Original Article

Multidrug resistant *Escherichia coli* Harboring Extended-spectrum β-Lactamase-encoding genes isolated from clinically healthy pigs

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Abstract

A total of 292 *E. coli* isolates obtained from fecal samples from pigs in Central (n = 103) and Northeastern (n = 189) provinces of Thailand were included in this study. Eighty-six *E. coli* isolates were phenotypically confirmed to be β-lactamases producers and screened for the presence of β-lactamase genes. The CTX-M family was most frequently identified (90.7%). The $bla_{\text{CTX-M-15}}$ gene belonging to CTX-M group I (59.3%) was the most predominantly identified CTX-M genotype followed by $bla_{\text{CTX-M-14}}$ (31.4%) and $bla_{\text{CTX-M-4}}$ (25.6%). The $bla_{\text{TEM-1}}$ gene was prevalent (75.6%). The $bla_{\text{CTX-M-4}}$ and $bla_{\text{CTX-M-14}}$ genes were located on conjugative plasmid. The results highlighted healthy pigs as reservoirs of ESBL-producing *E. coli* carrying ESBL genes that could be horizontally transferred.

Keywords: ESBLs, *Escherichia coli*, MDR, Pigs, Thailand

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Introduction

The rise in the occurrence and spread of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* is currently a global emergency. These pathogens constitute a significant threat to the efficacy of many antimicrobials including 3rd and 4th generation cephalosporins (Geser *et al*., 2012; Afema *et al*., 2018; Kamaruzzaman *et al*., 2020). Evidently, acquisition of antimicrobial resistance (AMR) genes arises due to continuous and indiscriminate use of antimicrobials in food animal production (Blanc *et al*., 2006). The occurrence and dissemination of the CTX- M group of enzymes in the environment, humans, companion animals and a variety of food producing animals including pigs, cattle and chickens is on the increase (Duan *et al*., 2006; Pietsch *et al*., 2017), thus paving way for animal to human transmission and vice versa (Michael *et al*., 2017; Norizuki *et al*., 2017).

E. coli acquired resistance to extended-spectrum cephalosporins is through horizontal transfer of a conjugative plasmid carrying gene that encodes ESBL or Ampicillin class C (AmpC) β-lactamases (Liebana *et al*., 2006). These ESBL genes are commonly found in *E. coli* strains isolated from humans and animals, possibly because of plasmid transfer or the spread of unique clonal lineages (Allen *et al*., 2010). ESBL belonging to the CTX-M-9 family is the most predominant ESBL gene circulating among *E. coli* strains in Asia (Trongjit *et al*., 2016). Occurrences of ESBL-producing *Enterobacteriaceae* from stool and fecal samples collected from apparently healthy individuals and pigs in Thailand have been reported (Sasaki *et al*., 2010; Trongjit *et al*., 2016), thus, highlighting the possible animal to human transmission and vice versa. ESBL-producing *E. coli* were initially observed in human clinical isolates but are now increasingly found in food animals. (Lay *et al*., 2012; Roca *et al*., 2015). It has been suggested that food-producing animals may be an important source of ESBL-producing bacteria that can potentially transfer ESBL genes to humans through the food chain (Trongjit *et al*., 2016). The prevalence of ESBL-producing *E. coli* isolated from food-producing animals has been reported in France, Denmark, Spain, China and Nigeria (Blanc *et al*., 2006; Zheng *et al*., 2012; Randall *et al*., 2013).

These studies highlight the role of food producing animals especially pigs as a reservoir for ESBL-producing *E. coli* and the resistance determinants that may be transferred to humans and contaminate the environment. In Thailand, Boonyasiri *et al*., (2014) reported a high prevalence of ESBL-producing *E. coli* phenotypes from healthy pigs (77%), healthy humans (76%), chickens (40%) and the environment (25%) respectively. Occurrence of ESBL-producing *E. coli* is now a global phenomenon. The burden of ESBL-producing *E. coli* from most developing countries, Thailand included has been recently receiving much attention. Report from previous studies have indicated that the burden of AMR is mostly focused on resistance phenotypes of *Salmonella* (Trongjit *et al*., 2016). Awareness of the threat of the evolution and spread of AMR in Thailand is limited and only a few countries in South-East Asia have a well-structured platform in place for monitoring this spread. As with many other developing countries, knowledge on ESBL in bacteria from food animals and its distribution is crucial and this study aimed to characterize ESBL-encoding genes in *E. coli* isolated from clinically healthy pigs in Thailand.

Materials and Methods

Confirmation of Bacterial isolates: A total of 292 *E. coli* isolates were obtained from the bacterial culture stock of the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University. All were collected from fecal samples from clinically healthy pigs between 2015-2016. The pig farms were in different provinces in central (n = 104) and northeastern Thailand (n = 188) (Table 1). All *E. coli* isolates were confirmed and identified using bacteriological culture, biochemical test and PCR assay. Briefly, presumptive *E. coli* isolates were cultured on MacConkey agar (Difco, Sparks, MD, USA). Five distinct colonies resembling *E. coli* from each culture plate were confirmed on eosin methylene blue agar (EMB) and by Indole test. A single colony from each positive sample was collected and stored in cryobeads containing 20% glycerol stock at -80°C.

Table 1 *Escherichia coli* isolates collected from central and northeastern Thailand

Regions	Provinces	Number of isolates
Central Thailand	Ratchaburi	92
	Chonburi	4
	Suphan Buri	4
	Aung Thong	2
	Chachoengsao	2
Northeastern Thailand	Nakhonratchasrima	137
	Udon Thani	49
	Buriram	2
Total		292

Test for antimicrobial susceptibility and ESBL production: All *E. coli* isolates were tested for susceptibility (CLSI, 2013) against eight antimicrobial agents (the recommended clinical breakpoints are in parentheses): ampicillin (AMP, 32 μg/ml), chloramphenicol (CHP, 32 μg/ml), ciprofloxacin (CIP, 4 μg/ml), gentamicin (GEN, 8 μg/ml), streptomycin (STR, 32 μg/ml), sulfamethoxazole (SUL, 512 μg/ml), tetracycline (TET, 16 μg/ml) and trimethoprim (TRI, 16 μg/ml) by determination of minimum inhibitory concentrations (MICs) using two-fold agar dilution method (Wayne, 2013). ESBLs production was examined by the disc diffusion method using ceftazidime (30 μg), cefotaxime (30 μg) and

cefpodoxime (10 μg) (Oxoid, Hampshire, UK). All *E. coli* isolates that showed resistance to any of the three cephalosporins were subjected to confirmation for ESBLs production using a combination disk diffusion method with ceftazidime (30 μg) and cefotaxime (30 μg) alone and in combination with clavulanic acid (30 μg/10 μg) (Difco/BD, Franklin Lakes, NJ, USA). The *E. coli* isolates were phenotypically considered as ESBL producers, when an increase in the size of inhibition zone was greater than (≥) 5 mm for antimicrobial agents with or without clavulanic acid was observed. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

Detection of ESBLs-encoding genes: ESBL-producing *E. coli* isolates were screened for the presence of ESBL genes (i.e. bla_{TEM}, bla_{CTX-M} group I, II, III and IV) and other β- lactamase (*bla*) genes (i.e. bla_{SHV}, bla_{CMY-1}, bla_{CMY-2}, bla_{PSE}) using PCR (Table 2). The DNA template was whole cell DNA freshly prepared from overnight cultures (Luvsansharav *et al*., 2012). PCR reaction was performed using GeNei™ mastermix (Merck, Munich, Germany) according to the manufacturer's instruction. PCR products were purified using PCR DNA and a Gel Band Purification Kit (Nucleospin™; Machery-Nagel, Duren, Germany) prior to DNA sequencing. The resultant nucleotide sequences were compared to previously established sequences from the GenBank Database using BLAST (http://www.ncbi.nlm.nih.gov/).

Conjugation experiments: Conjugation transfer of resistance genes was tested by the biparental mating method described by Lay *et al*., (2012). All ESBL-producing *E. coli* isolates (n=86) were used as donors and the spontaneous rifampicin resistant derivatives of *Salmonella* Enteritidis strain SE12rif^R (rifampicin MIC = 256 μg/ml) were used as recipients. The SE12 rif^R does not carry plasmid and is susceptible to all antimicrobial agents tested. Briefly, eighty microliters (80 μl) of the overnight culture of the donor and recipient strains were sub-cultured in 4 mL fresh Luria Bertani broth (Difco®, MD, USA)) and grown at 37^{o}C to log phase. The donor and recipient cultures were mixed at 1:1 ratio (750 μl of each) in a microcentrifuge. The bacterial cells were collected and placed on a 0.45-µm-pore-size filter (Millipore™, Merck, MS, USA) on LB agar plates and incubated at 37°C overnight. The conjugation cells were collected and spread onto LB agar plates containing rifampicin (32 μg/ml) and ampicillin (100 μg/ml). Transconjugants were confirmed as *Salmonella* by patching on xylose lysine deoxycholate (XLD) agar (Difco/BD) supplemented with rifampicin (32 μg/ml) and AMP (100 μg/ml). The efficiency of conjugation was estimated as the number of transconjugants per donor cell (Table 5). The number of transconjugants was calculated by counting the colonies in LB and XLD containing rifampicin (32 μg/ml) and AMP (100 μg/ml) plates. All transconjugants were tested for ESBL production and the presence of ESBL genes as described above.

Statistical analysis: Occurrence of phenotypic ESBL-producing *E. coli* and AMR was analyzed by descriptive statistics. Pearson's Chi Square test was performed to determine the association between ESBL-producing *E. coli* isolates and phenotypic antimicrobial resistance using SPSS version 22.0. A p-value of < 0.05 was statistically significant. The odds ratios (OR) and their 95% confidence intervals (CI) were also calculated.

Results

ESBL-producing E. coli isolates and antimicrobial resistance profiles: Antimicrobial susceptibility testing results and ESBL production are shown in Table 3. The results show that 29.5% (86/292) of *E. coli* isolates were ESBL producers. Among ESBL-producing *E. coli* isolates, 98.8% (85/86) were resistant to both AMP and TET, followed by STR (95.3%, 82/86), GEN (93%, 80/86), CHP (70.9%, 61/86), SUL (64%, 55/86), CIP (57%, 49/86) and TRI (55.8%, 48/86). A statistically significant association (p < 0.05) between ESBL-producing *E. coli* and resistance to these four antimicrobial agents was observed (Table 3). There was a positive association between the occurrence of ESBL-producing *E. coli* isolates and resistance to AMP, GEN and STR.

ESBL-producing *E. coli* isolates showed multidrug resistance (MDR) against at least three different classes of antimicrobial agents (Table 3). One ESBLs producing *E. coli* isolate was susceptible to ampicillin (Table 3) and this isolate was negative to all ESBL genes tested in this study.

ESBL-encoding genes and their transfer: Eighty-four ESBL-producing isolates (97.7%) carried at least one of the *bla* genes tested, while two isolates (2.3%) carried none of these genes. As the most predominant ESBL genotypes were bla_{TEM-1} and $bla_{CTX-M-15}$ (57%), one isolate (1.2%) carried bla_{TEM} and $bla_{CTX-M-14}$. Additionally, 10.5% of the isolates harbored bla_{TEM-1}, $bla_{CTX-M-4}$ and $bla_{CTX-M-15}$ and 15.1% were positive to $bla_{CTX-M-4}$ and $bla_{CTX-M-14}$ (Table 4). None of the isolates was found to carry the β-lactamase genes tested (i.e. bla_{CMY-1}, bla_{CMY-2}, bla_{PSE} and bla_{SHV}). The most common type of ESBL gene identified was bla_{CTX-M} (90.7%). The CTX-M group I gene was detected in 59.3% of the *E. coli* isolates, all of which were identified as $bla_{CTX-M-15}$, followed by $bla_{CTX-M-14}$ (31.4%) and $bla_{CTX-M-4}$ (25.6%) (Table 4). The bla_{TEM} gene was observed in 75.6% of the ESBL-producing *E. coli* isolates and all were identified as bla_{TEM-1}. More than ninety percent (90.8%) of the bla_{TEM-1} positive *E. coli* isolates also carried CTX-M type ESBL.

ESBL gene transfer: Based on the conjugation experiment, only 5 out of 13 ESBL producers carrying $bla_{CTX-M-4}$ and $bla_{CTX-M-14}$ yielded ampicillin-resistant *Salmonella* transconjugants (Table 4) with a conjugation efficacy of 10^{-7}-10^{-8}. The *Salmonella* transconjugants were confirmed to be ESBL producers and harbored the corresponding ESBL encoding genes ($bla_{CTX-M-4}$ and $bla_{CTX-M-14}$). Therefore, the $bla_{CTX-M-4}$ and $bla_{CTX-M-14}$ genes in these 5 isolates were horizontally transferred to the *Salmonella* recipient strains. None of the $bla_{CTX-M-15}$ was conjugally transferred.

Two randomly selected transconjugants from each donor/recipient combination were additionally tested for their antimicrobial susceptibilities (Table 5). All transconjugants were resistant to streptomycin. Transconjugants of one donor/recipient combination exhibited resistance to gentamicin.

Table 2 Primer sequences for *bla* genes used in this study.

Gene	Primer sequences (5′-3′)	Tm (°C)	Amplicon (bp)	Reference
bla_{TEM}	F- GCGGAACCCCTATTT R- TCTAAAGTATATATGAGTAAACTTGGTCTGAC	50	964	Olesen *et al*. (2004)
bla_{SHV}	F- TTCGCCTGTGTATTATCTCCCTG R- TTAGCGTTGCCAGTGYTCG	50	854	Hasman *et al*. (2005)
bla_{CMY-1}	F- GTGGTGGATGCCAGCATCC R- GGTCGAGCCGGTCTTGTTGAA	58	915	Hasman *et al*. (2005)
bla_{CMY-2}	F- GCACTTAGCCACCTATACGGCAG R- GCTTTTCAAGAATGCGCCAGG	58	758	Hasman *et al*. (2005)
bla_{PSE}	F- GCTCGTATAGGTGTTTCCGTTT R- CGATCCGCAATGTTCCATCC	55	575	Li *et al*. (2013)
bla_{CTX-M} group I	F- GACGATGTCACTGGCTGAGC R- AGCCGCCGACGCTAATACA	55	499	Pitout *et al*. (2004)
bla_{CTX-M} group II	F- GCGACCTGGTTAACTACAATCA R- CGGTAGTATTGCCCTTAAGCC	55	351	Pitout *et al*. (2004)
bla_{CTX-M} group III	F- CGCTTTGCCATGTGCAGCACC R- GCTCAGTACGATCGAGCC	55	307	Zheng *et al*. (2012)
bla_{CTX-M} group IV	F- GCTGGAGAAAAGCAGCGGAG R- GTAAGCTGACGCAACGTCTG	62	474	Zheng *et al*. (2012)

Table 3 Antimicrobial resistance phenotype and ESBL-production of the *Escherichia coli* isolates (n=292)

Antimicrobial agents	No. (%) of resistance isolates (*n* =292)	ESBL positive *E. coli* isolates (n=86)	ESBL negative *E. coli* isolates (n=206)	*p*-value	OR[a] (95% CI)
		No. (%) of resistance isolates	No. (%) of resistance isolates		
Ampicillin	265 (90.8)	85 (98.8)	180 (87.4)	<0.01	12.3 (1.6-91.9)
Gentamicin	187 (64.0)	80 (93)	107 (51.9)	<0.001	12.3 (5.2-29.5)
Streptomycin	232 (79.5)	82 (95.3)	150 (72.8)	<0.001	7.7 (2.7-21.9)
Trimethoprim	222 (76.0)	48 (55.8)	174 (84.5)	<0.001	0.2 (0.1-0.4)
Tetracycline	281 (96.2)	85(98.8)	196 (95.1)	0.13	4.3 (0.5-34.4)
Sulfamethoxazole	191 (65.4)	55(54.0)	136 (66.0)	0.74	0.9 (0.5-1.5)
Chloramphenicol	224 (76.7)	61(70.9)	163 (79.1)	0.13	0.64 (0.4-1.1)
Ciprofloxacin	161 (55.1)	49(57.0)	112 (54.4)	0.68	1.11 (0.7-1.8)

[a] OR > 1 represent positive association and OR < 1 represent negative association.

Table 4 Genotypes of β-lactamase-producing *Escherichia coli* from healthy pigs (n=86).

No.	*bla* genes	No. of isolates (%)
1	None of the bla_{TEM-1}, bla_{SHV} and bla_{CTX-M}	2 (2.3)
2	bla_{TEM-1}	6 (7.0)
3	$bla_{CTX-M-15}$	2 (2.3)
4	$bla_{CTX-M-14}$	4 (4.7)
5	bla_{TEM-1} and $bla_{CTX-M-15}$	49 (57.0)
6	bla_{TEM-1} and $bla_{CTX-M-14}$	1 (1.2)
7	$bla_{CTX-M-4}$ and $bla_{CTX-M-14}$	13 (15.1)[a]
8	bla_{TEM-1}, $bla_{CTX-M-4}$ and $bla_{CTX-M-14}$	9 (10.5)

[a] Five were conjugally transferred.

Table 5 Efficiency of conjugation and AMR phenotype of transconjugants (n=5).

Donor (ESBL producer)			Transconjugants		Conjugation efficiency
No	**Cell number in 750 µl**	**Resistance phenotype**	**No of colonies**	**Resistance phenotype of selected transconjugants[a]**	
1	1.5 x 10^8	AMP-CHP-CIP-GEN-STR-SUL-TET	8	· AMP-STR-TET · AMP-STR-TET	5.3 x 10^{-8}
2	1.9 x 10^8	AMP-CHP -GEN-STR-SUL-TET-TRI	6	· AMP-CHP-STR · AMP-CHP-STR	3.2 x 10^{-8}
3	2.4 x 10^8	AMP-CHP -GEN-STR-SUL-TET-TRI	26	· AMP-CHP-STR-TET · AMP-STR-TET	1.1 x 10^{-7}
4	1.5 x 10^8	AMP-CIP-GEN-STR-SUL-TET-TRI	21	· AMP-GEN-TET · AMP-GEN-TET	1.4 x 10^{-7}
5	1.2 x 10^8	AMP- CHP-CIP-GEN-STR-SUL-TET-TRI	13	· AMP-CHP-STR · AMR-CHP-STR	1.1 x 10^{-7}

[a]Two transconjugants from each donor/recipient combination

Discussion

The results show that 29.5% (86/292) of *E. coli* isolates from clinically selected pigs were ESBL producers (Table 3). This is lower than in other similar studies carried out in Thailand. Boonyasiri *et al*. (2014), reported a 40% and 76.7% prevalence of ESBL-producing *E. coli* from healthy chickens and pigs in an eastern and a northern province in Thailand. Nuangmek *et al*., (2018) also reported the occurrence of 36.7% (n = 588) EBSL-producing *E. coli* from 107 pig farms and 89 layer farms in the country. The difference in these two studies may be attributed to many factors such as the density of pigs on each farm, the difference in sensitivity of detection methods, farm management practices and the frequency of antimicrobial usage. Taken together, the outcomes of these studies suggest that pig farms in Thailand might serve as a hub for ESBL producing *E. coli*, hence the need for some concerted effort towards prudent use of antimicrobial agents.

The ESBL-producing *E. coli* isolates were additionally resistant to other antimicrobials with statistically significant association ($p < 0.05$) to resistance to STR, GEN, CHP and SUL (Table 3). This is consistent with the positive association between the occurrence of ESBL-producing *E. coli* isolates and resistance to AMP, GEN and STR. The positive correlation between ESBL production and AMP resistance supports the suggestion that ESBL producers displayed significantly higher rates of resistance to ampicillin, in agreement with a previous study (Xu *et al*., 2018). This was likely associated with the high catalytic activity of ESBLs on ampicillin. Co-resistance to GEN and STR suggests that ESBL genes and genes encoding resistance to gentamicin and streptomycin may be co-located either on the same plasmid or on different plasmids with different incompatibility groups within the same isolate. In contrast, a negative association was observed between ESBL-producing *E. coli* isolates and resistance to trimethoprim.

The observation of ESBL-producing *E. coli* isolates with MDR phenotype is in agreement with a previous study and demonstrates the occurrence of MDR-ESBL producing *E. coli* from hospitalized patients, livestock wastewater and the environment (Runcharoen *et al*., 2017). Most ESBL genes are usually co-localized on plasmids with various resistance determinants (Xu *et al*., 2018). This is supported by the observations in the conjugation experiment demonstrating that transconjugants were additionally resistant to non-cephalosporin antibiotics (i.e. chloramphenicol, streptomycin, gentamicin and tetracycline) (Table 3). Therefore, the frequent use of extended spectrum cephalosporins for prophylaxis or treatment purposes can facilitate the emergence of MDR *E. coli*.

Interestingly, resistance to TET (P=0.13) and CHP (P=0.13) was not significantly associated with ESBL producers (Table 3). However, the resistance phenotype to these two antibiotics was horizontally transferred in the conjugation experiment. It is likely that ESBL genes and the genes encoding tetracycline and/or chloramphenicol resistance were located on different plasmids that were in different Inc groups and co-selected by ampicillin. Therefore, limiting or suspending the use of a single antimicrobial does not effectively reduce the selective pressure of the development and spread of AMR. Action to contain AMR must be strengthened to ensure stewardship of all antibiotics.

In this study, $bla_{\text{CTX-M-15}}$ (59.3%) was the most common CTX-M group I gene identified, followed by $bla_{\text{CTX-M-14}}$ (31.4%) and $bla_{\text{CTX-M-4}}$ (25.6%) (Table 4). This agrees with previous studies conducted in food animals, humans and the environment in Thailand (Luvsansharav *et al*., 2012; Nakayama *et al*., 2015). However, previous studies reported CTX-M-14 as the most prevalent ESBLs gene identified from food animals, healthy and hospitalized individuals and environmental waste-water in China, Korea and Thailand (Hu *et al*. 2013; Tamang *et al*., 2013; Zheng *et al*., 2012). Interestingly, another study reported CTX-M-1 as the most predominant ESBL gene in food producing animals in UK, Germany, Tunisia and Switzerland (Valentin *et al*., 2014). These results showed that genotypes of ESBL vary across different geographical locations. However, it is just a matter of time, before genotypes that were usually restricted to one geographical area will be isolated from areas and sources where they have not previously been reported to occur. The phenomenon is a result of clonal proliferation and horizontal spread. On the other hand, the occurrence of CTX-M-15 in pigs reported in this study indicated healthy pigs served as carriers for ESBL-producing *E. coli* that may be transmitted to

humans. A previous study showed that CTX-M-15 is commonly associated with ESBL producing *E. coli* isolated from humans (Valentin *et al*., 2014). Both CTX-M-1 and CTX-M-15 belong to the same lineage, indicating the widespread of the CTX-M group in different sectors.

The $bla_{\text{TEM-1}}$ gene was predominant among the ESBL-producing *E. coli* isolates (75.6%). This is not surprising because TEM is the most common β-lactamase produced by Gram-negative bacteria particularly the Enterobacteriaceae family. TEM-1 is an enzyme that confers resistance to narrow and broad spectrum β-lactams such as penicillin and amoxicillin, two of the most commonly used antimicrobial agents in pig production whose resistance has been frequently observed (Lugsomya *et al*., 2018). A previous study in Thailand reported an occurrence rate of bla_{TEM} among ESBL producing *E. coli* in clinically healthy pigs (87.9%, 282/321) (Lugsomya *et al*., 2018). Our finding showed that most ESBL-positive isolates (83.7%) carried more than one *bla* gene. Production of more than one type of ESBLs in an isolate is not uncommon and has been previously reported (Nakayama *et al*., 2015). Almost all the $bla_{\text{TEM-1}}$ positive *E. coli* isolates (90.8%) additionally carried CTX-M type ESBL, in agreement with a previous study (Tamang *et al*., 2013). The fact that it is a common finding highlights that it may have considerable impact on public health.

Interestingly, one ESBLs producing *E. coli* isolate susceptible to ampicillin and lacking all ESBL genes tested was identified. This phenomenon agrees with previous studies that were conducted in ESBL-producing *E. coli* from various clinical samples (Das *et al*., 2012; Kumar *et al*., 2014; Hassuna *et al*., 2020). However, it is still unclear about the genetics underlying ESBL production in this ampicillin-resistant ESBL producing strain. Taken together, these results demonstrated that ESBL-producing *E. coli* can harbor various types of ESBLs with different substrate (antibiotic) profiles and do not always exhibit an ampicillin-resistance phenotype.

The $bla_{\text{CTX-M-4}}$ and $bla_{\text{CTX-M-14}}$ genes in 5 isolates were conjugally transferred consistent with previous studies (Woodall, 2003; Wang *et al*., 2013). This supports the predominance of CTX-M type among the isolates in this study and the spread of these genes among bacterial strains that are naturally shared between humans and animals. The occurrence of ESBL-producing *E. coli* from stool samples of people in close contact with pigs had been previously demonstrated (Hammerum *et al*., 2014). The findings of this study further suggest that CTX-M type ESBL from *E. coli* strains isolated from pigs can be transferred to humans and *vice versa*.

The conjugation experiment showed that all transconjugants were resistant to streptomycin, supporting the positive association that was observed between the occurrence of ESBL-producing *E. coli* isolates and resistance to STR. However, transconjugants of only one donor/recipient combination exhibited resistance to gentamicin, suggesting that the genes encoding gentamicin-resistance and ESBLs ($bla_{\text{CTX-M-4}}$ and $bla_{\text{CTX-M-14}}$) were not located on the same plasmid. These genes possibly coexist on different plasmid in different incompatibility groups.

In conclusion, this study revealed the occurrence of a significant proportion of ESBL-producing *E. coli* in healthy pigs in Thailand. It also represents one of the first reports of CTX-M type ESBL including $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-4}}$ and $bla_{\text{CTX-M-14}}$ in *E. coli* isolates from healthy pigs in Thailand with the CTX-M-15 subtype of CTX-M group I being the most predominant ESBL genotype. The outcomes of this study also indicated that pigs serve as reservoirs for ESBL genes that will perpetuate the spread and maintenance of ESBL genes in Thailand.

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