CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacteria Strains

Forty one MDR *Salmonella* strains (resistant to at least two classes of antimicrobials) of human origin were selected from Microbiology Laboratories of University Malaya Medical Center (UMMC) (2007-2008) and Penang Hospital. All except two (urine culture) were from stool culture. The purity of the strains was checked by plating the culture on selective media (XLD). The strains are listed in APPENDIX I.

3.1.2 Chemicals and Reagents

Chemicals and reagents that used in this study are listed in APPENDIX II.

3.1.3 Growth Media

Agar and broth that used in this study for bacterial culture are listed in APPENDIX III.

3.1.4 Buffers and Solutions

Buffers and solutions that used in this study are listed in the APPENDIX IV.

3.2 Methods

3.2.1 Detection of resistance genes using Polymerase Chain Reaction (PCR)

3.2.1.1 Preparation of DNA template

A loopful of bacterial colonies on a LB agar plate was picked and suspended into a 0.5 ml microcentrifuge tube that containing 100 µl of sterile deionized water. Then, the cell suspensions were boiled for five minutes at 99 ^{o}C using thermal cycler (Perkin Elmer). After that, the DNA template was kept in ice immediately for at least 10 minutes. The DNA template was spun down at 13000 rpm for three minutes before use for PCR. The lysate can also be stored at -20 ^{o}C for several weeks and only 5 µl (approximately 100 ng) of the template will be used for each PCR reaction.

3.2.1.2 Specific Oligonucleotide Primers for amplification of resistance genes

Specific oligonucleotide primers were used to amplify DNA fragments with different resistance genes that confer resistance to β-lactams and fluoroquinolones, as indicated in Table 3.1.

Table 3.1: Oligonucleotide sequences used for amplification of antimicrobial resistance gene

Primer	Size(bp)	Nucleotide sequence (5'-3')	Reference
bla$_{TEM}$	859	ATG AGT ATT CAA CAT TTC CG ACC AAT GCT TAA TCA GTG AG	Archambault *et al.*, 2006
bla$_{PSE-1}$	438	TGC TTC GCA ACT ATG ACT AC AGC CTG TGT TTG AGC TAG AT	Chen *et al.*, 2004
bla$_{SHV}$	795	TTA TCT CCC TGT TAG CCA CC GAT TTG CTG ATT TCG CTC GG	Archambault *et al.*, 2006
bla$_{CTX-M}$	593	ATG TGC AGY ACC AGT AAR GT TGG GTR AAR TAR GTS ACC AGA	Archambault *et al.*, 2006
bla$_{CMY-2}$	758	GCA CTT AGC CAC CTA TAC GGC AG GCT TTT CAA GAA TGC GCC AGG	Archambault *et al.*, 2006
bla$_{OXA-1}$	813	ACA CAA TAC ATA TCA ACT TCG C AGT GTG TTT AGA ATG GTG ATC	Brinas *et al.*, 2003
qnrA	580	AGA GGA TTT CTC ACG CCA GG TGC CAG GCA CAG ATC TTG AC	Cattoir *et al.,* 2007
qnrB	264	GGA ATC GAA ATT CGC CAG TG TTT GCT GCT CGC CAG TCG AA	Cattoir *et al.,* 2007
qnrS	428	GCA AGT TCA TTG AAC AGG GT TCT AAA CCG TCG AGT TCG GCG	Cattoir *et al.,* 2007

3.2.1.3 Reaction Mixture and Cycling Condition for PCR

PCR was performed in 25 µl of reaction mixture containing 1X PCR buffer, 1.4 mM of $MgCl_2$, 140 µM of each dNTPs, 0.4 µM of each primer, 1 U of *Taq*, sterilized distilled water and 5 µl of DNA template. PCR amplification conditions used in resistance gene detection are shown in Table 3.2.

Table 3.2: Conditions used in resistance gene detection

Resistance gene	Conditions	Reference
bla_{TEM}	3 min at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C; 10 min at 72 °C	Archambault *et al.*, 2006
bla_{PSE-1}	10 min at 95 °C; 30 cycles of 30 sec at 95 °C, 1 min at 55 °C, 1 min at 72 °C; 7 min at 72 °C	Chen *et al.*, 2004
bla_{SHV}	10 min at 94 °C; 35 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 1 min at 72 °C; 10 min at 72 °C	Archambault *et al.*, 2006
bla_{CTX-M}	7 min at 94 °C; 35 cycles of 50 sec at 94 °C, 40 sec at 50 °C, 1 min at 72 °C; 5 min at 72 °C	Archambault *et al.*, 2006
bla_{CMY-2}	5 min at 94 °C; 30 cycles of 45 sec at 94 °C, 45 sec at 58 °C, 1 min at 72 °C; 10 min 72 °C	Archambault *et al.*, 2006
bla_{OXA-1}	5 min at 96 °C; 35 cycles of 1 min of 96 °C, 1 min at 60 °C, 2 min at 72 °C; 10 min at 72 °C	Brinas *et al.*, 2003
qnrA, B, S	10 min at 95 °C; 35 cycles of 1 min at 95 °C, 1 min at 54 °C, 1 min at 72 °C; 10 min at 72 °C	Cattoir *et al.,* 2007

3.2.1.4 Detection of PCR products by Agarose Gel Electrophoresis

After PCR amplifications, 3 µl of PCR products was analyzed on a 1.5 % agarose gel subjected in 0.5 X TBE buffer at 100 V for around 35 minutes in a gel electrophoresis system. A 100 bp ladder (Promega) was used as the molecular size marker. Then, the agarose gel was stained with ethidium bromide (EtBr) (0.5 µg/ml) for 5 minutes. After that, the gel was destained using distilled water. Finally, the gel was visualized and photographed under UV light using Gel Doc ^{TM} XR system.

3.2.2 Detection of Class 1 Integron

3.2.2.1 Preparation of DNA template

DNA template preparation was similar to section 3.2.1.1.

3.2.2.2 PCR reaction

Primers (intI1F, 5'-GGT CAA GGA TCT GGA TTT GG-3'; intI1R, 5'-ACA TGC GTG TAA ATC ATC GTC-3') and (5'CS, 5'-GGC ATC CAA GCA GCA AG-3'; 3'CS, 5'-AAG CAG ACT TGA CCT GA-3') (Machado *et al.*, 2005) were used for detection of integrase 1 and class 1 integrons, respectively. PCR was performed in 25 µl of reaction mixture containing 1X PCR buffer, 1.4 mM of $MgCl_2$, 120 µM of each dNTPs, 0.3 µM of each primer, 0.5 U of *Taq*, sterilized distilled water and 5 µl of DNA template. The assays were performed according to the conditions in Table 3.3.

Table 3.3: PCR amplification conditions used in Class 1 Integron detection

Primers	Conditions	Reference
intI1	12 min at 96 °C; 35 cycles of 1 min at 96 °C, 1 min at 57 °C, 1min at 72 °C; 10 min at 72 °C	Machado *et al*., 2005
5'CS/3'CS	10 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 54 °C, 2 min at 72 °C; 8 min at 72 °C	Machado *et al*., 2005

3.2.2.3 Detection of PCR products

Five µl of the PCR product was analyzed by electrophoresis on a 1.5% agarose gel. A 100 bp ladder (Promega) was used as the molecular size marker. Then, the gel was stained with EtBr (0.5 µg/ml) for five minutes and destained in distilled water. After destaining, the gel was visualized and photographed under UV light using Bio Rad, Gel Doc ™ XR system.

3.2.3 Detection of *Salmonella* Genomic Island 1 (SGI 1)

3.2.3.1 Preparation of DNA template

DNA template preparation was identical to section 3.2.2.1. Specific oligonucleotide primers were used to amplify left junction and right junction of SGI 1, which were indicated in Table 3.4.

Table 3.4: Primers used for amplification of SGI 1

Primer	Amplification	Size (bp)	Nucleotide sequences (5'-3')	Reference
UJ-L12	Left junction	500	ACACCTTGAGCAGGGCAAG	Doublet *et al.,* 2003
LJ-R1			AGTTCTAAAGGTTCGTAGTCG	Doublet *et al.,* 2003
104-RJ	Right junction		TGACGAGCTGAAGCGAATTG	Doublet *et al.,* 2003
C9-L2		515	AGCAAGTGTGCGTAATTTGG	Doublet *et al.,* 2003
104-D		500	ACCAGGGCAAAACTACACAG	Doublet *et al.,* 2003

3.2.3.2 Reaction Mixture and Cycling Condition for SGI 1 detection using PCR

PCR was performed in 25 µl of reaction mixture containing 1X PCR buffer, 2 mM of $MgCl_2$, 200 µM of each dNTPs, 0.4 µM of each primer, 1 U of *Taq* and 5 µl of DNA template. The PCR conditions for SGI 1 detection was indicated in Table 3.5.

Table 3.5: PCR amplification conditions used in SGI1 detection

Condition	Temperature (°C)	Duration	Cycle
Prenaturation	**95**	**5 min**	**1**
Denaturation	**95**	**30 sec**	
Annealing	**57**	**30 sec**	**35**
Extension	**72**	**1 min**	
Final extension	**72**	**5 min**	**1**
Hold	**4**		

3.2.3.3 Detection of PCR products

After amplifications, five µl of the PCR product was analyzed by electrophoresis on a 1.5 % agarose gel. A 100 bp ladder (Promega) was used as the molecular size marker. Then, the gel was stained with EtBr (0.5 µg/ml) for five minutes destained for 20 minutes. After destaining, the gel was visualized and photographed under UV light using Bio Rad, Gel Doc ™ XR system.

3.2.4 DNA Preparation for sequencing

3.2.4.1 Purification of PCR products

After the electrophoresis, PCR products were extracted by using PCR and Agarose Gel DNA Extraction system, MEGA quick-spin ™, iNtRON Biotechnology, Inc. Purification was done by following the protocol that provided by the manufacturer.

3.2.4.2 Sequencing

The selected amplified DNA products were verified by the DNA sequencing. The amplicons were purified and sent to a commercial company for sequencing. The resulting DNA sequence data were compared with the GenBank database by using the BLAST algorithm available at the website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.5 Plasmid Profiling

3.2.5.1 Plasmid Extraction

Plasmid extractions of testing strains were carried out by using alkaline lysis method (Birnboim & Doly, 1979). Some modifications and optimizations were applied to extract plasmids. A single bacterial colony was transferred into 10 ml of LB broth that containing proper antibiotic as a selective pressure. The culture was then incubated at 37 ^{o}C overnight with vigorous shaking. One ml of the culture was transferred to a sterile 1.5 ml microcentrifuge tube and centrifuged at 13400 rpm for five minutes at 4 ^{o}C to harvest the cells. The supernatant was discarded, and the pellet was resuspended in 200 µl of ice-cold solution I. The mixture was then incubated at 0 ^{o}C for 20 minutes. After that, 400 µl of solution II was added to the mixture, and the mixture was mixed gently by gentle inversion until the cell lysed and kept on ice for five minutes. Then, 300 µl o ice-cold solution III was added, and the mixture was mixed gently by gentle inversion and keep on ice for 20 minutes. After 20 minutes, the mixture was centrifuged at 13400 rpm for 20 minutes. The clear supernatant (approximately 400 µl) was transferred into a clean and sterile 1.5 ml microcentrifuge tube. Then, 20 µg/ml of RNAase was added to the supernatant and incubated at 37 ^{o}C for 20 minutes. 200 µl of phenol-chloroform was added and mixed thoroughly. Centrifugation at 13400 rpm was carried out for 20 minutes at 4 ^{o}C. The upper layer aqueous phase was then transferred to a sterile centrifuge tube by using cut tips. Therefore, one tenth of sodium acetate and twice the volume of cold absolute ethanol were added and kept at -20 ^{o}C overnight. The mixture was then centrifuged at 13400 rpm for 30 minutes at 4 ^{o}C to collect the pellet and the supernatant was discarded. The collected pellet was washed with 70 % ethanol and centrifuged at 13400 rpm for 20 minutes at 4 ^{o}C. Then,

ethanol was discarded. The final DNA pellet was dissolved in 50 µl of pre-heated 1X TE buffer and incubates for 20 minutes at 37 °C before keep at -20 °C until for use.

3.2.5.2 Detection of Plasmid DNA by Agarose Gel Electrophoresis

A 0.6 % agarose gel was prepared by dissolving 0.6 g of LE, Analytical Grade agarose in 100 ml of 0.5X TBE buffer. The mixture was then cooled down to 50-55 °C and was poured into a gel-forming block. 15 µl of plasmid DNA product was mixed with 2 µl of gel loading dye. Then, the mixture was loaded into the wells of the gel. Electrophoresis was carried out at 90 V. *Escherichia coli* 39R and *E. coli* V517 were used as plasmid size markers and qualitative control. The gel was stained with EtBr (0.5 µg/ml) for five minutes and destaining in distilled water. Then, the gel was visualized and photographed under UV light using Bio Rad, Gel Doc ™XR system.