institute (CLSI), 2008 (183) ; BSAC guidelines (British society for antimicrobial chemotherapy) (182), isolates with an MIC of ≤0.25 µg/ml for erythromycin, clindamycin and clarithromycin were defined a susceptible *S. pneumoniae*, those with an MIC of 0.5 µg/ml as intermediated, and those with an MIC ≥1 µg/ml a resistant (183). *Streptococcus pneumoniae* reference strain ATCC 49619 was used for quality control. The MIC for the control organisms should be within the acceptable limits as shown in Table 3.

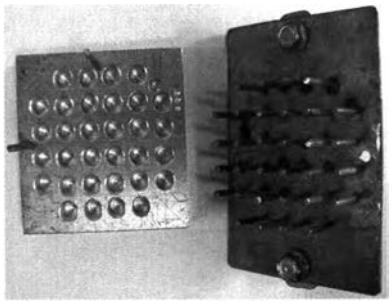

Figure 16 A multipoint inoculator

Figure 17 The MIC plates of *S. pneumoniae* isolates.
(A : control plate (no antibiotic) , B : MIC plate with erythromycin.)

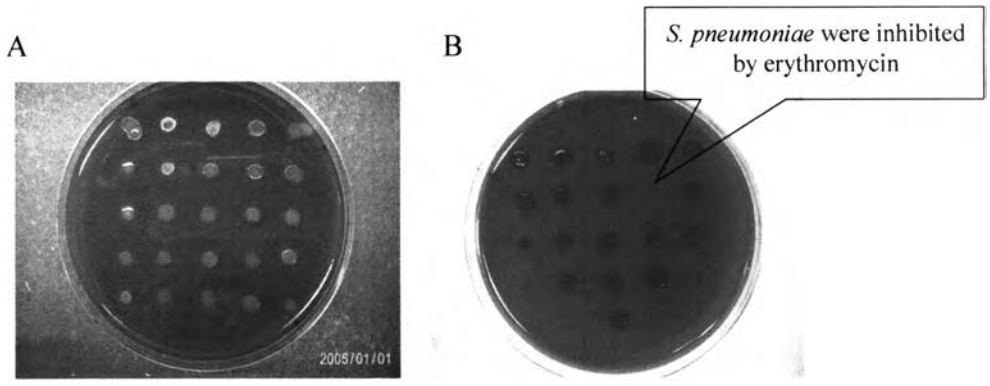

Table 3 Acceptable limits for quality control strains used to monitor accuracy of minimal inhibitory concentrations (MICs) (μg/ml) (183).

Strains	MICs (μg/ml)		
	Erythromycin	Clarithromycin	Clindamycin
S.pneumoniae **ATCC49619**	0.03-0.12	0.03-0.12	0.03-0.12
S. aureus **ATCC25923**	0.25-1	0.12-0.5	0.0625-0.25

Table 4 Scheme for preparing dilutions of antimicrobial agents to be used in agar dilution susceptibility tests (183).

Antimicrobial Solution							
Step	**Conc. (µg/mL)**	**Source**	**Vol. in agar**	**Diluent (µg/L)**	**Intermediate Concentration**	**Final Conc. at 1:10 Dilution**	**Log2**
5,120	Stock µg/mL (mg/L)		-	-	5,120	512	9
1	5,120	Stock	2ml	2ml	2,560	256	8
2	5,120	Stock	1ml	3	1,280	128	7
3	5,120	stock	1ml	7	640	64	6
4	640	step 3	2	2	320	32	5
5	640	step 3	1	3	160	16	4
6	640	step 3	1	7	80	8	3
7	80	step 6	2	2	40	4	2
8	80	step 6	1	3	20	2	1
9	80	step 6	1	7	10	1	0
10	10	step 9	2	2	5	0.5	-1
11	10	step 9	1	3	2.5	0.25	-2
12	10	step 9	1	7	1.25	0.125	-3
13	1.25	step 10	2	2	0.625	0.0625	-4
14	1.25	step 10	1	3	0.3125	0.03125	-5

NOTE : This table is modified from Ericsson HM. Sherris JC. Antibiotic sensitivity testing. Report of an international collaborative study. (Acta Pathol Microbiol Scand. 1971; 217 (suppl B) : 1-98).

PART III : DETECTION OF MACROLIDE RESISTANCE PHENOTYPE

1. Detection of M and MLS_B phenotypes by double disk diffusion test with erythromycin and clindamycin

The strains were grown overnight on trypticase soy agar supplemented with 5% (V/V) sheep blood. Several colonies were removed using a sterile swab, resuspended in 0.85% NaCl, and turbidity was adjusted to a 0.5 McFarland standard. Within 15 min after preparation of the suspension, a sterile swab was dipped into the suspension and rotated against the sides of the tube to remove excess fluid. A Mueller-Hinton agar plate containing 5% sheep blood was then inoculated with the wet swab in three directions to completely cover the plate, which was allowed to dry for 10 to 15 min. An 15 µg erythromycin disc (BBL, Becton Dickinson and Company, Coskeysville, MD) and 2 µg clindamycin disc (BBL, Becton Dickinson and Company, Coskeysville, MD) were placed 15-20 mm apart on Muller-Hinton agar supplemented with 5% sheep blood. All plates were incubated in 5% CO_2 incubator at 37°C for 20-24 hour, previously describeded by Roland Leclercq. *et al.*(184).

Resistance to two antibiotics disks defined the constitutive MLS_B ($cMLS_B$) phenotype. Resistance to erythromycin and a "D"-shaped zone around the clindamycin disc defined the inducible MLS_B ($iMLS_B$) phenotype. Resistance to erythromycin, with susceptibility to clindamycin defined the M phenotype.

PART IV : SCREENING FOR THE PRESENCES OF *MEF* AND *ERM* (B) GENES BY MULTIPLEX POLYMERASE CHAIN REACTION (PCR)

Erythromycin-resistant *S. pneumoniae* (MIC ≥ 1 μg/ml) were investigated for the presence of *mef* and *erm* (B) genes. Isolates were analyzed by using multiplex PCR.

1. DNA extraction

S. pneumoniae strains were resuspended in 200 μl of steriled nuclease-free water and boiled for 10 min and centrifuged at 12,000 rpm for 10 min. The supernatant was used as the DNA template in the PCR experiments.

2. Primers

The primers for the detection of *erm* (B) and *mef* genes are described in Table 5 and are based on those previously described by Sutcliffe *et al.*(41). Sequence of the oligonucleotides used as primers for PCR the *mef* gene (accession no. AF274302) and *erm* (B) gene of *S. pneumoniae* (accession no. AM490850).

Table 5 Sequences of the oligonucleotides used as primers for PCR

Primers name	Sequence (5' to 3')	Location of gene	Product size (bp)
***mef* forward**	5'-AGTATCATTAATCACTAGTGC-3'	57	346
***mef* reverse**	5'-TTCTTCTGGTACTAAAAGTGG-3'	402	
***erm* (B) forward**	5'- GAAAA(AG)GTACTCAACCAAATA -3'	1,635	639
***erm* (B) reverse**	5'- AGTAA(CT)GGTACTTAAATTGTTTAC -3'	2,273	

3. Amplification of *mef* and *erm* (B) genes by multiplex PCR

mef and *erm* (B) genes were amplified by multiplex PCR. The PCR was performed in a final volume of 25 µl containing 1X buffer, 1.5 mM $MgCL_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 10 mM for *mef* forward and *mef* reverse primers, 20 mM for *erm* (B) forward and *erm* (B) reverse primers, 0.125U of *Taq* polymerase (Fermentas, USA) and 2 µl of bacterial DNA template. Cycling conditions were 1 cycle 94°C for 3 min ; 35 cycles of 94°C for 1 min , 53°C for 1 min , and 72°C for min ; and 1 cycle at 72°C for 10 min. *mef*-positive *S. pneumoniae* and *erm* (B)-positive *S. pneumoniae* were used as positive control and erythromycin-susceptible *S. penumoniae* ATCC 49619 were used negative control.

4. Analysis of amplified DNA

The PCR products were analyzed on 1.5% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). Eight microliters of PCR products were mixed with 2 µl of loading buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light transiluminator. The sizes of PCR products were 346 and 639 bp for *mef* and *erm* (B) genes, respectively. A 100 bp DNA ladder (Fermentus, USA) was used as a DNA size marker.

Screening for the presence of the *mel* gene

S. pneumoniae isolates carrying the *mef* gene were determined for the presence of the *mel* gene by PCR. The primers for the detection of *mel* were designed by Primer 3 program at (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) base on the sequence data in GenBank under accession no. AF274302. The PCR was performed in a final volume of 25 µl containing 1X buffer, 1.5 mM $MgCL_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 10 mM for *mel* forward primers (*mel*-F5'- GCCATTTAGACCGTGAAGGA-3') and *mel* reverse primers (*mel*-R5'-TACGAGCCTGTTTTCGCTTT-3'), 0.125U of *Taq* polymerase (Fermentas, USA) and 2 µl of bacterial DNA template. Cycling conditions were 1

cycle 94°C for 3 min ; 35 cycles of 94°C for 1 min , 53°C for 1 min , and 72°C for min; and 1 cycle at 72°C for 10 min. The sizes of PCR products were 278 bp.

PART V : DETECTION OF *MEF* GENES TYPE

1. Detection of *mef* gene type by PCR-restriction fragment length polymorphism (RFLP)

In order to discriminate between *mef* (A) and *mef* (E), a PCR-RFLP analysis was performed, as described by Oster P. *et al.*,1999 (165). The primer pair used were *mef* forward (5'-AGTATCATTAATCACTAGTGC-3') and *mef* reverse (5'-TTCTTCTGGTACTAAAAGTGG-3'), both of which were used for screening *mef* gene in Part IV (41) to generate a 346 bp PCR product. The amplicon was digested with the *Bam*HI restriction enzyme (Promega, USA). The *mef* (A) amplicon contains one *Bam*HI site, so restriction generates two fragments of 64 and 282 bp, respectively, whereas the *mef* (E) amplicon contains no *Bam*HI restriction sites. The presence of *mef* (E) gene was confirmed by *Dra*II digestion (Promega, USA). The *mef* (E) amplicon contains one site, so restriction generates two fragments of 112 and 234 bp.

2. Quality Control

A *mef* (A)- postitive *Streptococcus pyogenes* clinical isolate was used as control. A *mef* (E)- postitive *Streptococcus pneumoniae* clinical isolate was used as control

PART VI : DETECTION OF EFFLUX PUMP

1. Detection of efflux pump in macrolide-resistant *S. pneumoniae* by efflux inhibitor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)

To study inhibitory effects of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) against efflux, susceptibility testing was carried out using agar dilution method. MIC changes were observed in either the absence or the presence of CCCP (Sigma, USA) at concentrations of 10 μg/ml, as described by Kaatz GW *et al.*, 2002 (159). The Mueller Hinton agar containing the two-fold dilutions of erythromycin (0.015-32 μg/ml) were inoculated with $5x10^4$ cfu/ml of each isolate. Plates were incubated for 20-24 hour at 37°C in a 5% CO_2 incubator. A phenotype for positive efflux was detectable after at least four fold dilutions of erythromycin MICs in the the presence of CCCP. A *mef*-positive *S. pneumoniae* was used as a positive control and *erm* (B)-positive *S. pneumoniae* was used as a negative control.

PART VII : ANALYSIS OF ENTIRE *MEF* AND *MEL* GENES BY AUTOMATED SEQUENCING

1. DNA Extraction

S. pneumoniae DNA was extracted and purified by QIAGEN DNA minicolumns kit (QIAGEN, Germany), according to the manufacturers's protocol. *S. pneumoniae* were grown at 37°C on trypticase soy agar supplemented with 5% (V/V) sheep blood and were removed from the culture plate with inoculation loop and suspended with 180 µl of ATL. Twenty microliters of proteinase K were added into the bottom, mixed by vortexing, and incubated at 56°C until bacterial cell were completely lysed. The 200 µl of buffer AL were added to the samples and mixed by pulse-vortexing for 15 S. After well mixing the suspensions were heated at 70°C for 10 min, and briefly centrifuged to remove drops from the inside of the lid. DNA were precipitated with 200 µl of absolute ethanol and mixed again by pulse-vortexing for 15 S. After mixing, the supernatants were transferred into spin columns and centrifuged at 8,000 rpm for 1 min. The Mini QIAamp spin columns was placed in a clean 2 ml collection tube and the tubes containing the filtrates were discard. The 500 µl of buffer AW1 were added into the filtrates tubes and centrifuged at 8,000 rpm for 1 min. The filtrates were then discarded. QIAamp spin columns were washed with 500 µl of AW2 and centrifuged at 14,000 rpm for 3 min and for 1 min to dry the membrane completely. The QIAamp mini columns were placed in clean 1.5 ml microcentrifuge tubes. The collection tubes containing the filtrate were discarded. Buffer AE were added into the mini column and incubated at room temperature for 1 min and then centrifuged at 8,000 rpm for 1 min. Extracted DNA samples were stored at -20°C.

2. Primers for PCR and DNA sequencing

The primers for the detection of upstream of *mef*, entire *mef* and entire *mel* genes were designed by Primer 3 program (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) based on the sequence data in GenBank under accession no. AF274302. A fragment of 630, 1,646 and 1,955 bp

was amplified using upstream of *mef* (F/R), entire *mef* (F/R) and entire *mel* (F/R) primers, respectively. The primers used for sequencing are shown in Table 6.

Table 6 Sequence of the oligonucleotides used as primers for PCR and DNA sequencing.

Primer name	Sequence (5' to 3')	PCR product (bp)	Positions in MEGA	
upstream of *mef*-forward	5'- GAA AAT AAT CCA GAG CGT TAC AGTC-3'	630	644	PCR
upstream of *mef*-reverse	5'- GATG GTC TTG TCT ATG GCT TCA-3'		1,273	
entire *mef*-forward	5'-TGC AGA CCA AAA GCC ACA-3'	1,646	1,019	
entire *mef*-reverse	5'-GGC AAG TTC ACC CAG CG-3'		2,664	
entire *mel*- forward	5'- AGT TTAGCC GTT TCG GGA AT-3'	1,955	2,016	
entire *mel*- reverse	5'- AGT CGC CAA CCA GAA CAG AC-3'		3,970	
upstream *mef*- forward	5'- GAA AAT AAT CCA GAG CGT TAC AGTC-3'	630	644	Sequencing
upstream *mef*-reverse	5'- GATG GTC TTG TCT ATG GCT TCA-3'		1,273	
entire *mef*-forward	5'-TGC AGA CCA AAA GCC ACA-3'	1,646	1,019	
entire *mef*-reverse	5'-GGC AAG TTC ACC CAG CG-3'		2,664	
entire *mel*- forward	5'- AGT TTAGCC GTT TCG GGA AT-3'	1,955	2,016	
entire *mel*- reverse	5'- AGT CGC CAA CCA GAA CAG AC-3'		3,970	
mel – F2	5'-TTC CCC AGT TGG ACG AAG-3'		2,670	

3. Amplification of the entire *mef* and *mel* genes by PCR

Upstream of *mef*, entire *mef* and entire *mel* genes were amplified by PCR. The PCR was performed in a final volume of 100 µl containing 1X buffer with 2 mM $MgSO_4$, 0.1 mM of each deoxynucleotide triphosphates (dNTPs), 5 mM of each forward and reverse primers and 0.125U of *Pfu* polymerase (Fermentas, USA) and 8 µl of bacterial DNA template. Cycling conditions were 1 cycle 94°C for 3 min ; 35 cycles of 94°C for 1 min, 58°C for 1 min , and 72°C for 2 min ; and 1 cycle at 72°C for 10 min.

4. Analysis of amplified DNA

The PCR products were analysed on 1.0% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). Eight microliters of PCR products were mixed with 2 µl of loading buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 90 minutes. The amplified products were visualized and photographed under UV light transilluminator. The sizes of PCR products were 630, 1,646 and 1,955 bp for upstream of *mef*, entire *mef* gene and entire *mel* genes, respectively. A 100 base pair plus DNA ladder (Fermentus, USA) was used as a DNA size marker.

5. Purification of PCR products

The PCR products of 630 bp for upstream of *mef* gene, 1,646 bp for entire *mef* gene and 1,955 bp for entire *mel* genes were purifired using QIAquick PCR purification kit as described by the manufacturers (QIAGEN , Max-Volmer-StraBe4 , Hilden, Germany). Five volume of Buffer PBI were added into the 1 volume PCR products and mixed by pulse-vortexing for 15 S. After well mixing the suspensions were placed into the 2 ml QIAquick column and centrifuged 13,000 rpm for 1 min. DNA was absorbed to the siliga-membrane in the presence of high salt while contaminants pass through the column. To wash, 750 ml of Buffer PE were added into the QIAquick column and centrifuged 13,000 rpm for 1 min. Discarded flow-through and placed the QIAquick column back in the same tube. The QIAquick columns were centrifuged for 60 S and placed the QIAquick column in a clean 1.5 ml microcentrifuge tube. The pure DNA was eluted with 30 µl of 10mM Tris-Cl buffer, pH 8.5. The concentration of DNA was measured by spectrophotometer (BIO RAD, Smart Spec tm 3000, U.S.A) and approximately adjusted to 50-100 ng/µl for preparation of sequencing reaction. The purified PCR products were stored at -20°C.

6. Preparation of sequencing reaction

Automated sequencing was done at the Macrogen Inc. (Seoul, Korea). Sequencing was done by the chain termination method. DNA samples were sequenced using four primer sets, upstream of *mef* gene, entire *mef* gene and entire *mel* gene (Table 6). Sequencing was conducted under BigDye™ terminator cycling conditions. The reacted products were purified by ethanol precipitation and running using automatic sequencer, Applied Biosystems DNA sequencer model 3730xI (Rochester NY, USA).

7. Sequence analysis

The nucleotide sequence and the deduced protein sequence were analyzed with 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 Multilin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) or Bioedit program.