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Antimicrobial Resistance in ESBL-Producing *Escherichia coli* Isolated from Layer and Pig Farms in Thailand

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ABSTRACT

***Background*:** Study of drug resistance of commensal bacteria in both humans and animals can determine the scale of the drug resistance problem. Usage of antimicrobials to treat infections in humans and animals has generated extensive antimicrobial pressure not only on targeted pathogens but also on commensal bacteria. Commensal *Escherichia coli* appears to be the major reservoir for resistant genes implicated in the transmission of genetic traits from one bacterium to another. Antimicrobial resistance in Enterobacteriaceae has increased dramatically worldwide in the last decade. An increasing number of community-onset extended-spectrum beta-lactamase (ESBL)-producing bacterial infections, especially those caused by ESBL-producing *E. coli*, have been reported in many countries, including Thailand. Moreover, ESBL-producing *E. coli* have been widely detected in food-producing animals and the environment. The increased use of ESBLs in food animals is a serious public health problem. The objective of the study was to determine the prevalence and antimicrobial resistance pattern of ESBL-producing *E. coli* isolated from pigs, layers, farm workers and stagnant water, in order to increase awareness about antimicrobial usage on farms and to minimize the expansion of the antimicrobial resistance phenomenon in farm settings.

***Materials, Methods & Results*:** A total of 588 samples were collected from 107 pig farms and 89 layer farms in Chiang Mai–Lamphun and Chon Buri provinces during May 2015-April 2016. Double-disk diffusion method according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines was used for detection. The results demonstrated that 36.7% (216/588) of samples were ESBL-producing *E. coli*-positive, including rectal swabs 74.8% (80/107), pig farm worker stool swabs 57.0% (61/107), stagnant water on pig farms 21.5% (23/107), healthy layer rectal swabs 6.7% (6/89) and layer farm worker stool swabs 51.7% (46/89). Most of the isolates were resistant against ampicillin (99.5%), followed by erythromycin (98.6%) and ceftriaxone (96.3%). All of them were classified as multidrug-resistant strains. Moreover, AMP-CRO-E-TE-C-SXT-CN was the most frequent phenotype pattern detected in animals, humans and the environment, followed by AMP-CRO-E-TE-C-SXT-NA-CN.

***Discussion*:** The present study offers clear evidence that the prevalence of ESBL-producing *E. coli* in healthy pigs is higher than in layers. One possible explanation is that a large amount and variety of antimicrobials are used on pig farms, resulting in a common and significant source of drug-resistant ESBL-producing *E. coli*. The lower incidence of ESBL-producing *E. coli* in samples from a pig farm environment than in samples of animal origin indicate that pigs are a reservoir of a reservoir for resistant bacteria and a source of environmental contamination. Antimicrobial resistance patterns of ESBL-producing *E. coli* detected in all sample types and study locations were quite similar. In almost all ESBL-producing *E. coli* isolates, resistance was shown against ampicillin, erythromycin, ceftriaxone, tetracycline and chloramphenicol. Moreover, multidrug resistance was found in all isolates of ESBL-producing *E. coli*. The differences in antimicrobial agent resistance patterns can be used to differentiate sources by employing analytical tools such as discriminant function analysis. A molecular typing protocol is recommended for use in a discriminant function analysis for pattern determination of pathogen spreading. However, genetic fingerprinting techniques for microbial source tracking are more expensive, and facilities with appropriate equipment and expertise are required.

Keywords: ESBL-producing *E. coli*, prevalence, antimicrobial resistance, pig farm, layer farm.

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INTRODUCTION

Antimicrobial resistance has been recognized as an emerging global problem in both animals and humans, with enormous health and economic impacts [13,25]. This phenomenon has been caused mainly by the growing prevalence of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae [5,9,10] and has resulted in increased use of last-resort antimicrobial drugs, e.g. carbapenems and colistin [18]. ESBL-producing *Escherichia coli* have been isolated with increasing frequency from human and animal samples, a development that has drawn considerable attention worldwide [6,24]. Food-producing animals and their surrounding environment colonized with ESBL-producing *E. coli* have been considered to be potential sources of resistant bacterial infections in the community [24].

In Thailand, the pig and poultry industries have become the largest food-producing animal sectors. Pig production has the highest total consumption of antimicrobials (66.96%), followed by layer production (3.47%) [1]. Chiang Mai-Lamphun (northern Thailand) and Chon Buri provinces (eastern Thailand) were chosen as the focus of this study because they have a high density of animal raising, with 46.57% of the layers and 26.57% of the pigs in the country [11].

The objective of this study was to determine the prevalence and identify patterns of antimicrobial-resistant ESBL-producing *E. coli* isolated from pigs, layer chickens, farm workers and the environment (stagnant water), in order to increase awareness about antimicrobial usage and to understand the growth dynamics of antimicrobial resistance on farms.

MATERIALS AND METHODS

Study design and sample size determination

A cross-sectional study was conducted in layer and pig farms in Chiang Mai–Lamphun and Chon Buri provinces during May 2015 to April 2016. The sample size of this study was determined using Epi Info™ 7 software. Expected prevalence of 10%, with 95% confidence interval and 5% error, were used as the parameters for calculation [12]. Considered for the population on pig and layer farms, 515 and 225 are included, respectively. The sample sizes for pig and layer farms were 107 and 89. Fifty-nine pig farms and 54 layer farms in Chiang Mai-Lamphun and 48 pig farms and 35 layer farms in Chon Buri province were selected using a convenience sampling method. The data of antimicrobial use on farms were collected by questionnaire.

Sample collection

The samples in this study consisted of animal feces, stools of farm workers, and water from the environment. Fecal samples from pigs and layers were collected rectally from healthy animals using culture swabs (Culturette®)[1]. Five rectal swab samples from the same pig group were pooled into one sample, the same as with cloacal swab samples from layers. Stool samples were collected from healthy farm workers who had been working at the site for more than one year. Cary-Blair transport media (non-nutritive medium) tubes were used to store all swab specimens. Farm environment samples (stagnant water, 30 mL each) were collected from both pig and layer farms. All samples were kept in an icebox before transporting to the Faculty of Medicine Siriraj Hospital laboratory.

ESBL-producing E. coli *cultures and antimicrobial susceptibility testing*

Stool samples from healthy adult farm workers, rectal or cloacal swabs from healthy animals, and stagnant water samples from pig and layer farms were cultured on MacConkey agar and incubated at 35ºC for 24 h. Suspected colonies on MacConkey agar were red in color with a surrounding dark red area of precipitated bile salts; presumptive colonies were confirmed by a biochemical test. ESBL-producing *E. coli* isolates were detected using a double-disk diffusion method according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines.

An extra zone was determined between a disk of amoxicillin/clavulanate (20 mg/10 mg) and a 30 mg disk of each cephalosporin (ceftriaxone, ceftazidime and cefepime) placed at a distance of 20 mm from center to center of the disks on Mueller-Hinton agar plates. Clear extension of the edge of the cephalosporin inhibition zone toward the amoxicillin/clavulanate disk was interpreted as positive for ESBL production [12]. All of the ESBL-producing *E. coli* isolates were tested for antimicrobial susceptibility, using a disk diffusion method (BD BBL™ Sensi-Disc™ antimicrobial susceptibility test discs)[1]. Tests were conducted on ceftriaxone (CRO) 30 µg, cefoxitin (FOX) 30 µg, ceftazidime (CAZ) 30 µg, imipenem (IPM) 10 µg, meropenem (MEM) 10 µg, gentamicin (CN) 10 µg, amikacin (AK) 30 µg, nalidixic acid (NA) 30 µg, trimethoprim/sulfamethoxazole (SXT)

1.25/23.75 μg, tetracycline (TE) 30 μg, erythromycin (E) 15 μg, ampicillin (AMP) 10 μg, amoxicillin/clavulanate (AMC) 20/10 μg, chloramphenicol (C) 30 μg and colistin (CT) 10 μg. Antimicrobial susceptibility testing was performed according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) [8].

Data analysis

The data were described by descriptive statistics and associations between the sample collection sites (Chiang Mai-Lamphun provinces vs Chon Buri province). Antimicrobial drug resistance was expressed as odds ratios with 95% confidence interval using Epi Info™ 7 software [7].

RESULTS

A total of 588 samples were collected, from which 216 (36.7%) ESBL-producing *E. coli* isolates were retrieved. Most were pig-associated samples (51.1%; 164/321); of these, approximately 75% (80/107) originated from healthy pigs, followed by 57.0% (61/107) and 21.5% (23/107) from pig farm workers and stagnant water, respectively. In layer-associated samples, 19.4% (52/267) were positive; 51.7% (46/89) were from farm worker stool samples and 6.7% (6/89) were detected in healthy layer rectal swab samples. None of the stagnant water samples from layer farms were found to contain ESBL-producing *E. coli* isolates (Table 1).

Most of the ESBL-producing *E. coli* isolates were resistant to ampicillin (99.5%), followed by erythromycin (98.6%), ceftriaxone (96.3%), tetracycline (85.6%) and chloramphenicol (75.5%). Colistin-resistant isolates were present only in layer farm workers (2.2%), while imipenem and meropenem were 100% effective against ESBL-producing *E. coli* isolates from all types of samples (Table 2).

ESBL-producing *E. coli* isolates resistant to >3 antimicrobial agents were denoted as multidrug-resistant strains. Distribution of the ten most common antimicrobial resistance patterns of multidrug-resistant ESBL-producing *E. coli* isolates from animals (pigs and layers), farm workers and the environment (stagnant water) is shown in Fig. 1. Drug resistance patterns of major ESBL-producing *E. coli* phenotypes were AMP-CRO-E-TE-C-SXT-CN (14.4%), followed by AMP-CRO-E-TE-C-SXT-NA-CN (11.6%), AMP-CRO-E-TE-C-CN (5.6%) and AMP-CRO-E-TE-C-SXT-CN-CAZ (5.6%), respectively. The strains derived from animals, humans and the environment were grouped in identical drug resistance pattern phenotypes.

Table 1. Sampling schemes and distribution of ESBL-producing *E. coli* isolates recovered from pig and layer farms in Chiang Mai-Lamphun and Chon Buri provinces, Thailand.

Sample	Chiang Mai-Lamphun		Chon Buri		Overall	
	No. samples	No. with ESBL-producing *E. coli* (%)	No. samples	No. with ESBL-producing *E. coli* (%)	No. samples	No. with ESBL-producing *E. coli* (%)
Pig farms						
Rectal swab (pigs)	59	32 (54.2)	48	48 (100.0)	107	80 (74.8)
Stool swab (farm workers)	59	30 (50.8)	48	31 (64.6)	107	61 (57.0)
Stagnant water on farms	59	8 (13.6)	48	15 (31.25)	107	23 (21.5)
Subtotals	177	70 (39.5)	144	94 (65.3)	321	164 (51.1)
Layer farms						
Rectal swab (layers)	54	4 (7.4)	35	2 (5.7)	89	6 (6.7)
Stool swab (farm workers)	54	27 (50.0)	35	19 (54.3)	89	46 (51.7)
Stagnant water on farms	54	-	35	-	89	-
Subtotals	162	31 (19.1)	105	21 (20.0)	267	52 (19.4)
Grand totals	339	101 (36.1)	249	115 (46.2)	588	216 (36.7)

Tiamulin was the most common antimicrobial used on pig farms in Chiang Mai-Lamphun (83.1%), followed by amoxicillin (71.2%), tylosin (50.8%) and chlortetracycline (23.7%), while the antimicrobials most commonly used on pig farms in Chon Buri were amoxicillin (54.2%), tiamulin (45.8%), colistin (25.0%) and penicillin/streptomycin (25.0%). The most widely used antimicrobials on layer farms in Chiang Mai–Lamphun were tiamulin (75.0%), chlortetracycline (64.3%), tylosin (55.4%) and amoxicillin (25.0%), while the antimicrobials most commonly used in Chon Buri were tylosin (91.4%), amoxicillin (62.9%), tiamulin (28.6%) and colistin (28.6%) [Table 3]. Generally, macrolides were the most common agents used over our study areas.

Odds ratios between sample collection sites (Chiang Mai-Lamphun vs Chon Buri) and resistance results (resistant vs susceptible) against each antimicrobial agent most commonly reported in the study were analyzed. No significant differences were detected for all sample types (Table 4).

Table 2. Percent resistance against each antimicrobial agent of ESBL-producing *E. coli* isolates originating from different sources in pig and layer farms in Chiang Mai-Lamphun and Chon Buri provinces.

Antimicrobial agent	Percent resistance against each antimicrobial agent					
	Pig farms			Layer farms		Overall (n = 216)
	Pigs (n = 80)	Farm workers (n = 61)	Stagnant water (n = 23)	Layers (n = 6)	Farm workers (n = 46)	
Ampicillin	100.0	100.0	100.0	100.0	97.8	99.5
Erythromycin	100.0	98.4	95.7	100.0	97.8	98.6
Ceftriaxone	96.3	93.4	95.7	100.0	100.0	96.3
Tetracycline	83.6	80.3	95.7	66.7	93.5	85.6
Chloramphenicol	81.3	68.9	82.6	100.0	67.4	75.5
Gentamicin	77.5	54.1	95.7	100.0	60.8	69.9
Trimethoprim/Sulfamethoxazole	66.3	54.1	69.6	33.3	60.8	61.1
Nalidixic acid	45.0	49.2	43.5	50.0	58.7	49.1
Ceftazidime	20.0	16.4	13.0	16.7	32.6	20.8
Amoxicillin/Clavulanic acid	6.3	4.9	-	16.7	21.7	8.8
Cefoxitin	5.0	4.9	4.3	16.7	2.2	4.6
Amikacin	-	-	4.3	-	2.2	0.9
Colistin	-	-	-	-	2.2	0.5
Imipenem	-	-	-	-	-	-
Meropenem	-	-	-	-	-	-

Table 3. Antimicrobial agents used in routine practices on pig and layer farms in Chiang Mai-Lamphun and Chon Buri provinces during the preceding year.

Antimicrobial agent	No. of farms using each antimicrobial agent (%)			
	Pig farms		Layer farms	
	Chiang Mai-Lamphun (n = 59)	Chon Buri (n = 48)	Chiang Mai-Lamphun (n = 54)	Chon Buri (n = 35)
Tiamulin	49 (83.1)	22 (45.8)	42 (75.0)	10 (28.6)
Amoxicillin	42 (71.2)	26 (54.2)	14 (25.0)	22 (62.9)
Penicillin/Streptomycin	39 (66.1)	12 (25.0)	-	-
Tylosin	30 (50.8)	5 (11.4)	31 (55.4)	32 (91.4)
Oxytetracycline	28 (47.5)	4 (8.3)	-	-
Lincomycin	21 (35.6)	1 (2.1)	-	-
Colistin	18 (30.5)	12 (25.0)	4 (7.1)	10 (28.6)
Trimethoprim/Sulfamethoxazole	16 (27.1)	1 (2.1)	1 (1.8)	-
Chlortetracycline	14 (23.7)	7 (14.6)	36 (64.3)	6 (17.1)
Cefotaxime	9 (15.3)	7 (14.6)	-	-
Enrofloxacin	7 (11.9)	4 (8.3)	7 (12.5)	3 (8.6)
Kanamycin	3 (5.1)	-	-	-
Gentamicin	12 (0.3)	7 (14.6)	2 (3.6)	3 (8.6)
Doxycycline	-	1 (2.1)	1 (1.8)	3 (8.6)

Table 4. Odds ratios between sample collection sites and resistance results against each antimicrobial agent most commonly reported in the study.

Sample	Odds ratio (95% confidence interval)*					
	Ampicillin	Erythromycin	Ceftriaxone	Tetracycline	Chloramphenicol	Gentamicin
Pig farms						
Rectal swab (pigs)	NA	NA	NA	2.39 (0.60-9.50)	0.34 (0.11-1.08)	0.73 (0.26-2.13)
Stool swab (farm workers)	NA	NA	0.96 (0.13-7.33)	0.63 (0.18-2.27)	1.24 (0.40-3.83)	1.60 (0.58-4.41)
Stagnant water on farms	NA	NA	NA	NA	1.75 (0.15–20.23)	NA
Layer farms						
Rectal swab (layers)	NA	NA	NA	NA	NA	NA
Stool swab (farm workers)	NA	NA	NA	3.06 (0.26-36.42)	NA	0.85 (0.25-2.84)

NA = not available. *Chon Buri was used as the reference category for the sample collection sites.

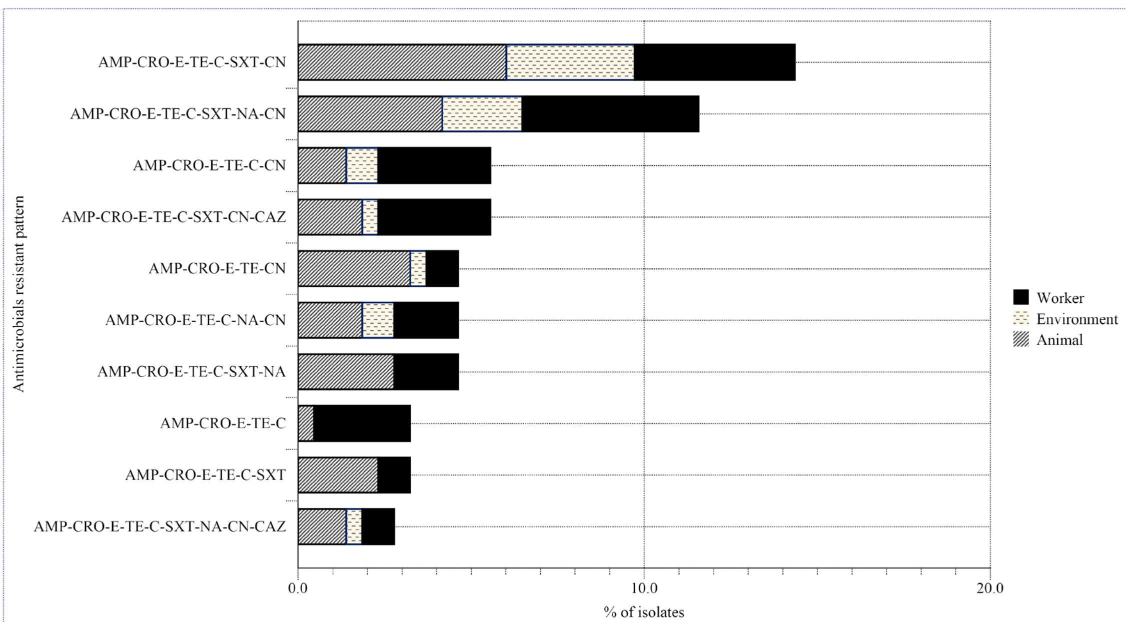

Figure 1. Distribution of ten most common antimicrobial resistance patterns of multidrug-resistant ESBL-producing E. coli isolates separated in each source. *amikacin (AK); ampicillin (AMP); amoxicillin/clavulanate (AMC); ceftazidime (CAZ); cefoxitin (FOX); ceftriaxone (CRO); chloramphenicol (C); colistin (CT); erythromycin (E); gentamicin (CN); imipenem (IPM); meropenem (MEM); nalidixic acid (NA); tetracycline (TE) and trimethoprim/sulfamethoxazole (SXT).

DISCUSSION

The prevalence of ESBL-producing *E. coli* in fecal samples from healthy pigs (74.8%) was higher compared with healthy layers (6.7%), which was similar to the prevalence found in a previous study [1,3]. The large amount and variety of antimicrobials used on pig farms could indicate that pig farms are a common major source of resistant ESBL-producing *E. coli* [4]. This is at variance with the results of studies by Hiroi *et al*. and Geser *et al*., in which layers are recognized as the major reservoir of ESBL-producing *E. coli* [14,15].

Upon the comparison of each sample type, the prevalence of ESBL-producing *E. coli* in samples from a farm environment was lower than in samples of animal origin. Indicating the livestock function is known to be a reservoir for resistant bacteria and a source of environmental contamination [22]. However, no stagnant water samples on layer farms were identified as containing ESBL-producing *E. coli*. Less water is utilized for a layer operation compared with the requirements for pigs. Water is also drained rapidly, so pathogen accumulation in stagnant water is not likely to occur in layer farms. Interestingly, in layer-associated samples, the prevalence of ESBL-producing *E. coli* in farm worker samples was found to be at a higher level compared with samples from healthy layers. Contamination may possibly be associated with non-farm sources.

When considering geographic areas, the ESBL-producing *E. coli* prevalence observed in the eastern region of the country was higher than in the north, in contrast with a previous report [3]. The higher prevalence in the north could be a result of variations in study design, different local environments and/or animal production practices [3,19,23].

Antimicrobial resistance profiles of ESBL-producing *E. coli* collected from all sample types were similar (Table 2). Most of the isolates were resistant against ampicillin, erythromycin, ceftriaxone, tetracycline and chloramphenicol. Moreover, ampicillin-, erythromycin- and ceftriaxone-resistant isolates were detected almost equally in animals, humans and the environment. These antimicrobials have been widely applied in animal production in Thailand. Improper use could have created selective pressure, resulting in a high antimicrobial resistance rate [16].

Tetracycline and chloramphenicol resistance in ESBL-producing *E. coli* was found at a low level. In Thailand, tetracycline usage in food-producing animals has been restricted since 2003 [21], while chloramphenicol usage has been prohibited since 2002 [20]. Consequently, selective pressure is unlikely. Horizontal gene transfer, facilitating the spread of resistant genes within and between various bacterial species, is possible [17]. Remarkably, ESBL-producing *E. coli* resistant to colistin were present in layer farm workers (2.2%). Colistin is a last-resort antimicrobial for treatment of severe infections caused by multidrug-resistant gram-negative bacteria, in particular those caused by carbapenem-resistant *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [2]. This situation makes it imperative to evaluate the magnitude of resistance to colistin in both humans and animals and to implement a system for surveillance of colistin resistance.

All ESBL-producing *E. coli* detected in the study were multidrug-resistant. AMP-CRO-E-TE-C-SXT-CN and AMP-CRO-E-TE-C-SXT-NA-CN were the major phenotypic patterns, including strains derived from animals, humans and the environment. The transmission of pathogens among originating sources (animal/human/environment) is a possibility. A molecular typing protocol is strongly recommended for use in a discriminant function analysis for pattern determination of pathogen spreading [22].

CONCLUSION

The prevalence of ESBL-producing *E. coli* on pig farms was higher than on layer farms; contamination rates in Chon Buri province tended to be higher. Antimicrobial resistance patterns of ESBL-producing *E. coli* detected in all types and sites of samples were comparable. Multidrug resistance was found in all isolates of ESBL-producing *E. coli*. Isolates originating from animals, farm workers and the environment were demonstrated to have identical drug resistance patterns. Awareness regarding antimicrobial usage on farms should be addressed. Furthermore, comprehensive surveillance systems to monitor antimicrobial resistance ought to be established in order to reduce the selective pressure downstream on humans.

MANUFACTURER

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***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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