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Repetitive sequence-based PCR fingerprinting and the relationship of antimicrobial-resistance characteristics and corresponding genes among *Salmonella* strains from pig production

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ABSTRACT

Objective: To investigate the relationship between antimicrobial resistance characteristics and corresponding genes, and to diversify repetitive element sequence-based PCR (rep-PCR) fingerprinting of three *Salmonella* serotypes: Rissen, Panama and Stanley, which were isolated from pig farms and slaughterhouses in Chiang Mai and Lumphun Provinces, Thailand.
Methods: A total of 90 *Salmonella* strains were identified using the Kauffman-White scheme. The Kirby-Bauer disk diffusion method was used to investigate resistance phenotypes of 10 antimicrobial agents. Conventional PCR was used to detect 10 antimicrobial resistance genes, additionally, rep-PCR typing method was applied to identify clonality among *Salmonella* isolates.
Results: The antimicrobial susceptibility testing found resistance to ampicillin (80.0%), streptomycin (65.6%), tetracycline (61.1%), sulfamethoxazole (53.3%), chloramphenicol (28.9%), nalidixic acid (6.7%) and cefotaxime (2.2%). All strains were sensitive to amoxicillin-clavulanic acid, ciprofloxacin and norfloxacin. The most common antimicrobial resistance patterns among the isolates were ampicillin, chloramphenicol, streptomycin, tetracycline, and sulfamethoxazole. The type and frequency of antimicrobial genes detected included bla_{TEM} (100.0%), *aad*A2 (52.2%), *cml*A (45.6%), *str*A (38.9%), *tet*A(B) (16.7%), *sul*1 (15.6%), $bla_{\text{OXA-2}}$ (14.4%), bla_{PSE1} (6.7%), *aph*A1-lab (2.2%) and $bla_{\text{CMY-2}}$ (1.1%).
Conclusions: Statistical analysis revealed no association between antimicrobial resistance genes and resistance profiles with the exception of *cml*A and chloramphenicol, *sul*1 and sulfamethoxazole, *aad*A2 and streptomycin, and *str*A and streptomycin ($P < 0.05$). Finally, the identical groups of clonal strains were detected by rep-PCR indicating similarity among *Salmonella* genotypes from farms and from slaughterhouses.

1. Introduction

Salmonella spp. are widespread in animals and the environment. They are prevalent in livestock animals such as pigs, poultry, and cattle. They can be transmitted through the food chain to humans who consume contaminated food. A common cause of human salmonellosis is *Salmonella enterica*. The characteristic sign of *Salmonella* infection is acute onset of fever, abdominal pain, diarrhoea, nausea and vomiting[1-5]. Every year, approximately 10 million people in the world are infected by *Salmonella* spp. resulting in more than hundred thousand deaths[6]. The highest risk of salmonellosis is in children under five years old, immunocompromized patients, and older individuals. Antibiotics are not recommended in mild to moderate cases in healthy individuals, but in severe salmonellosis cases and in high risk groups antimicrobial therapy is required.

The first antimicrobial resistance in *Salmonella* was observed in the early 1990s and is found in many serotypes[7,8]. The emergence of multidrug-resistance in *Salmonella* strains has been associated with the use of antimicrobials for various purposes in food animals[8-11]. In Thailand, the use of antimicrobials in livestock production for treatment of disease or as a growth promoter has been regulated by Department of Livestock Development and all antimicrobials used with food animals have been approved by the Thai Food and Drug Administration. However, increasing antimicrobial resistance is becoming a serious health problem due to the misuse and overuse of antimicrobials with food animals[12-15]. Resistance to penicillins,

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cephalosporins and other members of the beta-lactam group is regulated by the *bla* genes (bla_{TEM}, bla_{PSE-1}, bla_{CMY-2} and bla_{OXA-2}). Chloramphenicol resistance is encoded in the *cml*A genes, while *aad*A1 and *str*A confer resistance to spectinomycin and streptomycin. The *tet*A(B) genes encode the tetracycline efflux proteins, while sulfamethoxazole and kanamycin resistance are carried in *sul*1 and *aph*A1-lab[5,15]. Resistance genes are often transferred to other bacteria in many ways such as horizontal gene transfer (conjugation, transduction, transformation) and via mobile genetic elements (plasmids, bacteriophages, integrons, transposons), making this a serious problem and a public health issue[8,16,17].

Several epidemiological studies have developed many gold standards for *Salmonella* molecular typing such as pulsed-field gel electrophoresis, ribotyping, and multi-locus sequence typing all of which is expensive, time-consuming and restricted to national reference laboratories[18-20]. According to Weigel *et al.*[18], repetitive element sequence-based PCR (rep-PCR) is another technique for *Salmonella* molecular typing. This method uses a non-coding region of the bacterial DNA fingerprint for classifying the bacterial strain[18,21,22]. According to Chmielewski *et al.*[23], rep-PCR is able to discriminate among *Salmonella* serotypes. They concluded that all serotypes produce an unique fingerprint and that the isolates within one serotype have identical patterns[16,23]. Thus rep-PCR can also be used for *Salmonella* molecular typing. This method is not expensive and does not require a special laboratory and experts, yet its accuracy is similar to that of pulsed-field gel electrophoresis[24].

Several previous studies have examined antimicrobial resistance genes in *Salmonella* spp. in Thailand[12,13]. However, there is no information about the association between antimicrobial resistance genotypes and phenotypes of *Salmonella* and only limited data are available comparing the genetic makeup of *Salmonella* at various stages in the production chain. The objective of this study was to investigate the relationship between antimicrobial resistance characteristic and corresponding genes and to identify diverse sources of *Salmonella* isolates from pig farms and slaughterhouses using the rep-PCR fingerprinting technique.

2. Materials and methods

2.1. Sample collection and serotyping

A total of 90 *Salmonella* isolates were randomly selected from stock collected in previous studies (Project ID: P-11-00729 and P-10-10409) [25]. All the samples were collected from pigs and areas surrounding pig farms (n = 35) and slaughterhouses (n = 55) in Chiang Mai and Lumphun Provinces during 2012–2013. All experimental procedures involving animals in Project ID: P-11-00729 and P-10-10409 were conducted in accordance to experimental protocol No. R7/2554 and approved by Faculty of Veterinary Medicine Chiang Mai University-Animal Care and Use Committee. *Salmonella* serotypes were identified by the World Health Organization National *Salmonella* and *Shigella* Center, Department of Medical Science, Thailand. In this study, the isolates were comprised of 3 serotypes: *Salmonella* Rissen (*S.* Rissen) (n = 33), *Salmonella* Panama (*S.* Panama) (n = 30) and *Salmonella* Stanley (*S.* Stanley) (n = 27). These 3 serotypes are the most common types found in pig farms and slaughterhouses in Chiang Mai and Lumphun Provinces[25].

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was done with Mueller-Hinton agar (Oxoid, Milan, Italy) using the Kirby-Bauer disk diffusion method following guidelines of the National Committee for Clinical Laboratory Standards[26]. All the isolates were tested for sensitivity to ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), cefotaxime (30 μg), nalidixic (30 μg), norfloxacin (10 μg), streptomycin (10 μg), tetracycline (30 μg) and sulfamethoxazole (250 μg). The results were evaluated following Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards, 2011 recommendations.

2.3. Detection of antibiotic resistance genes

Genomic DNA was extracted by Chelex® (Bio-Rad Laboratories Inc.). Antimicrobial resistance genes were examined using conventional PCR. PCR amplification was accomplished using specific primers (Table 1), and PCR reaction was done in a final volume of 20 μL containing purified DNA 1 μL, 1× PCR buffer 0.5 mmol/L, $MgCl_2$ 200 μmol/L and deoxynucleoside triphosphate 0.25 μmol/L each of forward and reverse primers and 0.5 IU of *Taq* DNA polymerase (Vivantis Technologies, Malaysia). PCR conditions were as follows: 1 cycle of denaturation at 95 ˚C for 15 min, 35 cycles of denaturation at 95 ˚C for 30 s, annealing at 54 ˚C for 30 s, extension at 72 ˚C for 30 s, final extension at 72 ˚C for 10 min and cooling at 4 ˚C until removal of the PCR product. The agarose gel electrophoresis used 1% agarose gel (Vivantis Technologies, Malaysia) in 0.5× Tris-borate ethylene diamine tetraacetic acid running buffer under the following conditions: 100 V, 400 mA for 45 min. Ethidium bromide was used for staining agarose gels and the DNA bands were visualized under UV transillumination.

Table 1
Primers for antimicrobial resistance genedetection in this study.

Gene	Primer sequences (5' to 3')	Product size (bp)	Reference/GenBank accession No.	Drug resistant
*aad*A2	F: CGG TGA CCA TCG AAA TTT CG R: CTA TAG CGC GGA GCG TCT CGC	250	AF071555	Aminoglycosides
*aph*A1-lab	F: AAA CGT CTT GCT CGA GGC R: CAA ACC GTT ATT CAT TCG TGA	500	[27]	Kanamycin
bla_{CMY-2}	F: ATG ATG AAA AAA TCG TTA TGC R: TTG CAG CTT TTC AAG AAT GCG C	1 128	[28]	Beta-lactam group
bla_{PSE-1}	F: TTT GGT TCC GCG CTA G R: TACT CC GAG CAC CAA ATC CG	150	[29]	Beta-lactam group
bla_{TEM}	F: GCA CGA GTG GGT TAC ATC GA R: GGT CCT CCG ATC GTT GTC AG	318	[29]	Broadspectrum penicillinases
*cml*A	F: TGT CAT TTA CGG CAT ACT CG R: ATC AGG CAT CCC ATT CCC AT	435	[30]	Chloramphenicol
bla_{OXA-2}	F: ATG GCA ATC CGA ATC TTC GC R: TTA TCG CGC AGC GTCCGA GT	650	[30]	Beta-lactam group
*sul*1	F: CAC TGC CAC AAG CCG TAA R: GTC CGC CTC AGC AAT ATC	435	[30]	Sulfonamide
*str*A	F: CCT GGT GAT AAC GGC AAT TC R: CCA ATC GCA GAT AGA AGG C	548	[30]	Streptomycin and spectinomycin
*tet*A(B)	F: TTG GTT AGG GGC AAG TTT TG R: GTA ATG GGC CAA TAA CAC CG	659	[30]	Tetracyclines

2.4. Rep-PCR

Alkaline polyethylene glycol-based method was used for DNA extraction. Enterobacterial repetitive intergenic consensus (ERIC) (ERIC1: 5'-ATG TAA GCT CCT GGG GAT TCA C-3' and ERIC2: 5'-AAG TAA GTG ACT GGG GTG AGC G-3') and GTG (5'-GTG GTG GTG GTG GTG-3') primer were used in this study. PCR reaction of with ERIC primer was done in a final volume of 25 μL containing DNA 2 μL, 10× PCR buffer, 15 mmol/L $MgCl_2$ (Takara Bio Inc., Japan), 2.5 mmol/L deoxynucleoside triphosphates (Takara Bio Inc., Japan), 20 μmol/L each of ERIC1 and ERIC2 primer and 0.5 IU of *Taq* DNA polymerase (Takara Bio Inc., Japan). PCR reaction of GTG primer was done in a final volume of 25 μL containing DNA 2 μL, 10× PCR buffer, 15 mmol/L $MgCl_2$ (Takara Bio Inc., Japan), 2.5 mmol/L deoxynucleoside triphosphates (Takara Bio Inc., Japan), 20 μmol/L $(GTG)_5$ primer and 0.5 IU of *Taq* DNA polymerase (Takara Bio Inc., Japan). PCR conditions were as follows: 1 cycle of denaturation at 95 ˚C for 5 min, 30 cycles of denaturation at 94 ˚C for 45 s, annealing at 52 ˚C for 1 min, extension at 65 ˚C for 10 min, final extension at 65 ˚C for 20 min and cooling at 15 ˚C until removal of the PCR product.

Agarose gel electrophoresis used 1% agarose gel (UltraPure™ Agarose, Invitrogen Life Science Technologies, New Zealand) in 0.5× Tris-borate ethylene diamine tetraacetic acid running buffer under the following conditions: 150 V for 2 h and 20 min. Straining was done using ethidium bromide 0.5 μg/mL for 5 min followed by de-straining in tap water for 20 min. DNA bands were visualized under UV transillumination.

2.5. Statistical analysis

Epi Info™ statistical software was used for data management and analysis. Pearson *Chi*-square or Fisher's exact tests (where appropriated) in StatCalc modules were used to compare proportions in 2-by-2 contingency table. The results were considered as statistically significant when $P < 0.05$. Comparison of rep-PCR fingerprint and dendrogram were performed and generated using BioNumerics version 7.5 software (Applied Maths, Kortrijk, Belgium). All *Salmonella* strains within a similarity > 80% belong to the same patterns.

3. Results

The common resistance phenotype was resistant to ampicillin (80.0%), streptomycin (65.6%), tetracycline (61.1%), sulfamethoxazole (53.3%), chloramphenicol (28.9%), nalidixic (6.7%), and cefotaxime (2.2%). The highest resistance phenotype in pig farms and slaughterhouses was ampicillin (65.7% and 89.1%), followed by streptomycin (54.3% and 72.7%). *Salmonella* isolates collected from pig farms were susceptible to amoxicillin/clavulanic acid, ciprofloxacin, cefotaxime, nalidixic and norfloxacin. Fifty-five samples from slaughterhouses were susceptible to amoxicillin/clavulanic acid, ciprofloxacin and norfloxacin. Thus, overall the *Salmonella* isolates were susceptible to amoxicillin/clavulanic acid, ciprofloxacin and norfloxacin (Figure 1).

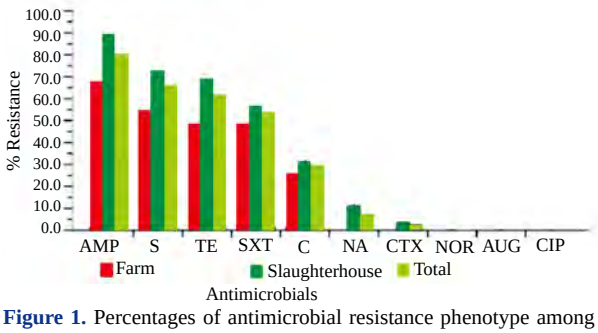

Figure 1. Percentages of antimicrobial resistance phenotype among three *Salmonella* serotypes.
AMP: Ampicillin; S: Streptomycin; TE: Tetracycline; SXT: Sulfamethoxazole; C: Chloramphenicol; NA: Nalidixic; CTX: Cefotaxime; NOR: Norfloxacin; AUG: Amoxicillin/clavulanic acid; CIP: Ciprofloxacin.

Nearly 84% of all *Salmonella* isolates were resistant to more than one antimicrobial agent, 13% were pan-susceptible, and 3% were resistant to only one antimicrobial agent. The most common antimicrobial resistance pattern among the isolates was ampicillin, chloramphenicol, streptomycin, tetracycline, sulfamethoxazole (20.00%). The most frequent antimicrobial resistance pattern among *Salmonella* isolates from pig farms was ampicillin, streptomycin, tetracycline, sulfamethoxazole (14.29%). Ampicillin, chloramphenicol, streptomycin, tetracycline, sulfamethoxazole (27.27%) was the highest most common antimicrobial resistance pattern in *Salmonella* isolates from slaughterhouses.

The most commonly found antimicrobial gene both in pig farms and in slaughterhouses was bla_{TEM} (100.0%), followed by *aad*A2 (52.2%) *cml*A (45.6%). No bla_{CMY-2} gene was found in samples from pig farms and no *sul*1 gene was found in samples from slaughterhouses. However, the *Salmonella* isolates collected from pig farms and slaughterhouses were found to contain at least one antimicrobial resistance gene (Table 2).

Table 2
Antimicrobial resistance genes among three *Salmonella* serotypes from pig production. *n* (%).

Gene	Farm (*n* = 35)	Slaughterhouse (*n* = 55)	Total (*n* = 90)
*aad*A2	20 (57.1)	27 (49.1)	47 (52.2)
*aph*A1-lab	1 (2.9)	1 (1.8)	2 (2.2)
bla_{CMY-2}	0 (0.0)	1 (1.8)	1 (1.1)
bla_{PSE1}	2 (5.7)	4 (7.2)	6 (6.7)
bla_{TEM}	35 (100.0)	55 (100.0)	90 (100.0)
*cml*A	18 (51.4)	23 (41.8)	41 (45.6)
bla_{OXA-2}	4 (11.4)	9 (16.4)	13 (14.4)
*sul*1	14 (40.0)	0 (0.0)	14 (15.6)
*str*A	5 (14.3)	30 (54.6)	35 (38.9)
*tet*A(B)	10 (28.6)	5 (9.1)	15 (16.7)

Association between antimicrobial resistance genes and phenotype was determined using *Chi*-square or Fisher's exact test which was included in the Epi Info™ program verson 7.0. Statistical analysis revealed associations between antimicrobial resistance genes and resistance profiles between *cml*A and chloramphenicol, between *sul*1 and sulfamethoxazole-trimethoprim, between *aad*A2 and streptomycin, and between *str*A and streptomycin ($P < 0.05$) (Table 3).

Table 3
Association between antimicrobial resistance genes and resistance profiles.

Antimicrobial resistance genes	Antimicrobial resistance agents	*P*-value
*cml*A	Chloramphenicol	< 0.05*
*sul*1	Sulfamethoxazole	< 0.05*
*aad*A2	Streptomycin	< 0.05*
*str*A	Streptomycin	< 0.05*
*aph*A1-lab	Streptomycin	> 0.05
*tet*A(B)	Tetracycline	> 0.05
bla_{CMY-2}	Amoxicillin/clavulanic acid	> 0.05
	Ampicillin	> 0.05
	Ciprofloxacin	> 0.05
	Cefotaxime	> 0.05
bla_{PSE1}	Amoxicillin/clavulanic acid	> 0.05
	Ampicillin	> 0.05
	Ciprofloxacin	> 0.05
	Cefotaxime	> 0.05
bla_{TEM}	Amoxicillin/clavulanic acid	> 0.05
	Ampicillin	> 0.05
	Ciprofloxacin	> 0.05
	Cefotaxime	> 0.05
bla_{OXA-2}	Amoxicillin/clavulanic acid	> 0.05
	Ampicillin	> 0.05
	Ciprofloxacin	> 0.05
	Cefotaxime	> 0.05

*: Statistically significant association between antimicrobial resistance genes and resistance profiles.

Figures 2–4 show the rep-PCR results for the *Salmonella* three serotypes. Based on an 80% similarity among *Salmonella* isolates, six rep-PCR patterns were identified in *S.* Rissen (Figure 2), two patterns were classified in *S.* Panama (Figure 3) and seven patterns were found in *S.* Stanley (Figure 4). The largest group of isolates, *S.* Rissen, were collected from pig farms and slaughterhouses as was the case for *S.* Panama and *S.* Stanley. This result indicated that *Salmonella* isolates from pig farms and slaughterhouses came from the same original source as 80% of the rep-PCR fingerprinting patterns were similar. Resistant type of antimicrobial susceptibility testing and antimicrobial resistance genes did not identify specific patterns. That indicated resistant type antimicrobial susceptibility testing and antimicrobial resistance genes cannot be used for classifying the source of *Salmonella*.

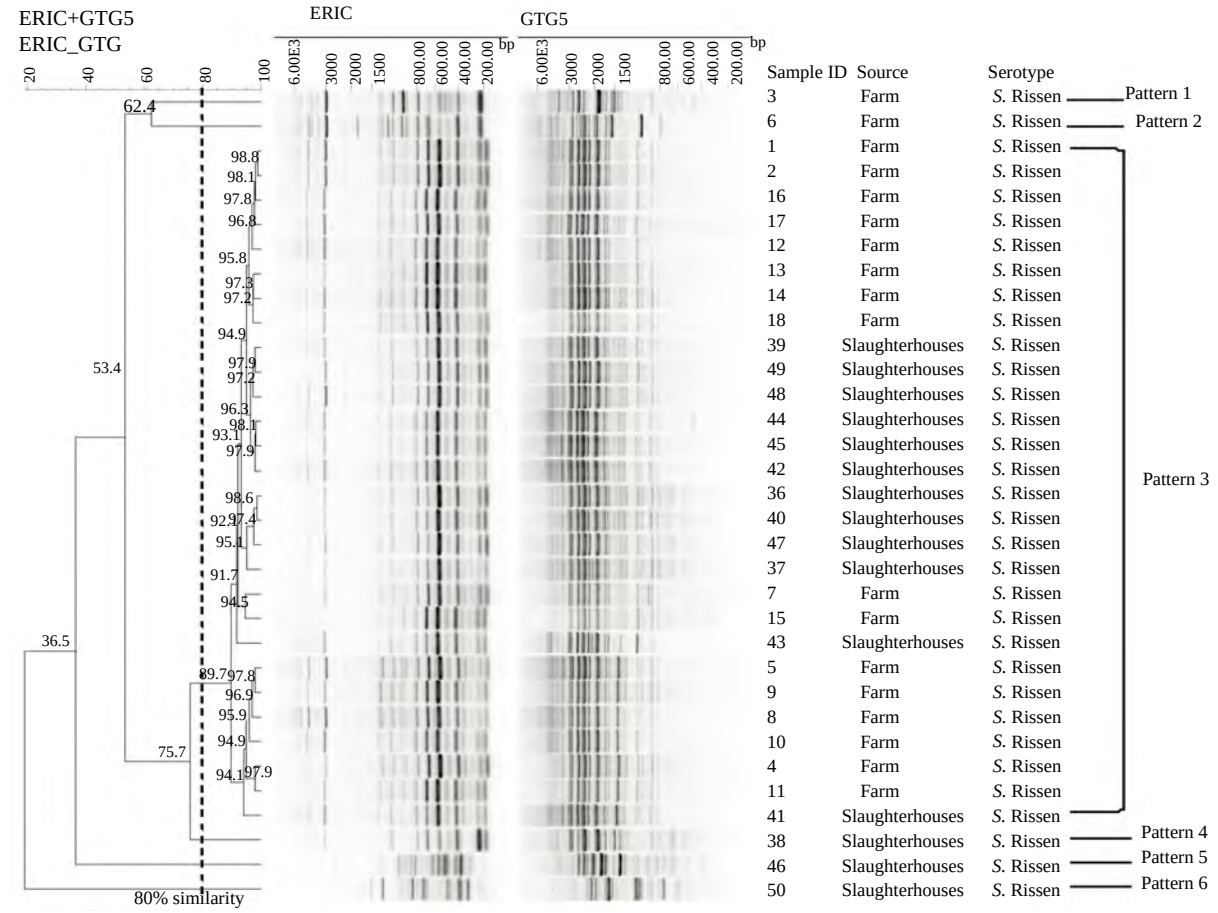

Figure 2. Rep-PCR fingerprint of *S.* Rissen. A dendrogram showed the similarities in rep-PCR fingerprint patterns observed among the *S.* Rissen DNA. Numbers on the right end of the dendrogram represented numbers of pattern which were classified by 80% similarity.

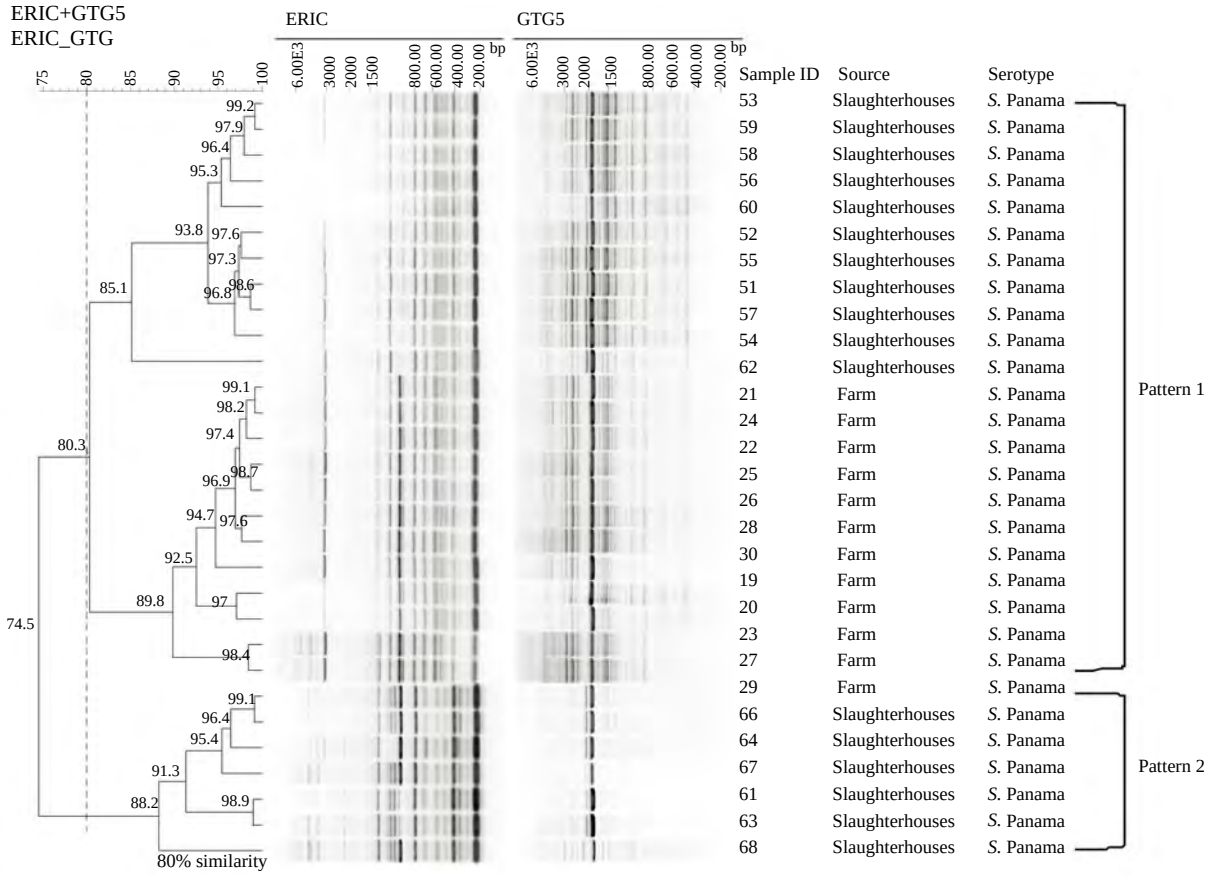

Figure 3. Rep-PCR fingerprint of *S.* Panama. A dendrogram showing the similarities in rep-PCR fingerprint patterns observed among the *S.* Panama DNA. Numbers on the right end of the dendrogram represent numbers of pattern which were classified by 80% similarity.

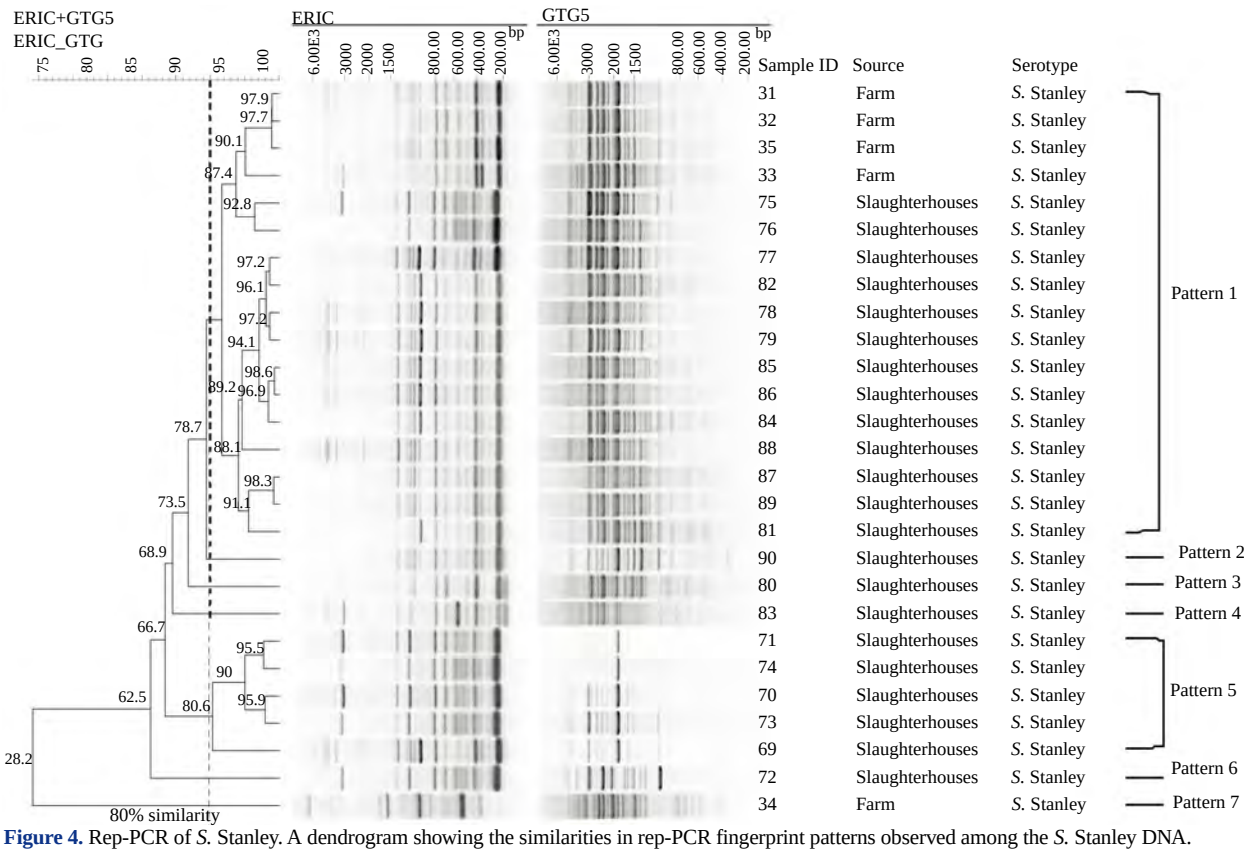

Figure 4. Rep-PCR of *S.* Stanley. A dendrogram showing the similarities in rep-PCR fingerprint patterns observed among the *S.* Stanley DNA. Numbers on the right end of the dendrogram represent numbers of pattern which were classified by 80% similarity.

4. Discussion

Salmonella isolates both from farms and from slaughterhouses in this study were found to have a high level of ampicillin resistance. That is because ampicillin is commonly used in pig production[25]. Intensive commercial units in pig farms are common in Thailand, making that a very important factor for emerging diseases on farms. The use of antimicrobial drugs is increasing in pig farms and has been extensively used in food animal production for decades. Ampicillin has been used in pig production as a cure for disease and as a growth promoter even though it has been prohibited in feed additives for a long time[12,13]. This study found ampicillin resistance in 80% of the samples.

The most common antibiotic resistance phenotype was ampicillin and was related to the bla_{TEM} gene which was found in all isolates. The bla_{TEM} gene was in the extended-spectrum beta-lactamases subgroup 2 serine beta-lactamases that regulate the beta-lactam resistance phenotype. Over the past four decades (1970–2009), the occurrence of extended-spectrum beta-lactamases has been increasing more than any other subgroup[31-34]. This study indicated that the antimicrobial resistance phenotype (ampicillin) is related to the antimicrobial resistance genotype (bla_{TEM}).

Using antimicrobial drugs in food animals is regulated by Department of Livestock Development of Thailand. Chloramphenicol has been banned for use in livestock production, but the chloramphenicol resistant phenotype and gene (*cml*A) were still found in this study due to the *cml*A gene which is a part of gene cassettes in class 1 integrons[8,12,34,35].

Statistical analyses have found that most antimicrobial resistance genes and resistance profiles have no significant association because the genes are one of many factors that regulate antimicrobial resistance phenotype. Misuse and overuse of antimicrobial drugs in livestock production is one factor that affects antimicrobial resistance in bacteria. Selective pressure in individual bacteria is another factor related to the presence of antimicrobial resistance characteristics[4,5,8,12,36,37].

Comparisons of molecular discrimination of *Salmonella enterica* isolates using the rep-PCR technique, the ERIC primer set and the $(GTG)_5$ primer set are effective in discriminating among *Salmonella* serotypes[16,18,19,21,23]. In the study of Sukroongruang *et al.*, the accuracy of the ERIC primer set, the $(GTG)_5$ primer set and the composite data set [ERIC plus $(GTG)_5$] for manual rep-PCR were 94.23%, 90.38% and 92.30%, respectively[24]. Thus using the ERIC primer set for rep-PCR is effective for discrimination of *Salmonella* serotypes[24]. In this study, we found that classification of *Salmonella* using rep-PCR with the ERIC primer set and the $(GTG)_5$ primer set, which provided an 80% similarity index, indicating that rep-PCR fingerprinting of *Salmonella* from farms and slaughterhouses is clonal, indicating that the *Salmonella* originates from the same source.

In conclusion, this study found no relationship between antimicrobial resistance genes and resistance profiles. Carrying antimicrobial resistance genes does not always show resistance characteristics because there are many factors which regulate antimicrobial resistance phenotypes. Our findings showed that the *Salmonella* in both pig farms and in slaughterhouses originated from the same source.

Conflict of interest statement

We declare that we have no conflict of interest.

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