MULTIDRUG RESISTANT AND EXTENDED SPECTRUM β-LACTAMASE PRODUCING *SALMONELLA ENTERICA* ISOLATED FROM FOOD ANIMALS IN PHATTHALUNG, THAILAND

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Abstract. This study investigated antimicrobial resistance profiles and genes of β-lactamase-producing *Salmonella enterica*, isolates from animal feces and meat samples at small-scale rural farms in Phatthalung Province, Thailand. Of 40 isolates from swine feces 50%, 47%, 17%, 17%, and 15% were resistant to ampicillin, streptomycin, nalidixic acid, tetracycline, and chloramphenicol, respectively; of 29 isolates from chicken feces 33%, 27%, 7%, and 3% were resistant to streptomycin, nalidixic acid, tetracycline, and ampicillin, respectively; and of 6 isolates from cattle feces 67% were resistant to sulfamethoxazole and tetracycline, and 33% resistant to ampicillin, nalidixic acid and streptomycin. Of the 23 isolates from chicken meat 96%, 96%, 78%, 73%, 61%, 30%, and 9% were resistant to sulfamethoxazole, streptomycin, ampicillin, tetracycline, nalidixic acid, chloramphenicol, and ciprofloxacin, respectively; and of 31 isolates from pork meat 87%, 77%, 39%, 32%, 10% and 10% were resistant to sulfamethoxazole, tetracycline, streptomycin, ampicillin, nalidixic acid, and chloramphenicol, respectively. Three ampicillin-resistant isolates from swine feces carried the same extended-spectrum β-lactamase gene belonging to bla_{CTX-M} group 1. The results of this study confirm the existence of ESBL in *S. enterica* isolated from food animals. Occurrence of an ESBL producing strain of *S. enterica* constitutes a public health threat through transmission of these strains to humans via contaminated food or transfer of antimicrobial resistant genes to human pathogens.

Keywords: *Salmonella enterica,* animal feces, animal meat, extended spectrum β-lactamase (ESBL), Thailand

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INTRODUCTION

Life threatening disease due to infection with antimicrobial-resistant non-typhoidal *Salmonella enterica* continues to be a serious public health problem worldwide, especially in underdeveloped and developing countries (Chuanchuen

et al, 2008; Lertworapreecha *et al*, 2013). This pathogen is one of the major causes of food-borne illness in humans, as it is able to colonize within the intestine of many food animals, and thereby causing contamination of meat products during carcass processing (Sanchez *et al*, 2002; Sivula *et al*, 2008). Moreover, dramatically increasing numbers of multidrug-resistant *Salmonella* and extended spectrum β-lactamase (ESBL)-producing strains, including resistance to 3rd generation cephalosporins, which have proven effective in treating *Salmonella* infection, are of particular concern (Hasman *et al*, 2005; Jure *et al*, 2010; Ibrahimagic *et al*, 2015). Furthermore, these antimicrobial resistant strains are likely to be a source of drug resistance genes transferred to other human pathogens (O'Brien, 2002; McEwen, 2012). Patients infected with antimicrobial-resistant *S. enterica* are associated with more frequent and longer hospitalization, with increased mortality rate compared to those infected with nonresistant strains (Helms *et al*, 2002, 2004).

ESBL-encoding genes are located mainly on mobile genetic elements and plasmids, all of which have significant abilities to be transferred among bacterial species. As a result, several ESBL genes have been reported to be distributed worldwide in Enterobacteriaceae isolated from humans and food animals (Kolar *et al*, 2010; Korzeniewska and Harnisz, 2013). The etiology of antimicrobial resistance in *S. enterica* is complicated; however, several studies indicated that overuse and misuse of antimicrobial drugs in livestock production are the major causes of the spread and continued presence of antibiotic-resistant *S. enterica* in animals and environment (Aarestrup, 2005; Landers *et al*, 2012).

Resistance to ESBLs in *S. enterica* in Thai patients have been reported, but only limited studies have investigated the responsible genes for ESBLs in *S. enterica* isolated from food animals, especially in rural areas (Kiratisin *et al*, 2008; Sasaki *et al*, 2010; Udomsantisuk *et al*, 2011). In the Thai countryside, animal production is mainly conducted by small-scale farmers who supply the majority of local foodstuffs. Animal production processes including animal husbandry, slaughtering, cutting and butchering are not always hygienic, and the issues of food safety and antimicrobial resistant pathogens have, to date, received insufficient attention.

In order to control this problem, epidemiological knowledge together with scientific data regarding antimicrobial resistance and their genes are need. This study examined antimicrobial resistance profiles (antibiograms) and responsible genes, in particular those encoding ESBLs, in *S. enterica* isolated from various animal food sources and produced by small-scale local farmers in Phatthalung Province, southern Thailand.

MATERIALS AND METHODS

Bacterial samples

S. enterica was isolated and identified from animal feces and meat samples. A total of 200 fecal samples (5 from each farm) were collected from 40 small-scale local farms, 100 from individual swine, 75 from pooled chicken (each pool sampled using cloacal swabs from 5-6 individual chickens), and 25 from individual cattle (fecal sample collected from Phatthalung Province during May to October 2015). Forty samples of both pork and chicken meat were collected from fresh markets in Phatthalung Province, Thailand during June to December 2014. Isolation and identification of *Salmonella* spp were

performed as previously reported (Lertworapreecha *et al*, 2013). In brief, isolates that exhibited biochemical characteristics of *Salmonella* spp were confirmed by slide agglutination for group O polyvalent *Salmonella* antibodies (S&A Reagents Lab, Bangkok, Thailand). Serotyping of all *Salmonella* spp was performed by slide agglutination according to Kauffman-White scheme (Grimont and Weill, 2007).

Antibiogram determination

Antimicrobial susceptibility tests were performed by determining minimal inhibitory concentrations (MICs) using a broth microdilution technique (CLSI, 2014). In brief, 7 antibiotics, namely, ampicillin, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, and tetracycline were two-fold serial diluted (0.125-1,024 µg/ml) in Mueller Hinton broth (HiMedia, Mumbai, India). The final concentration of bacteria in a total volume of 200 µl was approximately 5×10^5 CFU/ml. Microplates were incubated at 35°C for 16 to 20 hours. MIC was determined according to Clinical and Laboratory Standards Institute (CLSI, 2014). The susceptibility patterns were analyzed using WHONET 5.6 program (http://www.whonet.org/).

Phenotypic and genotypic detection of ESBL-producing *Salmonella* strains

Screening of ESBL production was performed by a disc diffusion method using cefpodoxime (10 mg) (HiMedia, Mumbai, India). All cefpodoxime-resistant strains were further confirmed for phenotype using a combination disc diffusion method with cefotaxime (30 µg), cefotaxime (30 µg) + clavulanic acid (10 µg), ceftazidime (30 µg), and ceftazidime (30 µg) + clavulanic (10 µg) (HiMedia).

In order to identify ESBL genes, ampicillin-resistant strains were grown on tryptic soy agar (TSA) (HiMedia,). Then, 2 to 3 colonies were boiled for 10 minutes in 200 µl of TE buffer (10 mM Tris-Cl, pH 7.5 and 1 mM EDTA) and supernatant used as DNA template in subsequent PCR using primers listed in Table 1. Reaction mixture (50 µl) contained 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 pmol of each primer, and 0.5 U Phusion High-Fedelity DNA polymerase (Thermo Scientific, Waltham, MA). Thermocycling was conducted in a Multigene™ mini (Labnet, Edison, NJ) as follows: 94°C for 10 minutes; followed by 40 cycles of 94°C for 1 minute, annealing temperature for each primer as listed Table 1 for 1 minute, and 72°C for 1 minute; with a final step at 72°C for 10 minutes. Amplicons were analyzed by 1.2% agarose gel-electrophoresis, gel-purified (E.Z.N.A.® Gel Extraction Kit; Omega Bio-tek, Doraville, GA) and directly sequenced (Bio Basic; Markham, NO, Canada).

Phylogenetic analysis

Alignment of amino acid deduce from nucleotide sequences was conducted by BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and sequences were deposited with GenBank (accession no. KT123259, KT123258 and KT123257). Phylogenetic tree was constructed by MEGA 6 program (http://www.megasoftware.net) using neighbor-joining method (bootstrap value = 1,000).

Conjugation assay

Conjugation transfer was performed as previously described (Oluduro *et al*, 2014). In short, ESBL-producing *S.* Typhimurium, *S.* Weltevreden and *S.* Stratford were used as donor strains and non ESBL-producing *Escherichia coli* (ATCC 25922) as recipient. Donor and recipient strains were grown overnight in 2X TSB medium and then adjusted to 0.5 McFarland standard prior

Table 1

β-lactamase gene-specific primers, annealing temperatures and amplicon sizes.

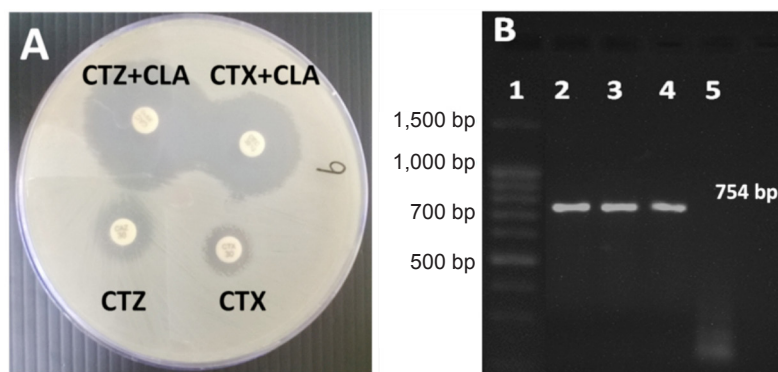| Gene	Sequences (5'-3') (F = forward, R = reverse)	Annealing temperature (°C)	Amplicon size (bp)	Reference
ESBL-TEM	F-TTTCGTGTCGCCCTTATTCC R-ATCGTTGTCAGAAGTAAGTTGG	50	404	Hassan et al, 2013
ESBL-SHV	F- CGCCTGTGTATTATCTCCCT R-CGAGTAGTCCACCAGATCCT	50	294	Hassan et al, 2013
ESBL-CTX	F-CGCTGTTGTTAGGAAGTGTG R-GGCTGGGTGAAGTAAGTGAC	50	754	Hassan et al, 2013
ESBL-OXA	F-ATGGCGATTACTGGDATAGATGG R-AGTCTTGGTCTTGGTTGTGAG	50	701	Bali et al, 2010
bla_CTX-M	F-ATGTGCAGYACCAGTAARGTKATGGC* R-TGGGTRAARTARGTSACCAGAAYSAGCGG*	54	593	Hasman et al, 2005
bla_SHV	F-TTATCTCCCTGTTAGCCACC R-GATTTGCTGATTTCGCTCGG	50	790	Arlet et al, 1997
bla_OXA	F-ACCAGATTCAACTTTCAA R-TCTTGGCTTTTATGCTTG	50	598	Gallardo et al, 1999
bla_TEM	F-CATTTCCGTGTCGCCCTTAT R-TCCATAGTTGCCTGACTCCC	55	793	Walker et al, 2001

*K = G or T; R = A or G; S = G or C; Y = C or T.

Fig 1–Phenotypic and genotypic determination of ESBL producing *Salmonella enterica* strain. A. Disc diffusion assay using cefotaxime (CTX; 30 μg), combination of cefotaxime+clavulanic acid (CTX+CLA; 30 μg + 10 μg), ceftazidime (CTZ; 30 μg), and combination of ceftazidime+clavulanic (CTZ+CLA; 30 μg + 10 μg). B. PCR amplification of bla_{CTX-M}. Lane 1, DNA size markers; lane 2, *S.* Typhimurium; lane 3, *S.* Weltevreden; lane 4, *S.* Stratford; lane 5, negative control.

to mixing at a 1:9 ratio (donor: recipient) with TSB medium. The bacterial suspensions were incubated for 3 hours before serial dilution, and then spread onto MacConkey plates supplemented with 100 μg/ml ampicillin. Phenotypic exhibition of ESBL of *E. coli* colonies on MacConkey agar plates was confirmed using a disc diffusion method. The existence of ESBL gene was determined by PCR using CTX-M primers.

RESULTS

Salmonella isolation and identification

A total of 129 isolates of *S. enterica* were isolated and identified, 40 (31%), 29 (22%), and 6 (5%) were isolated from swine, chicken and cattle feces, respectively and 31 (28%) and 23 (18%) from pork and chicken meat, respectively. The most common serovars from swine feces were *S.* Weltevreden (40%) and *S.* Typhimurium (17%), the most common from chicken feces were *S.* Braenderup (24%) and *S.* Weltevreden (17%), the most common from cattle feces were *S.* Bardo (67%) and *S.* Weltevreden (33%), the most common from chicken meat were *S.* Albany (30%) and *S.* Typhimurium and *S.* Give (17% each), and the most common from pork meat were *S.* Rissen (23%) and *S.* Weltevreden and *S.* Typhimurium (16% each) (Table 2).

Antibiogram profiles

Of the 40 isolates from swine feces 50% and 47% were resistant to ampicillin and streptomycin respectively; of the 29 isolates from chicken feces 34% and 27% were resistant to streptomycin and nalidixic acid, respectively; of the 6 isolates from cattle feces 67% were resistant to sulfamethoxazole and tetracycline, and 33% to ampicillin, nalidixic acid and streptomycin; of the 23 isolates of chicken meat 96%, 78%, 73% and 61% were resistant to sulfamethoxazole and streptomycin, ampicillin, tetracycline, and nalidixic acid, respectively; of the 31 isolates from pork meat 87% and 77% were resistant to sulfamethoxazole and tetracycline, respectively (Table 3).

ESBL phenotype and genotype

Of 21 ampicillin-resistant *S. enterica* isolates (20 from swine and 1 from chicken feces) screened for ESBL phenotype by a disc diffusion method with cefpodoxime (10 mg). Three (14%) isolates (*S.* Weltevreden, *S.* Typhimurium, and *S.* Stratford) from swine feces showed re-
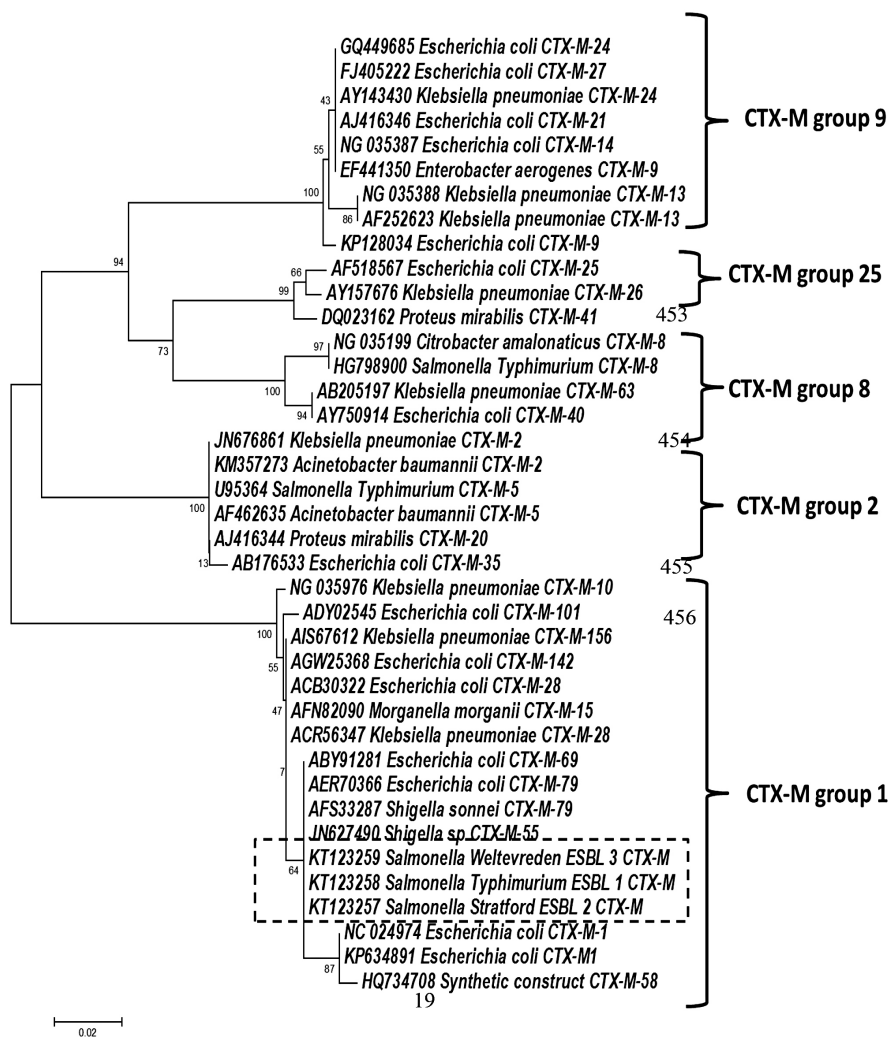
Fig 2–Phylogenetic tree of CTX-M gene. The phylogenetic tree was constructed using neighbor join-
ing method (bootstrap value = 1,000). Isolates of this study are in dash box. Number in front
of bacterial strain is GenBank accession number. (The numbers next to each node, represent
a measure of support for the node. The line segment with the number '0.02' shows the length
of branch that represents an amount genetic change).

sistance and this was confirmed using a combination disc diffusion method (Fig 1A). Antibiogram profiles of these three ESBL-producing strains showed that *S.* Typhimurium was resistant to ampicillin, chloramphenicol, nalidixic acid, strepto-mycin, sulfamethoxazole and tetracycline, whereas *S.* Stratford and *S.* Weltevreden showed similar antibiogram profile of resistance to ampicillin, chlorampheni-col, streptomycin, sulfamethoxazole and tetracycline (Table 3).

In order to determine ESBL geno-type, PCR was performed using primer pairs specific for ESBL genes (Table 1). Amplicon of approximately 750 bp were

Table 2
Salmonella serotypes isolated from animal fecal and meat samples.

Serovar	Fecal sample			Meat sample	
	Swine	Chicken	Cattle	Chicken	Pork
Agama	1				
Albany				7	
Amsterdam	2				
Anatum					4
Bardo		3	4		
Braenderup	2	7			
Bredeney					4
Choleraesuis		1			
Cremieu	1				
Derby					1
Fillmore		1			
Give				4	4
Glostrup		3			
Hadar		1			
Hvittingfoss				1	
Kalamu				1	
Kentucky				1	1
Magherafelt		1			
Mbandaka		1			
Muenchen		1			
Panama	1				
Istanbul		1			
Paratyphi B	2				
Rissen	5	1		3	7
Saintpaul	1				
Sandown	1	1			
Stratford	1				
Typhimurium	7	1		4	5
Virginia		1			
Weltevreden	16	5	2	2	5
Total	40	29	6	23	31

amplified from all three strains indicative of ESBL-CTX gene (Fig 1B). This was confirmed by nucleotide sequencing and phylogenetic analysis in comparison with ESBL genes retrieved from GenBank indicated that the ESBL gene is classified in the CTX-M group 1 (Fig 2). The remaining 18 isolates negative results for ESBL in both phenotype and genotype were investigated for the presence of β-lactamase genes (Table 1). Fourteen (67%) isolates carried bla_{TEM} and the remaining 4 isolates were negative for bla_{TEM}, bla_{CTX-M}, bla_{SHV} and bla_{OXA} (data not shown). Phylogenetic analysis showed the sequenced bla_{TEM} is closely related to bla_{TEM-1} family.

Table 3
Antibiogram of *Salmonella enterica* isolated from feces and meat samples.

Swine feces

Serovar	Antibiogram	Number of isolates (%)
Agama	ALT	1 (3)
Amsterdam	LT	1 (3)
	LS	1 (3)
Braenderup	LT	1 (3)
Cremieu	ALNS	1 (3)
Panama	CLT	1 (3)
Paratyphi B	ALST	2 (5)
Rissen	ALST	3 (8)
	T	1 (3)
Saintpaul	ALT	1 (3)
Sandown	ALNS	1 (3)
Stratford	ACLST*	1 (3)
Typhimurium	ALST	3 (8)
	ACLNST*	1 (3)
	CLST	1 (3)
	ALS	1 (3)
	AST	1 (3)
Weltevreden	GLNST	1 (3)
	ACLT	1 (3)
	ACLST*	1 (3)
	NLST	1 (3)
	ASL	1 (3)
	ALT	1 (3)
	LNS	2 (5)
	LT	1 (3)
	T	3 (8)
	L	3 (8)
Total		37

Chicken feces

Serovar	Antibiogram	Number of isolates (%)
Bardo	LNS	1 (3)
	LNST	2 (7)
Braenderup	LST	3 (10)
	L	3 (10)
	LNS	1 (3)
Choleraesuis	T	1 (3)
Fillmore	LST	1 (3)
Glostrup	LT	1 (3)
	T	1 (3)
Hadar	LT	1 (3)
	LNS	1 (3)
Magherafelt	LNST	1 (3)
Mbandaka	LT	1 (3)
Muenchen	L	1 (3)
Istanbul	LNS	1 (3)
Rissen	LNST	1 (3)
Typhimurium	LT	1 (3)
Weltevreden	L	2 (7)
	LT	2 (7)
	ALST	1 (3)
Virginia	LNST	1 (3)
Sandown	L	1 (3)
Total		29

Cattle feces

Serovar	Antibiogram	Number of isolates (%)
Bardo	NLST	1 (50)
	LS	1 (50)
Total		2

Chicken meat

Serovar	Antibiogram	Number of isolates (%)
Albany	ST	1 (5)
	NS	1 (5)
	ANS	2 (9)
	ACNS	1 (5)
	ACST	1 (5)
	ACNST	1 (5)
Give	ASN	1 (5)
	NSL	2 (9)
Hvittingfoss	ACNST	1 (5)
Kalamu	ACNS	1 (5)
Kentucky	ACNS	1 (5)
Rissen	ACNST	3 (9)
Typhimurium	NS	1 (5)
	ACNST	2 (9)
	ACST	1 (5)
Weltevreden	ACNST	1 (5)
Total		21

Pork

Serovar	Antibiogram	Number of isolates (%)
Anatum	AT	1 (5)
	ALST	1 (5)
Amsterdam	LNS	1 (5)
Bredeney	ALST	1 (5.2)
	LST	1 (5.2)
Give	LT	1 (5.2)
	LN	1 (5.2)
Rissen	ALST	1 (5.2)
Typhimurium	ALST	3 (15.6)
	T	1 (5.2)
Weltevreden	LT	4 (21)
	ST	2 (10.4)
	AST	1 (5.2)
Total		19

*ESBL–producing strain. A, ampicillin; C, chloramphenicol; G, gentamicin; L, sulfamethoxazole; N, nalidixic acid; P, ciprofloxacin; S, streptomycin; T, tetracycline.

Conjugation transfer

Only ESBL-producing *S.* Weltevreden strain showed evidence of horizontal gene transfer to the recipient *E. coli* strain as evidenced by the acquisition of the donor antimicrobial resistance profile and the presence of ESBL-CTX-M gene (data not shown).

DISCUSSION

This study investigated the prevalence of antimicrobial resistance and the types of ESBL genes responsible for β-lactam resistance in *S. enterica* isolated from feces and meat samples of food animals produced by local small-scale farmers in a province of southern Thailand. Prevalence of *S. enterica* in both swine and chicken feces was similar (40% and 38%, respectively), whereas only 24% prevalence were detected in cattle feces. This prevalence of *S. enterica* in feces samples was higher than reported in previous studies from other areas of Thailand, which indicated only 3% and 3.5% prevalence of *Salmonella* from rectal swabs of swine and live poultry, respectively (Pulsrikarn *et al*, 2012; Chotinun *et al*, 2014). The differences in prevalence may have resulted from sample collection method: this study used at least 25 g of individual swine and pooled chicken feces, which enhanced the probability of detecting *S. enterica* compared with rectal swab samples.

On the other hand, prevalence of *S. enterica* in pork and chicken meat samples was higher (57% and 77%, respectively) than found in feces samples. These prevalences of *S. enterica* in meat samples were similar to other reports (Angkititrakul *et al*, 2005; Pulsrikarn *et al*, 2012). High prevalence of *S. enterica* probably results from the multistep processes: from slaughterhouse, to carcass processing and finally to the butcher.

The predominant *S. enterica* serovars were different in each sample type. Main serovar found in swine, chicken and cattle feces was *S.* Weltevreden, *S.* Braenderup and *S.* Bardo, respectively, whereas predominant serovar in pork and chicken meat was *S.* Albany and *S.* Rissen, respectively. These findings are consistent with an earlier epidemiological survey of *S. enterica* in Thailand (Angkititrakul *et al*, 2005; Padungtod and Kaneene, 2006; Chotinun *et al*, 2014). Nevertheless, *S.* Weltevreden is the predominant serovar infecting humans in this country (Bangtrakulnonth *et al*, 2004; Sirichote *et al*, 2010). High prevalence of *S.* Weltevreden might suggest its potential to colonize a broader range of hosts and ability to persist in slaughterhouses and meat stalls for prolonged periods of time (Trung *et al*, 2016).

Almost all *S. enterica* isolates were multidrug resistant. The majority of isolates from chicken feces was resistant to nalidixic acid, implying that quinolone groups are extensively used in poultry production in Phatthalung area. According to a global survey from the year 2000 to 2010, consumption of quinolone drugs dramatically increased by 64% (Van Boeckel *et al*, 2014). Whereas only a few isolates from swine feces showed resistance to nalidixic acid, implying reduced exposure to this antibiotic. Previous studies reported that *S. enterica* isolated from food animals has a high resistance to nalidixic acid (White *et al*, 2001; Dallal *et al*, 2010).

β-Lactam antibiotics are the one that widely prescribed for treatment of infectious diseases in both human and veterinary medicine, and they are also used as feed additives to enhance the growth of food animals (Van Boeckel *et al*, 2014;

European Medicines Agency, 2015). Thus, this group of antibiotics continues to be a major cause of resistance among a number of gram-negative bacteria including *S. enterica*. We found that 3/21 ampicillin-resistant *S. enterica* from swine feces (*S. Typhimurium*, *S. Weltevreden*, and *S. Stratford*) were ESBL-producing strains, all carrying ESBL-CTX-M group 1 gene. Previous studies in Thai patients indicated that the majority of ESBL-producing Enterobacteriaceae carry bla_{CTX-M} group (Kiratisin *et al*, 2008; Sasaki *et al*, 2010). The high prevalence of CTX-M-producing Enterobacteriaceae in patients might be due to acquired horizontal gene transfer from food animal pathogens. This study demonstrated that ESBL-producing *S. Weltevreden* had the ability to transfer the gene-carrying plasmid to *E. coli*. In addition, all three ESBL-producing isolates had multiple antimicrobial antibiograms compared to other isolates, suggesting that they may carry class 1 integron (Jacoby and Sutton, 1991; Bonnet, 2004). Whereas, *S. Typhimurim* and *S. Stratford* did not transfer the CTX-M gene to the recipient cell, suggesting that the CTX-M gene might be located on the chromosome. A previous study indicated that bla_{CTX-M} in *S. Concord* is able to integrate into host chromosome (Fabre *et al*, 2009).

Fourteen ampicillin-resistant *S. enterica* isolates carried bla_{TEM-1}. This finding is inconsistent with previous studies, which demonsrated that the most prevalent β-lactamase gene in *S. enterica* is bla_{CTX-M} (Antunes *et al*, 2004; Aarestrup *et al*, 2005). This might be due to variations in sample size, geographic distribution and species of animals, as a previous report showed that ESBL types found in animals vary extensively among animal groups and across different geographical regions (Ewers *et al*, 2012). The four ampicillin-resistant *S. enterica* isolates that did not carry bla_{CTX-M}, bla_{SHV}, bla_{OXA}, or bla_{TEM} could be carrying other β-lactamase genes, such as AmpC β-lactamase gene and this requires further investigation.

In conclusion, this study demonstrated the existence of ESBL in *S. enterica* isolated from feces and meat of food animals from small rural farms in southern Thailand. The presence of ESBL-producing pathogens might be associated with over extensive use of antimicrobials in livestock production. The occurrence of ESBL-producing strains of *S. enterica* constitutes a public health threat through transmission of these strains to humans via contaminated food, or transfer of antimicrobial-resistant genes to other human pathogens. Thus, there is a need to limit the over dependence of antibiotics in livestock industry.

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