MATERIALS AND METHODS

Materials

1. Bacterial strains

- *Salmonella* strains were isolated from pigs which obtained from five farms in Thailand; Farm A (Suphan Buri province), Farm B and C (Ratchaburi province), Farm D (Chon Buri province), and Farm E (Chiang Mai province). These isolates were collected as cross-sectional (cross-x) samples from three types of sources including stools, feeds and environmental samples.
- *S.* Typhimuruim DT104 which contained class 1 integron was used as positive control in PCR screening for class 1 integrons.
- *E. coli* strain DH5α was used as recipient cell in conjugation.
- *E. coli* strain V517 was used as standard molecular weight (MW) for determining the MW of plasmids

2. Media for bacterial culture

- MacConkey agar
- XLD agar
- Mueller-Hinton agar
- Mueller-Hinton broth
- LB agar
- LB broth
- Skim milk

3. Reagents

- Tris-base
- Na-EDTA
- Sodium dodecyl sulfate (SDS)

- Sodium hydroxide (NaOH)
- Potassium acetate
- Sodium acetate
- Glacial acetic acid
- Chloroform
- Isopropanol
- Absolute ethanol
- Restriction endonuclease
- Agarose gel
- Ethidium bromide

4. Equipments

- 37^0C Incubator
- 37^0C Shaking incubator
- Laminar air flow
- Vibrator
- Microcentrifuge
- Dry bath incubator
- Thermocycler PCR machine
- Electrophoresis machine
- UV transilluminator

Methods

1. Isolation of *Salmonella* strains

Salmonella strains were isolated from pigs which derived from five farms in Thailand (Farm A, B, C, D and E). These isolates were collected as cross-x samples from three types of sources including stools, feeds and environmental samples. *Salmonella* isolates were selected according to conventional method, by using selective media (MacConkey and XLD agar; in appendix A) and biochemical

properties test (Murray *et al.*, 1999). Furthermore, these isolates were subjected to test for serogrouping. Only *Salmonella* serogroup B and C were selected for this study and were kept in skim milk (in appendix A) and stored at -20°C.

2. Antimicrobial susceptibility test by disc diffusion method

Pathogenic *Salmonella* isolates serogroup B and C were preliminary tested for antimicrobial resistance by using Kirby-Bauer disc diffusion assay on Mueller-Hinton agar (in appendix A) against sixteen antimicrobial agent discs (Table 4) which recommended by National Committee for Clinical Laboratory Standards (NCCLS). The criteria of resistant zones were evaluated following the recommendations of NCCLS (2000). *Salmonella* strains that conferred resistance to at least three drugs were designated as MDR strains.

Table 4 Antimicrobial agents used in the susceptibility test.

Antimicrobial class	Antimicrobial agent	Disc content
β-Lactams	Ampicillin	10 µg
β-Lactams/β-Lactamase inhibitor combination	Amoxicillin/clavulanic acid	30 µg
Cephalosporins	Ceftiofur	30 µg
	Ceftriaxone	30 µg
	Cephalothin	30 µg
Aminoglycosides	Amikacin	30 µg
	Apramycin	15 µg
	Gentamicin	10 µg
	Kanamycin	30 µg
	Streptomycin	10 µg
Phenicols	Chloramphenicol	30 µg
Tetracyclines	Tetracycline	30 µg
Quinolones and fluoroquinolones	Nalidixic acid	30 µg
	Ciprofloxacin	5 µg
Sulfonamides	Sulfamethoxazole	25 ug
	Sulfamethoxazole/trimethoprim	25 µg

3. Detection of class 1 integrons and resistance gene cassettes

3.1 Genomic DNA extraction

One colony of *Salmonella* was inoculated into 5 ml of LB broth (in appendix A) at 37°C overnight (18 hours) with shaking at 150 rpm. The overnight culture of 1.0 ml was transferred to microcentrifuge tube and genomic DNA was extracted by boiling method (Radu *et al.*, 2001). The cells were harvested by centrifugation at 13,000 rpm for 3 minutes. The supernatant was carefully removed and cell pellets were thoroughly suspended and washed two times with 200 µl of TE buffer, pH 8.0 (in appendix C) and centrifuged as before. The pellet was resuspended with TE buffer pH 8.0, 200 µl and then boiled for ten minutes. After boiling, the cell debris was precipitated by centrifugation at 13,000 rpm for 5 minutes. The supernatant was completely removed to new microcentrifuge tube and used as template for other experiments directly. The extracted DNA templates were stored at -20°C.

3.2 Class 1 integrons screening by PCR amplification

The extracted DNA templates were screening for class 1 integrons by using specific primer pair int1 F: 5'-CGGGCATCCAAGCAGCAAG-3' and int1 R: 5'-AAAGCAGACTTGACCTGATAG-3' (EMBL accession no. AY220520). This primer pair specified to 5' conserved segment and 3' conserved segment of class 1 integrons respectively (Figure 4). Each PCR reaction had a final concentration of 2.0 mM $MgCl_2$, 1X reaction buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 0.5 unit of *Taq* DNA polymerase, and 1 µl of DNA templates. For the amplification, the PCR condition included initial 5 minutes of incubation at 94°C, followed by incubations at 94°C for 30 seconds (denature), 55°C for 30 seconds (annealing) and 72°C for 1 minute (extension), for 30 cycles. The last step was incubated at 72°C for 7 minutes (Dalsgaard *et al.*, 2000). In order to check the yield and specificity of the product, DNA was separated on 1.0% agarose gel electrophoresis and 1X TAE gel stained with ethidium bromide (in appendix B) at 100 volts for 20 minutes. The λ DNA/*Hin*dIII +

ΦX/*Hae*III were used as standard molecular weight for determining the MW of PCR products. The expected size of PCR products were varied from different size of resistance gene cassettes inside class 1 integrons (Figure 4).

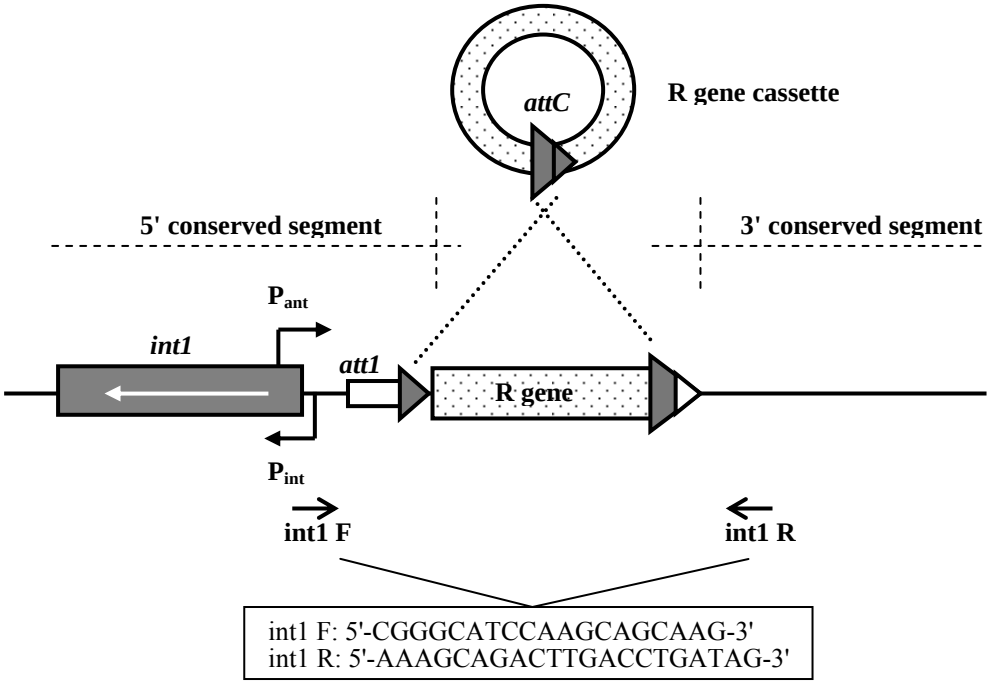

Figure 4 Schematic representation structural of class 1 integron containing R gene cassette and amplified fragment region. Specific primers for 5' conserved segment and 3' conserved segment used in PCR experiment were indicated in box.

3.3 Identification of resistance gene cassettes by sequencing technique

In order to identify the resistance gene cassettes in class 1 integron, the positive PCR products were subjected to nucleotide sequences. The PCR product was prepared for sequencing by using DNA Sequencing Kit (BigDye™ Terminator Cycle Sequencing v2.0 Ready Reaction) following to the manufacturer's recommendations. Briefly, the PCR products were precipitated in 2 µl of 3 M Sodium acetate, pH 4.6 (in appendix B) and 50 µl of absolute ethanol, gently mixed, then transferred to new microcentrifuge tube and maintained at 0°C for 15 minutes. After that the solution

was precipitated by centrifuged at 14,000 rpm for 15 minutes. After removing the supernatant completely, the DNA pellet was washed by 250 µl of 75% ethanol, centrifuged at 14,000 rpm for 5 minutes. The supernatant was discarded and then the DNA pellet was dried at 40°C and ready for further sequencing process.

4. Examination of antimicrobial resistance genes location and transferring by plasmid conjugation

4.1 Conjugation

Salmonella strains that had particular resistance patterns of interested were selected as donor cells. *E. coli* DH5α which conferred resistance to nalidixic acid was used as recipient cells in conjugation experiment. One colony of each donor cell and recipient cell were inoculated in 5 ml of LB broth separately. LB broth of donor was supplemented with 100 µg/ml ampicillin (in appendix B), while counter-part of recipient was supplemented with 30 µg/ml nalidixic acid (in appendix B), incubated at 37°C and shaking at 150 rpm for overnight. Then both cultures, 1 ml of donor and recipient cells were co-precipitated in microcentrifuge tube at 13,000 rpm for 3 minutes. The 900 µl of supernatant was discarded and 100 µl of suspension was dropped on LB agar (in appendix A) by at central region and incubated at 37°C for 4-6 hours. Transconjugants were harvested and selected on selective media (LB agar containing 100 µg/ml ampicillin and 30 µg/ml nalidixic acid) by making appropriated dilutions (10^{-1}, 10^{-2} and 10^{-3}). The dilutions were spread on LB agar containing 100 µg/ml ampicillin and 30 µg/ml nalidixic acid, incubated at 37°C for overnight.

In case of which donor cell conferred resistance to nalidixic acid, the transconjugants were subjected to select with morphological differentiation. The different morphology between donor cells and transconjugants were distinguished by growing on XLD agar. On such a chosen selective media, the donor cells gave black colonies whereas yellow colonies were given by transconjugants. In order to find out which genes were encoded on conjugative plasmid, the transconjugants were subjected to determine antimicrobial resistance by disc diffusion method as mentioned

above. Plasmid profiles analysis was performed for indication the size of conjugative plasmid.

5. Determination of diversity among the isolates by plasmid profiles and RFLP-PCR analysis

5.1 Plasmid profiles analysis

The selected *Salmonella* donor or transconjugant clones were grown in 5 ml of LB broth containing 100 µg/ml ampicillin then incubated at 37°C and shaking overnight at 150 rpm. After incubation, 1.5 ml of culture was transferred to microcentrifuge tube for plasmid extraction and was centrifuged at 13,000 rpm for 3 minutes. The supernatant was carefully removed and the cell pellet was thoroughly suspended in 200 µl of solution I (in appendix C). Then 200 µl of solution II (freshly prepare; in appendix C) was added and the tube was gently mixed, the suspension should become almost clear and slightly viscous. The tube was incubated at 0°C for 5 minutes, then 200 µl of solution III (in appendix C) was added and gently mixed by inversion for a few seconds in order to form a clot of DNA. The tube was further incubated at 0°C for 5 minutes and centrifuged at 13,000 rpm for 5 minutes. The supernatant was transferred to a new microcentrifuge tube, an equal volume of chloroform was added and vigorously mixed. Then the tube was centrifuged at 13,000 rpm for 5 minutes to yield an almost clear supernatant. The approximate 500 µl of supernatant was removed and transferred to a new microcentrifuge tube. Then 1 ml of absolute ethanol was added and incubated at -80°C for 2 hours. The precipitant is collected by centrifugation at 13,000 for 10 minutes and the supernatant was discarded. The pellet was dried at 40°C and dissolved in 20 µl of TE buffer containing RNase, incubated at 37°C for overnight (Ansary and Radu, 1992). Then plasmid was investigated by applied to 0.8% agarose gel electrophoresis at 100 volts for 40 minutes. The plasmid DNA of *E. coli* V517 was used as standard MW for determining the MW of plasmids. The remained solution was stored at -20°C.

5.2 RFLP-PCR analysis

The extracted genomic DNA of *Salmonella* isolates were amplified for *fliC* and *fljB* genes by using specific two primer pairs, FSa1: 5'-CAAGTCATTAAT ACAAACAGCC-3'; BSa1: 5'-TTAACGCAGTAAAGAGAGGAC-3' and FSa2: 5'-C AAGTAATCAACACTAACAGTC-3'; BSa2: 5'-TTAACGTAACAGAGACAGCAC -3' respectively (Dauga *et al.*, 1998). The two sets of each PCR reaction had a total volume of 35 µl and a final concentration of 3.0 mM $MgCl_2$, 1X reaction buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 0.5 unit of *Taq* DNA polymerase, and 4 µl of DNA templates. For the amplification, the PCR condition included initial 5 minutes of incubation at 94°C, followed by incubations at 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, for 40 cycles. The last step was incubated at 72°C for 7 minutes (Hong *et al.*, 2003). In order to check the yield and specificity of the product, DNA was separated on 1.0% agarose gel electrophoresis and 1X TAE gel stained with ethidium bromide at 100 volts for 20 minutes. The λ DNA/*Hin*dIII + ΦX/*Hae*III were used as standard molecular weight for determining the MW of PCR products. The expected size of PCR products of *fliC* and *fljB* genes were both 1.5 kbp approximately.

The amplified *fliC* and *fljB* genes were subjected to digest with two restriction enzymes *Hha*I and *Mbo*I in RFLP analysis. In both *Hha*I and *Mbo*I digestion, each four reactions included 15 µl of PCR products, 3 µl of distilled water (DW), 2X reaction buffer and 5 units of each enzyme and the reaction were incubated at 37°C for 2 hours. RFLP patterns of restriction enzyme digested PCR products were examined by 1.8% agarose gel electrophoresis and 1X TAE gel stained with ethidium bromide at 100 volts for 45 minutes. The 100 bp DNA ladder was used as standard molecular weight for determining the MW of DNA fragments (Hong *et al.*, 2003).

RESULTS AND DISCUSSIONS

1. Antimicrobial resistance in *Salmonella* strains

All of 230 *Salmonella* strains serogroup B and C isolated from pigs, which collected from five farms (A, B, C, D and E) in Thailand were tested for antimicrobial resistance by using disc diffusion assay against sixteen antimicrobial agents. The criteria of resistant zones were evaluated following the recommendations of NCCLS (2000). The result showed that *Salmonella* isolates either from fecal or environmental samples conferred resistance to several classes of antimicrobial agents. The percentages of resistance to antimicrobials were shown in Table 5. Among these antimicrobials, all isolates exhibited resistance to sulfamethoxazole (Su). More than 70% of isolates were resistant to ampicillin (A), streptomycin (S) and tetracycline (T). The *Salmonella* isolates also resisted to chloramphenicol (C), gentamicin (G), nalidixic acid (N) and sulfamethoxazole/trimethoprim (Sxt) approximately 30-60%. The isolates exhibited resistance to kanamycin (K) 27% and apramycin (Ap) 16%. While for amikacin (Ak), amoxicillin/clavulanic acid (Am) and cephalothin (Ct), the isolates showed resistance only 3%, 2% and 1% respectively. Prominently, all of the isolates were susceptible to ceftriaxone (Cx), ceftriofur (Cf) and ciprofloxacin (Cp). Hence, only three antimicrobial agents, which were ceftriaxone, ceftriofur and ciprofloxacin could inhibit growth of *Salmonella* strains completely. Furthermore, amikacin, amoxicillin/clavulanic acid and cephalothin were also useful for elimination bacterial infection. These antimicrobial agents were considered to be the efficient therapeutic drugs.

According to our knowledge, quinolones resistance cause by chromosomal mutation only. N-terminal point mutations of *gyrA* (at codon 87) in DNA gyrase reduce binding affinity of quinolones, which confer only nalidixic acid resistance (Choi, *et al.*, 2005). While, double mutations in *gyrA* (at codon 83 and 87) leading to amino acid substitutions confer resistance to nalidixic acid and ciprofloxacin (Chu *et al.*, 2005). Hence, nalidixic acid-resistant strains in this study could be implied that these strains probably had chromosomal mutation in *gyrA* gene at condon 87.

Furthermore, of 230 *Salmonella* isolates from five farms (A, B, C, D, and E), 211 isolates (91.74%) presented dramatically resistance to several classes of antimicrobial agents as MDR strains. These MDR strains resisted to at least three antimicrobial agents and exhibited different resistance patterns. As a result was shown in Table 6, the MDR strains were high prevalent in resistance patterns ASuT, ASSuT, AGSSuT, ACSSuSxtT, ACKNSSuT, ACKSSuSxtT, AApCGKNSSuT and AApCGNSSuSxtT. Moreover, these MDR strains also distributed to variable pig sources. The resistance patterns ASuT, ACKNSSuT, AApCGKNSSuT and AApCGNSSuSxtT predominated in *Salmonella* serogroup B, whereas most of *Salmonella* serogroup C had resistance pattern AGSSuT and ACKSSuSxtT. The resistance patterns SSuT, ASSuT, ACSSuSxtT, ACNSSuSxtT and ACGNSSuSxtT distributed to both serogroup B and C. Other resistance patterns including AApCKSSuSxtT, AAmApCSSuSxtT, ACCtGSSuSxtT and AGKNSSuT had small numbers among these isolates.

Among MDR *Salmonella* isolates from five farms, the resistance patterns ASuT, ASSuT, AGSSuT, ACKSSuSxtT and AApCGNSSuSxtT had high incidence in Farm A (Table 6). Especially, some resistance patterns had high incidence in particular type of pig, such as ASuT had high prevalent in dam and nursing period. While the AApCGNSSuSxtT pattern was vary into many types of pig including environmental samples. In contrast, only a few resistance patterns in Farm B and C were probably resulted from a small number of samples. In Farm D, the predominated resistance patterns were ACKNSSuT and AApCGKNSSuT, which fell into nursing period of pig (Table 6). While in Farm E, the resistance pattern ACSSuSxtT was the most prevalent pattern and also distributed to various pig types including sire, pregnant sow, rearing, replacement gilt and suckling period (Table 6).

This result indicated that MDR *Salmonella* strains already emerged in Thailand and would lead a serious public health problem. Especially, antimicrobial resistance pathogens in food-producing animals can infect humans via contaminated food. Similary to Carlson *et al.* (2000), these MDR strains also resisted to new types of antimicrobial including amoxicillin/clavulanic acid and cephalothin. The

emergence of these MDR *Salmonella* strains probably results from an inappropiate use of antimicrobials in food animals in order to promote growth and prevent from bacterial infection.

2. Class 1 integrons and resistance gene cassettes in *Salmonella* strains

2.1 Class 1 integron screening by PCR amplification

Class 1 integrons are genetic elements that act as site-specific recombination systems for recognition and acquisition of new resistance gene cassettes. Therefore, to find out class 1 integrons and the resistance gene cassettes, the 211 MDR *Salmonella* strains isolated from five pig farms were examined by using PCR amplification. The genomic DNA templates of the MDR *Salmonella* isolates were screening for class 1 integrons by using specific primer pair int1 F: 5'-CGGGCATCCAAGCAGCAAG-3' and int1 R: 5'-AAAGCAGACTTGACCTGAT AG-3' (EMBL accession no. AY220520). This primer pair specified to 5' conserved segment and 3' conserved segment of class 1 integrons. The PCR products of class 1 integron were analyzed in 1.0% agarose gel electrophoresis (Figure 5). The result revealed that only four isolates (1.89%) comprising of *S.* Stanley CC1, *S.* Panama CB2 and CB3, and *S.* Anatum EC3, yielded 1.0 kbp PCR products, which implied that these isolates had class 1 integrons and probably contained resistance gene cassettes.

2.2 Sequencing of resistance gene cassettes

In order to identify the resistance gene cassettes in class 1 integron, the positive PCR products were subjected to nucleotide sequences analysis. The PCR product was prepared for sequencing by using DNA Sequencing Kit (BigDye^{TM} Terminator Cycle Sequencing v2.0 Ready Reaction) following the manufacturer's recommendations. The obtained nucleotide sequences were illustrated in Figure 6-9. All of four class 1 integron nucleotide sequences were aligned with other related nucleotide sequences in GenBank database using BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) and multiple sequence alignment analysis ClustalW program (http://www.ebi.ac.uk/clustalw/). The alignment results revealed that each nucleotide

sequences were class 1 integrons, each of which contained *aadA* gene cassettes (Table 8). Nucleotide sequences of *S.* Stanley CC1 strain were similar to *aadA1* gene at 99% identity (Figure 10), while nucleotide sequences of *S.* Panama CB2 or CB3 strains were 98% identical to *aadA4* gene (Figure 11 and 12). In *S.* Anatum EC3 strain, nucleotide sequences were also similar to *aadA2* gene at 97% identity (Figure 13).

The nucleotide sequences of four isolates were analyzed for ORFs of gene cassette by using All in One Analyzer Program (http://www-personal.umich.edu/~ino/blast.html). The results represented that *S.* Stanley CC1 had nucleotide 792 bp and 262 amino acids of *aadA1* gene (Figure 6). As well as *S.* Anatum EC3 strains had nucleotide 792 bp and 262 amino acids of *aadA2* gene (Figure 9). While the *aadA2* gene derived from either *S.* Panama CB2 or CB3 strains had nucleotide 789 bp and 263 amino acids (Figure 7 and 8). All of *aadA* gene cassettes, which were inserted in the *attI* recombination site, had core site GTTAGGC. The inverse core site of *aadA1* and *aadA2* cassettes were GTCTAAC, while both *aadA4* cassettes were GCCTAAC (Figure 6-9). The *attC* site of *aadA1* and *aadA2* cassettes were 60 bp long, whereas, both *aadA4* cassettes were 57 bp long (Figure 6-9 and Table 8). These *aadA* genes encoded for aminoglycoside adenyltransferase, which conferred to streptomycin and spectinomycin resistance phenotype.

Moreover, all of four amino acid sequences were also compared with other related amino acid sequences in GenBank database. The comparative results revealed that each amino acid sequences were aminoglycoside adenyltransferase. The amino acid sequences (AadA1) of *S.* Stanley CC1 strain were similar to AadA(3") of *E. coli*, AadA1c of *Vibrio cholerae*, AadA of synthetic construct and AadA of binary vector pPZP (accession no. CAA26199, BAE66662, AAA93350 and AAX97761) at 98% identity. This AadA1 had amino acid substitution from V to G (positions 54), W to S (positions 195), V to E (positions 198) and M to K (positions 213) (Figure 14). While, the AadA4 amino acid sequences of both *S.* Panama CB2 and CB3 strains were similar to AadA4 of *E. coli* RA33.2, *A. baumannii*, *E. coli* and uncultured bacterium (accession no. CAB41476, AAN34365, AAT36683 and AAN41432) at 97, 96, 94 and 94% identity respectively. This AadA4 had substitution of amino acids

from S to F (positions 163), Y to S (positions 196), V to E (positions 199) and E to K (positions 114) (Figure 15 and 16). The AadA2 amino acid sequences of *S.* Anatum EC3 strain were similar to AadA2 of *E. coli* B7A, *Yersinia pestis* biovar Orientalis str. IP275, *V. cholerae* non-01/non-0139 and *E. coli* (accession no. ZP_00717087, ZP_01175305, BAE71360 and YP_449022) at 94% identity. This AadA2 had 13 amino acids substitution, which were from S to G (positions 9), I to X (positions 86), H to A (positions 93), D to E (positions 94), R to P (positions 114), P to L (positions 158), V to G (positions 187), W to C (positions 194), E to Q (positions 226), A to T (positions 227), H to N (positions 238), K to N (positions 254) and G to R (positions 255) (Figure 17).

Recently, only seven members of *aadA* gene family were known including *aadA1, aadA1b, aadA2, aadA3, aadA4, aadA5, aadA6 and aadA7* (White and Rawlinson, 2001). These genes encode for aminoglycoside adenyltransferase (Aad), which conferred resistance to streptomycin and spectinomycin. According to the results in this study, *S.* Stanley CC1, *S.* Panama CB2 and CB3 and *S.* Anatum EC3 strains carried class 1 integrons containing *aadA*1, *aadA4* and *aadA2* gene cassettes respectively. As well as in *S.* Newport isolated from animals in Pennsylvania, also represented class 1 integrons that harbored *aadA1* gene cassette (Rankin *et al.*, 2002). Additionally, *E. coli* isolates from pigs in Korea also found that class 1 integrons most frequently carried *aadA2* and *aadA1* cassettes (Kang *et al.*, 2005). These information suggesting that the frequently occurrence of *aadA* gene cassettes was attributed from the selective pressure of resistance strains. Especially, the isolates of food-producing animals, which usually derived antimicrobial supplemented feeding. Moreover, the new gene cassettes were found in other gram-negative bacteria. *A. baumannii* isolated from patient, presented a new integron-borne bla_{IMP-5} gene cassette (Da Silva *et al.*, 2002). A new bla_{VIM-2} gene cassette was also identified in *P. aeruginosa* isolates from patient in France (Poirel *et al.*, 2000). Although class 1 integron-borne gene cassettes in this study showed low incidence, however, these finding indicated that class 1 integrons are the potential genetic elements in dissemination and acquisition of resistance gene cassettes among gram-negative bacteria, especially, in public health isolates.

3. Antimicrobial resistance genes transferring in *Salmonella* strains

3.1 Transfer of resistance

In order to understand the widespread of resistance genes among MDR *Salmonella* strains, determination of resistance genes transferring was performed by plasmid conjugation. Among *Salmonella* isolates, 30 MDR isolates including *S.* Corvallis (serogroup C), *S.* Rissen (serogroup C) and *S.* 1,4,5,12:i:- (serogroup B) of Farm A were chosen for study resistance genes in this experiment. These isolates were endemic in many types of pigs and showed the correlation of the resistance phenotypes and serogroups. Of 30 isolates, ten isolates of each serotypes presented different resistance patterns. Interestingly, ten isolates of *S.* Corvallis had the same resistance pattern as AGSSuT. Ten isolates of *S.* Rissen exhibited resistance pattern ACKSSuSxtT and also ten isolates of *S.* 1,4,5,12:i:- had resistance pattern AApCGN-SSuSxtT (Table 7).

These isolates were examined their resistance genes transferring to *E. coli* DH5α by plasmid conjugation. Then the transconjugants were subjected to characterize antimicrobial resistance phenotype and compared with donor strains by disc diffusion assay. The result showed that all *S.* Corvallis isolates with resistance pattern AGSSuT could transfer resistance genes to *E. coli* transconjugants, which conferred ampicillin (A) and gentamicin (G) resistance phenotypes (Table 9 and Appendix Table D1). All of *S.* Rissen strains showing resistance pattern ACKS-SuSxtT also transferred resistance genes which showed resistance to ampicillin (A), chloramphenicol (C), kanamycin (K), sulfamethoxazole (Su) and sulfamethoxazole/ trimethoprim (Sxt) (Table 9 and Appendix Table D2), whereas, all of *S.* 1,4,5,12:i:- strains representing resistance pattern AApCGNSSuSxtT could not transfer resistance genes via conjugative plasmid (Table 9).

Plasmid profiles analysis of all *Salmonella* donor strains represented that different *Salmonella* serotypes and resistance patterns also had different plasmid profiles (Figure 18). Moreover, in each *Salmonella* serotypes and resistance patterns

could differentiate plasmid profiles into two groups. *S.* Corvallis exhibiting resistance pattern AGSSuT was designated plasmid profiles named M1 (54 and 10 kbp) and M2 (54, 10 and 4.3 kbp) patterns (Figure 18A and Table 11). For *S.* Rissen showing ACKSSuSxtT resistance pattern was designated as N1 (>54, 10, 9 and 5.6 kbp) and N2 (>54, 10, 9, 8, 5.1 and 4 kbp) patterns (Figure 18B and Table 11). Accordingly, *S.* 1,4,5,12:i:- representing AApCGNSSuSxtT resistance pattern was also designated as P1 (8.6, 8, 5.6, 5.1, 4 and 3 kbp) and P2 (8 and 5.1 kbp) patterns (Figure 18C and Table 11). Therefore, two donor strains of *S.* Corvallis and *S.* Rissen that had different plasmid profiles and their transconjugants were chosen to examine for conjugative plasmids. The result revealed *S.* Corvallis donors exhibiting resistance patterns AGSSuT and *S.* Rissen donors representing ACKSSuSxtT resistance pattern, contained conjugative plasmids of approximately 54 kbp and larger than 54 kbp respectively (Figure 19A, B and Table 9).

The conjugation results among these *Salmonella* isolates indicated that genes encoding for ampicillin and gentamicin resistance phenotypes were located on a 54 kbp transferable plasmid in *S.* Corvallis. Additionally, a large transferable plasmid (>54 kbp) of *S.* Rissen also carried multi-resistant genes mediated to ampicillin, chlorampenicol, kanamycin, sulfamethoxazole and trimethoprim resistance phenotypes. These results were similar to Michael *et al.* (2005) finding that *S.* Agona carried a 150 kbp conjugative plasmid. This conjugative plasmid contained all resistance genes detected in such strain. While in *S.* 1,4,5,12:i:- strains could not detect conjugative plasmid. This result probably due to these strains harbored only non-conjugative plasmids. In *S.* Corvallis and *S.* Rissen, the genes conferred resistance to streptomycin and tetracycline, which could not transfer via conjugation in this study, probably located on other small plasmids or chromosomal DNA. As well as in other study, *S.* Typhimurium isolates in France were found that the resistance genes for ampicillin (bla_{TEM-1}, bla_{PSE-1} and bla_{OXA-1}), chloramphenicol and tetracycline (flo_{ST}) resistance phenotypes were also located on plasmids (Biendo *et al.*, 2005).

3.2 Transferable of class 1 integron

Integrons are genetic elements that facilitate the occurrence and movement of resistance gene cassettes by site specific recombination systems. Most of integrons are often found associated with transposons and conjugative plasmids. Hence, in this study, resistance genes transferring in four *Salmonella* isolates that contained class 1 integrons (*S.* Stanley CC1, *S.* Panama CB2 and CB3, and *S.* Anatum EC3 strains), were also determined. As a result from plasmid conjugation, only one of *Salmonella* strains CC1 with resistance pattern ACGSSuSxtT could transfer resistance genes to *E. coli* that conferred ACSSuT pattern (Table 10 and Appendix Table D3). Whereas, the remaining strains CB2, CB3 and EC3 could not transfer resistance genes by conjugative plasmid (Table 10). Plasmid profiles analysis of *S.* Stanley CC1 strain and its transconjugants were examined for conjugative plasmids. The result revealed that *Salmonella* donor strain CC1 harbored only one plasmid. It was conjugative plasmid of approximately larger than 54 kbp, which mediated resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (Figure 20). In order to confirm the existing of class 1 integron on conjugative plasmid, the transconjugants were also detected for class 1 integron by using PCR amplification. The amplification showed that all of the transconjugants contained class 1 integron which carried *aadA1* gene (Figure 21).

This conjugation experiment represented similar result to *S.* Agona, which its conjugative plasmid carried class 1 integron containing a new variant *aadA23* gene cassette (Michael *et al.*, 2005). In addition, class 1 integrons were mostly found in conjugative plasmids in *E. coli* isolated from either humans or animals in Korea (Kang *et al.*, 2005). Therefore, these plasmid conjugation results confirmed that the conjugative plasmid could disseminate resistance genes among these MDR strains. In addition, the existing of class 1 integron on conjugative plasmid probably also play an important role in acquisition and widespread of antimicrobial resistance profiles. Especially, when bacterial chromosome already consisted of class 1 integron, the gene cassette could mobilize from class 1 integron on conjugative plasmid to secondary recombination site on chromosome (Taylor *et*

al., 2004). This situation would lead a stable expression of antimicrobial resistance genes. Moreover, the conjugation experiment in this study described that the conjugative plasmids could disseminate resistance genes among these MDR *Salmonella* strains. Apparently, the conjugative plasmids and resistance phenotype in *E. coli* and *Salmonella* explained the possible of resistance genes dissemination among different genus of enteric bacteria.

4. Diversity of *Salmonella* isolates by plasmid profiles and RFLP-PCR analysis

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4.1 Plasmid profile analysis

Plasmid profiles analysis of all 30 *Salmonella* strains *S.* Corvallis, *S.* Rissen and *S.* 1,4,5,12:i:- already mentioned above in conjugation issue (Table 7). As a result of this analysis, *Salmonella* strains represented different serotypes and resistance patterns also had different plasmid profiles and vary in size from 3 to >54 kbp (Figure 18 and Table 11). Each serotypes and resistance patterns could differentiate plasmid profiles into two groups. Ten isolates of *S.* Corvallis exhibiting resistance pattern AGSSuT presented plasmid profile patterns M1 and M2 (Figure 18A and Table 11). *S.* Rissen ten isolates with ACKSSuSxtT resistance pattern showed plasmid profiles N1 and N2 (Figure 18B and Table 11). While ten isolates of *S.* 1,4,5,12:i:- showing resistance pattern AApCGNSSuSxtT had plasmid profiles P1 and P2 (Figure 18C and Table 11). The plasmid profiles result exhibited the diversity of these isolates, even though among the strains represented identical resistance phenotype.

4.2 RFLP-PCR analysis

Due to RFLP analysis of *fliC* and *fljB* genes, the digestion of each PCR products with restriction endonucleases have clearly reflected the antigenic variances in the phase 1 and 2 flagellar antigens (Dauga *et al.*, 1998). This molecular method has been proven to be an alternative approach to serotype *Salmonella* strains. This study also demonstrated RFLP-PCR analysis of *fliC* and *fljB* genes among those 30

Salmonella isolates that represented AGSSuT and ACKSSuSxtT and AApCGN-SSuSxtT resistance patterns (Table 7). Genomic DNA of *Salmonella* isolates were amplified for *fliC* and *fljB* genes by using specific two primer pairs (FSa1: 5'-CAAG TCATTAATACAAACAGCC-3'; BSa1: 5'-TTAACCAGTAAAGAGAGGAC-3' and FSa2: 5'-CAAGTAATCAACACTAACAGTC-3'; BSa2: 5'-TTAACGTAACAGAGA CAGCAC-3') respectively (Dauga *et al.*, 1998). The amplified flagellin genes were analyzed by RFLP-PCR with *Mbo*I and *Hha*I.

A 1.5 kbp *fli*C fragment was successfully amplified from 20 isolates representing ACKSSuSxtT and AApCGNSSuSxtT resistance patterns. Moreover, there was a variation size of 1.24 kbp *fliC* fragment was amplified from ten isolates exhibiting AGSSuT resistance pattern. While, a *flj*B amplification was failed in all isolates exhibiting AGSSuT, ACKSSuSxtT and AApCGNSSuSxtT resistance patterns (data not shown). Restriction profiles of *fliC* gene were assigned with letters E and X for *Mbo*I and *Hha*I digestions respectively. The results revealed that all *Salmonella* isolates in each resistance phenotypes yielded the same RFLP pattern, and the isolates representing different resistance patterns also gave different RFLP profiles (Table 11). The resistance pattern AGSSuT exhibited RFLP profiles as E1 and X1 patterns (Figure 22). Whereas, the resistance pattern ACKSSuSxtT represented E4 and X4 patterns of RFLP profile (Figure 23). While all of isolates exhibited resistance pattern AApCGNSSuSxtT gave E3 and X3 RFLP patterns (Figure 24).

RFLP-PCR is considered as a rapid, cost-effective approach with good reproducibility among various bacterial molecular typing methods in epidemiologic studies. According to this RFLP-PCR analysis results, classification among these *Salmonella* strains could be achieved as pre-serotyping scheme. However, the conventional serotyping method was still performed in order to exactly identify of *Salmonella* serotypes. To confirm the accuracy and discriminatory of this RFLP-PCR analysis, all 30 *Salmonella* strains were submitted to serotype at WHO National *Salmonella* and *Shigella* Center Laboratory. The serotyping result showed that all of 10 *Salmonella* isolates exhibited AGSSuT resistance pattern were *S.* Corvallis. Each of 10 isolates of *Salmonella* strains, which exhibited ACKSSuSxtT and AApC-

GNSSuSxtT were *S.* Rissen and *S.* 1,4,5,12:i:- respectively (Table 11). This result also confirmed with RFLP analysis of *fliC* and *fljB* genes that was considered as a powerful approach in epidemiologic studies.

In PCR amplification of *fliC* and *fljB* genes, the smaller size (1.24 kbp) of *fliC* fragment from 10 isolates of *S.* Corvallis can presumably be attributable to a deletion of 261 bp in the flagellin C gene as found in *S.* Typhi (Frankel *et al.*, 1989). Besides, the fact that no *fljB* gene could be amplified from all 30 *Salmonella* isolates in this study is in agreement with the previous studies which showed some serotypes do not possess the *fljB* gene (Dauga *et al.*, 1998). No differences in RFLP patterns for *Salmonella* serotypes Corvallis and Rissen between this study and Jong *et al.* (2005) were detected, suggesting the good reproducibility of this RFLP system.

4.3 Molecular epidemiology of *Salmonella* strains

The classification of *Salmonella* strains into specific serotypes is essential to the epidemiologic studies and tracing the source of salmonellosis outbreaks. In order to understand the epidemiology, therefore, the combination of many techniques such as serotyping, resistance phenotype and molecular techniques probably provide more powerful epidemiology data. Particularly, the purpose for identify specific strains and outbreak of salmonellosis (Liebana, 2002). Hence, in this study, serotyping, resistance phenotype characterization, plasmid profile analysis and applied RFLP-PCR analysis of *fliC* and *fljB* genes were demonstrated to classify among these 30 MDR *Salmonella* strains.

4.3.1 Comparison of resistance and RFLP patterns

According to a combination of antimicrobial resistance and RFLP-PCR patterns reflected serotypes of the 30 MDR strains in Farm A. Therefore, this information capable used as preliminary assessment for epidemiologic study of these isolates. As a result was shown in Table 12, the isolates showing AGSSuT and RFLP

profile E1 and X1 patterns distributed in variety sources of pigs and environmental samples including dam, suckling period and lizard. Similarly, the isolates belonging to ACKSSuSxtT and RFLP profile E4 and X4 patterns also isolated from dam, nursing and fattening pigs. Moreover, the isolates representing AApCGNSSuSxtT and E3 and X3 patterns propagated in suckling period, nursing, fattening, feed and water. These data suggested the particular strain of *Salmonella* that yielded specific resistance and RFLP pattern, distributed among different source of pigs in Farm A.

Although, RFLP analysis was unable to differentiate some sero-types from one another (Jong *et al.*, 2005). However, this technique was confirmed as an easier and rapid alternative molecular marker for serotyping *Salmonella* isolates, especially, in large number of samples. Particularly, in non-motile *Salmonella* serotype Gallinarum could not differentiate biotype Gallinarum from Pullorum by serotyping. However, these strains were successfully differentiated by RFLP-PCR of *fliC* gene (Kwon *et al.*, 2000). Alternatively, the traditional serotyping is still required to confirm as a standard method. The serotyping revealed all 10 *Salmonella* isolates exhibited AGSSuT resistance pattern were *S.* Corvallis. Each of 10 isolates of *Salmonella* strains exhibiting ACKSSuSxtT and AApCGNSSuSxtT resistance patterns were *S.* Rissen and *S.* 1,4,5,12:i:- respectively. Therefore, the combination of the conventional serotyping with molecular screening approach will offer more accurate for shorter time and with less effort in epidemiologic study.

4.3.2 Epidemiology of *Salmonella* strains in Farm A

Accordingly, the combination of resistance profile, RFLP pattern and serotyping in this study, revealed *S.* Corvallis, *S.* Rissen and *S.* 1,4,5,12:i:- had high incidence in Farm A. As showed in Table 11 and 12, *S.* Corvallis isolates showing AGSSuT resistance pattern distributed in variety sources of pigs and environmental samples including dam, suckling period and lizard. Similarly, *S.* Rissen isolates belonging to ACKSSuSxtT resistance pattern also spread into dam, nursing and fattening pigs. Likewise, *S.* 1,4,5,12:i:- isolates representing resistance pattern AApCGNSSuSxtT propagated in suckling period, nursing, fattening, feed and

water. These data indicated that the particular *Salmonella* strain yielded specific resistance pattern and also distributed among different source of pigs. These results suspected the possible tracing back of original source of infection of these enteric pathogens among pigs in Farm A. Thus, in order to prove the doubtful point, further study would be performed. In this study, plasmid profile among these strains was also examined.

As a result of plasmid profile analysis (Table 11), all of *S.* Corvallis isolates representing AGSSuT resistance pattern, plasmid profiles could be divided into patterns M1 and M2. All *S.* Rissen isolates showing resistance pattern ACKSSuSxtT could be classified plasmid profiles into two patterns, N1 and N2. Also, all *S.* 1,4,5,12:i:- isolates exhibiting resistance pattern AApCGNSSuSxtT yielded plasmid profiles P1 and P2 patterns. These results suggested *Salmonella* isolates in this study, which were identical serotype could be further differentiated by plasmid profile analysis. Moreover, the identical plasmid profiles of particular strain showing specific resistance pattern probably assumed as the same clones of the isolates. These information possibly use in tracing back to the source of *Salmonella* infection among pig samples, nevertheless, this data was an inadequate conclusion. Other discriminatory molecular approach including PFGE, IRS-PCR (infrequent-restriction-site PCR) (Su *et al.*, 2002), RFLP and AFLP (amplified fragment length polymorphism) (Liebana, 2002) would be further performed, in order to classify into specific subtype.