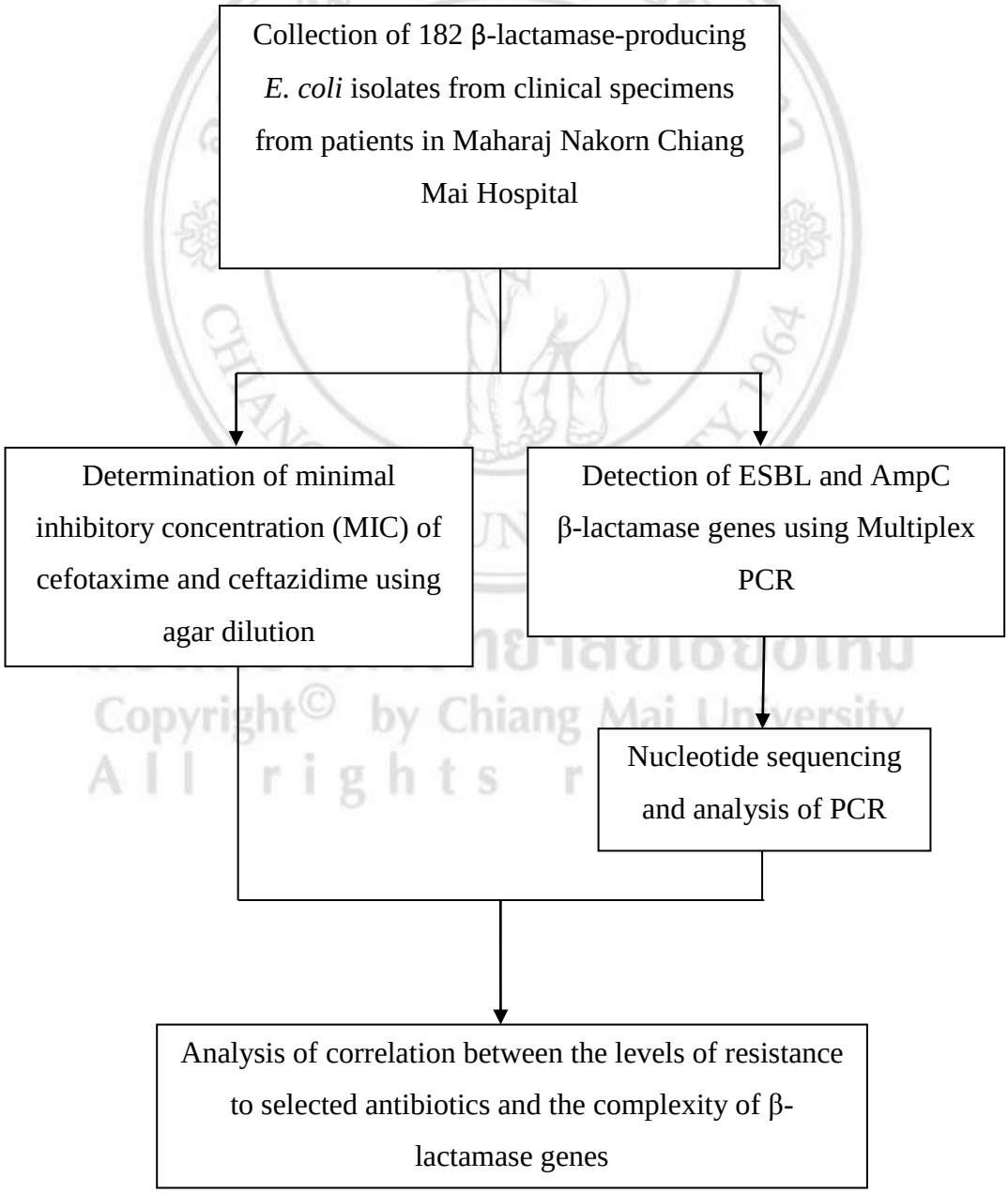
Chapter 4

Materials and Methods

4.1 Experimental design

The objectives and scope of research study are shown in the schematic diagram below.



4.2 Material and Method

4.2.1 Bacterial isolates

One hundred and eighty two ESBL-producing *E. coli* isolates from clinical specimens were previously characterized for their phenotypic resistance to third generation cephalosporins (Wicheannoi *et al*., 2010). They were collected from patients in Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand during April to May 2010. *E. coli* isolates were identified by the standard microbiological methods, and tested as ESBL producers using the combined disc test and double disc synergy test based on the criteria provided by the Clinical and Laboratory Standards Institute (CLSI). Twenty seven form 182 isolates showed cefoxitin resistant phenotype (inhibition zone <18 mm). They were tested AmpC β-lactamase production by Double disc synergy test (cefoxitin and cloxacillin). There were 11 isolates (40.7%) that produced AmpC β-lactamase.

4.2.2 Minimal inhibitory concentration (MIC)

The MIC of ceftazidime and cefotaxime for 182 ESBL-producing *E. coli* isolates were determined by 2- fold serial dilution method in agar based on CLSI guidelines. Beginning prepares Mueller-Hinton agar (MHA), MHB were added 1.7% agar before autoclaving. Around 25 ml was necessary to pour one 15 x 100 mm Petri dish to produce the required depth of 3-4 mm. After autoclaving, the medium were cooled to 50^{o} C. After that, antibiotic solution (100, 10 and 1 mg/l) according to table 4.1 was added to the liquefied MHA. Colonies of overnight culture of *E. coli* was inoculated in MHB and incubated in a shaker incubator at 37°C and 150 rpm for 4 hr. Then bacterial suspension was adjusted to the turbidity of 0.13 at OD_{600} (equal to McFarland Standard No. 0.5) by saline and diluted 10 times to obtain 10^7 CFU/ml. Next, transfer 700 µl of the inoculums suspension to the multi-point inoculator wells and inoculated plates starting with lowest concentration. The final inoculums on the agar were approximately 2 x 10^4 CFU/spot. The inoculums-spots should be dried before incubation at 37^{o} C for 16-18 h. Antimicrobial agent concentrations ranged from 0 to 1,024 mg/l for the agent tested. E. coli ATCC 25922 was used as quality control strains in each batch

of MIC test. The susceptibility rates of ESBL-producing isolates to cefotaxime and ceftazidime were determined according to CLSI-2012. For cefotaxime, MIC interpretive criteria for defining susceptible, intermediate and resistant strains were ≤1, 2 and ≥ 4 μg/ml, respectively and ≤4, 8 and ≥16 μg/ml, respectively for ceftazidime

Table 4.1 Antibiotic dilution chart for agar dilution method

Antimicrobial concentration (mg/ml)	**Volume of antibiotic stock solution (μl)**	**Final concentration when adding 25 ml agar (μg/ml)**
100	256	1,024
100	128	512
100	64	256
10	320	128
10	160	64
10	80	32
10	40	16
1	200	8
1	100	4
1	50	2
1	25	1

4.2.3 DNA extraction

One hundred eighty two isolates of ESBL and/or AmpC β-lactamase producing *E. coli* and 7 positive control stain (TEM3, SHV5, CTX-M-14, and CTX-M-15 for ESBL, CMY8b, CMY2 and DHA1 for AmpC β-lactamase) were extracted for DNA by rapid DNA extraction. 2-3 colonies in 100 μl of distilled

water were heated at 95° C for 10 min, cooled on ice and centrifuged at 13,000 rpm for 2 min. These supernatants were used for Multiplex PCR assay. The final concentration of DNA should be 0.5-2 µg/µl and the 260/280 value should be above 1.8.

4.2.4 Amplification of ESBL and AmpC β-lactamase- encoding genes

Detection of the genes coding was performed by Multiplex PCR. Multiplex polymerase chain reaction (Multiplex PCR) was a modified form polymerase chain reaction. The Multiplex PCR was performed to detect ESBL and AmpC β-lactamase genes by using primers specific. The reaction mixture contained 1X PCR buffer (invitrogen), 200µM concentration of each deoxynucleotide triphosphate, specific-group primer [Multiplex 1 were MultiTS-T and MultiTS-S for detection of bla_{TEM} and bla_{SHV}, respectively. Multiplex 2 were MultiCTXM-G1, MultiCTXM-G2 and CTXM-G9 for detection of $bla_{CTX\text{-}Msubgroup1}$, $bla_{CTX\text{-}Msubgroup2}$ and $bla_{CTX\text{-}M\text{-}subgroup9}$, respectively. Multiplex 3 were MultiCD-CMY1, MultiCD-CMY1 and MultiCD-DHA for detection of bla_{CMY1}, bla_{CMY2} and bla_{DHA} (Table 2)], 1.5U/µl of Taq polymerase (invitrogen, Germany) and 50-200 ng of total genomic DNA in a final volume of 25 µl. Amplification was performed in the TProfessional Thermocycler: initial denaturation at 94° C for 3 min; 30 cycles of 94° C for 40 s, 60° C for 30 s and 72° C for 90 s; and a final elongation step at 72° C for 7 min. PCR with Taq polymerase (invitrogen, Germany) was used for optimization of the three multiplex specific-group primers. The goal of optimization was to obtain the highest and specific yields of individual specific-group primers. The conditions to be optimized included the annealing temperature and primer concentration.

Table 4.2 Specific primer for ESBL and AmpC β-lactamase genes (Dallenne *et al*., 2010)

Gene	Primer name	sequence(5'—3')	Annealing position	Amplicon size (bp)
bla$_{TEM}$	MultiTS-T-for	CATTTCCGTGTCGCCCTTATTC	13-34	800
	MultiTS-T-rev	CGTTCATCCATAGTTGCCTGAC	812-791	
bla$_{SHV}$	MultiTS-S-for	AGCCGCTTGAGCAAATTAAAC	71-91	713
	MultiTS-S-rev	ATCCCGCAGATAAATCACCAC	783-763	
bla$_{CTX-M}$ subgroup1	MultiCTXM-G1-for	TTAGGAARTGTGCCGCTGYA	61-80	688
	MultiCTXM-G1-rev	CGATATCGTTGGTGGTRCCAT	748-728	
bla$_{CTX-M}$ subgroup2	MultiCTXM-G2-for	CGTTAACGGCACGATGAC	345-362	404
	MultiCTXM-G2-rev	CGATATCGTTGGTGGTRCCAT	748-728	
bla$_{CTX-M}$ subgroup9	MultiCTXM-G9-for	TCAAGCCTGCCGATCTGGT	299-317	561
	MultiCTXM-G9-rev	TGATTCTCGCCGCTGAAG	859-842	
bla$_{CMY1}$	MultiCD-CMY1-for	GCAACAACGACAATCCATCCT	3-23	895
	MultiCD-CMY1-rev	GGGATAGGCGTAACTCTCCCAA	900-879	
bla$_{DHA}$	MultiCD-DHA-for	TGATGGCACAGCAGGATATTC	113-133	997
	MultiCD-DHA-rev	GCTTTGACTCTTTCGGTATTCG	1109-1088	
bla$_{CMY2}$	MultiCD-CMY2-for	CGAAGAGGCAATGACCAGAC	570-589	538
	MultiCD-CMY2-rev	ACGGACAGGGTTAGGATAGY	1107-1088	

Note: MultiCTX-Msubgroup1: CTX-M1, CTX-M3, CTX-M15
MultiCTX-Msubgroup2: CTX-M2, CTX-M4, CTX-M5, CTX-M6, CTX-M7
MultiCTX-Msubgroup9: CTX-M9, CTX-M14
MultiCD-CMY1: MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11 and CMY-19
MultiCD-DHA: DHA-1 and DHA-2
MultiCD-CMY2: LAT-1 to LAT-3, BIL-1, CMY-2 to CMY-7, CMY-12 to CMY-18 and CMY-21 to CMY-23

4.2.5 Agarose Gel Electrophoresis

Gel electrophoresis separates DNA fragments by size in a solid support medium (agarose gel). Initially, 0.7 g of Agarose was dissolved in 70 ml TBE buffer (1% agarose gel). The mixture was heated using microwave at medium power for a few minutes to completely dissolve. Agarose was poured into gel tray with a comb in place. The agarose gel was completely fromed after 15-20 minutes. DNA sample were mixed 5:1 with agarose gel loading buffer. Amplicons were visualized after running at 100 V for 1 h on a 1% agarose gel in 0.5X TBE buffer. Agarose gel was stained with ethidium bromide. Band sizes were estimated from comigration of VC 100 bps Plus DNA ladder (Vivantis) molecular size standard. The DNA was visualized by UV illumination.

4.2.6 Nucleotide sequence analysis of PCR product

The PCR products were purified by using the NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany). Initially, the PCR product was analyzed by electrophoresis on 1% agarose gel and stained with ethidium bromide, followed by excision of the gel containing the DNA fragment of expected sizes under UV transilluminator. After weighing the gel slice, 2 volumes (µl) Buffer NTI (binding buffer) were added to 1 volume (mg) of the gel slice and incubated at 50 °C for 5-10 min (or until the gel slice had completely dissolved). A NucleoSpin Extract II Column was put into a Collection Tube (2ml). To bind DNA, the samples were applied to the NucleoSpin Extract II Column and centrifuged for 1 min at 13,400 rpm. After that, flow-through solution was discarded and placed the column back into the collection tube. To wash, 0.7 ml Buffer NT3 was added to the NucleoSpin Extract II Column and centrifuged for 1

min at 13,400 rpm then, flow-through solution was discarded and placed the column back into the collection tube. NucleoSpin Extract II Column in the 2 ml collection tube was centrifuged for 2 min at 13,400 rpm to remove Buffer NT3 completely. Make sure the spin column did not come in contact with the flow-through while removing it from the centrifuge and the collection tube. NucleoSpin Extract II column was transfered into a clean 1.5 ml microcentrifuge tube. To elute DNA, added 30 µl Buffer NE (5 mM Tris/HCl, pH 8.5) to the center of the NucleoSpin membrane and incubated at room temperature for 1 min and then centrifuged for 1 min at 13,400 rpm. Finally, the purified DNA was rechecked on a gel. Then, the purified PCR products and specific primer were sent to 1st BASE (Selangor Darul Ehsan, Malaysia). Sequences were edited and aligned with Sequence Scanner v1.0 software (Applied Biosystems), and http://ncbi.nilh.gov/blast/ used for a BLAST search (National Center for Biotechnology Information, Washington, D.C.) of the GenBank database.

4.2.7 Statistical analysis

The correlation between high-levels of resistance to cefotaxime and ceftazidime (Oteo *et al*., 2006) and the presence of ESBL and AmpC β-lactamase genes was examined by using Fisher's Exact Test Value in SPSS (version 16.0). A P-value of <0.05 was considered significant.