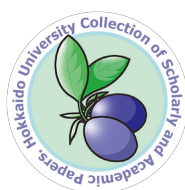




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Class 1 integrons characterization and multilocus sequence typing of *Salmonella* spp. from swine production chains in Chiang Mai and Lamphun provinces, Thailand

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Abstract

Pigs and pork products are well known as an important source of *Salmonella*, one of the major zoonotic foodborne pathogens. The emergence and spread of antimicrobial resistance is becoming a major public health concern worldwide. Integrons are genetic elements known to have a role in the acquisition and expression of genes conferring antibiotic resistance. This study focuses on the prevalence of class 1 integrons-carrying *Salmonella*, the genetic diversity of strains of those organisms obtained from swine production chains in Chiang Mai and Lamphun provinces, Thailand, using multilocus sequence typing (MLST) and comparison of genetic diversity of sequence types of *Salmonella* from this study with pulsotypes identified in previous study. In 175 *Salmonella* strains, the overall prevalence of class 1 integrons-carrying-*Salmonella* was 14%. The gene cassettes array pattern “*dfrA12-orfF-aadA2*” was the most frequently observed. Most of the antimicrobial resistance identified was not associated with related gene cassettes harbored by *Salmonella*. Six sequence types were generated from 30 randomly selected strains detected by MLST. *Salmonella* at the human-animal-environment interface was confirmed. Linkages both in the farm to slaughterhouse contamination route and the horizontal transmission of resistance genes were demonstrated. To reduce this problem, the use of antimicrobials in livestock should be controlled by veterinarians. Education and training of food handlers as well as promotion of safe methods of food consumption are important avenues for helping

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prevent foodborne illness.

Key words: *Salmonella*, Integrons, MLST, Swine production, Thailand

Introduction

Salmonella is an important zoonotic foodborne pathogen which is recognized as a major public health concern worldwide^{11,27}. The organism is usually found in the digestive tracts of animals such as birds and reptiles as well as mammals¹⁶. Humans can become infected by consuming products contaminated with *Salmonella* from the digestive tract contents of those animals⁴⁵. Foods obtained from farm animals are considered to be the main source of salmonellosis, especially pig products^{4,26}. Pig farms are notorious as the initial point of contamination with *Salmonella* in the production chain²⁹. The slaughtering process is acknowledged as another critical point that can result in the spread of the organism from pig guts to carcasses or other pig products^{36,41}.

Antimicrobial resistant pathogens are a serious and growing phenomenon in human and veterinary medicine, and have become a major public health concern²⁰. *Salmonella* has developed resistance to various types of antimicrobials, and multidrug resistant strains have been documented⁴⁰. Consequently, choices of treatment, including first-line treatment options, are limited⁴⁰. Moreover, antimicrobial resistant *Salmonella* may induce genetic mutations. In addition, acquisition of resistant genes from the same organism species or another organism species by horizontal gene transfer via mobile genetic elements such as integron gene cassettes, transposon gene and R-plasmid is possible^{17,43}. Class 1 integrons are genetic elements known for their role in the acquisition and expression of genes conferring antibiotic resistance²². The structures are linear double strands of DNA, which are arranged in the 5' variable region and the 3' conserved region. The integrase gene (*int*), *attI* and a promoter are located in the 5' variable

region. In addition, the quaternary compound resistant gene (*QacEΔ1*) and sulfonamide resistant gene (*SuII*) are located at the end of the 3' conserved region. Integrons can play a role in multidrug resistance as gene cassettes can be inserted into them by integrase^{13,22,31}.

Molecular techniques are essential for bacterial typing. They can provide the information needed to determine an infectious-disease transmission pattern, to allow tracking, as well as to distinguish between strains⁸. Pulsed field gel electrophoresis (PFGE) is considered the gold standard for *Salmonella* typing³⁷. However, PFGE has several limitations: the method is technically demanding, and time-consuming, specialist. In addition, data obtained are limited compared to sequence-based methods³⁰.

Multilocus sequence typing (MLST) may be more appropriate for investigation of evolutionary and population biology relationships. MLST is a molecular technique based on allelic differences in the nucleotide sequences of the housekeeping genes of various bacterial strains¹⁴. This method can be used to identify and evaluate interrelationships among *Salmonella* isolates as part of disease surveillance and outbreak investigations³⁹. This study focuses on the prevalence of class 1 integrons-carrying *Salmonella*, the genetic diversity of strains of those organisms obtained from swine production chains in Chiang Mai and Lamphun provinces, Thailand, using multilocus sequence typing (MLST) and comparison of genetic diversity of sequence types of *Salmonella* from this study with pulsotypes identified in previous study³⁷.

Materials and methods

Salmonella strains: One hundred and seventy-

Table 1. Number of *Salmonella* strains tested

<i>Salmonella</i> serotype	Number of <i>Salmonella</i> strain		
	Farm	Slaughterhouse	Total
<i>S.</i> I,4,5,12:i:-	12	5	17
<i>S.</i> Agona	0	2	2
<i>S.</i> Lexington	0	1	1
<i>S.</i> Panama	13	32	45
<i>S.</i> Rissen	27	31	58
<i>S.</i> Stanley	5	23	28
<i>S.</i> Typhimurium	10	2	12
<i>S.</i> Weltevreden	6	5	11
Total	73	102	175

One hundred and seventy-five *Salmonella* strains, initially identified as appropriate 8 serotypes were tested. These strains were recovered from pig farms ($n = 73$) and pig slaughterhouses ($n = 102$) Chiang Mai and Lamphun provinces, Thailand, during 2011–2013.

five *Salmonella* strains, initially identified as appropriate serotypes, were tested. Eight confirmed *Salmonella* serotypes, *Salmonella* group I. 4,5,12:i:-, *Salmonella* Agona, *Salmonella* Lexington, *Salmonella* Panama, *Salmonella* Rissen, *Salmonella* Stanley, *Salmonella* Typhimurium, and *Salmonella* Weltevreden were included in the study. These strains were recovered from pig farms ($n = 73$) and pig slaughterhouses ($n = 102$) Chiang Mai and Lamphun provinces, Thailand, during 2011–2013. The number of strains of each serotype is shown in Table 1.

Identification of class 1 integrons by polymerase chain reaction (PCR): A total of 175 *Salmonella* DNA samples were extracted by boiling in a suspension of 5% Chelex® 100 (Bio-Rad Laboratories, California) following the manufacturer's recommended protocol⁵⁾. All *Salmonella* DNA samples were screened for class 1 integrons by PCR using the Class 1 integrons primers (F: GGC ATC CAA GCA GCA AG and R: AAG CAG ACT TGA CCT GA). PCR amplification was done using a final volume of 20 µL containing 10X PCR buffer, 1.5 mM MgCl₂, 300 µM dNTP, 0.2 µM each of the forward and reverse primers, 1 µl of purified DNA and 0.5 U of *Taq* DNA Polymerase (Vivantis® Vivantis Technologies Sdn Bhd,

Malaysia). Cycling conditions consisted of a 5 min hold at 95°C, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 7 min. Products were separated by 1% agarose gel electrophoresis and visualized with ethidium bromide staining. The expected product size was variable, depending on the insertion of antimicrobial resistant gene cassettes²³⁾.

Sequencing of Class 1 integrons and their cassette genes: PCR products were submitted to an on-line DNA sequencing service (1st Base, Selangor, Malaysia) to purify and detect gene cassettes following the Sanger method using class 1 integrons forward and reverse primer, adding dideoxynucleotides (ddNTPs: ddATP, ddCTP, ddGTP, ddTTP) into the reaction. Sequencing of the amplicons was continued until the resistance genes were inserted by automated DNA sequencer. As a final step, sequences attained were BLAST analyzed via <http://www.ncbi.nih.gov> and compared with those registered in GenBank⁴⁴⁾.

Similarly, the results of the cassette gene arrays were compared with an antimicrobial resistance phenotype. All resistance phenotype profiles were acquired from data provided by the

National Science and Technology Development Agency (NSTDA) Project ID: P-10-10409 and P-11-00729⁶⁾. Ten different antimicrobial agents were interpreted by agar disk diffusion testing, including ampicillin (AMP) 10 µg, amoxicillin-clavulanic acid (AUG) 30 µg, sulfamethoxazole-trimethoprim (SXT) 23.75/1.25 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. The quality control organism, *Escherichia coli* ATCC 25922, was used to ensure that all antimicrobial agents were appropriately quality controlled⁹⁾.

MLST genotyping: Representatives of 30 *Salmonella* strains, including *Salmonella* group I. 4,5,12:i:-, *Salmonella* Panama, *Salmonella* Rissen, *Salmonella* Stanley, *Salmonella* Typhimurium, and *Salmonella* Weltevreden, were randomly selected for further MLST analysis. To explore the genetic diversity by MLST, seven housekeeping genes were chosen for MLST profiling: *aroC* (chorismate synthase), *dnaN* (DNA polymerase III beta subunit), *hemD* (uroporphyrinogen III cosynthase), *purE* (phosphoribosylaminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase), *hisD* (histidinol dehydrogenase) and *thrA* (aspartokinase I/homoserine dehydrogenase). Amplification protocols detailed in the database, including primer sequences and annealing temperatures, were followed in this study as specified in the MLST database website (<http://mlst.warwick.ac.uk/mlst/>). PCR amplification of seven housekeeping genes was done with a final volume of 20 µL containing 10X PCR buffer, 1.5 mM MgCl₂, 200 µM dNTP, 0.2 µM each of the forward and reverse primers, 1 µl of purified DNA and 1.25 U of *Taq* DNA Polymerase (Thermo Scientific® Thermo Fisher Scientific Inc.). PCR cycling conditions were a 2 min hold at 95°C, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min/kb, and a final extension at 72°C for 7 min. Products were separated by 1%

agarose gel electrophoresis and visualized with ethidium bromide staining. The PCR products were purified using GF-1 AmbiClean Kit Gel & PCR (Vivantis® Vivantis Technologies Sdn Bhd, Malaysia) and were sequenced using a housekeeping gene primer recommended by the MLST database website (<http://mlst.warwick.ac.uk/mlst/>).

The sequences were submitted to the MLST database website (<http://mlst.warwick.ac.uk/mlst/>) and assigned allele numbers and sequence type numbers defined by the database. A phylogenetic tree was created using Bionumerics 7.1 software (Applied Maths, Belgium).

Genetic diversity comparison among MLST and PFGE: Some of the *Salmonella* strains in this study were submitted sequence type for sequence type to compare the genetic diversity with existing pulsotype characteristic information about those strains from a previous study³⁷⁾ which had been obtained using pulse field gel electrophoresis. Simpson's index of diversity in MLST and in PFGE was calculated to determine the ability to differentiate between related and non-related organisms. Discriminatory power was measured using an online tool for quantitative assessment of classification agreement available at <http://darwin.phyloviz.net/ComparingPartitions/>. In addition, the adjusted Rand coefficient and the Wallace coefficient were analyzed to determine the concordance of the two typing techniques and the relative ability of the two techniques to predict directional information¹⁵⁾. Both concordance coefficients were calculated using the online tool mentioned above.

Results

Prevalence of class 1 integrons and associated gene cassettes

The overall prevalence of class 1 integrons-carrying *Salmonella* in tested samples was 13.71% (24/175). The prevalence in farm strains

and slaughterhouse strains were 9.58% (7/73) and 16.67% (17/102), respectively. Only 4 serotypes carried class 1 integron genes: *Salmonella* Rissen, Typhimurium, Stanley and Agona. The *dfrA12* gene was the most prevalent gene cassette detected in class 1 integrons-carrying *Salmonella* in this study (75.00%; 18/24), followed by *aadA1* gene (62.50%; 15/24). This finding was related to the gene cassette array pattern “*dfrA12-orfF-aadA2*” which was the most frequency found (62.50%; 15/24). Most of the integron-carrying *Salmonella* strains were resistant to at least two antimicrobial agents with the exception of “strain 26” which was susceptible to all antimicrobials. Regarding the concordance of resistant phenotypes and their concomitant gene cassettes, associations between sulfamethoxazole-trimethoprim and the *dfrA12* gene as well as between streptomycin and the *addA1* and *addA2* genes were observed in many strains. However, most antimicrobial resistance phenotypes were not associated with related gene cassettes harbored by *Salmonella* (Table 2.).

MLST genotyping

Multilocus sequence typing (MLST) generated profiles using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Thirty *Salmonella* strains were grouped into six sequence types: ST 469, 48, 1500, 34, 19 and 29. Most of the strains with similar sequence types belonged to the same serotype with the exception of ST 34, which was comprised of *S.* I,4,5,12:i:- and *S.* Typhimurium. In general, ST 469 was the predominant grouped in this study, involved with 10 *Salmonella* strains, followed by ST 34 (7 strains), and ST 48 (5 strains). The origins of 30 strains were verified. Common sequence type *Salmonella* strains with several phenotypic expressions were recovered from various sources (Fig. 1.).

Comparison of molecular typing methods between MLST and PFGE

Twenty *Salmonella* strains submitted in

sequence type in this study were selected for comparison of genetic diversity with existing PFGE characteristic information from the study by Tadee *et al.*, (2015)³⁷. Thirteen pulsotypes could be grouped into five sequence type profiles. Most of the strains with similar pulsotypes belonged to type ST with the exception of the “D9” pulsotype which was comprised of ST 19 and ST 34 (both classified as *S.* Typhimurium) (Table 4.). Comparison of the discriminatory power and concordance index of both techniques was carried out. The Simpson’s Diversity Index values of MLST and PFGE were 0.753 and 0.932, respectively. In addition, the Adjusted Rand coefficient was 0.253. The Wallace coefficient of PFGE to MLST was 0.769 and the Wallace coefficient of MLST to PFGE was 0.213 (Table 3.).

Discussion

The overall prevalence of class 1 integron-carrying *Salmonella* in swine production chains in Chiang Mai and Lamphun provinces of Thailand was found to be 14%. However, our study revealed a noticeably lower prevalence than the 46% prevalence of class 1 integron-carrying *Salmonella* isolated from pork and human in Chiang Mai Province in 2011⁴¹. The difference might be due to sample types, time period and location of sampling. The isolates from humans can be exposed to numerous types of antimicrobial agents. However, some antimicrobial agents are limited use in livestock. The occurrence of resistance gene expansion in organisms resulting from several selective pressures has been reported^{10,39}. Greater differences in prevalence have also been detected by studies conducted in other regions of the world. A prevalence of 53.1% was isolated from slaughtered animals and animal-origin food in Ethiopia²⁰, and 39.1% was reported in Egypt, isolated from meat and dairy products¹. The reason for the variance might be due to differences in sample type as well as the season and temperature at the time the samples

Table 2. Characteristics of class 1 integrons-carrying *Salmonella* isolated from swine production chains in Chiang Mai and Lamphun provinces, Thailand

Strain	Serotype	Location	Antimicrobial susceptibility testing pattern										Gene cassette pattern	Class 1 integrons product size (bp ^d)
			AMP ^f	AUG ^g	C ^h	CIP ⁱ	CTX ^j	NA ^k	NOR ^l	S ^m	TE ⁿ	SXT ^o		
542003	Rissen	Farm											<i>dfrA12^a-orfF^b-aadA2^c</i>	2000
542012	Rissen	Farm											<i>dfrA12-orfF-aadA2</i>	2000
542075	Typhimurium	Farm											<i>aadA1</i>	1000
542076	Typhimurium	Farm											<i>aadA1</i>	1000
542077	Typhimurium	Farm											<i>aadA1</i>	1000
543036	Rissen	Farm											<i>dfrA12-orfF-aadA2</i>	2000
543046	Stanley	Farm											<i>dfrA1-orfC</i>	1500
26	Stanley	SLH ^e											<i>dfrA12-orfF-aadA2</i>	2000
37	Rissen	SLH											<i>dfrA12-orfF-aadA2</i>	2000
65	Rissen	SLH											<i>dfrA12-orfF-aadA2</i>	2000
69	Rissen	SLH											<i>dfrA12-orfF-aadA2</i>	2000
81	Panama	SLH											<i>dfrA12-orfF-aadA2</i>	2000
107	Rissen	SLH											<i>dfrA12-orfF-aadA2</i>	2000
114	Rissen	SLH											<i>dfrA12-orfF-aadA2</i>	2000
126	Rissen	SLH											<i>dfrA12-orfF-aadA2</i>	1200
134	Rissen	SLH											<i>dfrA12-orfF-aadA2</i>	2000
139	Rissen	SLH											<i>dfrA12-orfF-aadA2</i>	2000
143	Agona	SLH											<i>aadA1</i>	1200
148	Stanley	SLH											<i>aadA1</i>	1200
151	Rissen	SLH											<i>aadA1</i>	2000
154	Rissen	SLH											<i>dfrA12-orfF-aadA2</i>	2000
181	Rissen	SLH											<i>dfrA12-orfF-aadA2</i>	2000
182	Rissen	SLH											<i>dfrA12-orfF</i>	2000
190	Rissen	SLH											<i>dfrA12-orfF</i>	2000

^a*dfrA12* (dihydrofolate reductase) confers resistance to sulfamethoxazole-trimethoprim^b*orfF*, *orfC* (hypothetical protein) unknown function^c*aadA1*, *aadA2* (aminoglycoside 3'-adenylyltransferase) confer resistance to aminoglycoside (streptomycin, spectinomycin)^dbp (base pair)^eSLH Slaughterhouse^fAMP (ampicillin) 10 µg^gAUG (amoxicillin-clavulanic acid) 30 µg^hC (chloramphenicol) 30 µgⁱCIP (ciprofloxacin) 5 µg^jCTX (cefotaxime) 30 µg^kNA (nalidixic acid) 30 µg^lNOR (norfloxacin) 10 µg^mS (streptomycin) 30 µgⁿTE (tetracycline) 30 µg^oSXT (sulfamethoxazole-trimethoprim) 25 µg

were collected. Animal products provide a suitable matrix for these organisms. Sub-lethal stress in storage conditions could also play a role

in increasing resistance ability^{19,32}.

The gene cassette array pattern “*dfrA12-orfF-aadA2*” was the most frequently found in

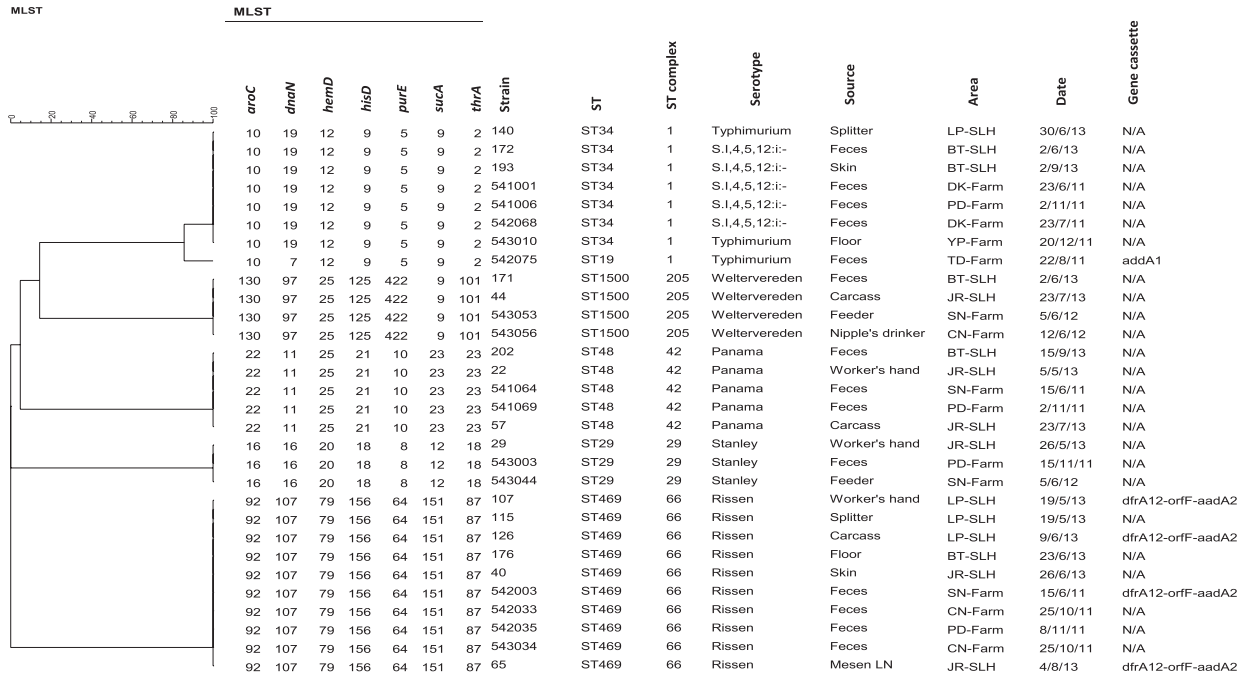


Fig. 1. Dendrogram representing 6 sequence types (ST) from 6 major *Salmonella* serotypes isolated from swine production chains in ChiangMai and Lamphun provinces, Thailand. MLST generated profiles using the UPGMA. Thirty *Salmonella* strains were grouped into six sequence types.: ST 469, 48, 1500, 34, 19 and 29. Strains belonging to similar sequence type (strains sharing common sequence types) were obtained from various sample types.

our study. Most were observed in *S. Rissen*, similar to the study by Caleja et al. (2011)⁷. *dfrA12-orfF-aadA2* gene cassette arrays were also detected in the highest frequency in *Salmonella* Rissen. However, many *Salmonella* serotypes have been found in class 1 integrons, including monophasic Typhimurium¹², Heidelberg²³, Typhimurium, Enteritidis, Derby, Saintpaul, Bredeney, Brandenburg, Muenchen, Brikama, IIIb:65:lv:enxz15³³, Emek, Haifa, Mbandaka, Newport, Ohio and Virchow²⁸.

Most of the antimicrobial resistant phenotypes and gene cassettes in our study were not related. Some resistant strains were not carried associated gene cassettes. That situation could be related to the genes harboring other mobile genetic elements which might take place such as transposons or plasmids³⁵. Additionally, *Salmonella* can develop the ability to neutralize an antibiotic by point mutations³³. On the other hand, some strains harbored resistant gene cassettes which did not show resistance ability in

their phenotypes. A possible cause could be the occurrence of an off function in the gene cassettes due to environmental interaction².

Six sequence types were generated from 30 *Salmonella* strains belonging to six serotypes. Almost all the sequence types were correlated with one serotype. However, ST 34 and ST 19 did not match well because *S. I,4,5,12:i:-* and *S. Typhimurium* were grouped in ST 34, while, only one strain of *S. Typhimurium* belonged in ST 19. About these both ST types, their allele number is different only in *dnaN* gene. Predictably, high genetic relatedness was found. Considering in antigenic formula of *S. Typhimurium* using the Kauffman-White classification showed that this serotype belongs in 4,5,12:i:1,2²⁴. This is close to the antigenic formula of *S. I,4,5,12:i:-* but that existent in second phase of flagella antigens are existed. The explanation is that both serotypes originated from a common ancestor; therefore, the genetic commonality found is not surprising³⁴.

Strains belonging to similar sequence type

Table 3. Simpson's diversity and coefficient indexes comparing MLST and PFGE methods

Typing method	Simpson's index of diversity (95% CI)	Adjusted Rand coefficient (95% CI)		Wallace coefficient (95% CI)	
		MLST	PFGE	MLST	PFGE
MLST	0.735 (0.646–0.859)	1.000 (1.000–1.000)	0.253 (0.013–0.524)	1.000 ^a (1.000–1.000)	0.213 (0.010–0.416)
PFGE	0.932 (0.868–0.995)		1.000 (1.000–1.000)	0.769 ^b (0.524–1.000)	1.000 (1.000–1.000)

^aWallace coefficient of MLST to PFGE^bWallace coefficient of PFGE to MLST**Table 4. Comparison of molecular typing methods between MLST and PFGE from 20 *Salmonella* strains isolated from the swine production chains in Chiang Mai and Lamphun provinces, Thailand.**

Strain	Sequence type	PFGE pattern
40	469	F1
65	469	F1
542035	469	F3
542003	469	F5
107	469	F5
115	469	F5
126	469	F5
542033	469	F9
44	1500	G1
543056	1500	G2
172	34	D1
193	34	D3
542068	34	D9
543010	34	D9
140	34	D9
541006	34	D10
542075	19	D9
543044	29	A2
543003	29	A3
29	29	B1

Twenty *Salmonella* strains submitted in sequence type in this study were selected for comparison of genetic diversity with existing PFGE characteristic information from the study by Tadee *et al.*, (2015)³⁶⁾. Thirteen pulsotypes could be grouped into five sequence type profiles. Most of the strains with similar pulsotypes belonged to type ST with the exception of the “D9” pulsotype which was comprised of ST 19 and ST 34 (both classified as *S. Typhimurium*)

(strains sharing common sequence types) were obtained from various sample types, indicating the occurrence of infection cycle through pigs, workers and the farm environment, as well as

the present of carcasses contaminated with pig feces via dirty equipment from improper practices in slaughterhouses (from the strains sharing in common sequence types) were carried

out. In addition, strains belonging to similar sequence types were obtained at different production levels (e.g., farm or slaughterhouse) signifying that the farm-slaughterhouse contamination route is the fact of *Salmonella* dissemination pattern in this region (in “542003”-“107 or 126” route). Furthermore, spread of resistant genes between production levels was demonstrated, as well.

The Simpson's index of diversity of MLST and PFGE were 0.753 and 0.932, respectively, indicating the high discriminatory power of these two techniques. However, PFGE had a higher differentiation ability than MLST for *Salmonella* strains, comparable to the results in several other studies^{14,39}. The concordance between MLST and PFGE was examined by calculating the Adjusted Rand and Wallace coefficients. The Adjusted Rand coefficient was 0.253, which indicated a low congruence between MLST and PFGE (From 6 sequence types, thirteen pulsotype were generated. Disarranged outcome was found in the grouping of ST 34 and ST 19 with *S. Typhimurium* and *S. I. 4,5,12:i:-*). Moreover, the Wallace coefficient of PFGE to MLST was 0.769, which indicates that if the isolates were recognized as having the same PFGE type, those isolates had a 76.9% chance of being identified as the same sequence type. On the other hand, the Wallace coefficient of MLST to PFGE was 0.213, indicating that if the isolates were identified as having the same sequence type, those isolates had only a 21.3% chance of being identified as the same PFGE type²⁵). These findings indicate that the capability of typing PFGE was greater than MLST. However, PFGE can be difficult to compare across various laboratories for the same analysis²¹. Alternatively, the MLST database is available online worldwide, so that technique can be provide faster results for disease monitoring and investigation of global epidemiology than the PFGE technique¹⁸.

Conclusions

Antimicrobial resistant genes can have an important impact on public health efforts. Contamination and spread of resistant organisms originating at the beginning of the swine production chain was demonstrated using the MLST technique. The use of antimicrobials in livestock should be controlled by the best route in right dose at optimum intervals for the appropriate period by veterinarian. Education and training on food handling and food consumption are also important ways to help prevent foodborne illnesses.

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