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Article

Multidrug Resistant *Escherichia coli* Harboring Extended-spectrum β-lactamase-encoding Genes Isolated from Clinically-healthy Pigs

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Abstract: Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* is a serious-global public health issue. A total of 292 *E. coli* isolates obtained from fecal samples of 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-lactamase producers (29.5%) and screened for the presence of β-lactamase genes. The genes in CTX-M family was most frequently found (90.7%). The $bla_{CTX-M-15}$ gene (59.3%) was predominantly identified CTX-M genotype, followed by $bla_{CTX-M-14}$ (31.4%) and $bla_{CTX-M-4}$ (25.6%). The bla_{TEM-1} gene was prevalent (75.6%). The $bla_{CTX-M-4}$ and $bla_{CTX-M-14}$ genes were located on conjugative plasmid. The results highlight healthy pigs as reservoirs of ESBL-producing *E. coli* carrying ESBL genes that could be horizontally transferred.

Keywords: ESBL; *Escherichia coli*; Pigs; Thailand

1. 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 [1,2]. Evidently, acquisition of antimicrobial resistance (AMR) genes arises due to continuous and indiscriminate use of antimicrobials in food animal production [3]. The dissemination of CTX- M type ESBLs in the environment, humans, companion animals and food animals (including pigs, cattle and chickens) is on the increase [4,5] thus paving the way for animal to human transmission and vice versa [6,7].

E. coli acquire resistance to extended-spectrum cephalosporins through transfer of conjugative plasmids carrying genes for ESBLs or Ampicillin class C (AmpC) β-lactamases [8]. These ESBL genes are commonly found in *E. coli* isolated from humans and animals, possibly because of plasmid transfer or spread of unique clonal lineages [9]. Occurrence of ESBL-producing *Enterobacteriaceae* from stool and fecal samples collected from apparently healthy individuals and pigs in Thailand has been reported [10,11], 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 [12,13]. It was suggested that food-producing animals may be an important source of ESBL-producing bacteria that might spread through the food chain [11]. The prevalence of ESBL-producing *E. coli* isolated from food animals have been reported in France, Denmark, Spain, China,

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and Nigeria [3,14,15]. These studies highlighted role of food animals, especially pigs, as a reservoir of ESBL-producing *E. coli* and their resistance determinants that may be transferred to humans and contaminate the environment.

Spread of ESBL-producing *E. coli* is a worldwide phenomenon. The burden of ESBL-producing *E. coli* from most developing countries Thailand inclusive is until recently receiving much attention. Over the past few years, awareness into the threat of evolution and spread of AMR in Thailand has considerably increased. However, knowledge on ESBLs in bacteria from food animals and its distribution is still limited. This study aimed to explore the importance of food-animals as sources of infection to humans by characterizing ESBL phenotype and genotype in *E. coli* isolated from healthy pigs.

2. Results and Discussion

Antimicrobial susceptibilities and ESBL production are shown in Table 3. The results showed that 29.5% (86/292) of *E. coli* isolates were ESBL producers (Table 3). This was lower than other similar studies carried out in Thailand. Boonyasiri et al. [16] 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. [17] reported 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 in each farm, difference in sensitivity of detection methods, farm management practices and frequency of antimicrobial usage. Taken together, it is suggested that pig farms in Thailand might serves as a hub for ESBL-producing *E. coli* and concerted efforts towards prudent use of antimicrobial agents are needed.

Among ESBL-producing *E. coli*, 98.8% (85/86) were resistant to ampicillin and tetracycline, followed by streptomycin (95.3%, 82/86), gentamicin (93%, 80/86), chloramphenicol (70.9%, 61/86), sulphonamide (64%, 55/86), ciprofloxacin (57%, 49/86) and trimethoprim (55.8%, 48/86) (Table 3). Statistically-significant association ($p < 0.05$) between ESBL-producing *E. coli* and resistance to four antimicrobial agents was observed (Table 3). There was a positive association between the occurrence of ESBL-producing *E. coli* and resistance to ampicillin, gentamicin and streptomycin. The positive correlation between ESBL production and ampicillin resistance supports that ESBL producers displayed significantly higher rates of ampicillin resistance, in agreement with a previous study [18]. Co-resistance to gentamicin and streptomycin 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. Interestingly, resistance to tetracycline (p=0.13) and chloramphenicol (p=0.13) were not significantly associated with ESBL producers. However, the resistance phenotype to these two antibiotics was horizontally transferred in conjugation experiment. It is likely that ESBL genes and the genes encoding tetracycline and/or chloramphenicol resistance were located on different plasmids in different Inc groups and co-selected by ampicillin. Therefore, limiting or suspending the use of a single antimicrobials does not effectively lower the selective pressure of the development and spread of AMR. Actions to contain AMR must be strengthened to ensure stewardship of all antibiotics.

ESBL-producing *E. coli* showed multidrug resistance (MDR) against at least three different classes of antimicrobial agents. This agrees with a previous study demonstrating the occurrence of MDR-ESBL producing *E. coli* from hospitalized patients, livestock wastewater and the environment [19]. Most ESBL genes are usually located on plasmids with various resistance determinants [18]. 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 5). Therefore, the frequent usage of extended-spectrum cephalosporins for prophylaxis or treatment purposes can facilitate the emergence of MDR *E. coli*.

Eighty-four ESBL-producing isolates (97.7%) carried at least one of the *bla* genes tested. 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 were found to carry *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). This agrees with previous studies conducted in food animals, humans and environment in Thailand [20,21]. However, previous studies reported CTX-M-14 as the most prevalent ESBL gene identified from food animals, healthy and hospitalized individuals and environmental waste water in China, Korea, and Thailand [14, 22,23]. Interestingly, another study reported CTX-M-1 as the most predominant ESBL gene in food animals in UK, Germany, Tunisia and Switzerland [24]. These results showed that genotypes of ESBL vary across different geographical location. 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 that they have not previously been reported to occur. On the other hand, the occurrence of CTX-M-15 in pigs reported in this study, indicated the zoonotic potentials of ESBL producing *E. coli.* A previous study showed that CTX-M-15 is commonly associated with ESBL producing *E. coli* isolated from humans [24]. Both CTX-M-1 and CTX-M-15 belongs to the same lineage, indicating the widespread of the CTX-M group in different sectors.

The *bla*$_{TEM}$ gene was observed in 75.6% of the ESBL-producing *E. coli* isolates and all were identified as *bla*$_{TEM-1}$. 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 and whose resistance has been frequently observed [25]. A previous study in Thailand reported the occurrence rate of *bla*$_{TEM}$ among ESBL producing *E. coli* in clinically healthy pigs (87.9%, 282/321) [25]. 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 [21]. More than ninety percent (90.8%) of the *bla*$_{TEM-1}$ positive *E. coli* isolates also carried CTX-M type ESBL, in agreement with a previous study [23]. The fact that it is a common finding highlights that it may have considerable impact on public health.

In addition, one ESBL-producing *E. coli* isolate is susceptible to ampicillin (Table 3) and this isolate was negative to all ESBL genes tested in this study. This phenomenon agrees with previous studies that were conducted in ESBL-producing *E. coli* from various clinical samples [26-29]. However, it is still unclear about 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 ampicillin-resistance phenotype.

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 the conjugation efficacy of 10^{-7}-10^{-8}. The *Salmonella* transconjugants were confirmed to be ESBL producers and harbor the corresponding ESBL encoding genes (*bla*$_{CTX-M-4}$ and *bla*$_{CTX-M-14}$). Therefore, the *bla*$_{CTX-M-4}$ and *bla*$_{CTX-M-14}$ gene in these 5 isolates were horizontally transferred to the *Salmonella* recipient strains, in consistent with previous studies [29,30]. 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 was previously demonstrated [31]. The finding of this study further suggests that CTX-M type ESBL from *E. coli* strains isolated from pigs can be transferred to humans and *vice versa*.

Two randomly-selected transconjugants from each donor/recipient combination were additionally tested for their antimicrobial susceptibilities (Table 5). 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 streptomycin. However, transconjugants of only one donor/recipient combination exhibited resistance to gentamicin, suggesting that gentamicin-

resistance and ESBLs (*bla*$_{CTX-M-4}$ and *bla*$_{CTX-M-14}$) genes were not located on the same plasmid. The genes are likely to coexist on 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 report of CTX-M type ESBL including *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-4}$ and *bla*$_{CTX-M-14}$ in *E. coli* isolates from healthy pigs in Thailand with CTX-M-15 subtype of CTX-M group I being the most predominant ESBL genotype. It is indicated that pigs serve as reservoirs of ESBL genes that will perpetuate the spread and maintenance of ESBL genes in Thailand.

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.

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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 transconjugant from each donor/recipient combination

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3. Materials and Methods

Bacterial isolates

A total of 292 stock cultures of *E. coli* isolates collected from fecal samples of clinically healthy pigs were reconfirmed and used in this study. The pig farms were in different provinces in central (n = 104) and northeastern Thailand (n = 188) (Table 1). All *E. coli* strains were isolated as previously described [32]. 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 20% glycerol at -80°C.

Test for antimicrobial susceptibility and ESBL production

All *E. coli* isolates were tested for susceptibility against eight antimicrobial agents (the recommended clinical breakpoints are in parentheses) : ampicillin (32 μg/ml), chloramphenicol (32 μg/ml), ciprofloxacin (4 μg/ml), gentamicin (8 μg/ml), streptomycin (32 μg/ml), sulfamethoxazole (512 μg/ml), tetracycline (16 μg/ml) and trimethoprim (16 μg/ml) by determination of MICs using two-fold agar dilution method [33]. ESBL production was examined by disk diffusion method using ceftazidime (30 μg), cefotaxime (30 μg) and cefpodoxime (10 μg) (Oxoid, Hampshire, UK). All *E. coli* isolates resistant to any of the three cephalosporins were subjected to confirmation for ESBL production using 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 is greater than (≥) 5 mm for antimicrobial agent 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 ESBL-encoding genes

All ESBL-producing *E. coli* were screened for the presence of nine *bla* genes including bla_{TEM}, bla_{SHV}, bla_{CMY-1}, bla_{CMY-2}, bla_{PSE}, bla_{CTX-M} group I, II, III and IV using PCR (Table 2). DNA template was whole cell DNA freshly prepared from overnight cultures [20](Luvsansharav et al. 2012). PCR reaction was performed using GeNei™ mastermix (Merck, Munich, Germany) according to manufacturer's instruction. PCR products were purified using PCR DNA and Gel Band Purification Kit (Nucleospin™; McCherey–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 biparental mating method described by Lay et al. [12]. All ESBL-producing *E. coli* (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. Briefly, the overnight culture of the donor and recipient strains were grown in fresh Luria Bertani broth (Difco) at 37°C to log phase. The donor and recipient culture 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 and incubated at 37°C overnight. Transconjugants were selected on LB agar containing rifampicin (32 μg/ml) and ampicillin (100 μg/ml) and confirmed as *Salmonella* on xylose lysine deoxycholate (XLD) agar (Difco/BD). The conjugation efficiency was estimated as the number of transconjugants per donor cell (Table 5). Number of transconjugants was calculated by counting the colonies in LB and XLD containing rifampicin (32 μg/ml) and ampicillin (100 μg/ml) plates. All transconjugants were examined for ESBL production and ESBL genes as described above.

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Data analysis

Occurrence of ESBL-producing *E. coli* and AMR was analyzed by descriptive statistics. Pearson's Chi Square test was performed to determine the association between ESBL producers and AMR 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.

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

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	[34]
*bla*SHV	F- TTCGCCTGTGTATTATCTCCCTG R- TTAGCGTTGCCAGTGYTCG	50	854	[35]
*bla*CMY-1	F- GTGGTGGATGCCAGCATCC R- GGTCGAGCCGGTCTTGTTGAA	58	915	[35]
*bla*CMY-2	F- GCACTTAGCCACCTATACGGCAG R- GCTTTTCAAGAATGCGCCAGG	58	758	[35]
*bla*PSE	F- GCTCGTATAGGTGTTTCCGTTT R- CGATCCGCAATGTTCCATCC	55	575	[36]
*bla*CTX-M group I	F- GACGATGTCACTGGCTGAGC R- AGCCGCCGACGCTAATACA	55	499	[37]
*bla*CTX-M group II	F- GCGACCTGGTTAACTACAATCA R- CGGTAGTATTGCCCTTAAGCC	55	351	[37]
*bla*CTX-M group III	F- CGCTTTGCCATGTGCAGCACC R- GCTCAGTACGATCGAGCC	55	307	[14]
*bla*CTX-M group IV	F- GCTGGAGAAAAGCAGCGGAG R- GTAAGCTGACGCAACGTCTG	62	474	[14]

Author Contributions: R.C. and K.K.L. conceived and designed the study. R.C., K.K.L., H.E.T, A.A.B and W.M. performed the data collection and analysis. N.S. and K.K.L. were involved in recovery of stock cultures and performed the experiments, R. C., K.K.L and A.A.B. were involved in data interpretation. K.K.L. wrote the first draft of the manuscript, A.A.B. and R. C. revised the full paper. All authors have read and approved the final draft of the manuscript for Publication.

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Conflicts of Interest: The authors declare no conflict of interest.

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