CHAPTER 2

SERODIVERSITY AND ANTIMICROBIAL RESISTANCE PROFILES OF DETECTED *Salmonella* ON SWINE PRODUCTION CHAIN IN CHIANG MAI AND LAMPHUN, THAILAND

2.1 Abstract

Foodborne illness is growing public health problem worldwide. *Salmonella* is recognized as a major cause of this problem. *Salmonella* serotyping is a phenotypic characteristics which provided useful epidemiological markers for primary discrimination. The emergence and spread of antimicrobial resistant of this pathogen have become a major public health concern. The objectives of this study were to determine *Salmonella* serotypes, and antimicrobial resistance profiles on swine production chain (Farm-to-slaughtering process) in Chiang Mai and Lamphun, Thailand. A total of 300 *Salmonella* strains were randomly selected from isolates recovered in 2011-2013 in Chiang Mai and Lamphun, Thailand, including the isolates from fecal, lymph node, pest and environmental samples. Serotyping and antimicrobial susceptibility testing was performed by WHO National *Salmonella* and Shigella center (Thailand). *Salmonella* isolates were serotyped by agglutination tests with antisera (S&A Reagents Lab Ltd., Thailand) on the basis of somatic-O, as well as phase 1 and phase 2 flagellar antigens according to the Kauffmanne-White scheme. In addition, all serotyping *Salmonella* isolates were detected for antimicrobial susceptibility testing by using the disk diffusion method of the Clinical and Laboratory Standard Institute. Twenty-one *Salmonella* serotypes were detected in both farms and slaughterhouses. *Salmonella* Rissen is the highest frequency found in both farms and slaughterhouses (30.7% and 38.0%, respectively). Thirty three antimicrobial resistance patterns were demonstrated. There were including 10 common patterns isolated from pig farms and slaughterhouses. The highest frequency antimicrobial resistant pattern was "AMP, S, TE" (47 isolates, 15.7%) followed by "AMP, SXT, C, S, TE" (44 isolates, 14.7%) and

"AMP, SXT, S, TE" (36 isolates, 12.0%). Considering in each antibiotic agent, the highest frequency found was ampicillin (83.33%) followed by tetracycline (75.67%) and streptomycin (64%). The resistance of ciprofloxacin and norfloxacin of *Salmonella* isolates were not observed. There were no statistical differences in numbers of *Salmonella* found in different sources in each antimicrobial agent except cefotaxime and sulfamethoxazole-trimethoprim. Finally, ciprofloxacin and norfloxacin resistant strains were not found in both farms and slaughterhouses. Resistance to amoxicillin- clavulanic acid was not observed in *Salmonella* strains isolated from slaughterhouses. *Salmonella* Rissen was also the majority serotypes in this region. Contrasting with the study in Germany, *S*. Rissen was found few in pig farms and slaughterhouses. Almost 90% of *Salmonella* spp. tested were resisted to antimicrobial at least 1 drug and 72% were multi drug resistance. Even though, we could not conclude the contamination from farms to slaughterhouses linked to the common source, but this study indicated that antimicrobial resistance- *Salmonella* can contaminate any steps of pig production line. Good practices and hygiene should be implemented to minimize this problem. Nevertheless, molecular epidemiology could further confirm the linkage of the contamination. The studies of antimicrobial resistance gene and molecular epidemiology should be performed.

2.2 Introduction

Foodborne illness is growing public health problem worldwide [5] *Salmonella* are recognized as a major cause of this problem [40,41] . More than 2500 *Salmonella* serotypes have been identified to date [13]. Pork is determined to be the important reservoir of salmonellosis in humans [15]. In farm levels, sub-clinically pigs may excrete *Salmonella* in their feces or keep the bacteria in their digestive tract, the closely associated lymph nodes or tonsils [2]. The pork was contaminated with this pathogen from dirty sites by improper slaughtering processes [42].

Salmonella serotyping is a phenotypic characteristics providing useful epidemiological markers for primary discrimination [26]. The majority *Salmonella* serotypes are different in various regions. *Salmonella* Enteritidis is the most common in the United

States [43] while *Salmonella* Rissen is the most common found in pork in northern region of Thailand [44].

Furthermore, the emergence and spread of antimicrobial resistant pathogens have become a major public health concern [45]. *Salmonella* developed resistance to the various type of antibiotic [14]. Excessive and incorrect using of antibiotic in pre-harvest level are probably a primary cause of increasing bacterial resistance [15,16]. Improper storage condition at the end of slaughtering-process was also considered as sub-lethal stress factor, enhancing resistant ability of the organism. The objectives of this study were to determine *Salmonella* serodiversity and antimicrobial resistance profiles of 10 antimicrobial drugs of *Salmonella* isolated from swine production chain in Chiang Mai and Lamphun, Thailand in 2011-2013.

2.3 Materials and methods

2.3.1 Sampling

A total of 300 *Salmonella* isolates, recovered in Chiang Mai and Lamphun provinces were tested (150 from pig's farms and 150 from pig's slaughterhouses), these were included strains isolated from feces, lymphnode, pest and environmental samples, financially by the National Science and Technology Development Agency (NSTDA) Project ID: P-10-10409 and P-11-00792

2.3.2 Serotyping and Antimicrobial susceptibility testing

Three hundred *Salmonella* isolates were serotyped by agglutination tests with antisera 1 on the basis of somatic-O, as well as phase 1 and phase 2 flagellar antigens according to the Kauffmanne-White scheme. In addition, all serotyping *Salmonella* isolates were detected for antimicrobial susceptibility testing by using the disk diffusion method of the Clinical and Laboratory Standard Institute. Ten antimicrobial agents were determined including ampicillin (AMP) 10 μg, amoxicillin-clavulanic acid (AUG) 30 μg, sulfamethoxazole-trimethoprim (SXT) 25 μg, ciprofloxacin (CIP) 5 μg, chloramphenicol (C) 30 μg, streptomycin (S) 30 μg, nalidixic acid (NA) 30 μg, norfloxacin (NOR) 10 μg, cefotaxime (CTX) 30 μg and tetracycline (TE) 30 μg. If

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isolates showed intermediate resistance, they were grouped with the susceptible isolates to avoid overestimation of resistance. Serotyping and antimicrobial susceptibility testing in this study were performed by WHO National *Salmonella* and Shigella Center Laboratory (NSSC), Nonthaburi, Thailand.

2.3.3 Statistical analysis

The data were collected and analyzed for descriptive statistical analysis by Epi Info™72. Fisher's test was also used to compare the proportion of antimicrobial resistance among *Salmonella* isolates from pig farms and slaughterhouse by the computer program mentioned above. A P < 0.05 value was considered statistically significant.

2.4 Results

From 300 *Salmonella* isolates which were randomly selected from a pig production line in Chiang Mai and Lumphun recovered in 2011- 2013, the serodiversity was demonstrated in figure 2.1. Twenty-one *Salmonella* serotypes were detected in both farms and slaughterhouses. Nineteen serotypes were observed for farm investigations. *Salmonella* Rissen is the highest frequency found (46 isolates, 30.7%) followed by S.I.4,5,12:i:- (25 isolates, 16.7%) and *S*. Typhimurium (19 isolates, 12.7%), respectively. Twelve serotypes were observed in slaughterhouses. *Salmonella* Rissen was also the highest frequency found (57 isolates, 38.0%) followed by S. Panama (36 isolates, 24.0%) and S. Stanley (22 isolates, 14.7%), respectively.

No. of isolates
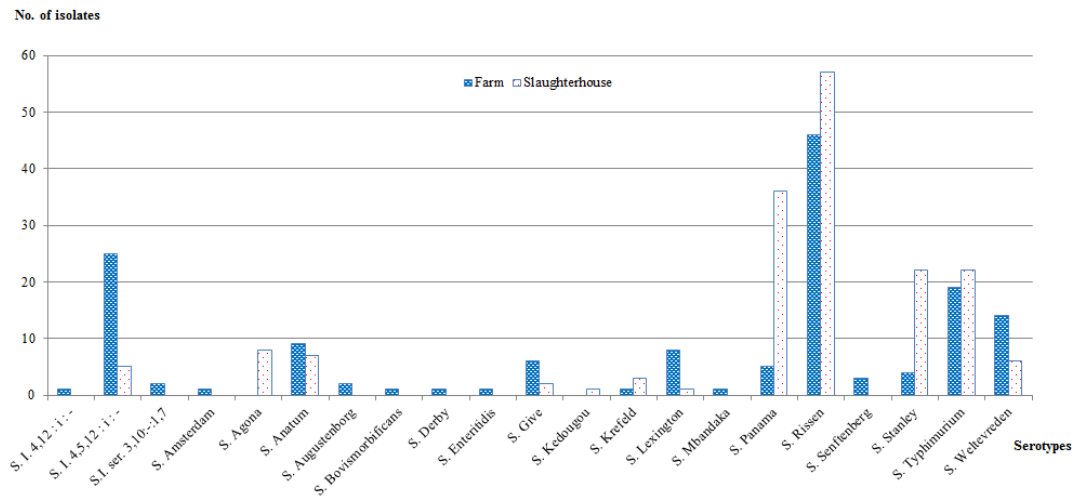
Figure 2.1 Distribution of *Salmonella* Serotypes among farms and slaughterhouse in Chiang Mai - Lamphun, Thailand, 2011-2012

Thirty three antimicrobial resistance patterns were demonstrated. There were including 10 common patterns isolated from pig farms and slaughterhouses. The highest frequency pattern was "AMP, S, TE" (47 isolates, 15.7%) follow by "AMP, SXT, C, S, TE" (44 isolates, 14.7%) and "AMP, SXT, S, TE" (36 isolates, 12.0%). While 14 and 9 antimicrobial resistance patterns were only observe in *Salmonella* isolates from pig farms and slaughterhouses, respectively (Table 2.1), and 72.3% (217/300) of all isolates were resistant up to 3 antimicrobials, they would be identified as multidrug-resistant (MDR). Figure 2.2 demonstrated a result of individual antimicrobial resistance against *Salmonella* isolates, the highest frequency of antibiotic resistance were ampicillin (250 isolates, 83.3%) follows by tetracycline (227 isolates, 75.7%) and streptomycin (192 isolates, 64.0%), which were related to the results of antimicrobial patterns found in this study. Interestingly, resistance of amoxicillin-clavulanic acid was observed only in slaughterhouse, and the resistance of ciprofloxacin and norfloxacin were not observed in this study. In addition, considering in antimicrobial resistance from *Salmonella*'s sources (between farms and slaughterhouses), there were no statistical differences in numbers of *Salmonella* found in differences source in each antimicrobials. Except cefotaxime and sulfame-thoxazole-trimethoprim, the statistical difference was detected (P < 0.05). Indicating the antimicrobial resistance ability between *Salmonella* isolates in

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difference source was not similar to these 2 antimicrobials. Finally, resistant to ciprofloxacin and norfloxacin were not found in both farms and slaughterhouses. And resistant to amoxicillin-clavulanic acid was not observed in a slaughterhouse. So the Fisher's test was therefore not possible (Table 2.2).
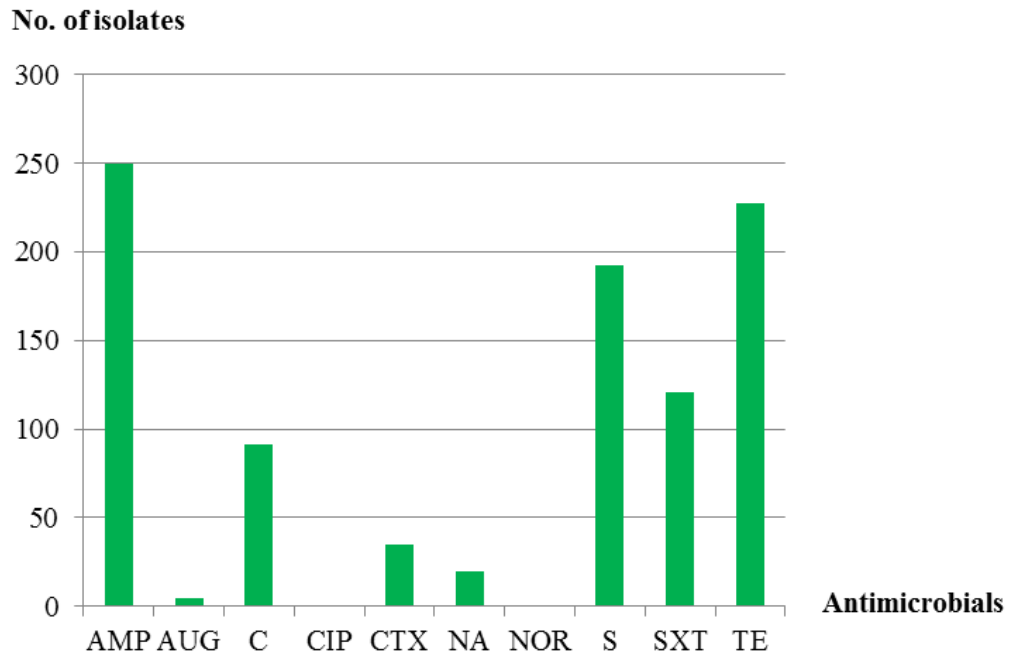
Figure 2.2 The number of antimicrobial resistance in *Salmonella* isolates (n=300) from pig production chain in Chiang Mai - Lamphun, Thailand, 2011-2012

Abbreviation for antimicrobial agents : AMP (Ampicillin); AUG (Amoxicillin-clavulanic acid); C (Chloramphenicol); CIP (Ciprofloxacin); CTX (Cefotaxime); NA (Nalidixic acid); NOR (Norfloxacin); S (Streptomycin); SXT (Sulfamethoxazole-Trimethoprim); TE (Tetracycline

Table 2.1 Distribution of antimicrobial resistance patterns from *Salmonella* isolates (n=300) in pig production chain in Chiang Mai - Lamphun, Thailand in 2011-2013

Resistant patterns	No. of *Salmonella*'s isolates			%
	Farm	Slaughterhouse	Total	
A. Common pattern in isolates from farms and slaughterhouse				
AMP[1],C[2],S[3],CTX[4],TE[5]	12	2	14	4.67
AMP,SXT[6],C,NA[7],TE	4	2	6	2.00
AMP,SXT,C,S,TE	6	38	44	14.67
AMP,SXT,S,TE	20	16	36	12.00
AMP,S,TE	34	13	47	15.67
AMP,SXT,S	1	2	3	1.00
AMP,SXT,TE	11	12	23	7.67
AMP,S	3	10	13	4.33
AMP,TE	2	14	16	5.33
All susceptible	22	14	36	12.00
Subtotal (A)	115	123	238	79.33
B. Pattern of isolates only observed in farms				
AMP,SXT,C,S,TE,CTX	1	-	1	0.33
AMP,SXT,C,S,NA,TE	3	-	3	1.00
AMP,CTX,NA,S,TE	1	-	1	0.33
AMP,SXT,S,CTX,TE	1	-	1	0.33
AMP,SXT,S,NA,TE	1	-	1	0.33
AMP,C,CTX,TE	1	-	1	0.33
AMP,CTX,S,TE	8	-	8	2.67
AMP,NA,S,TE	4	-	4	1.33
AMP,SXT,C,S	7	-	7	2.33
SXT,C,S,TE	1	-	1	0.33
AMP,C,S	2	-	2	0.67

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Table 2.1 (continued)

Resistant patterns	No. of *Salmonella*'s isolates			%
	Farm	Slaughterhouse	Total	
AMP,SXT,C	3	-	3	1.00
AMP,CTX	1	-	1	0.33
NA	1	-	1	0.33
Subtotal (B)	35	-	35	11.67
C. Pattern of isolates only observed in slaughterhouse				
AMP,AUG[8],C,S,NA,CTX,TE	-	5	5	1.67
AMP,C,S,CTX	-	1	1	0.33
AMP,C,S,TE	-	1	1	0.33
AMP,SXT,C,TE	-	3	3	1.00
SXT,S,TE	-	1	1	0.33
CTX,TE	-	1	1	0.33
SXT,S	-	1	1	0.33
AMP	-	5	5	1.67
TE	-	9	9	3.00
Subtotal (C)	-	27	27	9
Grantotal (A+B+C)	150	150	300	100.00

[1]Ampicillin; [2]Chloramphenicol; [3]Streptomycin; [4]Cefotaxime; [5]Tetracycline;
[6]Sulfamethoxazole-Trimethoprim; [7]Nalidixic acid; [6]Amoxicillin-clavulanic acid

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Table 2.2 Odds ratio and p-value of the antibicrobial resistance which difference source (farm vs slaughterhouse) in pig production line in Chiang Mai and Lamphun, Thailand in 2011-2013

Antibiotics	Source	No. of resistance isolates	Odds ratio	P-value
Ampicillin	Farm	126	1.10	0.87
	Slaughterhouse	124		
Amoxy-Clavulanic acid	Farm	0	-	-
	Slaughterhouse	5		
Choramphenicol	Farm	39	0.66	0.13
	Slaughterhouse	52		
Ciprofloxacin	Farm	-	-	-
	Slaughterhouse	-		
Cefotaxime	Farm	24	3.01	<0.01*
	Slaughterhouse	9		
Nalidixic acid	Farm	13	1.93	0.24
	Slaughterhouse	7		
Norfloxacin	Farm	-	-	-
	Slaughterhouse	-		
Streptomycin	Farm	102	1.41	0.18
	Slaughterhouse	90		
Sulfamethoxazole-Trimethoprim	Farm	46	0.44	<0.01*
	Slaughterhouse	75		
Tetracycline	Farm	110	0.77	0.41
	Slaughterhouse	117		

*Cefotaxime and sulfamethoxazole-trimethoprim, the statistical difference was detected ($P < 0.05$). Indicating the antimicrobial resistance ability between *Salmonella* isolates in difference source was not similar to these 2 antimicrobials.

2.5 Discussion

In this study, serodiversity of *Salmonella* in farm were higher than those found in slaughterhouses. Farms were opened ecosystem. The equipments were easy to contaminate from pest carrying *Salmonella* from other places. In contrast, slaughterhouses were nearly closed ecosystem. Cleaning program was directed in routine, the opportunity of contamination with other outside pathogens was lower than those found in farms. In addition, the number of target farms was twice as much as the slaughterhouses. Consequently, variety of *Salmonella* origin was different. Interestingly, *S*. Rissen was demonstrated as the major serotype. Similar to the survey on pre-slaughter pigs [46,47] and healthy human [44] in Thailand, indicated that S. Rissen was also the major serotypes in this region. Contrasting with the study in Germany from Visscher et al. [48], *S*. Rissen was found few in pig farms and slaughterhouses. Of the 300 tested *Salmonella*, almost all were resistant to at least one antimicrobial agent. The three-fourth of them was resistant to three or more antimicrobials. Ampicillin, tetracycline and streptomycin showed in high resistance rate considering in individual antimicrobial agent, and their patterns was closed to the report from Germany [14], Belgium [16] and Ireland [49]. Therefore, betalactam, aminoglycoside and tetracycline group were not recommended to salmonellosis treatment in pigs. The excessive or inadequate using in livestock to treatment or prophylactic are considered to be a key aspects of this current situation [50]. The absence of norfloxacin and ciproflxacin resistant strains were observed in this study was similar to the study in Sakaew, Thailand. [47]. It might be due to the limited use of these antimicrobial drugs in pig production in Thailand [51]. Moreover, amoxy clavulanic acid and cefotaxime resistance were shown in this study. Although, they were not often used on livestock, *Salmonella* may be harboured resistance genes which were transferred from other bacteria by horizontal gene transfer. *AmpC* beta-lactamases are clinically important cephalosporinases of many enterobacteriaceae and a few other organisms, where they mediate resistance to 1st-3rd generation of cephalosporins, most penicillins, and beta-lactamase inhibitor [52]. As well, extended-spectrum beta-lactamases (ESBLs) is enzymes recognizing a cause of resistance to 1st-4th generation of cephalosporins and aztreonam [53]. In addition, integrons which are mobile genetic elements play role on

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multidrug resistant [54]. The study of resistance gene, which were mentioned above will be investigated in further study.

Most of the resistance rate between *Salmonella* isolated from farms and slaughterhouses were not a statistical difference except cefotaxime and sulfamethoxazole-trimethoprim. In this study, the number of cefotaxime-resistant *Salmonella* in farms was higher than slaughterhouses. Most of them were found in Lamphun. Eighteen from twenty-four and eight from nine cefotaxime-resistant *Salmonella* were detected in this area from farms and slaughterhouses, respectively (data not shown). Lumphun might be a high density area of cephalosporin resistant *Salmonella*. In contrast, the number of sulfamethoxazole-trimethoprim-resistant *Salmonella* in farms was lower than slaughterhouses. The meat is a particularly suitable matrix for bacterial growth. Stress factor such as cooling or pH as well as other sub-lethal stress conditions in slaughterhouse could play the role to enhance the antimicrobial resistance ability of the organisms [14,17]. In contrast, considering in antimicrobials demonstrated in high resistance rate, ampicillin, tetracycline and streptomycin-resistant *Salmonella* were not quite different between farms and slaughterhouses. All of them were often used in livestock. Mutation of *Salmonella* to all of them might be occurred.

The same serotypes demonstrated similar antimicrobial resistance patterns were found in this study. These might be identified as the same strain. Phenotypic finding was provided only primary investigation. Phenotypic characterizations are not enough to conclude into the same strain. Finally, molecular epidemiology such as Pulse Field Gel Eletrophoresis and Multi Locus Sequence Typing should be conducted in further studies.

2.6 Conclusions

This study was carried out phenotype characteristic of *Salmonella* spp. which were isolated from farms and slaughterhouses in Chiang Mai and Lamphun, Thailand. Almost 90% of *Salmonella* spp. were resistant to at least 1 antimicrobial and 72% were multidrug resistance. Even though, we could not conclude that the contamination from

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farms to slaughterhouses linked to the common phenotypic-expression strains, this study indicated that antimicrobial resistance-Salmonella can be contaminated in any step of pig production line. Good practices and hygiene should be implemented to minimize this problem. Nevertheless, molecular epidemiology comparing the DNA fingerprint or gene sequencing of *Salmonella* serotypes could be further confirmed the linkage of the contamination. The studies of antimicrobial resistance gene and molecular epidemiology should be performed.

2.7 Acknowledgements

Manufacturers

[1]S & A Reagents Lab. Ltd. Lat Phrao, Bangkok, Thailand.
[2]Centers for Disease Control and Prevention (CDC). Atlanta, GA, USA.