CONCLUSION

In this study, the total *Salmonella* strains isolated from pigs, which derived from five farms (A, B, C, D and E) in Thailand conferred resistance to several classes of antimicrobial agents. Of 230 *Salmonella* isolates, 211 isolates (91.74%) presented dramatically resistance phenotype as MDR strains. The resistance patterns ASuT, ASSuT, AGSSuT, ACKNSSuT, ACKSSuSxtT and AApCGNSSuSxtT predominated among these MDR strains. The emergence of these MDR *Salmonella* strains probably resulted from the use of antimicrobials in pig production in order to promote growth and prevent from bacterial infection.

The detection of class 1 integrons and gene cassettes among 211 MDR strains revealed that only four isolates contained class 1 integron carrying *aadA* gene cassettes, which conferred resistance to streptomycin and spectinomycin. Class 1 integrons of *S.* Stanley CC1 strain harbored *aadA1* gene cassette, while *S.* Panama CB2 and CB3 strains contained *aadA4* gene cassette. The *aadA2* gene cassette existed in *S.* Anatum EC3 strain. These information suggesting that the frequently occurrence of *aadA* gene cassettes probably resulted from the selective pressure of resistance strains. Although, streptomycin is not currently used as therapeutic antimicrobial agent, however, these strains containing class 1 integrons could potentially driven the occurrence of the new resistance gene cassettes.

Moreover, resistance genes transferring by plasmid conjugation of 30 MDR isolates of Farm A included *S.* Corvallis representing resistance patterns AGSSuT (10 isolates), *S.* Rissen showing ACKSSuSxtT resistance pattern (10 isolates) and *S.* 1,4,5,12:i:- exhibiting resistance pattern AApCGNSSuSxtT (10 isolates), were examined. All isolates *S.* Corvallis and *S.* Rissen with resistance pattern AGSSuT and ACKSSuSxtT respectively, could transfer resistance genes to *E. coli* transconjugants via conjugative plasmid approximately 54 kbp and >54 kbp respectively. Whereas, *S.* 1,4,5,12:i:- showing resistance pattern AApCGNSSuSxtT could not transfer resistance genes by conjugative plasmid. Furthermore, resistance genes transferring in *S.* Stanley CC1 strain containing class 1 integron revealed the large conjugative plasmid

size >54 kbp could also transfer resistance genes to *E. coli*. That conjugative plasmid was confirmed the existing of class 1 integron carrying *aadA1* gene cassette. These results indicated that the conjugative plasmids mediated resistance phenotype and could disseminate resistance genes among these MDR strains. The existing of class 1 integron on conjugative plasmid probably also play an important role in acquisition and widespread of antimicrobial resistance profiles. Prominently, the conjugative plasmids could also disseminate between different genus of enteric bacteria.

Plasmid profile and RFLP-PCR analysis among 30 MDR strains represented diversity of these isolates. *Salmonella* isolates showing particular antimicrobial resistance pattern yielded the respective RFLP pattern. In contrast, the isolates exhibiting different resistance patterns gave different RFLP profiles. Additionally, these isolates were confirmed serotypes by serotyping and the results showed that *Salmonella* isolates exhibited particular resistance patterns including AGSSuT, ACKSSuSxtT and AApCGNSSuSxtT were *S.* Corvallis, *S.* Rissen and *S.* 1,4,5,12:i:- respectively. Furthermore, each of the serotypes showed correlation to specific RFLP patterns. These results suggested RFLP of flagellin genes in this study reflected the serotypes of the isolates and was considered as an alternative molecular markers scheme for pre-serotyping. Furthermore, the combination of the conventional serotyping with molecular screening approach will offer more accurate in epidemiologic study.

The combination of resistance profile, RFLP pattern and serotyping in this study, revealed *S.* Corvallis, *S.* Rissen and *S.* 1,4,5,12:i:- were endemic in Farm A. The particular *Salmonella* strain yielded specific resistance pattern and also distributed among different sources of pigs. However, *Salmonella* isolates in this study, which were identical serotype could be further differentiated by plasmid profile analysis. These information possibly use in tracing back to the source of *Salmonella* outbreaks among pig samples. Nevertheless, this data was an inadequate conclusion, other discriminatory powerful molecular approach such as PFGE, IRS-PCR, RFLP and AFLP would be further performed, in order to trace back the original source of *Salmonella* infection in this farm.

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APPENDIXES

APPENDIX A

Media preparation

1. Luria-Bertani (LB) broth (1 L)

- Tryptone	10.0	g
- Yeast extract	5.0	g
- NaCl	5.0	g
- Dissolve in DW	950.0	ml

Shake until the solutes have dissolved then adjust pH to 7.0 by adding 5 N NaOH (~0.2 ml). Adjust the volume of the solution to 1 L. The media is sterilized by autoclaving at 121°C, 15 lb/inch^2 for 15 minutes.

2. LB agar (1 L)

- Tryptone	10.0	g
- Yeast extract	5.0	g
- NaCl	5.0	g
- Agar	15.0	g
- Dissolve in DW	950.0	ml

The agar should be completely dissolved by boiling then adjust pH to 7.0 by adding 5 N NaOH (~0.2 ml). Adjust the volume of the solution to 1 L. The media is sterilized by autoclaving at 121°C, 15 lb/inch^2 for 15 minutes.

3. MacConkey agar (1 L) (Merck, Germany)

- MacConkey agar	50.0	g
- Dissolve in DW	1.0	L

Before sterilization, the agar should be completely dissolved by boiling. The media is sterilized by autoclaving at 121°C, 15 lb/inch^2 for 15 minutes.

4. <u>Xylose-lysine-deoxycholate (XLD) agar (1 L)</u> (Merck, Germany)

- XLD agar 55.0 g
- Dissolve in DW 1.0 L

Before sterilization, the agar should be completely dissolved by boiling. There is no need to sterilize the media by autoclaving at 121°C, 15 lb/inch^2 for 15 minutes.

5. <u>Mueller-Hinton broth (1 L)</u> (Hardy Diagnostics, USA)

- Mueller-Hinton 21.0 g
- Dissolve in DW 1.0 L

Before sterilization, the agar should be completely dissolved by boiling. The media is sterilized by autoclaving at 121°C, 15 lb/inch^2 for 15 minutes.

6. <u>Mueller-Hinton agar (1 L)</u> (Merck, Germany)

- Mueller-Hinton agar 34.0 g
- Dissolve in DW 1.0 L

Before sterilization, the agar should be completely dissolved by boiling. The media is sterilized by autoclaving at 121°C, 15 lb/inch^2 for 15 minutes.

7. <u>Skim milk (1 L)</u> (Oxoid, England)

- Skim milk powder (10%) 100.0 g
- Dissolve in DW 1.0 L

The media is sterilized by autoclaving at 121°C, 15 lb/inch^2 for 5 minutes.

APPENDIX B

Stock solutions preparation

1. 1 M Tris-HCl, pH 8.0 (1 L)

- Tris-base 121.0 g
- Dissolve in DW 800.0 ml
- Adjust pH to 8.0 by adding conc. HCl (~42 ml)

Allow the solution to room temperature before making the final adjustments to the pH 8.0. Adjust volume to 1 L with DW and sterilize by autoclaving at 121°C, 15 $lb/inch^2$ for 15 minutes.

2. 0.5 M Na-EDTA, pH 8.0 (1 L)

- Na_2-EDTA.$2H_2O$ 186.0 g
- Dissolve in DW 800.0 ml
- Adjust pH to 8.0 by adding NaOH pellets (~20 g)

Adjust volume to 1 L with DW and sterilize by autoclaving at 121°C, 15 $lb/inch^2$ for 15 minutes.

3. 10% Sodium dodecyl sulphate (SDS) (1 L)

- SDS 100.0 g
- Dissolve in DW 900.0 ml

The solution is heat to 68°C in order to dissolve and then adjust the pH to 7.2 by adding a few drops of conc. HCl. Adjust volume to 1 L and there is no need to sterilize the solution.

4. 10 N Sodium hydroxide (NaOH) (1 L)

- NaOH 400.0 g
- Dissolve in DW 1.0 L

There is no need to sterilize the solution.

5. 3 M Sodium acetate, pH 4.6 (1 L)

- Sodium acetate.$3H_2O$ 408.0 g
- Dissolve in DW 800.0 ml
- Adjust pH to 4.6 by adding glacial acetic acid

Adjust volume to 1 L with DW and sterilize by autoclaving at 121°C, 15 lb/inch^2 for 15 minutes.

6. 5 M Potassium acetate (1 L)

- Potassium acetate 490.0 g
- Dissolve in DW 800.0 ml

Adjust volume to 1 L with DW and sterilize by autoclaving at 121°C, 15 lb/inch^2 for 15 minutes.

7. 50X Gel electrophoresis buffer (500 ml)

7.1 0.5 M Na-EDTA (50 ml)

- Na_2-EDTA.$2H_2O$ 9.3 g
- Dissolve in DW 40.0 ml

Adjust volume to 50 ml with DW in order to further prepare 50X TAE buffer.

7.2 50X Tris-Acetate EDTA (TAE) buffer, pH 8.0

- Tris-base 121.0 g
- Glacial acetic acid 28.5 ml
- 0.5 M Na-EDTA 50.0 ml
- Dissolve in DW 300.0 ml
- Adjust pH to 8.0 by adding NaOH

Adjust volume to 500 ml with DW and sterilize by autoclaving at 121°C, 15 lb/inch^2 for 15 minutes.

8. 10 mg/ml Ethidium bromide (100 ml)

- Ethidium bromide 0.2 g
- Dissolve in DW 20.0 ml

Mix well and store at 4°C in dark.

Caution: Ethidium bromide is mutagen and must be handled carefully.

9. 100 mg/ml Ampicillin (10 ml)

- Ampicillin 1.0 g
- Dissolve in sterile DW 10.0 ml

The solution is sterilized by filtration then aliquot to sterile microcentrifuge tube and store at -20°C.

10. 30 mg/ml Nalidixic acid (10 ml)

- Nalidixic acid 0.3 g
- Dissolve in sterile DW 10.0 ml
- Adjust pH to 11 by adding NaOH to dissolve

The solution is sterilized by filtration then aliquot to sterile microcentrifuge tube and store at -20°C.

APPENDIX C

Reagents preparation

1. Tris-EDTA buffer (TE buffer), pH 8.0

- 10 mM Tris-HCl, pH 8.0
- 1 mM Na-EDTA, pH 8.0

2. Solution I

- 25 mM Tris-HCl, pH 8.0
- 10 mM EDTA, pH 8.0
- 50 mM glucose

3. Solution II

- 0.2 N NaOH
- 1% SDS

Prepare fresh from stock solutions of 10 N NaOH and 10% SDS.

4. Solution III (100 ml)

- 5 M potassium acetate	60.0	ml
- Glacial acetic acid	11.5	ml
- Sterile DW	28.5	ml

APPENDIX D

Inhibition zone of *Salmonella* donors and transconjugants

Appendix Table D1 Inhibition zone of *S.* Corvallis donors represented AGSSuT resistance pattern and their transconjugants using disc diffusion method.

Donor strain[a] / Transconjugant[b]	Antimicrobial agent[c] and inhibition zone (mm)					
	A	G	N	S	Su	T
AC11	**0**	**0**	**20**	**0**	**0**	**0**
T11.1	0	12	10	24	30	25
T11.2	0	11	9	24	29	26
T11.3	0	11	9	24	30	26
AC23	**0**	**0**	**19**	**0**	**0**	**0**
T23.1	0	12	10	24	30	26
T23.2	0	12	10	23	30	26
T23.3	0	12	10	24	29	27
AC26	**0**	**0**	**19**	**0**	**0**	**0**
T26.1	0	11	9	24	31	27
T26.2	0	12	10	25	30	26
T26.3	0	11	10	24	29	26
AC30	**0**	**0**	**20**	**0**	**0**	**0**
T30.1	0	11	10	24	29	27
T30.2	0	11	10	24	30	26
T30.3	0	11	10	25	29	27
AC74	**0**	**0**	**19**	**0**	**0**	**0**
T74.1	0	11	9	24	30	27
T74.2	0	11	10	24	29	27
T74.3	0	12	9	24	29	27
AC119	**0**	**0**	**21**	**0**	**0**	**0**
T119.1	0	12	10	23	29	25
T119.2	0	12	10	24	31	26
T119.3	0	12	10	24	30	26
AC125	**0**	**0**	**19**	**0**	**0**	**0**
T125.1	0	12	9	25	30	27
T125.2	0	12	10	24	29	26
T125.3	0	11	10	24	29	26
AC135	**0**	**0**	**19**	**0**	**0**	**0**
T135.1	0	12	9	24	30	26
T135.2	0	11	9	24	30	27
T135.3	0	12	10	23	31	26
AC149	**0**	**0**	**19**	**0**	**0**	**0**
T149.1	0	12	9	24	30	26
T149.2	0	12	10	24	30	26
T149.3	0	12	9	24	30	25
AC151	**0**	**0**	**19**	**0**	**0**	**0**
T151.1	0	12	10	25	30	26
T151.2	0	11	10	25	30	26
T151.3	0	11	10	24	31	27

Note: [a] Isolates name: 1st letter A = *Salmonella* isolated from pigs of Farm A, 2nd letter C = *Salmonella* serogroup C, number = Lab number of the isolates.

[b] Transconjugants of each donor cells.

[c] A = ampicillin, G = gentamicin, N = nalidixic acid, S = streptomycin, T = tetracycline, Su = sulfamethoxazole.

Appendix Table D2 Inhibition zone of *S*. Rissen donors represented ACKSSuSxtT resistance pattern and their transconjugants using disc diffusion method.

Donor strain[a] / Transconjugant[b]	Antimicrobial agent[c] and inhibition zone (mm)							
	A	C	K	N	S	Su	Sxt	T
AC40	**0**	**7**	**0**	**25**	**9**	**0**	**0**	**0**
T40.1	0	9	0	9	19	0	0	28
T40.2	0	9	0	10	19	0	0	28
T40.3	0	9	0	10	19	0	0	27
AC41	**0**	**7**	**0**	**27**	**11**	**0**	**0**	**0**
T41.1	0	9	0	9	18	0	0	28
T41.2	0	9	0	10	18	0	0	27
T41.3	0	10	0	10	19	0	0	28
AC46	**0**	**7**	**0**	**24**	**9**	**0**	**0**	**8**
T46.1	0	8	0	10	19	0	0	27
T46.2	0	9	0	9	19	0	0	28
T46.3	0	9	0	10	19	0	0	28
AC49	**0**	**7**	**0**	**23**	**9**	**0**	**0**	**0**
T49.1	0	9	0	10	18	0	0	27
T49.2	0	9	0	10	19	0	0	28
T49.3	0	10	0	10	19	0	0	27
AC50	**0**	**7**	**0**	**24**	**9**	**0**	**0**	**8**
T50.1	0	9	0	10	19	0	0	27
T50.2	0	9	0	10	19	0	0	27
T50.3	0	9	0	9	18	0	0	28
AC69	**0**	**7**	**0**	**25**	**10**	**0**	**0**	**8**
T69.1	0	9	0	10	18	0	0	28
T69.2	0	10	0	10	19	0	0	28
T69.3	0	9	0	10	18	0	0	28
AC80	**0**	**7**	**0**	**23**	**10**	**0**	**0**	**8**
T80.1	0	9	0	9	19	0	0	27
T80.2	0	9	0	10	19	0	0	28
T80.3	0	9	0	10	19	0	0	28
AC105	**0**	**7**	**0**	**23**	**0**	**0**	**0**	**0**
T105.1	0	8	0	10	19	0	0	27
T105.2	0	9	0	10	18	0	0	27
T105.3	0	9	0	10	18	0	0	27
AC106	**0**	**7**	**0**	**22**	**0**	**0**	**0**	**0**
T106.1	0	8	0	9	19	0	0	28
T106.2	0	9	0	9	19	0	0	28
T106.3	0	9	0	10	19	0	0	27
AC108	**0**	**7**	**0**	**23**	**0**	**0**	**0**	**0**
T108.1	0	9	0	9	18	0	0	28
T108.2	0	10	0	9	19	0	0	27
T108.3	0	9	0	10	19	0	0	28

Note: [a] Isolates name: 1st letter A = *Salmonella* isolated from pigs of Farm A, 2nd letter C = *Salmonella* serogroup C, number = Lab number of the isolates.

[b] Transconjugants of each donor cells.

[c] A = ampicillin, C = chloramphenicol, K = kanamycin, N = nalidixic acid, S = streptomycin, T = tetracycline, Su = sulfamethoxazole and Sxt = sulfamethoxazole/ trimethoprim.

Appendix Table D3 Inhibition zone of *S*. Stanley CC1 donor represented ACGSSuSxtT resistance pattern and their transconjugants using disc diffusion method.

Donor strain[a] / Transconjugant[b]	Antimicrobial agent[c] and inhibition zone (mm)							
	A	C	G	N	S	Su	Sxt	T
S. Stanley CC1	**0**	**9**	**11**	**26**	**0**	**0**	**0**	**9**
TCC1.1	0	9	15	11	10	0	27	11
TCC1.2	0	9	15	11	10	0	28	11
TCC1.3	0	9	14	11	10	0	27	11
TCC1.4	0	9	15	11	10	0	28	11

Note: [a] Isolates name: 1st letter C = *Salmonella* isolated from pigs of Farm C, 2nd letter C = *Salmonella* serogroup C, number = Lab number of the isolates.

[b] Transconjugants of each donor cells.

[c] A = ampicillin, C = chloramphenicol, G = gentamicin, N = nalidixic acid, S = streptomycin, T = tetracycline, Su = sulfamethoxazole and Sxt = sulfamethoxazole/trimethoprim.