CHAPTER IV

MATERIALS AND METHODS

1. Microorganism

1.1 Samples

Three-hundred strains of *Salmonella* Schwarzengrund were isolated from human patients and chicken meat samples. All samples were previously submitted to the WHO National *Salmonella* and *Shigella* Center, the National Institute of Health, Department of Medical Science, Ministry of Public Health, Thailand for serotyping in the year 2000-2002.

1.2 Control strains

Escherichia coli ATCC 25922

Staphylococcus aureus ATCC 25923

Pseudomonas aeruginosa ATCC 27853

2. Identification of *Salmonella* Schwarzengrund

2.1 Biochemical tests

All the isolates obtained from the previous step were identified according to the methods described in Bergey's Manual of Systemic bacteriology. The identification steps were as followed: The isolates were tested for the biochemical characteristics as shown in **Table 4**.

Table 4 Biochemical reaction tests and characteristics of *Salmonella* Schwarzengrund

Biochemical tests	**Characteristics of *Salmonella***
Triple Sugar iron (TSI)	acid butt/alkaline slant (K/A), gas
H_2S	positive
Motility	positive
Indole	negative
Citrate	positive
Urease	negative
LDA	negative
LDC	positive
Dextrose fermentation	positive
Lactose fermentation	negative
Manitol	positive
Malonate	negative

2.2 Serological tests

The isolates were tested for the serotypes by slide agglutination test according to the Kauffman-White serotyping scheme (5).The serological characteristics are shown in **Table 5**.

Table 5 Serological test and result of *Salmonella* Schwarzengrund

Antisera	**Serological characteristics of *Salmonella* Schwarzengrund**
Day 1 Slide agglutination between *Salmonella* Schwarzengrund and A-antiserum were as followed:	Used 0.85 % NaCl as negative control
Salmonella group A-67	positive
Salmonella group A-I	positive
Salmonella group O:17-O:67	negative
Salmonella group A	negative
***Salmonella* group B**	**positive**
Salmonella group C	negative
Salmonella group D	negative
Salmonella group E	negative
Salmonella group F	negative
Salmonella group G	negative
Salmonella group H	negative
Salmonella group I	negative
Vi	negative
O:1	positive
O:4	positive
O:(5)	positive
O:27	positive
Day2 After known O antigen in group B then test H antigen by culture *Salmonella* Schwarzengrund on swarm agar 18-24 hours and test slide agglutination between *Salmonella* Schwarzengrund and H antiserum were as followed.	Used 0.85% NaCl as a negative control
H:Unspecific	positive
H:1 H:7 H:d	positive positive positive
Concentrate Antiserum: H:1, 7, d	no spreading

Antiserum to Salmonella species

The antiserum using in Thailand are following :

1. *Salmonella* polyvalent A – 67 antiserum : composed of group A, group B, groupC, group D and every group to group 67 antisera.

2. *Salmonella* polyvalent A –I antiserum : composed of group A, group B, group C, group D group E, group F, group G, group H, group I antiserum

3. *Salmonella* polyvalent O : 17 – O : 67 antiserum : composed of group J (O :17) , group K (O : 18) to group O : 67

4. *Salmonella* polyvalent H : H antiserum : composed of all of flagella both phase I and II which can described as H:a,H: b,H:c,H: d, H:eh toH: z_{61}

5. *Salmonella* polyvalent H : L antiserum : composed of flagella as described as : H : l,v , H:v, H:w, H:z_{13} , H:z_{28}

6. *Salmonella* polyvalent H : G antiserum : composed of flagella as described as : H:f, H:g, H:s, H:t, H:m, H:p, H:q

7. *Salmonella* polyvalent H : unspecific : composed of all phase 2 flagella as described as : H:1,2 , H:2, H:5, H:6, H:7 and H: z_6

4. Susceptibility testing: Determination of phenotypic resistance

4.1 Disk diffusion methods

All 300 isolates were tested for antimicrobial susceptibility against 2 antimicrobial agents which have been commonly used in the treatment of infections due to *Salmonella*. The antimigrobial disks were cefotaxime (30µg/mL) and cefotaxime(30µg/mL) +clavalunic acid (10µg/mL) The test procedure was performed according to Kirby-Bauer disk diffusion method (51).

Standardization of inoculum was prepared by using a loop that touched the top of 3 or 4 individual colonies and transfer to a tube of saline. Emulsify the inoculum on the inside of the tube to avoid lumps.Adjust to McFarland 0.5 (approximately 10^8 CFU/mL) : Compare turbidity to that in the 0.5 McFarland standard using paper with black lines. Adjust turbidity of inoculum to match that standard.

Inoculate agar plate by swab plate within 15 minutes of preparing the adjusted inoculum: Check purity of Mueller Hinton II agar plates. Dip a sterile cotton swab into the inoculum and pulling out slightly, rotate the swab several times against the inside of the tube above the fluid level to remove excess liquid. Streak the swab over the entire surface of the Mueller Hinton II agar plate. Rotate the plate approximately 60° then repeat streaking motion. Rotate 60° again and repeat streaking. Complete leave the lid of the plate ajar for 5 minutes (no more than 15 minutes) to allow any excess moisture to be absorbed before applying disks. Inoculation by running the swab around the rim of the agar. Leave the lid of the plate ajar for 5 minutes (no more than 15 minutes) to allow any excess moisture to be absorbed before applying disks.To pick material from more than one colony is a deviation from normal microbiologic practice. It is done to minimise the risk of picking bacteria, which have lost their resistance. Dispense disks to the agar surface with the disk dispenser or forceps. Do not move any disks after contact to the agar. Make sure the disk have complete contact to the agar-surface by touching the disk with forceps. Standardised inoculum is essential, because the zone size of inhibition depends on the growth density, and because the interpretation of the results is based on a confluent lawn of growth (51). To avoid further growth before inoculation homogeneously plating is important to yield reliable results. Moving disk yield oval zones, which are difficult to read and might give unreliable results. Check purity of the inoculum: Transfer with a 10 loop inoculum from the tube onto a nutrient agar plate. Incubate plates at 37 °C over night. After 24 hours of incubation, the diameter of the zone of complete inhibition were measured. The size of the zones of inhibition were interpreted by referring to table of zone diameter standard of National Committee of Clinical Laboratory Standards (51) as shown in the **Table 6.** The organisms were reported as either susceptible, intermediate susceptible, or resistant to the agents tested.

Table 6 Zone diameter standard of National Committee of Clinical Laboratory Standards(NCCLS,2000)

Antimicrobial agent	**Disc content** (µg/mL)	**Inhibition zone diameter (mm)** R	I	S
Cefotaxime	30	<14	15-22	>23
Cefotaxime+clavulanic acid	30	<14	15-22	>23

4.2 MIC determination by agar dilution

All 300 isolates were tested for antimicrobial susceptibility against 10 antimicrobial agents which have been commonly used in the treatment of infections due to Salmonella in animals. The antimigrobial drug were ampicillin , gentamicin (30µg/mL), streptomycin (30µg/mL), sulfamethoxazone (30µg/mL), sulfa+ trimethoprim (30µg/mL), nalidicid acid (30µg/mL), ciprofloxacin (30µg/mL), and chloramphenical (30µg/mL) followed this step by step.

Standardization of inoculum was prepare from a pure overnight culture material from at least 3-4 colonies and dissolved in 4 mL NaCl in tubes. After mixing the colonial-suspension, the suspension was adjusted it turbidity equal to McFarland 0.5 (a nephelometer which is approximately 10^8 CFU/mL). Calibrating the nephelometer before use and gently turn all suspensions upside-down before measuring. After adjusting the turbidity of inoculum to McFarland 0.5, the suspension is diluted 10-fold to yield the final inoculum suspension and transferring 0.1 mL into an eppendorf tube contained 0.9 mL normal saline. Turning the eppendorf tube up-side-down two times. The inoculum suspension should be used for inoculation within 15 minutes. After that transfer 400 mL of inoculum suspension to the multi-point inoculator wells. Place the control strains and write down the orientation of the other isolates too. This is done to minimize the risk of picking bacteria, which have lost their resistance. Inoculate plates starting with the lowest concentration (The concentration of antimicrobial for use in agar dilution that was shown in **Table 7**). Remember to inoculate one of the growth control plares before and after. It is important, that all plates are dry before inoculation. Allow the inoculum-spots to dry before incubation (37 °C for 16-20 hrs., bottoms up). Most multipoint inoculators applies 1-2 µl of the suspension to the agar surface. The final inoculum on the agar will then be approximately 10^4 CFU/ spot. Prepare purity control: Spread 10 mL of the inoculation-suspension on a nutrient agar plate. Incubate at 37°C overnight as shown in **Figure2**. (The inoculum suspension approximately 10^7 CFU/mL). After 24 hours of incubation, The MIC is read as the lowest concentration without visible growth. A faint haze, pinpoint colonies or growth of a single colony should be ignored. Further interpretation of the MIC were interpreted by referring to table of MIC of reference control of National Committee of Clinical Laboratory Standards (44) as shown in the **Table 8**. The organisms

were reported as either susceptible, intermediate susceptible, or resistant to the agents tested.The MIC were interpreted by referring to table of MIC standard of National Committee of Clinical Laboratory Standards(44) for MIC analysis as shown in the **Table 9.**

Table 7 The concentration of antimicrobial for use in agar dilution (From NCCLS Guidelines, 1999)

Step	**Concentration** (μg/mL)	**Source**	**Volume use** (mL)	**Add Distilled Water** (mL)	**Intermediate Conc.** (µg/mL)	**1:10 Dilution in Agar**	**Log 2**
1	5,120	Stock	-	-	5,120	512	9
2	5,120	Step 1	1	1	2,560	256	8
3	5,120	Step 1	1	3	1,280	128	7
4	1,280	Step 3	1	1	640	64	6
5	1,280	Step 3	1	3	320	32	5
6	1,280	Step 3	1	7	160	16	4
7	160	Step 6	1	1	80	8	3
8	160	Step 6	1	3	40	4	2
9	160	Step 6	1	7	20	2	1
10	20	Step 9	1	1	10	1	0
11	20	Step 9	1	3	5	0.5	-1
12	20	Step 9	1	7	2.5	0.25	-2
13	2.5	Step 12	1	1	1.25	0.125	-3

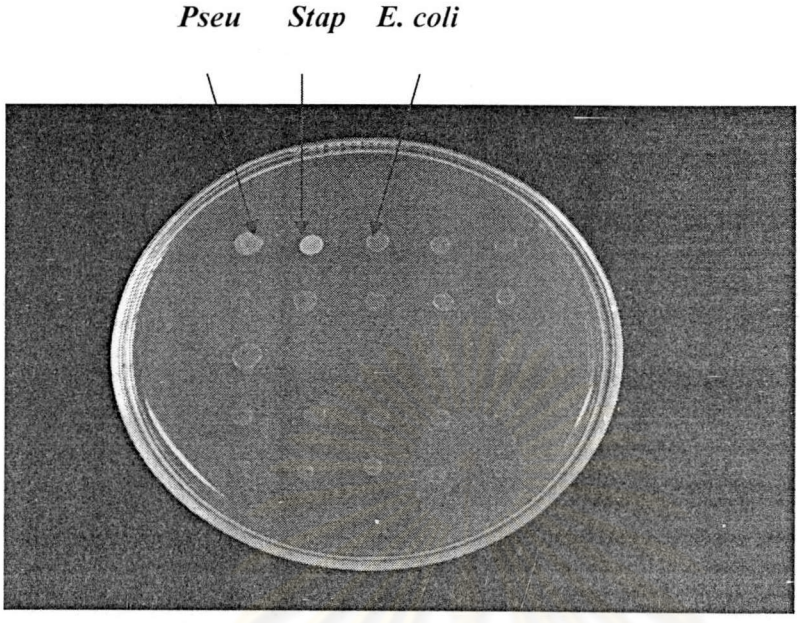

Figure 3. Inoculum plate of agar dilution method
Reference control: *Escherichia coli* ATCC 25922 = *E. coli*
Staphylococcus aureus ATCC 25923 = *Stap*
Pseudomonas aeruginosa ATCC 27853 = *Pseu*

Table 8 MIC of reference control for MIC determinations (µg/mL)

Antimicrobial Agents	***Enterococcus faecalis* ATCC 29212**	***Staphylococcus aureus* ATCC 29213**	***Pseudomonas aeruginosa* ATCC 27853**	***Escherichia coli* ATCC 25922**
Ampicillin	0.5-2	0.25-1	>32	2-8
Chloramphenicol	4-16	2-8	> 64	2-8
Ciprofloxacin	0.25-2	0.12-0.5	0.25-1	0.004-0.015
Florfenicol	2-8	2-8	>16	2-8
Gentamicin	4-16	0.12-1	0.5-2	0.25-1
Nalidixic Acid	>128	16-64	≥ 128	1-4
Sulphamethoxazole	>512	>512	>512	8-32
Tetracycline	8-32	0.25-1	8-32	0.5-2
Trimethoprim	≤1	1-4	>64	0.5-2

Grey area: NCCLS recommendations
White area: Quality control range assigned by the Danish Veterinary Laboratory

Table 9 MIC standard range and their interpretation for the antimicrobial agents

Antimicrobial agent	Drug content (□g/mL)	MIC breakpoint		
		R	I	S
Ampilicillin	30	<8	16	>32
Ciprofloxacin	30	<1	2	>4
Chloramphinicol	30	<8	16	>32
Gentamicin	30	<4	8	>16
Nalidixic acid	30	<16	-	>32
Neomycin	30	<16	32	>64
Streptomycin	30	<8	16	>32
Sulfametoxazone	30	<4	8	>16
Sulfa+Trimethoprim	30	<2/38	-	>4/76
Tetracyclin	30	<4	8	>16

5. Analysis of restricted fragments of *Salmonella* Schwarzengrund By Pulsed-Field Gel Electrophoresis (PFGE)

5.1 Preparation of PFGE agarose plugs from Salmonella cell suspensions

Organism were harvested into SE buffer adjust density to Mcfarland No. 5 and transfer 400 µl (0.4 mL) of each cell suspensions to labeled microcentrifuge tubes and incubate in the 37°C water bath for 5 minutes. Keep tubes containing rest of the cell suspensions on ice bath until plugs are made. Remove cell suspensions in 1.5 mL tubes form water bath; add 20 µl proteinase K (20 mg/mL stock solution) to each 0.4 mL cell suspension. Add 400 µl (0.4 mL) melted 1% SeaKem Gold: 1% SDS agarose to one of the 0.4 mL cell suspension and Immediately dispense part of mixture into appropriate well in plug molds. Lysis of Cell in Agarose Plugs by add 25 µl Proteinase K stock solution to the tube that contains the 5 mL cell lysis buffer to each of the other 50 mL plastic tubes. Remove agar blocks and place into proteinase K / cell lysis buffer after that incubate for 2 hours at 54 °C warm water bath(with shaking). Then washing of agarose plugs by add 10 mL of prewarmed (54 °C) distilled water and incubate at 54 °C for 15 min (in water bath with shaking). Repeat wash step with distilled water two times after that

wash the blocks three times in TE buffer incubate at 54 °C for 15 minutes (in water bath with shaking) and store blocks in 5 mL TE buffer (52-54).

5.2 Restriction digestion of DNA in agarose plug with *Xba* I

Carefully remove a block from TE buffer to aglass slide and cut ablock 1 mm wide using a clean scalpel (ensure both edges are straight and parallel). Place sample into relevant enzyme buffer 100 µl incubate at 37 °C for 10 min and then remove buffer with pipette. Add 50 µl enzyme mix (enzyme 20 units+relevant enzyme buffer+distiled water that shown in table 10).

Table 10 Restriction enzyme mixture

Reagents	µl/Plug Slice	µl/13 Plug Slice
Distilled water	175 µl	2275 µl
Relevant enzyme buffer	20 µl	260 µl
Enzyme (20U/ µl)	5µl	65 µl
Total Volume	200 µl	2600 µl

5.3 Gel preparation and gel running

The running agarose gel was prepared by dissolving 1 g of Ultrapure High-melting temperature agarose in 100 mL of 0.5x TBE buffer, melted by microwave and then cool at 56 °C. One percent running agarose gel was poured into the block and let gel to solidify for 30 min at room temperature. After the gel has hardened, the comb was removed and the plug was placed onto the horizontal side of the comb The gel was placed in the PFGE box containing 0.5x TBE buffer 2 L enough to cover the gel to a depth of about 1 mm or just until the tops of the wells are submered. CHEF DNA size standards Lambda ladder (BioRad, USA) was used as the molecular standard markers. PFGE was performed at 200 v constant voltage by using a contour-clamped homogenous electric field apparatus (CHEF-DR III system) with an initial switch time of 10 sec and a final switch time of 20 sec for 22 hours.

5.4 Gel visualization

The gel was stained with 0.5 µg/mL of ethidium bromide for 15 min. After that it was rinsed and destained with deionized water for 30 min. The gel was then photograpghed under UV illumination. The result was interpreted by Bionumeric Software version 3.0 with a position tolerance of 1.0 % and optimization is 0.5 %.