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\(^1\)\(^{11,27}\). The organism is usually found in the digestive tracts of animals such as birds and reptiles as well as mammals\(^16\). Humans can become infected by consuming products contaminated with Salmonella from the digestive tract contents of those animals\(^4\)\(^{45}\). 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 chain\(^29\). 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 concern\(^20\). Salmonella has developed resistance to various types of antimicrobials, and multidrug resistant strains have been documented\(^4\)\(^{40}\). Consequently, choices of treatment, including first-line treatment options, are limited\(^50\). 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 resistance\(^22\). 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), attl and a promoter are located in the 5’ variable region. In addition, the quaternary compound resistant gene (QacE\(\Delta\)1) and sulfonamide resistant gene (SulI) 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 strains\(^8\). Pulsed field gel electrophoresis (PFGE) is considered the gold standard for Salmonella typing\(^37\). However, PFGE has several limitations: the method is technically demanding, and time-consuming, specialist. In addition, data obtained are limited compared to sequence-based methods\(^30\).

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 strains\(^14\). This method can be used to identify and evaluate interrelationships among Salmonella isolates as part of disease surveillance and outbreak investigations\(^39\). 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\(^37\).

Materials and methods

Salmonella strains: One hundred and seventy-
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.

### Table 1. Number of *Salmonella* strains tested

<table>
<thead>
<tr>
<th>Salmonella serotype</th>
<th>Number of <em>Salmonella</em> strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Farm</td>
</tr>
<tr>
<td>S. I.4,5,12:i:-</td>
<td>12</td>
</tr>
<tr>
<td>S. Agona</td>
<td>0</td>
</tr>
<tr>
<td>S. Lexington</td>
<td>0</td>
</tr>
<tr>
<td>S. Panama</td>
<td>13</td>
</tr>
<tr>
<td>S. Rissen</td>
<td>27</td>
</tr>
<tr>
<td>S. Stanley</td>
<td>5</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td>10</td>
</tr>
<tr>
<td>S. Weltevreden</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>73</strong></td>
</tr>
</tbody>
</table>

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.

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 \(\mu\)L containing 10X PCR buffer, 1.5 mM MgCl₂, 300 \(\mu\)M dNTP, 0.2 \(\mu\)M each of the forward and reverse primers, 1 \(\mu\)l 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-11-00729. 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, ceftaxime (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 controlled.

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 (uroporphyrinogenIII cosynthase), purE (phosphoribosylaminomimidazole 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 amplification 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 study 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 information. 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 reported10,39. 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
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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Location</th>
<th>Antimicrobial susceptibility testing pattern</th>
<th>Gene cassette pattern</th>
<th>Class 1 integrons product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>542003</td>
<td>Rissen</td>
<td>Farm</td>
<td>AMP$^{a}$ AUG$^{b}$ CIP$^{c}$ CTX$^{d}$ NA$^{e}$ NOR$^{f}$ S$^{g}$ TE$^{h}$ SXT$^{i}$</td>
<td>dfrA12-orfF-aadA2$^{a}$</td>
<td>2000</td>
</tr>
<tr>
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<td>Farm</td>
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<td>2000</td>
</tr>
<tr>
<td>542075</td>
<td>Typhimurium</td>
<td>Farm</td>
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<td>aadA1</td>
<td>1000</td>
</tr>
<tr>
<td>542076</td>
<td>Typhimurium</td>
<td>Farm</td>
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<td>aadA1</td>
<td>1000</td>
</tr>
<tr>
<td>542077</td>
<td>Typhimurium</td>
<td>Farm</td>
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<td>aadA1</td>
<td>1000</td>
</tr>
<tr>
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<td>Rissen</td>
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<td>SLH$^{v}$</td>
<td>AMP$^{a}$ AUG$^{b}$ CIP$^{c}$ CTX$^{d}$ NA$^{e}$ NOR$^{f}$ S$^{g}$ TE$^{h}$ SXT$^{i}$</td>
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<td>2000</td>
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<td>aadA1</td>
<td>1200</td>
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<tr>
<td>148</td>
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<td>dfrA12-orfF-aadA2$^{a}$</td>
<td>2000</td>
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<tr>
<td>182</td>
<td>Rissen</td>
<td>SLH$^{v}$</td>
<td>AMP$^{a}$ AUG$^{b}$ CIP$^{c}$ CTX$^{d}$ NA$^{e}$ NOR$^{f}$ S$^{g}$ TE$^{h}$ SXT$^{i}$</td>
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<td>190</td>
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<td>SLH$^{v}$</td>
<td>AMP$^{a}$ AUG$^{b}$ CIP$^{c}$ CTX$^{d}$ NA$^{e}$ NOR$^{f}$ S$^{g}$ TE$^{h}$ SXT$^{i}$</td>
<td>dfrA12-orfF</td>
<td>2000</td>
</tr>
</tbody>
</table>

$^{a}$dfrA12 (dihydrofolate reductase) confers resistance to sulfamethoxazole-trimethoprim
$^{b}$orfF, orfC (hypothetical protein) unknown function
$^{c}$aadA1, aadA2 (aminoglycosid -3’- adenyltransferase) confer resistance to aminoglycoside (streptomycin, spectinomycin)
$^{d}$bp (base pair)
$^{e}$SLH Slaughterhouse
$^{f}$AMP (ampicillin) 10 μg
$^{g}$AUG (amoxicillin-clavulanic acid) 30 μg
$^{h}$C (chloramphenicol) 30 μg
$^{i}$CIP (ciprofloxacin) 5 μg
$^{j}$CTX (ceftoxime) 30 μg
$^{k}$NA (nalidixic acid) 30 μg
$^{l}$NOR (norfloxacin) 10 μg
$^{m}$S (streptomycin) 30 μg
$^{n}$TE (tetracycline) 30 μg
$^{o}$SXT (sulfamethoxazole-trimethoprim) 25 μg
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Fig. 1. Dendogram 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 types (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 Typhimurium\(^{12}\), Heidelberg\(^{23}\), Typhimurium, Enteritidis, Derby, Saintpaul, Bredeney, Brandenburg, Muenchen, Brikama, IIIb:65:lv:enxz15\(^5\), Emek, Haifa, Mbandaka, Newport, Ohio and Virchow\(^{28}\).

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 plasmids\(^{33}\). Additionally, Salmonella can develop the ability to neutralize an antibiotic by point mutations\(^{33}\). 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 interaction\(^{22}\).

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:1,2\(^{24}\). 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 surprising\(^{34}\).

Strains belonging to similar sequence type
Epidemiological Characteristic of *Salmonella* spp.

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(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 studies14,39). 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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