CHAPTER IV

MATERIALS AND METHODS

1. Organisms: All strains of *Salmonella* Enteritidis in this study were obtained from the WHO National *Salmonella* and *Shigella* Center (NSSC), the National Institute of Health (NIH), Department of Medical Science, Ministry of Public Health, Thailand.

2. Sources of *Salmonella* Enteritidis

2.1 *Salmonella* Enteritidis isolated from human patients. *Salmonella* Enteritidis were samples sent from hospitals and Centers of Medical Science in Thailand to the WHO National *Salmonella* and *Shigella* Center (NSSC), the National Institute of Health (NIH), Department of Medical Science, Ministry of Public Health, Thailand for serotyping in the year 2002. All of them were selected for this study.

2.2 *Salmonella* Enteritidis isolated from chicken meat. *Salmonella* Enteritidis isolated from chicken meat were samples submitted to the WHO National *Salmonella* and *Shigella* Center (NSSC), the National Institute of Health (NIH), Department of Medical Science, Ministry of Public Health, Thailand for serotyping in the year 2002. All of them were selected for this study.

3. Serovar confirmation of all the samples

All samples were reconfirmed for *Salmonella* Enteritidis by the following process.

3.1 Isolation: Each sample was streaked on a TSA and incubated at 37°C for 18-24 hours. Pick up single colony and inoculated on TSA, incubated at 37°C for 18-24 hours.

3.2 Biochemical reaction test: The identification of *Salmonella* Enteritidis was performed according to Bailey and Scott's, 1998. The tests and the results were shown in Table 11.

3.3 Serovar confirmation for *Salmonella* Enteritidis. Inoculate culture on TSA, incubate at 37^{O}C for 18-24 hrs. Testing of biochemical reaction and followed by serological test with O – antisera, start test with polyvalent A – 67, positive; test with polyvalent A – I, positive; test with group D, positive, Test with O – factor O:1, negative; O:9, positive; O:12, positive; O : 46,negative. Pour melted swarm agar into petridish, allow agar to cool. Transfer a loopful of culture from TSA to the center of swarm agar surface, incubate at 37^{O}C for 18 – 24 hrs. Pick culture from surface of swarm agar to test with H-antisera should be positive with polyvalent H:G complex , and should be negative with H:L complex,H: unspecific. Then further test with H:f, negative; H:g, positive; H:m, positive; H:p, negative; H:s, negative and H:t, negative factor. If be positive with H:g and m factor. And go to next step. Drop H:g, m concentrated antiserum titer at 1:800 approximately for 0.09 ml. in petridish and pour melted swarm agar, mix well, allow to cool and pick culture from step five to spot on the surface of swam agar. Incubate at 37 ^{O}C for 18-24 hrs. If the strains don't spread on, test with O group D antiserum must be positive. The tested isolate positive for O:9, 12 in Compose of O:9 and 12 antigen and single phase of H-antigen is g, m. Compare the test results to Antigetic formula table, can summarize the strain is *Salmonella* Enteritidis 9, 12:g, m. The tests and the results were shown in table 12.

4. Analysis of restricted fragments of chromosomal DNA from *Salmonella* Enteritidis by pulsed-field gel electrophoresis (PFGE)

Chromosomal DNA analysis by Pulsed-Field Gel Electrophoresis was

performed according to the method as followed (Maslow *et al.*, 1993; Thong *et al.*, 1994; and Carson *et al.*, 1995):

4.1. Sample DNA plug preparation

Each sample isolate of *Salmonella* Enteritidis was streaked onto TSA to yield a single colony and was then inoculated onto TSA, streaked and incubated for 18 to 24 hours at 37°C. Organism cells were harvested into the SE buffer (75 mM NaCl pH 8.0, 25 mM EDTA pH 8.0) and adjusted the turbidity to McFarland No 4., 1 ml. was sucked and transferred to 1.5 ml eppendorp tube. Then the washing step by centrifugation at 13,000 rpm at room temperature for 90 seconds, 2-3 times. Cells were resuspended in the 0.3 ml SE buffer and mixed. Prepare 1% low melting point agarose (chromosomal grade agarose) and cool to 56 °C before use. The 0.3 ml. of suspension were mixed with 0.7 ml of 1% low melting point agarose. And dispense into 5 chambers of the mould. Add 0.1 mg/ml of proteinase K into 5 ml of lysis buffer (50 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 1% Sarcosyl) in 50 ml plastic tube , and prewarmed at 56^{o}C for 15 min. Remove 5 agar plugs of each sample into 50 ml of plastic tube. Incubate 50 ml of plastic tube containing 5 ml of lysis buffer and 5 agar plugs at 54^{o}C for 1.5-2 hours in water bath with shaking. Remove lysis buffer from 50 ml of plastic tube and add 10 ml of distilled water. Incubate at 54^{o}C for 10-15 min in water bath with shaking, repeat this step 2 times, and wash the agar plugs with TE buffer (10 mM Tris pH 8.0, 1mM EDTA pH 8.0) at 54^{o}C for 10-15 min in water bath with shaking 3 times. Store agar plugs in 1.5 ml eppendorf tube containing 1.0 ml of fresh TE buffer at 4^{o}C.

4.2 Restriction endonuclease enzyme digestion

4.2.1 Pre-incubation

Carefully remove agar plug from TE buffer to glass slide. After that the agar plug was sliced in to a small piece about 1 mm thick with sterile surgical blade and place into 1.5 eppendorf tube containing 1X buffer and deionized water, total volume per reaction is 100 µl. and then incubated at room temperature for 10 min and then buffer removed

4.2.2 Restriction enzyme digestion (*Xba* I, 5'- TCTAGA - 3')

Add 50 µl of restriction enzyme *X*baI solution (20 units of *X*baI, 1X restriction enzyme *X*baI buffer, 0.01X bovine serum albumin, and sterile deionized water) and incubated at 37°C for 4 hours.

4.3 Gel preparation and preelectrophoresis

The running gel was prepared by dissolving 0.9 g of ultrapure high-melting temperature agarose (1%wt/vol) in 90 ml of 0.5x TBE buffer. The agarose was melted until completely dissolved and was cooled down to approximately 50°C, and then poured into the gel casting. The 15-well comb was placed in the gel to make 15-well running agarose gel. The gel was placed in the gel casting until solidified. The gel was then transferred to the electrophoresis tank (CHEF-DR®III system, BioRad, USA). The running gel was preelectrophoresed for 0.5 hour in 0.5x TBE buffer to improve the clarity and resolution of the gel using the temperature was 14 °C. The running gel was removed from the tank.

4.4 Sample loading and running of gel electrophoresis

Each sliced plug sample including a plug of lambda ladder marker were loaded into each well of the preelectrophoresis gel. All the wells of the gel were filled with 1% low-melting point agarose to protect the sliced plug from floating out of the well. The gel was then placed in the PFGE tank with 0.5x TBE buffer and electrophoresed using the

following condition; the temperature was 14°C, 6 V/cm, angle was 120°, initial switch time was 2.0 s, final switch time was 40.0 s, and running time was 20.0 hours.

4.5 Gel visualization

The gel was stained with 0.5 μg/ml of ethidium bromide for 30 min. After that it was rinsed and destained with deionized water for 30 min. The gel was then photographed under UV illumination. Result was interpreted by BioNumeric program.

5. Interpreting of chromosomal DNA restrictions patterns

PFGE profiles are analyzed by using BioNumerics software version 3.0 with a position tolerance of 1.0 % and optimization is 0.5%.