CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial Strains

A total of 49 *Salmonella* Typhimurium strains including 35 from animals and 14 from humans were investigated in this study. These strains were selected from 1969 to 2006. The animal strains recovered from variety of animals (chicken, n = 18; cattle, n = 8; swine, n = 1; fish, n = 1; frog n = 1) and unknown animal hosts (n = 6), were provided by the Veterinary Research Institute, Ipoh, Perak. The human strains isolated from 1969 to 2006 were from Local teaching hospital (University Malaya Medical Centre).All the biochemical and serological tests were performed at the Microbiology Laboratory, UMMC for the clinical strains and Bacteriology Unit, Veterinary Research Institute for the animal strains. The purity of strains was carried out at the Biomedical Science Laboratory, IPS, University Malaya. The strains are listed in APPENDIX I.

3.1.2 Chemical and Reagents

Chemicals and Reagents used in this study are listed in APPENDIX II.

3.1.3 Growth Media

Broth and agar used in this study for bacterial culture are listed in APPENDIX III.

3.1.4 Buffers and Solutions

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

3.2 Methods

3.2.1 Bacterial Cultures and Purity Check

All isolates were labeled accordingly and checked for purity before analysis. One loopful of the culture was streaked onto LB (Luria Bertani) agar plate for purification checking. Next, a single colony was taken out and streaked on Xylose-Lysine-Desoxycholate (XLD) Agar to check the isolates as *Salmonella* strains. After 12-18 hours incubation at 37 ºC, the red with black centre *Salmonella* colonies were streaked onto Hektoen Enteric Agar to purify as *S*. Typhimurium strains. After 12-18 hours incubation at 37 ºC, the blue-green coloured with black centres *Salmonella* Typhimurium colonies were streaked onto LB agar plates. The pure cultures were kept as stocks by preparing both nutrient agar slants at room temperature and 50% glycerol stocks at -20 ºC.

3.2.2 Confirmation of the *S*. Typhimurium strains using Polymerase Chain Reaction (PCR)

A confirmation PCR was used in this study for confirmation of 55 *Salmonella* strains as *Salmonella* Typhimurium isolated from clinical and animal sources.

3.2.2.1 Preparation of DNA template

A single bacterial colony on LB agar plate was picked and was suspended into 0.5 ml microcentrifuge tube containing 50 µl of sterile deionised water. The cell suspension was mixed well to ensure homogeneity. The thermal cycler (Eppendorf) was set up and

the cell suspensions were boiled for 5 minutes at 99 ºC. After that DNA template was kept in ice immediately for 10 minutes. The 5 µl PCR of template was spun down at 10,000 x g for 2 minutes before used for PCR. PCR templates can be stored for several weeks in -20 ºC for each reaction.

3.2.2.2 Specific oligonucleotide primers for confirmation of *S.* Typhimurium strains

The primers used for confirmation of *Salmonella* Typhimurium strains are TYPHF (5'TTGTTCACTTTTTACCCCTGAA 3') and TYPHR (5'CCCTGACAGCCGTTAGATATT 3') as reported by Olsen *et al.* (1995). A sequence specific for *Salmonella* Typhimurium is approximately 401bp.

3.2.2.3 Reaction Mixture and Cycling Condition for PCR

PCR was performed in a 25 µl reaction mixture containing 1 µl of 5X PCR Buffer, 1.5 µl of 25mM $MgCl_2$, 0.5 µl of 40mM dNTPs, 0.1 µl of each primer (100 pmol), 0.2 µl of *Taq* DNA polymerase (iNtRON) and 5 µl of template DNA. The mixture was vortexed for few seconds and spun down; a negative control containing the same reaction mixture except the DNA template was replaced with water was included. The entire reaction was performed in an Eppendorf thermal cycler and cycling conditions were as previously described by Alvarez *et al.,* (2004) as described in Table 3.1.

Table 3.1: PCR amplification conditions used in PCR confirmation test

Condition	Temperature (ºC)	Time (minute)	Number of Cycles
Initial Denaturation	95	2	1
Denaturation	95	1	35
Annealing	55	1	35
Extension	72	2	35
Final Extension	72	5	1
Hold at	4	-	-

3.2.2.4 Detection of PCR product by Agarose Gel Electrophoresis

After PCR amplification, 5µl of PCR products was analyzed on 1.5% agarose gel in 0.5X TBE buffer at 80V for approximately one hour in a gel electrophoresis system. A 100 bp ladder (Promega) was used as the molecular size marker. Then gel was stained in ethidium bromide (0.5µg/ml) for 10 min and after destaining the gel was visualized and photographed under UV light using Gel Doc system (Bio-RAD).

3.2.3 Extraction of genomic DNA

DNA was extracted using a commercial genomic DNA extraction Mini Kit (i-genomic CTB DNA Extraction Mini Kit, iNtRON Biotechnology, South Korea).

Extraction was carried out according to the manufacturer's instructions. To this instructions 6 different steps were conducted; preparation step, sample sizing step, lysis step, DNA binding step, washing step and elution step.

Strains were grown overnight on Luria Bertani (LB) agar plates at 37 ºC. Then, one single colony of a plate was taken out and inoculated to 5 ml Luria Bertani (LB) broth and incubated at 37 ºC overnight. After incubation, 2 ml of cultured bacteria was transferred into 2 ml tubes and centrifuged at 13,000 rpm for 5 minute. The supernatant was discarded and pellet was resuspended by tapping or vigorously vortexing to disperse the cell pellets. 200 µl of buffer CG, 10 µl of Proteninase K, 3 µl of RNase A solution were added and mixed by votexing vigorously. The tube was incubated at 65 ºC for 30 minute. Then, 250 µl of buffer CB was added and mixed by inverting for 5-6 times. The mixture was spun down and then 250 µl of 80% ethanol was added and mixed by gently inverting. The mixture was spun down and it was transferred to spin column. Then, it was centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded and the spin column was placed into new 2.0 ml collection tube, 700 µl of buffer CW (including 40 ml of ethanol) was added to the spin column, and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded. Then, the spin column was paced into a new 1.5 ml tube and 100 µl of buffer CE was added directly into membrane and, it was incubated at room temperature for 1 minute. Then, it was centrifuged at 13,000 rpm for 1 minute. The spin column was thrown out and the template DNA was used for digestion, or frozen -20 ºC, to prevent degradation of DNA template. Extracted DNA was analyzed on 1.5% agarose gel in 0.5X TBE buffer at 80V for approximately one hour in a gel electrophoresis system by using Lambda

DNA/HindIII (Promega) as ladder. Then gel was stained in ethidium bromide (0.5µg/ml) for 10 min and after destaining the gel was visualized and photographed under UV light using Gel Doc system (Bio-RAD).

3.2.4 Restriction enzyme digestion

1 µg/ µl genomic DNA of each isolate was digested by *Hae*III endonuclease (GG/CC) restriction enzyme prior to amplification with three arbitrary oligonucleotide primers. The digestion was performed in a final volume of 20 µl on 1 µl (1 µg/ µl) of extracted genomic DNA according manufacturer's instruction (Promega, Medison, Wisconsin, USA).

The restriction was carried out in following step, the sterile tube was taken and 2 µl of 10X RE Buffer, 0.2 µl of 10 µg/ µl acetylated BSA and 1 µl of 1 µg/ µl DNA were added into the tube and mixed by inverting vigorously. Then, 0.5 µl of 10 µ/µl restriction enzyme was added into the mixture. The mixture was mixed gently by inverting and centrifuged for a few seconds in microcentrifuge and it was incubated at 37 ºC for 3 hours. Digested DNA was used for PCR amplification.

3.2.5 Restricted AP-PCR

Restricted AP-PCR amplification was performed using three various primer sets for enzyme digested product. These 9-mer oligonucleotide primers have been selected based on genome sequence of *S*. Typhimurium using the *in silico* resAP-PCR program (*http://insilico.ehu.es/resAP-PCR/* using *S*. Typhimurium genome as a template). Primers used by Bikandi *et al*. (2008) are listed in Table 3.2.

Table 3.2: Primers used for restricted AP-PCR

Primer	Sequence (5'→ 3')	Reference
ResAP-PCR I	GGCGAACTG	Bikandi *et al.*, 2008
ResAP-PCR II	GGCGGCATT	Bikandi *et al.*, 2008
ResAP-PCR III	CAGGCGATG	Bikandi *et al.*, 2008

PCR amplification was performed in a volume of 25 µl included 1 µl of template DNA (digested genomic DNA, 10 ng), 2 µl of 5U *Taq* DNA polymerase (iNtRON), 0.5 µl of 10 mM dNTPs (iNtRON),, 2.5 µl of 2.5 mM $MgCl_2$ (iNtRON), and 0.2 µl of each three primers (100 pmol) in 2.5 µl 5X PCR buffer. The amplification was carried out in an eppendorf thermal cycler.

The cycling conditions for restricted AP-PCR amplification were described previously by Bikandi *et al.* (2008): an initial denaturation step at 95ºC for 2 min and then 30 cycles with denaturation at 95ºC for 1 min, primer annealing at 32ºC for 30 s,

and extension at 72ºC for 1 min. To ensure complete strand extension, the reaction mixture was kept at 72ºC for 4 min after the final cycle. A negative control (without DNA) was included in each run. PCR products reaction tubes were electrophoresed immediately after amplification, or frozen -20 ºC, to prevent degredation of the PCR products (Gibson and McKee, 1993). PCR products were analyzed on 2% agarose gel (wt/vol) in 0.5X TBE buffer at 80V for approximately three hours in a gel electrophoresis system by using 100bp as ladder (Promega). Then gel was stained in ethidium bromide (0.5µg/ml) for 10 min and after destaining for 10 min the gel was visualized and photographed under UV light using Gel Doc system.

3.2.6 Data Analysis

An assessment of which typing method is the most efficient has to be based on a number of factors: typeability, reproducibility, and discrimination. Of these characteristics, typability and reproducibility are relatively easy to quantify and are often expressed as simple percentages. Thus, the typeability of a method is the percentage of distinct bacterial strains which can be assigned a positive typing marker, and the reproducibility is the percentage of strains that give the same result on repeated testing.

PCR data were analyzed by using GelCompare software (version 4, Applied Maths, Belgium). The profiles were scored for the presence and absence of bands, and strains that differed by one band were assigned different profiles. Clustering was based on the unweighted pair group average method (UPGMA) with the position tolerance of 0.10. The discriminatory ability of the different techniques was determined by the Discriminatory Index, (*D*) (Hunter and Gaston, 1988).

The discriminatory power of a typing method is its ability to distinguish between unrelated strains. It is determined by the number of types defined by the test method and the relative frequencies of these types. These two facets of discrimination are not generally presented as a single numerical value and therefore cannot be used for a straightforward comparison of different methods. In the literature, most workers present the frequency of the most common types and the number of types and it is often left to the interested reader to make a subjective assessment of how one system compares with another (Hunter and Gaston, 1988). Thus, a D value of 1.0 would indicate that a typing method was able to distinguish each member of a strain population from all other members of that population. Conversely, an index of 0.0 would indicate that all members of a strain population were of an identical type (Hunter, 1990).

In the present study, the fingerprints were grouped according to their similarities by use of the UPGMA algorithm. Discriminatory Index (*D*) and similarity coefficient between the fingerprints was obtained from dendrogram generated by GelCompare II software.