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Characterization of Multiple-Antimicrobial Resistant *Salmonella* Isolated from Pig Farms in Thailand

Pannatee Sanpong[1], Gunjana Theeragool[2], Worrawit Wajjwalku[3] and Patamaporn Amavisit[4]*

ABSTRACT

A cross-sectional study was conducted to characterize *Salmonella enterica* serogroup B and C in five intensive pig farms in central Thailand. Of 230 *Salmonella* isolates, 211 isolates (91.74%) were resistant to three or more antimicrobials and were defined as MDR isolates. Every isolate (100%) was resistant to sulfamethoxazole. The isolates with high resistance rates (95, 91 and 78%) were resistant to tetracycline, ampicillin and streptomycin, respectively. All of the isolates were sensitive to ceftriaxone, ceftriofur and ciprofloxacin. Only four of the 211 MDR isolates harbored class 1 integrons that carried *aadA* gene cassettes, which conferred resistance to streptomycin. Each isolate of serovar *S*. Stanley and *S*. Anatum harbored the *aadA1* and the *aadA2* gene cassette, respectively, and two isolates of serovar *S*. Panama contained the *aadA4* gene cassette. These four isolates could transfer resistance genes and class 1 integron carrying the *aadA* gene to *E. coli* by conjugative plasmids. The common antimicrobial resistances that were found in transconjugants were ampicillin, chloramphenicol, kanamycin, sulfamethoxazole and tetracycline. The high occurrence of *Salmonella* on one farm presented three common serovars, namely *S*. Corvallis, *S*. Rissen, and *S*. 1,4,5,12:i-. The antimicrobial resistance pattern was the same in each serovar. Their PCR-RFLP of flagellin genes and plasmid profiles showed that these three serovars were possibly endemic strains on this farm and had spread by cross contamination.

Keywords: antimicrobial resistance, pig farm, *Salmonella*

INTRODUCTION

Salmonella spp. are gram-negative bacterial pathogens causing food-borne diseases that can result in public health concerns worldwide. These pathogens regularly infect humans after the consumption of contaminated food or direct contact with carrier animals (Pang, *et al*., 1995; Soto *et al*., 2003). The development of antimicrobial resistance among *Salmonella* species is becoming a serious problem, especially the emergence of multi-drug resistant (MDR) *Salmonella* strains (Lee *et al*., 1994; Glynn *et al*., 1999; Duijkeren *et al*., 2003; Kristiansen *et al*., 2003). Many studies have revealed the major mechanisms in dissemination of antimicrobial resistance genes that are related to mobile genetic elements, including conjugative plasmids,

1 Interdisciplinary Graduate Program in Genetic Engineering, Kasetsart University, Bangkok 10900, Thailand.
2 Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
3 Department of Pathology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand.
4 Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: fvetpaa@ku.ac.th

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transposons and integrons (Lawley *et al*., 2004; Hsu *et al*., 2006).

This study aimed to characterize *Salmonella* isolated from samples from pig farms by focusing on their antimicrobial resistance phenotypes, their class 1 integrons and resistance genes cassettes, and the transferring capability of the antimicrobial resistance genes. Subtyping was also performed to find out the possible source of *Salmonella* contamination on one pig farm.

MATERIALS AND METHODS

Bacterial strains

A total of 230 *Salmonella enterica* serogroup B and C isolates were obtained from five intensive pig farms. The farms were located in different provinces in central Thailand. Cross sectional sampling was conducted between June 2003 and December 2003. The animal samples were divided into eight groups of boar, sow, suckling, nursing, fattening, lactating sow, gestating sow and replacement gilt. Fifty-five fecal samples were collected from each animal group. Twenty environmental samples from water, feed and carrier animals (such as rats, lizards, flies, cockroaches or spiders) were randomly collected on each farm. *Salmonella* spp. were detected following the ISO 6579:2002 standard process.

The isolate origins are described in more detail in Table 1 (216 isolates from pig feces, 4 isolates from feeds and 10 isolates from environmental samples). Particular isolates were confirmed for their serotypes by the WHO National *Salmonella* and *Shigella* Center Laboratory, Thailand, following the Kauffman-White scheme.

All statistical analysis was carried out using NCSS software (Number Cruncher Statistical Systems, Kaysville, UT), using the Pearson χ^2 test to determine the association between R-types and sources of the isolates. Differences were considered significant at $P < 0.05$.

Antimicrobial susceptibility test

All isolates were tested for antimicrobial resistance using the Kirby-Bauer disc diffusion assay on Mueller-Hinton agar against 16 antimicrobial agent discs (Oxoid Ltd., UK). The antimicrobial agents used for R-types were ampicillin (10 μg), amikacin (30 μg), amoxicillin/clavulanic acid (30 μg), apramycin (15 μg), ceftiofur (30 μg), ceftriaxone (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), sulfamethoxazole (25 μg), sulfamethoxazole/trimethoprim (25 μg) and tetracycline (30 μg). *E. coli* ATCC 25922 was used as a control strain and inhibition zones were evaluated following the recommendations of CLSI (NCCLS, 2000).

Detection of class 1 integrons by PCR amplification

The 211 MDR *Salmonella* isolates were selected for class 1 integron detection. Genomic DNA extractions were performed with the boiling method as described by Radu *et al*. (2001). The extracted DNA templates were screened for class 1 integrons with specific modified/ the specifically modified primer pair int1 F: 5'-CGGGCATCC AAGCAGCAAG-3' and int1 R: 5'-AAAGCAG ACTTGACCTGATAG-3' (EMBL accession no. AY220520). This primer pair was specified to a 5' conserved segment and a 3' conserved segment of class 1 integrons, respectively. The PCR reactions were conducted according to Dalsgaard *et al*. (2000) and amplifications were performed in a PTC-200 thermocycler (MJ Research, USA.). The PCR products obtained were detected on 1.0% agarose gel electrophoresis at 100 V for 20 min. The PCR products were varied due to the different sizes of resistance gene cassettes inside the class 1 integrons.

Table 1 Percentage of *Salmonella* isolates from pig farms conferring resistance to antimicrobial agents using the disc diffusion method.

Antimicrobial agent	Percentage of *Salmonella* isolates resistant to antimicrobial agents									
	Fecal sample source								Environmental samples	**Total**
	Boar	Sow	Gestating sow	Lactating sow	Replacement gilt	Suckling period	Nursing	Fattening		
	(4/1.45%)[a]	(48/17.75%)	(12/4.36%)	(12/4.36%)	(3/1.09%)	(25/9.09%)	(73/26.54%)	(39/14.18%)	(14/14%)	**(230/100%)**
Ampicillin	100	96	58	58	100	100	96	85	100	**91**
Amikacin	0	4	0	0	0	0	0	10	0	**3**
Amoxicillin/clavulanic acid	0	4	8	0	0	0	0	0	14	**2**
Apramycin	0	0	8	8	0	16	22	21	43	**16**
Ceftriaxone	0	0	0	0	0	0	0	0	0	**0**
Ceftriofur	0	0	0	0	0	0	0	0	0	**0**
Cephalothin	0	2	0	0	0	0	0	5	0	**1**
Chloramphenicol	25	15	58	67	100	48	66	64	43	**51**
Ciprofloxacin	0	0	0	0	0	0	0	0	0	**0**
Gentamicin	25	48	0	8	0	68	23	33	71	**36**
Kanamycin	0	8	25	33	0	12	53	25	0	**27**
Nalidixic acid	0	0	17	33	67	36	63	46	100	**38**
Streptomycin	75	69	67	83	100	88	75	80	79	**78**
Sulfamethoxazole	100	100	100	100	100	100	100	100	100	**100**
Trimethoprim/sulfameth[b]	50	19	67	58	100	48	29	67	43	**41**
Tetracycline	100	94	83	100	100	100	92	97	100	**95**

[a] (number of *Salmonella* isolates or rates of *Salmonella* found in each specimen); [b]Trimethoprim/sulfamethoxazole; 275 fecal specimens in each animal group and in 100 environmental samples were collected.

Identification of resistance gene cassettes by DNA sequencing

The positive PCR products generated by four isolates, comprising serovars *S*. Stanley CC1, *S*. Anatum EC3 and *S*. Panama CB2 and CB3, were subjected to nucleotide sequencing. The PCR products were prepared for sequencing with a DNA sequencing kit (BigDye™ Terminator Cycle Sequencing v2.0 Ready Reaction, Applied Biosystems, USA.) following the manufacturer's recommendations. The sequencing reactions were carried out using a DNA sequencer ABI Prism model 377 (GMI, USA.). The derived sequences were analyzed by the BLAST algorithm and aligned with other related sequences in the GenBank database by the ClustalW multiple sequence alignment analysis program (Thompson *et al*., 1994).

Conjugation experiment of plasmids

Four *Salmonella* isolates containing class 1 integrons were selected as donor cells for conjugation. Nalidixic acid-resistant *E. coli* DH5α were used as recipient cells. The cultures of donor and recipient cells (1:10) were co-precipitated, suspended and harvested on LB agar coated with ampicillin and nalidixic acid (Curtiss *et al*., 1968). The antimicrobial resistance of transconjugants was determined by the disc diffusion method mentioned above.

RFLP and plasmid profiles

The flagella genes *fljB* and *fliC* were amplified using two primer pairs (Dauga *et al*., 1998) and the PCR products were digested with *Mbo*I and *Hha*I restriction enzymes (Finnzymes, Finland) to yield PCR-RFLP patterns. Plasmid isolation was carried out as described by Ansary and Radu (1992). The plasmid DNA of *E. coli* V517 was used as a standard marker for determining the size of plasmids.

RESULTS

Antimicrobial resistances in *Salmonella* isolates

All 230 *Salmonella* isolates conferred resistance to several classes of antimicrobial agents (Table 1). Every isolate (100%) was resistant to sulfamethoxazole. Tetracycline, ampicillin and streptomycin resistance rates were 95, 91 and 78%, respectively. Less than 3% of the isolates showed resistance to amikacin, amoxicillin/clavulanic acid and cephalothin. Obviously, all isolates were susceptible to ceftriaxone, ceftriofur and ciprofloxacin. Among the isolates from the eight pig groups, resistance to amikacin, amoxicillin/clavulanic acid, cephalothin, streptomycin and tetracycline was not significantly different in each original source. However, the isolates from each animal group presented different levels of resistance to apramycin, chloramphenicol, gentamicin, kanamycin, nalidixic acid and trimetroprim/sulfamethoxazole ($P < 0.05$).

Of all the isolates, 211 isolates (91.74%) were resistant to several classes of antimicrobials as MDR strains. These MDR strains resisted at least three antimicrobial agents and exhibited 32 different resistance patterns. The major five (59% of the MDR isolates) of MDR resistance patterns and their isolate origins are presented in Table 2.

Class 1 integrons and resistance gene cassettes

The 211 MDR *Salmonella* isolates were examined for class 1 integrons through PCR amplification. Only four isolates (1.89%) of *S*. Stanley CC1, *S*. Anatum EC3, and *S*. Panama CB2 and CB3, were PCR positive. Each of the PCR products was about 1.0 kb and was subjected to nucleotide sequence analysis. Each nucleotide sequence alignment was compared with the GenBank database and revealed that all PCR products were inserted with class 1 integrons, each of which contained *aadA* gene cassettes. The nucleotide sequences of four isolates were analyzed for their ORFs of gene cassettes, using

Table 2 Top five antimicrobial resistance patterns of MDR *Salmonella* isolates and their sample origin.

Resistance pattern	Serogroup	Number of *Salmonella* isolates																							
		Fecal sample																Environ-mental sample		**Total**					
		Boar		Sow		Pregnant sow		Lactating sow		Replacement gilt		Suckling period		Nursing		Fattening									
		B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	**B**	**C**	**%**[a]			
AMP-SUL-TET		1		9									1	14	2	4		3		**31**	**3**	**16**			
AMP-GEN-STR-SSS-TET			1		17								9						4	**0**	**31**	**15**			
AMP-CHL-KAN-NAL-STR-SSS-TET														22		2				**24**	**0**	**11**			
AMP-APM-CHL-GEN-NAL-STR-SSS-SXT-TET												4		4	2	5		6		**19**	**2**	**10**			
AMP-CHL-KAN-STR-SSS-SXT-TET					1	1			2					8		3				**1**	**14**	**7.1**			

AMP, ampicillin; APM, apramycin; CHL, chloramphenicol; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline; SSS, sulfamethoxazole; and SXT, trimethoprim/sulfamethoxazole; [a] the rates of multi-drug resistant strains (n = 211)

the All in One Analyzer Program, Version 1.36 (used on line at http://www-personal.umich.edu/~ino/blast.html). The results showed that *S.* Stanley CC1 had nucleotide 792 bp and 262 amino acids of the *aadA1* gene. In addition, *S.* Anatum EC3 strains had nucleotide 792 bp and 262 amino acids of the *aadA2* gene, while the *aadA4* gene derived from either *S.* Panama CB2 or CB3 strains had nucleotide 789 bp and 263 amino acids. All *aadA* gene cassettes, which were inserted in the *attI* recombination site, had core site GTTAGGC. The inverse core sites of *aadA1* and *aadA2* cassettes were GTCTAAC, while both *aadA4* cassettes were GCCTAAC. The *attC* sites of the *aadA1* and *aadA2* cassettes were 60 bp long, whereas both *aadA4* cassettes were 57 bp long. These *aadA* genes were encoded for aminoglycoside adenyltransferase, which conferred to streptomycin and spectinomycin resistance phenotypes.

Antimicrobial resistance gene transfer

Among four isolates harboring class I integrons, the common antimicrobial resistances that could be transferred to *E. coli* were ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline. The frequency of conjugation was approximately 10^{-3}. In order to confirm the existence of class 1 integrons on conjugative plasmid, the transconjugants were detected for class 1 integrons with PCR. The amplification confirmed that all the transconjugants contained class 1 integrons that carried the *aadA* gene.

Subtyping of *Salmonella* in farm A

Three common serovars were identified as isolates from farm A and each serovar had the same MDR patterns, comprising *S.* Corvallis (AMP-GEN-STR-SSS-TET), *S.* Rissen (AMP-CHL-KAN-STR-SSS-SXT-TET) and *S.* 1,4,5,12:i- (AMP-APM-CHL-GEN-NAL-STR-SSS-SXT-TET). The subtyping profiles of the isolates, including PCR-RFLP patterns of flagella

genes (data not shown) and plasmid profiles (Table 3), showed that these three serovars were possibly endemic strains on this farm. *S*. Corvallis samples were isolated from the sow and suckling animal groups and from lizards as a carrier animal. *S*. Rissen samples were isolated from the sow, nursing and fattening animal groups, while *S*. 1,4,5,12:i- samples were isolated from the suckling, nursing and fattening animal groups and from feed and water.

DISCUSSION

The study demonstrated that the rates of resistance were high in several antimicrobials commonly used in both medical treatments and

Table 3 Isolate origin and plasmid profiles of three common *Salmonella* serovars isolated from farm A.

Isolate[a]	Origin	Plasmid profile (kbp)
S. Corvallis C11	Sow	54, 10
S. Corvallis C23	Suckling	54, 10, 4.3
S. Corvallis C26	Suckling	54, 10
S. Corvallis C30	Suckling	54, 10
S. Corvallis C74	Sow	54, 10, 4.3
S. Corvallis C119	Sow	54, 10
S. Corvallis C125	Sow	54, 10
S. Corvallis C138	Sow	54, 10
S. Corvallis C149	Lizard	54, 10
S. Corvallis C151	Lizard	54, 10
S. Rissen C40	Nursing	> 54, 10, 9, 5.6
S. Rissen C41	Nursing	> 54, 10, 9, 5.6
S. Rissen C46	Nursing	> 54, 10, 9, 5.6
S. Rissen C49	Nursing	> 54, 10, 9, 8, 5.1, 4
S. Rissen C50	Nursing	> 54, 10, 9, 8, 5.1, 4
S. Rissen C69	Nursing	> 54, 10, 9, 8, 5.1, 4
S. Rissen C80	Sow	> 54, 10, 9, 5.6
S. Rissen C105	Fattening	> 54, 10, 9, 5.6
S. Rissen C106	Fattening	> 54, 10, 9, 5.6
S. Rissen C108	Fattening	> 54, 10, 9, 5.6
S. 1,4,5,12:i:- B37	Nursing	8.6, 8, 5.6, 5.1, 4, 3
S. 1,4,5,12:i:- B44	Nursing	8, 5.1
S. 1,4,5,12:i:- B51	Fattening	8.6, 8, 5.6, 5.1, 4, 3
S. 1,4,5,12:i:- B87	Fattening	8.6, 8, 5.6, 5.1, 4, 3
S. 1,4,5,12:i:- B93	Fattening	8, 5.1
S. 1,4,5,12:i:- B116	Suckling	8, 5.1
S. 1,4,5,12:i:- B117	Suckling	8.6, 8, 5.6, 5.1, 4, 3
S. 1,4,5,12:i:- B139	Feed	8, 5.1
S. 1,4,5,12:i:- B141	Feed	8, 5.1
S. 1,4,5,12:i:- B144	Water	8.6, 8, 5.6, 5.1, 4, 3

[a] letter B or C indicates *Salmonella* serogroup B or C and the number following this indicates the lab number of the isolate.

agriculture. Pigs in the nursing group contained the highest antimicrobial resistance rates. The recently used antimicrobials, such as the third generation of cephalosporins and ciprofloxacin, were still considered efficient drugs for the treatment of animals. Correspondingly, a report on *Salmonella* isolated from animal origins in Thailand showed high sensitivity to these drugs (Padungtod and Kaneene, 2006).

In Thailand, the antimicrobials most commonly used as feed medication in the pig industry have been penicillins, such as amoxicillin. Even though cephalexin (3rd generation) has been used in pig farms for a long time, it has been only occasionally used as an alternative to amoxicillin in particular cases. Ceftiofur and enrofloxacin have been parenterally administered to sows and piglets for specific treatments. In general, the other 3rd and 4th generations of cephalosporins have not been used as feed medication because of their higher prices and scarce availability in the market.

The *aadA* gene cassettes in this study were similar to other reports of class 1 integrons found among *Salmonella* isolates from food-producing animals and in other genera of Enterobacteriaceae (Rankin *et al*., 2002; Kang *et al*., 2005; Michael *et al*., 2005). In this study, class 1 integron-borne gene cassettes showed low prevalence and clonal relevance, and were not related to resistance phenotypes among the tested isolates. However, using the same set of primers, without the presence of gene cassettes, most of the isolates showed the amplification products of the integrase gene at approximately size 200 bp (data not shown).

The conjugation experiment showed similar results to the study of conjugative plasmid that carried class 1 integrons in different *Salmonella* serovars and in *E. coli* (Tosini *et al*., 1998; Kang *et al*., 2005; Michael *et al*., 2005; Hsu *et al*., 2006). The results of the current study confirmed that the conjugative plasmid could disseminate resistance genes among these MDR strains. In addition, it is suggested that the existence of class 1 integrons on conjugative plasmid probably also plays an important role in acquisition and the widespread antimicrobial resistance profiles. Importantly, when the bacterial chromosome already consisted of class 1 integrons, the gene cassette could mobilize from class 1 integrons on the conjugative plasmid to a secondary recombination site on a chromosome and integrative mobilizing elements, such as *Salmonella* genomic island 1 (Carattoli *et al*., 2002; Taylor *et al*., 2004).

Subtyping profiles including R types, PCR-RFLP and plasmid profiles of three common serovars found on farm A implied their clonal origins in each serovar had spread by cross contamination from sows to their offspring and the environment. Though the source of *Salmonella* introduction to farm A could not be identified, the high number of isolated *Salmonella* and the dissemination of particular clones on the farm revealed an inappropriate hygiene program. Furthermore, one of the serovars spreading on farm A was MDR *S.* 1,4,5,12:i- that was reported as one of the top ten serovars found in humans in Thailand (Bangtrakulnonth *et al*., 2004) and was thought to be a monophasic variant of serovar Typhimurium related to phage type DT 104 (Amavisit *et al*., 2005). The dissemination of this serovar on animal farms and to humans revealed that this serovar is another multi-host serovar, like Typhimurium, and requires careful consideration.

In general, the surveillance of MDR *Salmonella* strains presented useful information to update the changing profiles of MDR bacteria over different periods and in different feed medications on pig farms. The characterization of MDR *Salmonella* in this study may provide the descriptive data and trends of particular strains to provide a better understanding of *Salmonella* contamination on pig farms.

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