Characterization of antimicrobial resistant *Escherichia coli* isolated from food producing-animals in Thailand.

（タイの食料生産用動物から分離された薬剤耐性大腸菌の性状解析）

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CONTENTS

CHAPTER II

Antimicrobial Resistance, Extended Spectrum beta-lactamase Productivity and Class 1 Integrons in *Escherichia coli* from Healthy Swine

ABBREVIATIONS

tet(A)	tetracycline resistance protein TetA
tet(B)	tetracycline resistance protein TetB
tet(C)	tetracycline resistance protein TetC
tet(D)	tetracycline resistance protein TetD
tet(E)	tetracycline resistance protein TetE
tet(G)	tetracycline resistance protein TetG
intI	class I integron integrase gene
sul	dihydropteroate synthase genes
qacE	quaternary ammonium compound-resistance protein qacE
dfrA	dihydrofolate reductase genes
aadA	aminoglycoside adenyltransferase genes
bla	beta-lactamase genes
CLSI	Clinical and Laboratory Standards Institute
AMP	ampicillin
CTX	cefotaxime
CRO	ceftriazone
CAZ	ceftazidime
STR	streptomycin
KAN	kanamycin
TET	tetracycline
NAL	nalidixic acid
CHL	chloramphenicol

TMP	trimethoprim
GEN	gentamicin
SXT	trimethoprim-sulphamethoxazole
CIP	ciprofloxacin
NOR	norfloxacin
MEM	meropenem
ESBL	extended-spectrum beta-lactamase
MDR	multidrug resistance

PREFACE

Food contamination with antimicrobial-resistant bacteria is a public health concern because the resistant organisms can be transferred to humans through the consumption of contaminated food and can thus compromise human health. The extensive use of antimicrobials in food-producing animals has potential to raise antimicrobial-resistance in enteric commensal bacteria. These bacteria may constitute a significant reservoir of antibiotic resistance determinants, which can be transferred to pathogenic bacteria for animals and/or humans (73).

Beta-lactam antimicrobials are one of the important antimicrobial agents in veterinary medicine and food animal production. These antimicrobial agents can be divided into several groups, three of which are used in veterinary medicine, namely, penicillins, first- to fourth-generation cephalosporins, and beta-lactamase inhibitors (62). The occurrence of beta-lactam resistance in *Enterobacteriaceae* in different food-producing animals has been increasingly reported (23, 43, 57). The production of extended-spectrum beta-lactamases (ESBLs) constitutes one of the most spread and relevant antimicrobial resistance mechanisms, compromising the use of several beta-lactam antimicrobials (16). ESBL-producing bacteria are resistant to most of the beta-lactams and, more often than not, to other antimicrobials such as aminoglycosides, tetracyclines, chloramphenicol, trimethoprim, sulphonamides or quinolones. These resistances relate to co-selection by antibiotics (26, 54, 67, 69).

The horizontal transfer of resistance genes, especially mobile genetic elements, has led to the rapid emergence of multidrug-resistant bacteria in the food-producing animals, which may deliver to humans. Integrons are gene-capturing systems located on the bacterial chromosome or

plasmid that incorporate gene cassettes and make them functional. Integrons play an important role in the carriage and dissemination of antimicrobial resistance genes (18). Class 1 integrons (figure 1) are most frequently found in multidrug-resistant gram-negative bacteria and have been identified in bacterial strains recovered from food-producing animals, their products, and the environment (27, 34, 36, 51). In recent years, class 1 integrons have been found in ESBL-producing clinical isolates of *Enterobacteriaceae* (11, 48) and food-producing animals (6, 14, 28, 42, 44).

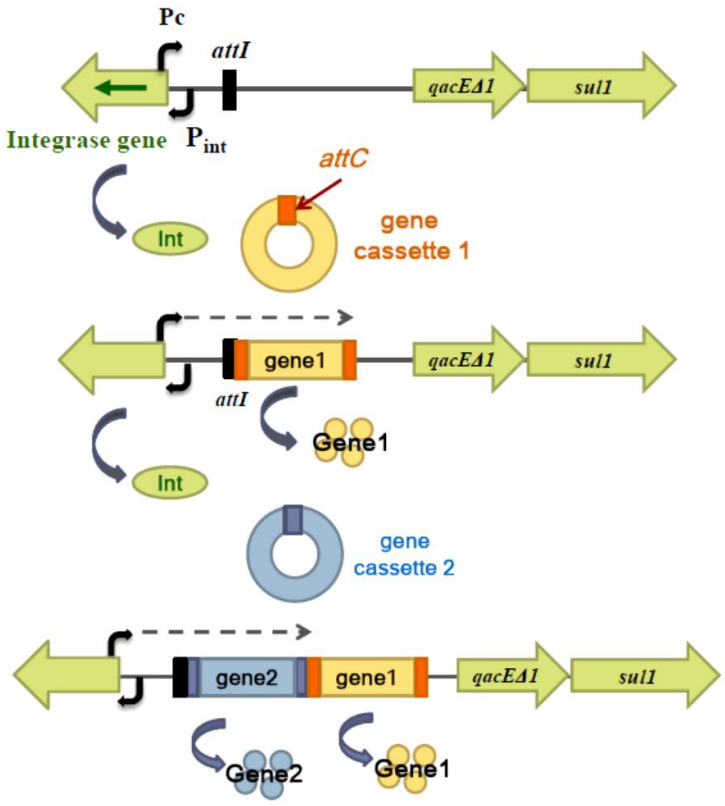

Figure 1. Representation of class 1 integrons and a model for gene cassette acquisition. The process by which circularized gene cassettes (resistance gene 1 and 2) are inserted at the *attI* site in a class 1 integrons. Genes and open reading frames in the 5'-conserved segment (5'-CS) and 3'-conserved segment (3'-CS) of a schematic class 1 integron are indicated by boxes. Resistance gene cassettes inserted within the integron are indicated by yellow and blue boxes and vertical dark blue and orange bars represent *attC* recombination sites. The *qacEΔ*1 and *sulI* genes in the 3'-CS confer resistance to quaternary ammonium compounds and sulfonamides, respectively. Pc is cassette promoter; Pint is the integrase gene (*intI1*) promoter. Multiple insertion events at an *attI* site are common and cassette-associated genes are usually inserted in the same orientation, producing tandem arrays of co-transcribed genes expressed from Pc.

Escherichia coli is the predominant member of normal intestinal flora of food animals but possesses the potential to cause a serious pathogenicity in human. This bacterium has been introduced as an indicator species in surveillance studies of antimicrobial resistance, since it easily acquires resistance potentials (68). According to antimicrobial resistance monitoring data, the high prevalence of antimicrobial resistant *E. coli* in food-producing animals has become a worrisome problem. Kaesbohrer *et al.* (30) reported that 68% of *E. coli* isolated from pigs, poultry and cattle in slaughterhouses in Germany had the drug resistance phenotype. In Korea, 91.4% of *E. coli* isolates from commercial foods (raw meat, fish, and processed foods) were resistant to at least one antimicrobial (36). In Thailand, 98% of *E. coli* isolated from healthy swine was reported to have multidrug resistance (MDR) (40). In Thailand, little is known about phenotypic drug resistance and genetic mechanisms of resistance in *E. coli* isolates from food-producing animals. The present thesis consist of two chapter; in chapter I, I have investigated drug resistance profiles and characterized class 1 integrons in *E. coli* isolates from shrimps and their environment. In addition, the resistance determinants for the major resistance phenotype were also identified. In chapter II, I have investigated antimicrobial susceptibility and ESBL-producing strains, and characterised class 1 integrons in *E. coli* isolates from healthy swine in Thailand.

CHAPTER I

Characterization of Antibiotic Resistance in *Escherichia coli* Isolated from Shrimps and Their Environment

Antimicrobial use in aquaculture results in a risk of the evolution of antimicrobial-resistant bacteria in aquatic animals and the environment (25). In shrimp farming industries, large amounts of antimicrobial agents are used as feed additives and thrown into the pond water as therapeutic and prophylactic agents for shrimp. Consequently, antimicrobials persisting in shrimp, water, and sediment in the shrimp pond may contribute to the acquisition of drug resistance among shrimp pathogens and the intestinal flora of the shrimp.

In recent studies in various countries, the prevalence of antimicrobial-resistant bacteria in shrimp farms and shrimp products has been reported (17, 34, 52, 56, 65, 66). The microbiological condition of shrimp is mainly dependent on the transportation, handling, and processing conditions (53). Antimicrobial-resistant bacteria can contaminate shrimp products through improper handling of shrimp between harvesting and marketing, which is a particular problem in markets where raw shrimp are sold and handled by sellers and customers (38, 53).

Tetracyclines are therapeutic agents most commonly used for human and animal treatment. Oxytetracycline is one of the antibiotics approved by the US FDA and has been widely used to treat diseases in aquaculture animals (59). The widespread use of tetracycline and its derivatives has resulted in the selection of resistant bacteria. Among tetracycline resistance genes, the genes associated with an efflux mechanism such as *tet*(A)-(E) and *tet*(G) occur frequently in Gram-negative bacteria (58).

MATERIALS AND METHODS

E. coli isolates and DNA preparation.

A total of 312 *E. coli* isolates were collected from shrimp farms and markets in a province in southern Thailand from February, 2007 to February, 2008. The isolates were revived and reconfirmed by biochemical tests (35). Briefly, one loopfull of *E. coli* stock was streaked directly on MacConkey agar and incubated at 37^{o}C for 24 h. Single lactose-positive colony was picked and then the IMViC (indole, methyl red, Voges-Proskauer, and citrate) test was performed. The isolate demonstrating IMViC pattern of positive-positive-negative-negative was considered to be *E. coli* strain. Drug resistant strains were cultured in Luria broth at 37 °C for 24 h for DNA extraction. DNA was prepared by phenol-chloroform extraction based on the procedure by Maniatis and Fristsch (46)

Antimicrobial susceptibility tests.

Antimicrobial susceptibility tests were carried out using agar disc diffusion method in accordance with the standard and interpretive criteria recommended by the Clinical and Laboratory Standards Institute (15). The following commercially available antimicrobial discs (Oxoid, Basingstoke, UK) were employed: ampicillin (AMP) 10 µg; cefotaxime (CTX) 30 µg; ceftriazone (CRO) 30 µg; ceftazidime (CAZ) 30 µg; streptomycin (STR) 10 µg; kanamycin (KAN) 30 µg; tetracycline (TET) 30 µg; nalidixic acid (NAL) 30 µg; chloramphenicol (CHL) 30 µg; and trimethoprim (TMP) 5 µg. *E. coli* ATCC 25922 was used to verify the quality and accuracy of the tests.

Characterization of tetracycline resistance genes.

Tetracycline efflux genes were amplified by PCR. Amplification of *tet*(B), *tet*(D), *tet*(E) and *tet*(G) was carried out in 20 µl volumes consisting of 1 µl of DNA template (10 ng/µl), 1 x Green GoTaq reaction buffer, 0.2 mM each dNTP, 0.4 µM of each primer and 0.5 U of Go*Taq* DNA polymerase (Promega, Fitchburg, WI). For *tet*(A) and *tet*(C), a PCR reaction mixture (total 20 µl) consisting of 1 x standard *Taq* reaction buffer, 0.2 mM each of dNTP, additional 0.5 mM $MgCl_2$, 0.4 µM of each primer and 0.5 U *Taq* DNA polymerase (New England BioLabs, Ipswich, MA) were used. DNA amplification was carried out in a thermal cycler (iCycler, Bio-Rad, Hercules, CA). The primer sequences and PCR condition are presented in Table 1. PCR products were analyzed by electrophoresis using 2% agarose gel with 0.5 µg/ml ethidium bromide in 1 x TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0) at 50 mA for 25 min, followed by visualization under UV light (ATTO Co., Tokyo, Japan). The sizes of the PCR products were calculated using 50-bp DNA marker. The predicted sizes were 210 bp, 659 bp, 418 bp, 787 bp, 278 bp and 467 bp for *tet*(A), *tet* (B), *tet*(C), *tet*(D), *tet*(E) and *tet*(G), respectively.

Identification of class 1 integrons.

Resistant isolates were tested for the presence of class 1 integrase genes by PCR using primer INT-1U and INT-1D (Table 1). PCR reaction mixture (total 20 µl) consisted of 1 µl DNA template, 1 x Green GoTaq reaction buffer, 0.2 mM each of dNTP, 0.4 µM of each primer and 0.5 U GoTaq DNA polymerase. PCR products were analyzed by electrophoresis using 1% agarose gel and the sizes of the PCR products were determined using 100-bp DNA ladder and lambda *Hind* III digest as size markers.

Characterization of gene cassettes in integrons.

All class 1 integrase PCR-positive strains were further analyzed for their gene cassette pattern by amplifying the variable region between the 5'-conserved segment (5'CS) and 3'-conserved segment (3'CS) or *sul* genes. The following four primer sets using one forward primer and four different reverse primers were designed and employed: 5'CS2 (F) with 3'CS2 (R), Sul1 (R), Sul2 (R) or Sul3 (R), as shown in Table 1. PCR reaction mixture (total 20 µl) consisted of 1 µl DNA template (10 ng/µl), 1 x LA PCR buffer (Mg^{2+} free), 0.375 mM each of dNTP, 2.5 mM $MgCl_2$, 0.4 µM of each primer and 1 U LA Taq (Takara Bio, Shiga, Japan). After agarose gel electrophoretic separation, PCR products were purified by Wizard SV Gel and PCR Clean-Up System Kits (Promega). The concentration and quality of the purified products were measured using ND-1000 V3.1.0 (Thermo Fisher Scientific, Waltham, MA). The purified products were sequenced by primer walking using the BigDye terminator cycle sequencing ready reaction kit and analyzed using the 3130 Genetic Analyzer (Technologies, Carlsbad, CA). Contiguous sequences were analyzed by the blast search engine (http://www.ncbi.nih.gov) and compared with those registered in the GenBank database. The primer sets designed in this study and used for primer walking are shown in Table 2.

Data analysis.

The prevalence of antimicrobial resistant *E. coli* isolated from various samples in shrimp farms and markets were presented as percentage. Antimicrobial resistance rate were compared by a chi-square and Fisher's exact test. Differences were considered significant at $P < 0.05$.

Table 1. Oligonucleotide primers used in this study

Name	Primer sequence (5'-3')	PCR condition			Reference
		Denaturing	Annealing	Extension	
tetAF (643-662)	GGCGGTCTTCTTCATCATGC	95 °C, 30 s	55 °C, 30 s	72 °C, 15 s[a]	
tetAR (882-863)	AAGCAGGATGTAGCCTGTGC				
tetB (F)	TTGGTTAGGGGCAAGTTTTG	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[b]	*(37)*
tetB (R)	GTAATGGGCCAATAACACCG				
tetC (F)	CTTGAGAGCCTTCAACCCAG	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[b]	*(37)*
tetC (R)	ATGGTCGTCATCTACCTGCC				
tetD (F)	AAACCATTACGGCATTCTGC	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[b]	*(37)*
tetD (R)	GACCGGATACACCATCCATC				
tetE (F)	AAACCACATCCTCCATACGC	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[b]	*(37)*
tetE (R)	AAATAGGCCACAACCGTCAG				
tetG (F)	GCTCGGTGGTATCTCTGCTC	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[b]	*(37)*
tetG (R)	AGCAACAGAATCGGGAACAC				
INT-1U	GTTCGGTCAAGGTTCTG	94 °C, 30 s	50 °C, 30 s	72°C, 2 min[c]	*(37)*
INT-1D	GCCAACTTTCAGCACATG				
5' CS2 (F)	TGTACAGTCTATGCCTCGGGCATCC	98 °C, 10 s	60 °C, 10 s	72°C, 5 min[d]	
3' CS2 (R)	AGCAGACTTGACCTGATAGTTTGGC				
Sul1 (R)	CTAGGCATGATCTAACCCTCGGTCT				
Sul2 (R)	CGAATTCTTGCGGTTTCTTTCAGC				
Sul3 (R)	CATCTGCAGCTAACCTAGGGCTTTGGA				

[a] After 30 cycles, final extension step of 5 min at 72 °C was performed.

[b] After 35 cycles, final extension step of 10 min at 72 °C was performed.

[c] After 30 cycles, final extension step of 7 min at 72 °C was performed.

[d] After 30 cycles, final extension step of 10 min at 72 °C was performed.

Table 2. Oligonucleotide primers used for primer walking

Name	Primer sequence (5'-3')	Target
5'CS-RV (R)	CTTTGTTTTAGGGCGACTG	5´-CS
aadA2(202-182) (R)	TGAGCAATGCTCGCCGCGTCG	*aadA2*
aadA5 (25-2) (F)	AACGATTGAGAGAATCTTGCGTTG	*aadA5*
dfr17 (274-287) (F)	GATCATGTATATGTCTCTGGCGGG	*dfr17*
dfr17-Cap (606-625) (F)	TCCGCCACGACATCCTTTCC	*dfr17*
Sul1 (284-263) (R)	CGCTTGAGCGCATAGCGCTGGG	*sul1*
Sul2 (179-160) (R)	GAAACAGGCGCGGCGTCGGG	*sul2*
Sul2 -5'side noncode-1 (R)	GATTTTATTCCCGCGCCTCG	*sul2*
Sul2-5'side (244)	GCAGGGAATACTCAGCAAGC	*sul2*
Sul2-5'side (230)	ACCTCCCGTGCTGTACTGTC	*sul2*
QacE delta1-Cap (272-292) (R)	CCCTCGCTAGATTTTAATGCG	*qacE Δ1*

RESULTS

Antimicrobial susceptibility of *E. coli* isolates.

A total of 312 *E. coli* isolates derived from shrimps and their environment were tested for their susceptibility to ten antimicrobial agents. Among these, 149 were obtained from shrimp farms and 163 were from related markets. From shrimp farms, 27, 54, 55, 11 and 2 isolates were from shrimp (*Penaeus vannamei*), surface water, shrimp pond waste (accumulated waste in the center of the culture pond), shrimp pond sediment (pond soil around the edge of the culture pond) and shrimp feed, respectively. From markets, 37 and 126 were from shrimp and water (ice water in shrimp container), respectively. The results showed that 17.6% (55/312) of isolates were resistant to at least one antimicrobial agent (Table 3). High resistance rates were observed against TET (14.4%; 45/312), AMP (8.0%; 25/312) and TMP (6.7%; 21/312). Resistance to NAL (2.9%; 9/312), CHL (2.2%; 7/312), KAN (1.6%; 5/312), STR (1.3%; 4/312), CTX (0.3%; 1/312), CRO (0.3%; 1/312), and CAZ (0.3%; 1/312) was less common.

Twenty-two different resistance patterns were found among resistant isolates (Table 3). The rate of double drug-resistant isolates was the highest, 38.2% (21/55), followed by that of single drug-resistant (32.7%; 18/55), triple drug-resistant (14.5%; 8/55), quadruple drug-resistant (9.1%; 5/55) and quintuple drug-resistant (5.5%; 3/55). The prevalence of multidrug-resistant (MDR) isolates (resistant to more than three antibiotics) was 29.1% (16/55). Regarding the source of the samples, the resistant isolates were found in shrimp (29.6%, 8/27), water (7.4%; 4/54) and shrimp pond waste (25.5%, 14/55) in the farms and shrimp (13.5%, 5/37) and water (19.0%; 24/126) in the markets.

Table 3. Antimicrobial resistance patterns of *E. coli* isolated from shrimp farms and markets

Resistance pattern	Shrimp farms										Markets				Total	
	Shrimp (n=27)		Water (n = 54)		Pond waste (n = 55)		Pond sediment (n = 11)		Feed (n = 2)		Shrimp (n = 37)		Water (n = 126)		(N = 312)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
AMP	1	3.7													1	0.3
NAL											1	2.7	1	0.8	2	0.6
STR											1	2.7			1	0.3
TET	1	3.7	1	1.9	9	16							3	2.4	14	4.5
AMP-TMP											1	2.7	2	1.6	3	1
AMP-TET			2	3..7									6	4.8	8	2.6
CHL-TMP	1	3.7													1	0.3
NAL-TMP											1	2.7			1	0.3
STR-TET													1	0.8	1	0.3
TMP-TET			1	1.9	3	5.5							3	2.4	7	2.2
AMP-CHL-TET	1	3.7													1	0.3
AMP-KAN-TET	2	7.4													2	0.6
AMP-NAL-TET	1	3.7									1	2.7			2	0.6
AMP-STR-TET													1	0.8	1	0.3
KAK-TMP-TET													1	0.8	1	0.3
NAL-TMP-TET					1	1.8									1	0.3
AMP-CHL-TMP-TET													2	1.6	2	0.6
AMP-NAL-CHL-TMP													1	0.8	1	0.3
AMP-NAL-TMP-TET													2	1.6	2	0.6
AMP-CRO-CTX-CAZ-TET					1	1.8									1	0.3
AMP-KAN-CHL-TMP-TET													1	0.8	1	0.3
STR-KAN-CHL-TMP-TET	1	3.7													1	0.3
Any drug	8	30	4	7.4	14	26					5	14	24	19	55	17.6
None	19	70	50	93	41	75	11	100	2	100	32	87	102	81	257	82.4

AMP, ampicillin; CTX, cefotaxime; CRO, ceftriazone; CAZ, ceftazidime; STR, streptomycin; KAN, kanamycin; TET, tetracycline; NAL, nalidixic acid; CHL, chloramphenicol; TMP, trimethroprim

The resistance rate of *E. coli* isolated from water (19%) in the markets was significantly higher than that in the shrimp farm water (7.4%, $P < 0.05$). Conversely, no statistically significant differences were observed between the resistant isolate rates in shrimp from both sources. The MDR isolate ratio was highest in shrimp in the farms (62.5%); however, the ratio that was resistant more than 4 drugs was predominantly higher in water in the markets (25%) than other samples.

Characterization of tetracycline resistance genes.

Tetracycline resistance genes were amplified by PCR with 6 different sets of primers targeting efflux genes. Among 55 resistant isolates, 47 isolates were positive for *tet* gene amplification (Table 4). The most common *tet* gene identified was *tet*(A) (69.1%; 38/55). The second common gene was *tet*(B), which was found in 31 (56.4%) and *tet*(C) was found in 2 isolates (3.6%). *tet*(D), *tet*(E) and *tet*(G) were not detected. Fifteen resistant isolates carried only *tet*(A), 7 isolates carried only *tet*(B), 23 isolates carried both *tet*(A) + *tet*(B) and 2 isolates contained *tet*(B) + *tet*(C).

Class 1 integron detection and characterization.

All 55 resistant isolates were tested for the presence of the class 1 integrase (*IntI1*) gene by PCR amplification. Sixteen isolates, eleven from water in the markets, four from shrimp pond waste and one from shrimp in a farm, were found to carry *IntI1* gene (Table 4), and further examined for the gene cassette.

Table 4. Phenotypes and genotypes of antimicrobial-resistant *E. coli* isolated from shrimp and its environments

Source	Sample	Area	Code	Antimicrobial resistance profile[a]		*intl* I PCR	Integron name	Promoter (Pc)	*tet* gene
				Resistant	Intermediate				
farm	shrimp	BA	N52B1	STR, KAN, CHL, TMP, TET	-	-	-	-	A
		BI	N347B4	AMP, KAN, TET	CRO, NAL, TMP	-	-	-	A
		BI	N362B4	AMP, KAN, TET	NAL	-	-	-	A + B
		BI	N230B3	AMP, CHL, TET	STR	+	In54[b]	PcW	A+B
		AI	N373B4	AMP, NAL, TET	-	-	-	-	B
		BA	N53B1	CHL, TMP	-	-	-	-	-
		BI	N222B3	TET	NAL	-	-	-	B + C
		AI	N37B1	AMP	-	-	-	-	-
	shrimp pond waste	AI	N93B1	AMP, CRO, CTX, CAZ, TET	-	-	-	-	A + B
		AI	N246B3	NAL, TMP, TET	AMP, STR	-	-	-	A + B
		AI	N244B3	TMP, TET	AMP, STR	+	In54[b]	PcH1	A
		AI	N245B3	TMP, TET	AMP, STR	+	-	-	A
		AI	N247B3	TMP, TET	AMP, STR	-	-	-	A + B
		AA	N270B3	TET	STR	-	-	-	A + B
		AA	N274B3	TET	STR	-	-	-	A
		AA	N275B3	TET	-	-	-	-	-
		AI	N266B3	TET	-	-	-	-	A + B
		AI	N267B3	TET	-	-	-	-	A + B
		AI	N268B3	TET	-	-	-	-	A + B
		AI	N276B3	TET	-	+	-	-	A + B
		AI	N277B3	TET	-	+	-	-	A + B
		AI	N278B3	TET	-	-	-	-	A + B
	water	AA	N21B1	AMP, TET	-	-	-	-	-
		AA	N71B1	AMP, TET	-	-	-	-	-
		AA	N218B3	TMP, TET	STR, NAL	-	-	-	A
		AI	N23B1	TET	-	-	-	-	A
market	shrimp	BI	N321B4	AMP, NAL, TET	-	-	-	-	A + B
		CI	N13B1	AMP, TMP	STR, TET	-	-	-	A
		CA	N16B1	NAL, TMP	-	-	-	-	B
		CI	N171B2	NAL	-	-	-	-	B + C
		CI	N173B2	STR	-	-	-	-	-
water		CA	N220B3	AMP, KAN, CHL, TMP, TET	STR	+	-	-	A + B
		CA	N106B2	AMP, NAL, CHL, TMP	STR	-	-	-	A
		CA	N369B4	AMP, NAL, TMP, TET	-	+	In27[c]	PcW TGN-10	A
		CA	N370B4	AMP, NAL, TMP, TET	-	+	In27[c]	PcW TGN-10	A
		CA	N360B4	AMP, CHL, TMP, TET	-	-	-	-	A + B
		CA	N33B1	AMP, CHL, TMP, TET	-	-	-	-	-

Table 4. (Continued)

Source	Sample	Area	Code	Antimicrobial resistance profile[a]		*intl* I PCR	Integron name	Promoter (Pc)	*tet* gene
				Resistant	Intermediate				
		CI	N229B3	AMP, STR, TET	-	-	-	-	A + B
		CA	N219B3	KAN, TMP, TET	STR, CHL	+	-	-	A + B
		CA	N361B4	AMP, TMP	STR	+	In27[c]	PcW TGN-10	B
		CI	N28B1	AMP, TMP	STR, TET	-	-	-	-
		CA	N48B1	AMP, TET	-	+	-	-	B
		CA	N100B1	AMP, TET	-	-	-	-	A
		CA	N104B2	AMP, TET	-	+	-	-	A
		CA	N166B2	AMP, TET	-	-	-	-	A + B
		CA	N150B2	AMP, TET	-	-	-	-	A + B
		CI	N85B1	AMP, TET	-	-	-	-	B
		CA	N368B4	TMP, TET	NAL	+	-	-	A
		CA	N24B1	TMP, TET	-	+	In54[b]	PcH1	A+B
		CA	N237B3	TMP, TET	-	+	In54[b]	PcH1	A + B
		CI	N116B2	STR, TET	-	+	-	-	B
		CA	N165B2	TET	-	-	-	-	A + B
		CA	N350B4	TET	-	-	-	-	A + B
		CA	N348B4	TET	-	-	-	-	A
		CA	N366B4	NAL	-	-	-	-	B

[a] Antimicrobials: TET, tetracycline; AMP, ampicillin; TMP, trimethoprim; NAL, nalidixic acid; CHL, KAN, kanamycin; STR, streptomycin; CTX, cefotaxime; CRO, ceftriazone; CAZ, ceftazidime.

[b] In54 consist of *dfrA17* and *aadA5*.

[c] In27 consist of *ddfrA12*, *orfF* and *aadA2*.

From 7 isolates, gene cassettes were successfully detected; however, it was failed in the remaining nine isolates, presumably because of alterations in the primer binding site. Seven isolates carried gene cassettes that included dihydrofolate reductase genes (*dfrA12* or *dfrA17*) conferring trimethoprim resistance and aminoglycoside adenyltransferase A genes (*aadA2* or *aadA5*) conferring streptomycin resistance. The figure 2 showed schematic representation of class 1 integrons identified in this study. Two different integrons (In54 and In27) were defined by their structural features (50). In54 was amplified from four isolates, N24B1, N230B3, N237B3 and N244B3, using a primer set, 5'CS2 (F) + Sul2 (R). This integron harbored two partial sequences of insertion elements IS*26* and IS*5075* in addition to *dfrA17*, *aadA5* and *sul2* (Fig. 2A). DNA fragments of 2.0 and 3.7 kb were amplified using primer sets 5'CS2 (F) + 3'CS2 (R) and 5'CS2 (F) + Sul1 (R), respectively, from three isolates, N361B4, N369B4 and N370B4. Sequencing results of these fragments demonstrated that those two sequences were derived from the same integron consisting of *dfrA12*, *orfF*, *aadA2*, *qacEΔ1* and *sul1* (In27) (Fig. 2B). The promoter region in each integron was also analyzed and shown in Table 4.

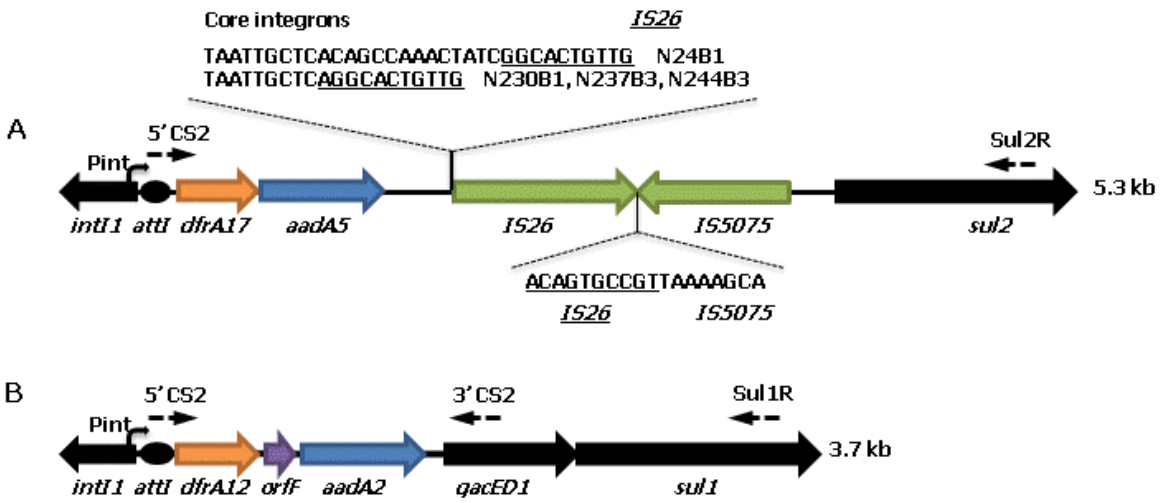

Figure 2. Schematic representation of class 1 integrons identified in *E. coli* isolates. A and B show the schematics of In54 and In27, respectively. Arrows with dotted line indicate primers and anneal sites. Open arrows indicate the open reading frames of the different genes.

DISCUSSION

Antimicrobials are used for the treatment of infectious diseases in humans and for nonhuman applications. The emergence of antimicrobial-resistant organisms is a major public health concern because these organisms may be circulating in the food chain, including shrimp farms and markets. In this study, high resistance rates were found for tetracycline, ampicillin, and trimethoprim. In Thai shrimp farms, quinolones, tetracyclines, and sulfonamides are used to prevent and treat *Vibrio* infections (22). Similar resistance phenotypes have been previously detected in *Salmonella* and *Vibrio* strains from shrimp farms in Thailand (34, 35). Similar results have been published for sea bass farms in Turkey and shrimp farms in Brazil and the Philippines (49, 56, 64, 66). The presence of antimicrobial-resistant organisms appeared to be due to the uncontrolled use of tetracycline, ampicillin, and trimethoprim and the spread of antibiotic-resistant *E. coli* strains in the aquatic environment.

Tetracyclines have been used commonly as growth promoters, and their residues have been carried over to animal husbandry and aquaculture (21). Long-term low level exposure to tetracyclines can increase the spread of resistant bacterial strains, which can occur in shrimp farms and markets where tetracyclines are regularly used, as indicated by the high prevalence of tetracycline-resistant *E.coli* strains in this study. In aquaculture farms in different regions of the world, *tet*(A), *tet*(B), and *tet*(E) are prevalent (4, 19). Two of these genes, *tet*(A) and *tet*(B), were found in the present study. The genetic background of tetracycline resistance among bacteria from this aquatic environment appeared to be diverse locally, probably because of differences in the drug resistance gene pool in the environment. In some tetracycline-resistant isolates, no genes for efflux proteins were detected. This resistance phenotype may have been caused by an

alternative mechanism of tetracycline resistance, such as ribosomal protection or target mutation (10, 12).

The high prevalence of ampicillin-resistant strains may reflect (i) the widespread clinical use of penicillins in both humans and animals, which results in selective pressure on bacterial populations in the environment, or (ii) some illegal use of penicillins in shrimp farms and markets, because beta-lactams are not officially used in shrimp farming (3). Alternatively, the wide distribution of penicillin resistance could be due to co-selection of antibiotics, as suggested by the frequent presence of several kinds of drug resistance genes on the same mobile element. In our study, 29% of resistant isolates were resistant to more than three drugs. Multidrug resistance has been observed in bacteria from aquaculture environments in association with the use of various drugs (2, 64, 66). Akinbowale *et al.* (2) found that use of one or more antibiotics in aquaculture provided selection pressure for the emergence of multidrug-resistant bacteria.

Class 1 integrons were detected in 16 resistant isolates. Of these isolates, seven carried gene cassettes that included dihydrofolate reductase genes (*dfrA12* or *dfrA17*) conferring trimethoprim resistance and aminoglycoside adenyltransferase A genes (*aadA2* or *aadA5*) conferring streptomycin resistance. Class 1 integrons are widely distributed among bacteria isolated from shrimp, fish, and shellfish (34, 60, 65). Five isolates in this study containing In54 were found in samples from different areas (AI, BI, and CA) (Table 4), suggesting they are widely spread in southern Thailand. Kang *et al.* (31) reported that the In54 integron is commonly found in *E. coli* isolates from humans in Korea. In Thailand, a study of class 1 integrons among *Salmonella enterica* isolates from poultry and swine revealed that the gene cassette array *dfrA12*-orfF-*aadA2* (In27) was the most prevalent among these isolates (32). Wannaprasat *et al.* (71)

also reported that *Salmonella* isolated from pork and humans in northern Thailand carried class 1 integrons and that the most frequently found profile was In27. In present study, *E. coli* isolates possessing In27 were detected from samples obtained in the markets, indicating that In27 is widespread among humans and food animals in Thailand.

Among the isolates carrying the integrons with the *dfrA* gene cassette, six were resistant to trimethoprim and one was susceptible to this agent. All isolates carrying the integrons with the *aadA* gene cassette were not resistant to streptomycin; however, four isolates expressed a streptomycin intermediate phenotype (Table 4). The MICs of isolates with the *aadA* gene cassette were 4 mg/liter (six isolates) and 8 mg/liter (one isolate). A similar result was reported by Sunde and Norstro¨m (63), who suggested that *E. coli* strains carrying *aadA* gene cassettes could have MICs far below the breakpoint values normally used to classify a strain as susceptible to streptomycin. Zhao *et al.* (74) reported that the integron-borne *aadA* gene was silent in *E. coli* O111:NM but became fully expressed when it was transferred to *Hafnia alvei*. This silent *aadA* gene can become expressed when transferred to a new host, and horizontal transfer may enhance the expression of a resistance gene. Variable expression of gene cassettes in integrons can be caused by several factors, e.g., whether the integron is located on a high-copy-number plasmid or the position of the cassette on the integron is near the 3' end (8). Several versions of the integron promoters located in the 5'-conserved segment of the integron cause differences in the strength of the promoter (41). Five predominant gene cassette promoter (Pc) variants possessing different expression efficacies have been identified (29) (weakest to the strongest): PcW, PcH1, PcWTGN-10, PcH2, and PcS. In the present study, three different Pc variants were found: PcW, PcH1, and PcWTGN-10 (Table 4). One isolate carrying In54 harbored PcW (N230B3), the

weakest promoter, and was susceptible to trimethoprim although it also carried a *dfrA17* cassette. In contrast, three other isolates carrying In54 with PcH1 (N244B3, N24B1 and N237B3) were resistant to trimethoprim (Table 4). These results suggest that gene cassettes of class 1 integrons may be differently expressed depending on the Pc promoter variant.

The presence of *E. coli* in water or food is an indicator of fecal contamination and suggests the possibility of exposure of consumers to potential pathogens. I postulated that drug-resistant *E. coli* strains on farms and in markets might come from a variety of sources, including workers' hands, animal waste, shrimp feed, and water. The highest rate of antibiotic resistance was found in shrimp samples. However, the prevalence of highly drug-resistant *E. coli* (i.e., resistant to more than four drugs) was significantly higher in shrimp storage water (ice water) in markets than that on shrimp farms. Contamination by microorganisms might occur after harvesting shrimps, during transportation and sale in the markets. These findings are in agreement with reports from various countries, in which drug-resistant *E. coli* contamination of shrimp and associated materials was also found in selling places, e.g., fresh markets, supermarkets, and retail stores (36, 38, 53, 65). Previous studies have indicated that water used for washing seafood and ice used for chilling seafood may be heavily contaminated with drug-resistant organisms that can grow on shrimps (39, 53).

SUMMARY

Antimicrobial resistance in bacteria associated with food and water is a global concern. To survey the risk, 312 *Escherichia coli* isolates from shrimp farms and markets in Thailand were examined for susceptibility to 10 antimicrobials. The results showed that 17.6% of isolates (55 of 312) were resistant to at least one of the tested drugs, and high resistance rates were observed to tetracycline (14.4%; 45 of 312), ampicillin (8.0%; 25 of 312), and trimethroprim (6.7%; 21 of 312); 29.1% (16 of 55) were multidrug resistant. PCR assay of the *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G) genes detected one or more of these genes in 47 of the 55 resistant isolates. Among these genes, *tet*(A) (69.1%; 38 of 55) was the most common followed by tet(B) (56.4%; 31 of 55) and *tet*(C) (3.6%; 2 of 55). The resistant isolates were further investigated for class 1 integrons. Of the 55 resistant isolates, 16 carried class 1 integrons and 7 carried gene cassettes encoding trimethoprim resistance (*dfrA12* or *dfrA17*) and aminoglycosides resistance (*aadA2* or *aadA5*). Two class 1 integrons, In54 (*dfrA17-aadA5*) and In27 (*dfrA12*-orfF-*aadA2*), were found in four and three isolates, respectively. These results indicate a risk of drug-resistant *E. coli* contamination in shrimp farms and selling places.

CHAPTER II

Antimicrobial Resistance, Extended Spectrum beta-lactamase Productivity and Class 1 Integrons in *Escherichia coli* from Healthy Swine

INTRODUCTION

Swine is one of the major food-producing animals in several countries including Thailand. More importantly, swine has been implicated as a source of antimicrobial-resistant bacteria (36, 37, 40, 51) indicating the importance of surveillance and monitoring of antimicrobial resistance in food-producing animals. However, data on the occurrence of ESBL-producing strains and/or class 1 integrons in *E. coli* from healthy swine were still limited in Thailand. In this study, I investigated antimicrobial susceptibility and ESBL-producing strains, and characterised class 1 integrons in *E. coli* isolates from healthy swine in Thailand.

MATERIALS AND METHODS

Isolation of bacterial strains.

A total of three swine farms in Nakhon Pathom, Thailand were included in this study. Forty-five fecal swab samples were obtained from farm 1 (14 samples), farm 2 (11 samples) and farm 3 (20 samples). Each sample was inoculated on MacConkey agar and incubated for 24 h at 37 °C. Lactose fermentation-positive colonies were picked and confirmed by the triple sugar iron and IMViC tests. Any isolate appearing as fermenting lactose with gas production within 48 h, hydrogen sulfide negative and demonstrating an IMViC pattern of positive-positive-negative-negative was considered to be *E. coli*.

Antimicrobial susceptibility test and ESBL producing strains detection.

The antimicrobial susceptibility test was carried out using a Kirby-Bauer agar disk diffusion method (15). Clinical Laboratory Standards Institute (CLSI) guidelines were followed for inoculum standardization, medium and incubation conditions, and internal quality control organisms. The following commercially available antimicrobial discs (Oxoid, Basingstoke, UK) were employed: gentamicin (GEN), streptomycin (STR), trimethoprim-sulphamethoxazole (SXT), cefotaxime (CTX), ceftazidime (CAZ), ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), norfloxacin (NOR), nalidixic acid (NAL) and tetracycline (TET). In addition, susceptibility to meropenem (MEM) was tested in all ESBL-producing isolates. ESBL-producing strains were confirmed by the combination disk method (15) using cefotaxime (30 μg) or ceftazidime (30 μg) alone or in combination with clavulanic acid (10 μg). An increase in zone diameter of ≥ 5 mm for either cefotaxime or ceftazidime in combination with clavulanic acid indicated ESBL production. *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were

used as quality control organisms.

Beta-lactamase genes identification.

All ESBL-producing *E. coli* were screened for the existence of bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{OXA} genes by PCR amplification as previously described (1, 5, 55). The bla_{CTX-M}-carrying isolates were further characterized using CTX-M group specific primers. PCR reaction mixture (total 20 µl) consisted of 1 µl of DNA template, 1× Green GoTaq reaction buffer, each dNTP (0.2 mM), 1 µl of each primer (0.5 µM) and 0.5 U of GoTaq DNA polymerase (Promega, Fitchburg, WI). The reaction parameters were 94°C for 5 min (first cycle only), 94°C for 30 sec, the primer-specific annealing temperature for 30 sec, 72°C for 1.5 min (30 cycles) and 72°C for 10 min. The products from bla_{TEM} amplification were subjected for sequencing. The primers used are listed in Table 5.

Detection of class 1 integrons.

All resistant isolates were tested for the presence of class 1-integrase genes by PCR using primers INT-1U and INT-1D (Table 1). PCR reaction mixture (total 20 µl) consisted of 1 µl of DNA template, 1× Green GoTaq reaction buffer, each dNTP (0.2 mM), 1 µl of each primer (0.4 µM) and 0.5 U of GoTaq DNA polymerase. The reaction parameters were 35 cycles at 98, 56 and 72 °C for 10 sec, 10 sec and 1 min, respectively, followed by a final extension at 72 °C for 4 min. PCR products were analysed by electrophoresis using 1% agarose gel and the sizes of the PCR products were determined using a 100-bp DNA ladder and lambda *Hind* III digest as size markers.

Characterisation of gene cassettes in integrons.

All class 1 integrase PCR-positive strains were further analysed for their gene cassette patterns by amplifying the variable region between the 5'-conserved segment and 3'-conserved segment or using *Sul* genes. The following four primer sets were designed and employed: 5'CS2 (F) with 3'CS2 (R), Sul1 (R), Sul2 (R), and Sul3 (R). PCR reaction mixture (total 20 µl) consisted of 1 µl of DNA template, 1× Ex Taq buffer, each dNTP (0.25 mM), 1 µl of each primer (0.5 µM) and 0.5 U of Ex Taq (Takara Biomedicals, Tokyo, Japan). The reaction parameters were 35 cycles at 98, 56 and 72 °C for 30 sec, 30 sec and 2 min, respectively, followed by a final extension at 72 °C for 7 min. After agarose gel electrophoretic separation, PCR products were purified with Wizard SV Gel and PCR Clean-Up System kits. Purified products were sequenced by primer walking using a BigDye terminator cycle sequencing ready reaction kit, and analysed using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Contiguous sequences were analysed by the BLAST search engine and compared with those registered in the GenBank database.

Data analysis.

The rate of antimicrobial resistance and ESBL-producing isolates in farms 1, 2 and 3 were compared by the chi-square and Fisher's exact test. A comparison of antimicrobial resistance phenotype between ESBL-producing isolates and non ESBL-producing isolates was also conducted by the chi-square and Fisher's exact test. Differences were considered significant at $P < 0.05$.

Table 5. Oligonucleotide primers used in this study

Name	Primer sequence (5'-3')	Target	Annealing temperature (^{o}C)	Product size (bp)
Beta-Lactamase				
TEM-F	TCGGGGAAATGTGCG	*bla* $_{TEM}$ gene	60	1074
TEM-R	TGCTTAATCAGTGAGGCACC			
SHV-F	GCCGGGTTATTCTTATTTGTCGC	*bla* $_{SHV}$ gene	60	1016
SHV-R	ATGCCGCCGCCAGTCA			
CTX-M-uni-F	CGATGTGCAGTACCAGTAA	*bla* $_{CTX}$ gene	60	585
CTX-M-uni-R	TAAGTGACCAGAATCAGCGG			
OXA-uni-F	TCAACTTTCAAGATCGCA	*bla* $_{OXA}$ gene	55	610
OXA-uni-R	GTGTGTTTAGAATGGTGA			
CTX-M-group				
CTX-M-1-F[a]	GCTGTTGTTAGGAAGTGTGC	CTX-M-1 cluster	55	499
CTX-M-1-R	CCATTGCCCGAGGTGAAG			
CTX-M-2-F[b]	ACGCTACCCCTGCTATTT	CTX-M-2 cluster	55	399
CTX-M-2-R	CCTTTCCGCCTTCTGCTC			
CTX-M-8-F[c]	CGGATGATGCTAATGACAAC	CTX-M-8 cluster	62	550
CTX-M-8-R	GTCAGATTGCGAAGCGTC			
CTX-M-9-F[d]	GCAGATAATACGCAGGTG	CTX-M-9 cluster	62	474
CTX-M-9-R	CGGCGTGGTGGTGTCTCT			

[a]CTX-M-1 cluster includes CTX-M-1, -3, -10 to -12, -15 (UOE-1), -22, -23, -28, -29, and -30.

[b]CTX-M-2 cluster includes CTX-M-2, -4 to -7, and -20 and Toho-1.

[c]CTX-M-8 cluster CTX-M-8.

[d]CTX-M-9 cluster includes CTX-M-9, -13, -14, -16 to -19 and -21, and -27 and Toho-2.

RESULTS

Antimicrobial resistance phenotypes.

I obtained 17, 32 and 73 *E. coli* isolates from swine in farm1, 2 and 3, respectively. One hundred and twenty-two *E. coli* isolates were analysed for their susceptibility to 11 antimicrobials. Interestingly, all of the tested isolates had drug-resistant phenotypes. High resistance rates were observed to AMP (98.4%), CHL (95.9%), GEN (78.7%), STR (77.9%), TET (74.6%) and CTX (72.1%) (Table 6). MDR isolates (45) were found in 99% of all isolates. *E. coli* resistant to six drugs were most frequent, (20.5%, 25/122) followed by resistance to seven and eight drugs (14.8%, 18/122; each) drugs. As shown in Table 6, resistance to GEN, AMP, CHL and TET was common in all three farms and resistance to CAZ, CTX, SXT, CIP and NOR was most commonly found in farm 3, which showed a significantly higher rate of resistance than those in farm 1 or 2 ($P < 0.05$). However, resistance to NAL was significantly more common in isolates from farm 2 than in those from the other farms ($P < 0.05$).

ESBL producing *E. coli.*

A total of 54 *E. coli* (44.3%, 54/122) were confirmed as ESBL-producing isolates. ESBL-producing *E. coli* in farm 3 (63%, 46/73) showed a significantly higher rate than those in farm 1 (5.9%, 1/17) or farm 2 (21.9%, 7/32). All ESBL-producing *E. coli* showed the MDR phenotype and were resistant to some of the tested beta-lactams including AMP, CTX and CAZ. Regarding the rate of resistance to third-generation cephalosporins, ESBL-producing *E. coli* were much more resistant to CTX (93.3%, 52/54) than CAZ (66.7%, 36/54) (Table 7).

Table 6. Antimicrobial resistance phenotype of *E. coli* isolates obtain on different pig farms

Antimicrobial[a]	No. (%) of isolates							
	Farm 1 (n=17)		Farm 2 (n=32)		Farm 3 (n=73)		Total (n=122)	
	R[b]	I	R	I	R	I	R	I
GEN	12 (70.6) A[c]	0	24 (75) A	2 (6.3)	60 (82.2) A	0	96 (78.7)	2 (1.6)
STR	8 (47.1) A	7 (41.2)	24 (75) AB	5 (15.6)	63 (86.3) B	8 (11)	95 (77.9)	20 (16.4)
CAZ	2 (11.8) A	1 (5.9)	4 (12.5) A	7 (21.9)	55 (75.3) B	9 (12.3)	61 (50)	17 (13.9)
CTX	4 (23.5) A	1 (5.9)	13 (40.6) A	4 (12.5)	71 (97.3) B	2 (2.7)	88 (72.1)	7 (5.7)
SXT	12 (70.6) AB	0	15 (46.9) A	3 (9.4)	55 (75.3) B	6 (8.2)	82 (67.2)	9 (7.4)
AMP	16 (94.1) A	0	31 (96.9) A	0	73 (100) A	0	120 (98.4)	0
CHL	16 (94.1) A	0	30 (93.8) A	0	71 (97.3) A	1 (1.4)	117 (95.9)	1 (0.8)
CIP	1 (5.9) A	3 (17.6)	5 (15.6) A	4 (12.5)	30 (41.1) B	13 (17.8)	36 (29.5)	20 (16.4)
NOR	0 A	3 (17.6)	2 (6.3) A	3 (9.4)	31 (41.1) B	8 (11)	30 (24.6)	14 (11.5)
NAL	3 (17.6) A	4 (23.5)	23 (71.9) B	0	32 (41.1) C	21 (28.8)	67 (54.9)	25 (20.5)
TET	15 (88.2) A	0	11 (65.6) B	0	33 (41.1) B	9 (12.3)	91 (74.6)	9 (7.4)

[a]GEN, gentamicin; STR, streptomycin; CAZ, ceftazidime; CTX, cefotaxime;
SXT, trimethoprim-sulphamethoxazole; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; NOR, norfloxac
NAL,nalidixic acid; TET, tetracycline

[b]Results were interpreted as sensitive (S), intermediate resistant (I) or resistant (R) based on CLSI guidelines .

Table 7. Antimicrobial resistance of ESBL-producing *E. coli* and ESBL-negative *E. coli*

E. coli strains	No. of antimicrobial resistant isolate (%)										Class 1 integrons	
	GEN[a]	STR	CAZ	CTX	SXT	AMP	CHL	CIP	NOR	NAL	*int* +	Gene
ESBL(54)	**52*** **(96.3)**	43 (79.6)	**36*** **(66.7)**	**52*** **(96.3)**	**43*** **(79.6)**	54 (100)	52 (94.4)	**24*** **(44.4)**	**20*** **(37)**	34 (63)	37 (68.5)	17 (31.5)
ESBL-negative (68)	**44*** **(64.7)**	52 (76.5)	**25*** **(36.8)**	**36*** **(52.9)**	**39*** **(57.4)**	66 (97.1)	66 (97.1)	**12*** **(17.6)**	**10*** **(14.7)**	33 (48.5)	51 (75)	28 (41.2)

aGEN, gentamicin; STR, streptomycin; CAZ, ceftazidime; CTX, cefotaxime; SXT, trimethoprim-sulphamethoxazole; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; NOR, norfloxacin; NAL,nalidixic acid; TET, tetracycline.

[b]Values with the same column with asterisk differ sifgnificantly (p <0.05)

The results also revealed that all ESBL-producing *E. coli* were susceptible to MEM. In the comparison of resistance rates to non-beta-lactam drugs, ESBL-producing *E. coli* showed significantly higher resistance rates to GEN, SXT, CIP and NOR than did ESBL-negative *E. coli* (Table 7). PCR and sequence analysis revealed that ESBL-producing *E. coli* isolates carried $bla_{\text{TEM-1}}$ (38 isolates), $bla_{\text{TEM-135}}$ (2 isolates), $bla_{\text{TEM-176}}$ (1 isolate), $bla_{\text{CTX-M-1}}$-cluster (37 isolates) and $bla_{\text{CTX-M-9}}$-cluster (12 isolates) (Table 8). bla_{SHV} and bla_{OXA} genes were not identified. Almost all of ESBL-producing *E. coli* carried multiple types of beta-lactamase genes. The combination of $bla_{\text{TEM-1}}$+$bla_{\text{CTX-M-1}}$-cluster, $bla_{\text{TEM-1}}$+$bla_{\text{CTX-M-9}}$-cluster and $bla_{\text{TEM-135}}$ +$bla_{\text{CTX-M-9}}$-cluster was found in 26, 4 and 2 isolates, respectively. The combination of $bla_{\text{TEM-1}}$+$bla_{\text{CTX-M-1}}$-cluster+$bla_{\text{CTX-M-9}}$-cluster was found in 4 isolates. Genotypes and phenotypes of ESBL-producing *E. coli* of different farms are shown in Table 8.

Class 1 integrons and characterisation of gene cassettes.

PCR amplification for the class 1 integrase gene showed that 87 (71.3%) of all *E. coli* isolates carried class 1 integrons. Class 1 integrons-positive *E. coli* were found in farm 1 (14 isolates), farm 2 (21 isolates) and farm 3 (52 isolates) with no significant differences in frequency between the three farms. Drug resistance-associated gene cassettes were successfully detected in 43 of 122 integron-positive isolates. Gene cassettes were found to code for resistance to aminoglycosides (*aadA1, aadA2, aadA22* or *aadA23*), trimethoprim (*dfrA5*, *dfrA12* or *dfrA17*) and lincosamide (*linF*). Genes encoding for beta-lactamases were not found in class 1 integrons. Seven integrons were identified by their structural features, of which the *aadA22* gene cassette array (55.8%, 24/43) was the predominant type (Table 9). A number of class 1 integron-positive isolates showed no significant differences between ESBL-producing *E. coli* and ESBL negative *E. coli* (Table 7).

Table 8. Characteristics of of ESBL-producinf *E. coli* isolated from fecal samples of healthy swine

E. coli isolates	Farms	Antimicrobal resistance phenotypes		*bla* genes[a]	Class 1 Integrons	
		Betalactams	Non-betalactams		*intl*	Gene cassettes
R1/2	1	AMP	GEN-STR-SXT-CHL-TET	TEM-1, CTX-M-1-cluster	+	*aadA2-linF*
B15-15-4	2	CAZ-CTX-AMP	GEN-STR-SXT-CHL-NAL-TET	TEM-1, CTX-M-1-cluster	+	-
B16-16-3	2	CAZ-CTX-AMP	GEN-STR-CHL-TET	-	+	*aadA1*
B4-4-1	2	CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL-TET	TEM-1, CTX-M-1- and 9-cluster	+	-
B2-2-2	2	CTX-AMP	GEN-CHL-CIP-NAL-TET	TEM-1, CTX-M-9-cluster	-	-
B3-3-2	2	CTX-AMP	GEN-CHL-CIP-NAL-TET	-	+	-
B19-19-1	2	CTX-AMP	GEN-STR-CHL-NAL-TET	-	+	*aadA1*
B3-3-4	2	CTX-AMP	GEN-CHL-NAL-TET	-	-	-
C2-2-1	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL-TET	TEM-1, CTX-M-1-cluster	+	-
C2-9-5	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL-TET	TEM-1, CTX-M-1-cluster	-	-
C2-10-1	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL-TET	TEM-1, CTX-M-1-cluster	-	-
C1-5-3	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL-TET	TEM-1, CTX-M-1-cluster	+	-
C1-9-4	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL-TET	TEM-1, CTX-M-1-cluster	+	-
C1-6-3	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL-TET	TEM-1, CTX-M-1-cluster	+	-
C2-7-3	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL-TET	TEM-176	+	-
C1-5-2	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL	TEM-1, CTX-M-1-cluster	+	*aadA22*
C1-10-3	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL	TEM-1, CTX-M-1-cluster	+	*aadA22*
C2-1-2	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL	TEM-1, CTX-M-1-cluster	+	*aadA22*
C2-1-3	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NAL-TET	TEM-1, CTX-M-1-cluster	+	-
C2-4-10	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL	TEM-1, CTX-M-1-cluster	+	*aadA22*
C2-6-1	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL	TEM-1, CTX-M-1-cluster	+	*aadA22*
C2-10-2	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL	TEM-1, CTX-M-1-cluster	+	*aadA22*
C2-10-5	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL	TEM-1, CTX-M-1-cluster	-	-
C2-6-4	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-NOR-NAL-TET	CTX-M-1-cluster	+	-
C1-8-3	3	CAZ-CTX-AMP	GEN-STR-CHL-CIP-NOR-NAL	TEM-1, CTX-M-1-cluster	+	*aadA22*
C2-4-1	3	CAZ-CTX-AMP	GEN-STR-CHL-CIP-NOR-NAL	TEM-1, CTX-M-1-cluster	+	*aadA22*
C2-5-3	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-CIP-NAL	CTX-M-1-cluster	+	-

Table 8. (Continued)

E. coli isolates	Farms	Antimicrobal resistance phenotypes		*bla* genes[a]	Class 1 Integrons	
		Betalactams	Non-betalactams		*intl*	Gene cassettes
C1-8-5	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-NAL-TET	-	+	-
C2-5-4	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-NAL-TET	CTX-M-1-cluster	+	-
C1-5-4	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-TET	CTX-M-1-cluster	+	-
C1-10-1	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-TET	TEM-1, CTX-M-1-cluster	-	-
C2-4-9	3	CAZ-CTX-AMP	GEN-STR-STX-NAL-TET	TEM-1, CTX-M-1-cluster	+	*aadA22*
C2-5-1	3	CAZ-CTX-AMP	GEN-STR-SXT-CHL-TET	TEM-1, CTX-M-1- and 9-cluster	-	-
C2-2-2	3	CAZ-CTX-AMP	GEN-SXT-CHL-TET	TEM-1, CTX-M-1- and 9-cluster	+	*dfrA5*
C2-4-3	3	CAZ-CTX-AMP	GEN-STR-CHL-TET	TEM-1, CTX-M-1-cluster	+	-
C2-8-1	3	CAZ-CTX-AMP	GEN-STR-SXT-TET	CTX-M-1- and 9-cluster	+	-
C1-3-10	3	CAZ-CTX-AMP	SXT-CHL-TET	TEM-1, CTX-M-1-cluster	-	-
C1-7-7	3	CAZ-CTX-AMP	GEN-SXT-CHL	TEM-1, CTX-M-1-cluster	+	-
C1-10-5	3	CAZ-CTX-AMP	GEN-STR-CHL	TEM-1, CTX-M-1-cluster	+	*aadA22*
C2-4-5	3	CAZ-CTX-AMP	GEN-CHL-TET	TEM-1, CTX-M-1-cluster	+	-
C1-8-1	3	CAZ-CTX-AMP	CHL	TEM-1	-	-
C2-1-1	3	CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL-TET	TEM-1, CTX-M-1-cluster	+	-
C2-6-2	3	CTX-AMP	GEN-STR-SXT-CHL-CIP-NOR-NAL	CTX-M-1-cluster	-	-
C1-4-3	3	CTX-AMP	GEN-STR-SXT-CHL-NAL-TET	TEM-135, CTX-M-9-cluster	+	*dfrA12-orfF-aadA2*
C2-9-1	3	CTX-AMP	GEN-STR-SXT-CHL-NAL-TET	TEM-1	-	-
C2-9-3	3	CTX-AMP	GEN-STR-SXT-CHL-CIP-TET	TEM-1, CTX-M-9-cluster	-	-
C1-4-1	3	CTX-AMP	GEN-STR-SXT-CHL-TET	TEM-135, CTX-M-9-cluster	+	*dfrA12-orfF-aadA2*
C2-10-4	3	CTX-AMP	GEN-STR-SXT-CHL-TET	TEM-1, CTX-M-9-cluster	-	-
C2-6-5	3	CTX-AMP	GEN-SXT-CHL-NAL-TET	TEM-1	-	-
C2-3-1	3	CTX-AMP	GEN-STR-SXT-CHL	TEM-1, CTX-M-1- and 9-cluster	-	-
C1-10-2	3	CTX-AMP	GEN-STR-CHL-TET	TEM-135, CTX-M-9-cluster	+	*aadA22*
C2-8-2	3	CTX-AMP	GEN-SXT-CHL-TET	TEM-1	-	-
C1-6-6	3	CAZ-AMP	GEN-STR-SXT-CHL-NAL-TET	CTX-M-1-cluster	-	-
C1-9-5	3	CTX	GEN-SXT-CHL-TET	TEM-1, CTX-M-9-cluster	+	-

[a]CTX-M-1 cluster includes CTX-M-1, -3, -10 to -12, -15 (UOE-1), -22, -23, -28, -29, and -30. CTX-M-9 cluster includes CTX--13, -14, -16 to -19 and -21, and -27 and Toho-2.

Table 9. Class 1 integrons and antimicrobial resistance patterns of *E. coli* isolates from healthy swine (n=43)

Gene cassettes	Size (bp)	Antimicrobial resistance patterns[a]	Number of	(%)
aadA1	1,000	STR-AMP-CHL-NAL-TET	1	2.3
		GEN-STR-AMP-CHL-NAL-TET	2	4.7
		GEN-STR-CAZ-CTX-AMP-CHL-TET	1	2.3
		GEN-STR-CTX-AMP-CHL-NAL-TET	2	4.7
		GEN-STR-CAZ-CTX-AMP-CHL-NAL-TET	2	4.7
		GEN-STR-CTX-SXT-AMP-CHL-NAL-TET	1	2.3
aadA22	1,000	GEN-STR-SXT-AMP-CHL	2	4.7
		STR-AMP-CHL-NAL-TET	1	2.3
		GEN-STR-CAZ-CTX-AMP-CHL	1	2.3
		GEN-STR-CTX-AMP-CHL-TET	1	2.3
		STR-CTX-SXT-AMP-CHL-NAL	1	2.3
		GEN-STR-CAZ-CTX-AMP-CHL-TET	2	4.7
		GEN-STR-CAZ-CTX-SXT-AMP-CHL-TET	1	2.3
		GEN-STR-CAZ-CTX-STX-AMP-NAL-TET	1	2.3
		STR-CAZ-CTX-SXT-AMP-CHL-NAL-TET	1	2.3
		GEN-STR-CAZ-CTX-AMP-CHL-CIP-NOR-NAL	1	2.3
		GEN-STR-CAZ-CTX-SXT-AMP-CHL-CIP-NOR-NAL	6	14.0
		GEN-STR-CAZ-CTX-AMP-CHL-CIP-NOR-NAL-TET	4	9.3
		GEN-STR-CAZ-CTX-SXT-AMP-CHL-CIP-NOR-NAL-TET	2	4.7
aadA23	1,000	GEN-STR-SXT-AMP-CHL	3	7.0
		STR-CAZ-CTX-AMP-CHL-CIP-NOR-NAL-TET	1	2.3
dfrA5	800	GEN-CAZ-CTX-SXT-AMP-CHL-TET	1	2.3
		STR-CAZ-CTX-SXT-AMP-CHL-TET	1	2.3
dfrA17	800	GEN-STR-CTX-SXT-AMP-CHL-CIP-NOR-NAL-TET	1	2.3
dfrA12-orfF-aadA2	2,000	GEN-STR-CTX-SXT-AMP-CHL-TET	1	2.3
		GEN-STR-CTX-SXT-AMP-CHL-NAL-TET	1	2.3
aadA2-linF	2,000	GEN-STR-SXT-AMP-CHL-TET	1	2.3

[a]GEN, gentamicin; STR, streptomycin; CAZ, ceftazidime; CTX, cefotaxime; SXT, trimethoprim-sulphamethoxazole; AMP, ampicillin;CHL, chloramphenicol; CIP, ciprofloxacin; NOR, norfloxacin; NAL, nalidixicacid; TET, tetracycline.

DISSCUSSION

High resistance rates were observed for AMP, CHL, GEN, STR, TET and CTX. These resistance rates are in agreement with data from several previous studies in Thailand and other countries where resistance to tetracyclines, aminoglycosides and penicillins was revealed to be common amongst *E. coli* isolated from food animals (7, 37, 70, 72). Swine farms in this study are personal farms which under the supervision of livestock office. Farm 1 and 2 are medium size farm where raise 1,200 and 2,400 pigs, respectively. Farm 3 is small size farm where raise 500 pigs. All of them have facility for making self-mixed feed. Unfortunately, I could not access exactly information about the amount and type of antimicrobial mixed in feed by interview. In Thailand, the most commonly used antimicrobials as feed medication in the swine industry have been beta-lactams, aminoglycosides and tetracyclines (51). The high resistance rate to several antimicrobials found in this study was possibly due to selective pressures of antimicrobial usage in pig farming. Burow *et al*. (9) emphasised that oral administration of antimicrobials can increase antimicrobial resistance in commensal *E. coli*. They found that tetracyclines, aminoglycosides and quinolones had a strong effect on the development of antimicrobial resistance. Tetracyclines have been used as growth promoters, treatment and control of multiple diseases in livestock (pigs, poultry and cattle). In swine farms, GEN has been used for the treatment of colibacillosis and dysentery (48). In contrast, quinolones are not frequently delivered to livestock (9). Even though chloramphenicol has been withdrawn from use in food-producing animals in many countries including Thailand (13), a high resistance to chloramphenicol was found in this study. The wide distribution of chloramphenicol resistance could be due to co-selection by antibiotics, since several kinds of drug resistance genes are frequently present in the same mobile genetic element with CHL resistance-associated genes (7).

I identified 54 (44.3%) ESBL-producing *E. coli* that showed the MDR phenotype and all were resistant to beta-lactams. The rate of isolates resistant to GEN, SXT, CIP and NOR in ESBL-producing *E. coli* was significantly higher than those in ESBL-negative *E. coli* ($P < 0.05$). The prevalence and characteristics of ESBL-producing *Enterobacteriaceae* in food-producing animals were reported in many countries. In Germany, ESBL-producing *Enterobacteriaceae* was found in 88.6% and 72.5% of chicken carcasses and ceca, respectively (57). A longitudinal study conducted in Danish swine farms reported that 35% of *E. coli* isolated from sows was ESBL-producing (23). Machado *et al.* (44) reported ESBL-producing *Enterobacteriaceae* in 60% of chicken carcasses, 10% of healthy chickens and 5.7% of healthy swine samples in Portugal. However, in Thailand, only information of ESBL-producing *E. coli* in human clinical isolates is available. Hence, our study is the first to demonstrate ESBL-producing *E. coli* in healthy swine in Thailand. Penicillins are commonly used as feed medication on Thai swine farms, and third-generation cephalosporins have been approved as the second-choice treatment in particular cases such as respiratory diseases (61). Therefore, the use of beta-lactam antimicrobials in swine farms may play an important role for the selection of beta-lactam-resistant bacteria and also other drug-resistant bacteria. I also found that all ESBL-producing *E. coli* were MDR. The high rate of resistance to some of the non-cephalosporin drugs (GEN, SXT, CIP and NOR) in ESBL-producing *E. coli* seems to emphasise the potential rate of swine commensal *E. coli* as a reservoir of antimicrobial resistance, which could spread widely in the environment and food chains.

Among 54 ESBL-producing *E. coli*, 90.7% carried *bla* genes which responsible for beta-lactam resistant phenotypes. The $bla_{\text{CTX-M-1}}$ cluster and $bla_{\text{TEM-1}}$ was the majority *bla* genes identified in our study. In food-producing and companion animals, the most frequent *bla* genes is $bla_{\text{CTX-M}}$ followed by $bla_{\text{TEM-52}}$ and $bla_{\text{SHV-12}}$ (62). The study in human clinical isolates revealed

that *bla*$_{CTX-M}$ among ESBL-producing *E. coli* is highly endemic in Thailand (33). However, there was no study describing the prevalence and characteristics of ESBL-producing *Enterobacteriaceae* in healthy swine in Thailand. ESBL-producing *E. coli* carried multiple types of beta-lactamase genes were identified and the combination of *bla*$_{TEM-1}$+*bla*$_{CTX-M-1}$ cluster is predominance. ESBL-producing *E. coli* carried *bla*$_{CTX-M-1}$ cluster and additional *bla*$_{TEM}$ were reported in swine and cattle in Switzerland (20). Previous study has shown that ESBL-mediated plasmids may carry more than one beta-lactamase genes and may account for high-level of beta-lactam resistant phenotypes (33).

Integrons are genetic elements that have been found to play an important role in the carriage and dissemination of drug resistance genes. I found a high prevalence (73.2%) and widespread of class 1 integrons in commensal *E. coli* in healthy swine. This observation agreed well with previous studies that were conducted in diseased and healthy swine in Thailand (40, 51). The current study identified 69 integron-positive *E. coli* as carrying gene cassettes, including those associated with resistance to aminoglycosides (*aadA1, aadA2, aadA22* or *aadA23*), trimethoprim (*dfrA5, dfrA12* or *dfrA17*) and lincosamide (*linF*). The presence of these antimicrobial resistance-associated gene cassettes was found to correspond well with resistance phenotypes. Gene cassettes found in this study to encode resistance to aminoglycosides and trimethroprim have been frequently reported in class 1 integrons in different bacteria from various sources in many countries (36, 40, 72). The predominant gene cassette array in this study was *aadA22* (44.9%, 31/69), which contrasts with data from a previous study in Thailand that found *Salmonella enterica* isolates from poultry and swine harbouring the gene cassette array 1)*dfrA12*-orfF-*aadA2* as the most prevalent (32). Wannaprasat *et al.* (71) also reported that *Salmonella* isolated from pork meat and humans in northern Thailand carried class 1 integrons

with the same cassette array of *dfrA12*-orfF-*aadA2*. Lay *et al.* (36) reported that the *aadA1* gene cassette array was most commonly found in *E. coli* isolates from healthy swine. The accumulation of drug resistance gene cassettes carried by class 1 integrons seems to be locally diverse, probably because of differences in the drug resistance gene pool in the environment.

Here we reported a class 1 integron-harbouring gene cassette array *aadA2-linF* in commensal *E. coli*. The *linF* gene encodes a putative 273-amino acid lincosamide nucleotidyltransferase, which can transfer lincomycin and clindamycin resistance phenotypes to bacteria. This gene cassette linked to *aadA2* was demonstrated in class 1 integrons in *E. coli* isolated from humans with bloodstream infection in Norway (24) and healthy swine in Spain (47). As far as I know, this study is the first to identify the integron-carrying *aadA2-linF* cassette array in Thailand. Class 1 integron-carrying *linF* was rarely detected in *E. coli*, which indicates that resistance gene cassettes might disseminate through integrons between different bacteria species under antimicrobial selective pressure (9).

I revealed a high prevalence of MDR and ESBL-producing *E. coli* harbouring class 1 integrons. However, the class 1 integrons examined in this study did not support the total resistance phenotype observed. In addition, the gene cassettes encoding ESBL resistance were not identified in the variable region of class 1 integrons. Nevertheless, integrons may capture genetic determinants of drug resistance on farms using antimicrobials, which could become a concern in the future.

SUMMARY

Administration of antimicrobials to food-producing animals increases the risk of higher antimicrobial resistance in normal intestinal flora. Accordingly, the present cross-sectional study was conducted to investigate antimicrobial susceptibility and extended spectrum beta-lactamase (ESBL)-producing strains and to characterise class 1 integrons in *E. coli* in healthy swine in Thailand. Interestingly, all of the tested isolates (122 isolates) showed drug-resistant phenotypes. High resistance rates were observed to ampicillin (98.4%), chloramphenicol (95.9%), gentamicin (78.7%), streptomycin (77.9%) tetracycline (74.6%) and cefotaxime (72.1%). Fifty-four (44.3%) *E. coli* isolates were confirmed as ESBL-producing strains. Among them, $bla_{\text{CTX-M}}$ (45 isolates) and bla_{TEM} (41 isolates) were detected. Of all $bla_{\text{CTX-M}}$-carried *E. coli*, 37 isolates carried $bla_{\text{CTX-M-1}}$ cluster, 12 isolates carried $bla_{\text{CTX-M-9}}$ cluster and 5 isolates carried both of the clusters. Sequence analysis revealed that $bla_{\text{TEM-1}}$, $bla_{\text{TEM-135}}$ and $bla_{\text{TEM-175}}$ were found in 38, 2 and 1 isolates, respectively. Seventy-one percent (87/122) of the isolates carried class 1 integrons, in which eight distinct drug-resistance gene cassettes with seven different integron profiles were identified in 43 isolates. Gene cassettes were found to be associated with resistance to aminoglycosides (*aadA1, aadA2, aadA22* or *aadA23*), trimethoprim (*dfrA5, dfrA12* or *dfrA17*) and lincosamide (*linF*). Genes encoding for beta-lactamases were not found in class 1 integrons. This is the first study to report ESBL-producing *E. coli* and to identify a class 1 integron carrying *linF* gene cassette in swine in Thailand.

CONCLUSION

Antimicrobial use in food-producing animals results in a risk of the evolution of antimicrobial resistant bacteria. The present study has identified high prevalence of multidrug resistance in *E. coli* isolated from shrimp, shrimp environment and pigs. This reflects the selective pressure, caused by intense and less prudent use of the antimicrobials in animal production in Thailand. In addition, commensal *E. coli* can be a reservoir for antimicrobial resistance genes, which can be transferred to pathogenic bacteria. Hence, resistance genes transferring from farm to fork can compromise human health by the potential of producing difficult-to-treat pathogens.

In chapter I, I revealed that the environment of shrimp farms and selling places is the main source of drug-resistant *E. coli* strains that contaminate the shrimp. Therefore, the improvement of hygiene practices along the food chain and prudent use of antimicrobials on shrimp farms are essential. The occurrence of multidrug-resistant *E. coli* isolates carrying *tet* genes and class 1 integrons in this study suggests that multidrug-resistant pathogenic bacterial strains other than *E. coli* may occur in shrimp and associated materials and could be a significant public health concern..

In the chapter II, the data presented in this study confirmed the diversity and widespread occurrence of MDR and ESBL-producing commensal *E. coli* harbouring class 1 integrons in healthy swine. A great concern is that swine can be reservoirs for the antimicrobial resistance gene pool as a mixing vessel that develops antimicrobial-resistant pathogenic bacteria and causes health risks to humans. Hence, the results of present study call for urgent surveillance of the emergence of antimicrobial resistance in commensal bacteria and prompt countermeasures to

prevent the dissemination of antimicrobial-resistant genes to other bacteria in the same environment.

It is suggested by my data that the co-selection of multiple drug resistance genes by usage some antimicrobials greatly contributes to the wide distribution of MDR bacteria. Therefore, regulation of usage of all antimicrobials that can select the resistance might be needed in order to control antimicrobial resistant bacteria.

Finally, I believe that the finding in my study will be useful information for the development of antimicrobial resistance surveillance and monitoring programs in food-producing animals in Thailand.

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