OCCURRENCE AND DISSEMINATION OF MULTIDRUG-RESISTANT *Escherichia coli* IN POULTRY AND POULTRY MEAT PRODUCT

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has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy in Microbiology of Naresuan University

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ABSTRACT

The aims of this study were to determine the prevalence and dissemination of extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL-EC) in backyard poultry farms and poultry meat products in Phitsanulok province, Thailand. A total of 734 samples was obtained from backyard farms (n = 587) and poultry meat samples (n = 147) between September 2013–September 2014. Two-hundred and fifty (34.1%) *E. coli* isolates with reduced susceptibility to cefotaxime were recovered. Antimicrobial susceptibility testing revealed that all isolates were resistant to ampicillin, cephozolin, cefotaxime and cefpodoxime and were high resistant (> 60%) to many drugs. All isolates were confirmed as multidrug-resistant *E. coli* (MDR-EC). In addition, 214 MDR-EC (85.6%) were ESBL-EC. $bla_{\text{CTX-M group 1}}$ (54.8%) was the highest prevalence of $bla_{\text{ESBL/AmpC}}$, followed by $bla_{\text{CTX-M group 9}}$ (23.6%) and $bla_{\text{CMY-2}}$ (8.4%). Phylogenetic grouping showed that group A and B1, commensal strains, were the most common groups among MDR-EC (81.6%) while the prevalence of group B2 and D, pathogenic strains, was 18.4%. Multilocus sequence typing was performed on 14 isolates belonging to group B2. The results showed that sequence type (ST) 131, a highly virulent strain, was the predominant ST (n = 7) followed by ST95, ST127, ST219 and ST8180 (1 isolate each) while 3 isolates were untypeable. Conjugation experiment demonstrated that $bla_{\text{ESBL}}/bla_{\text{AmpC}}$ plasmids could be transferred to recipient with high frequencies (1.8×10^{-7}–8.2×10^{-2}) and contributed the increase cefotaxime MICs of respective transconjugant (8–64 folds compare with recipient).

Pulsed-field gel electrophoresis (PFGE) illustrated the clonal spread of ESBL-EC within the same and between farms, including cross-contamination between farmer and chicken as well as poultry and environment. It is interesting to observe that clonal spread of $bla_{CTX\text{-}M\text{-}27}$-positive ST131 between human and chicken on different farms had occurred. Location of $bla_{CTX\text{-}M\text{-}55}$ was investigated in 6 $bla_{CTX\text{-}M\text{-}55}$-positive isolates by S1-PFGE and gel hybridization. The results showed that $bla_{CTX\text{-}M\text{-}55}$ was located on plasmid with 30–150 kb in sizes in 5 isolates. Plasmid sequencing was performed on a $bla_{CTX\text{-}M\text{-}55}$-positive isolate, pEC278. Genetic context of $bla_{CTX\text{-}M\text{-}55}$ showed the presence of several antimicrobial-resistant genes associated with IS*26* (IS*26*-*catA2*-IS*26*-IS*Ecp1*-$bla_{CTX\text{-}M\text{-}55}$-Tn*3*-IS*3*-*qnrS1*-*res*-IS*26*-IS*4*-*aac(3)-IId*-IS*6*-Tn*3*-*mcr-3*-*dgkA*-IS*3*-IS*26*). These results suggest that backyard poultry farms and poultry meat products may be a reservoir of MDR-EC which could be disseminated among Thai communities.

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ABBREVIATIONS

Antimicrobial agents

AK	amikacin	AMP	ampicillin
AMC	amoxicillin/clavulanate	ATM	aztreonam
C	chloramphenicol	CAZ	ceftazidime
CIP	ciprofloxacin	CN	gentamicin
CPD	cefpodoxime	CTX	cefotaxime
CXM	cefuroxime	DO	doxycycline
ETP	ertapenem	FEP	cefepime
FOX	cefoxitin	IMP	imipenem
KZ	cefazolin	LEV	levofloxacin
MEM	meropenem	NA	nalidixic acid
SAM	ampicillin/sulbactam	S	streptomycin
SXT	trimethoprim/sulfamethoxazole		
TE	tetracycline		

Antimicrobial resistance

AmpC-EC	AmpC β-lactamase-producing *Escherichia coli*
ARGs	Antimicrobial-resistant genes
ARB	Antimicrobial-resistant bacteria
EC-CTX	*E. coli* with reduced susceptibility to cefotaxime
ESBL-EC	Extended-spectrum β-lactamase-producing *E. coli*
MDR-EC	Multidrug-resistant *E. coli*

Methods and related

cm	Centimeter
g	Gram
Inc	Plasmid incompatibility groups
L	Liter
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing

ABBREVIATIONS (CONT.)

µl	Microliter
µM	Micromolar
mM	Millimolar
mg	Milligram
ml	Milliter
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
S1-PFGE	S1-nuclease pulsed-field gel electrophoresis
ST	Sequence type

Nucleotides and genetic material

a	Adenine
bp	Base pair
c	Cytosine
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
g	Guanine
IS	Insertion sequence
kb	Kilobase
t	Thymine
Tn	Transposon

CHAPTER I

INTRODUCTION

Rationale for the study

The emergence of antimicrobial-resistant bacteria (ARB), particularly Gram-negative bacilli, has increasingly been reported and highlighted as a major public health problem globally. Infections caused by ARB result in high morbidity and mortality rates, over 2,000,000 and 80,000 cases per year, respectively. The highest concern is that there are no effective control measures, presently, to prevent the emergence and spread of ARB. It is generally accepted that selective pressure caused by the misuse of antimicrobial agents in both hospital and livestock industry contributes to the emergence of ARB. Hence, the restricted use of antimicrobial agents has recently been recommended as the best solution.

Food-producing production system has contributed the emergence of ARB because high antimicrobial agent consumption is used for growth promotion, disease prevention and treatment. This issue is currently being considered from public health organizations worldwide for several reasons. Firstly, the antimicrobial use in farm is not strictly controlled, especially in developing countries. Consequently, ARB is continuously emerged. Secondly, the dissemination of ARB within and between farms is probably wider than that in hospital because farms are generally large-sized area and found throughout the community. Thirdly, most farms in developing countries often use natural resources (soil and environmental water) to raise their animals, hence the contamination of ARB to environments may easily occur. Lastly, ARB from farm may disseminate to animal or human via a direct contact and consumption of contaminated meat. These may result in the increase number of ARB carriers and community-onset infections. Furthermore the antimicrobial-resistant genes are mostly located on transferable elements, e.g. plasmid and transposon, thereby enhancing the spread of ARB.

It has been documented that, in Asia, Thailand is the country with the high prevalence of antimicrobial-resistant *E. coli* among healthy population, animal and

environment. Extended-spectrum β-lactamase (ESBL) producers, especially those that are CTX-M-positive, have commonly been found in clinical, environmental and animal isolates. Backyard poultry farms and their products have become an interesting point due to the fact that backyard farm is frequently found in rural area in Thailand and antimicrobial use is not controlled. Therefore, it is possible that backyard poultry farm in Thailand could be the reservoir of antimicrobial-resistant *E. coli*, especially multidrug-resistant *E. coli*, and cause the dissemination of antimicrobial-resistant *E. coli* between human, animal, the environment. However, the study of ARB from food-producing animals in Thailand is limited. Most studies have reported the ARB from commercial farms and focused mainly on the antimicrobial-resistant phenotypes and antimicrobial-resistant genes. This study was designed to determine the occurrence of multidrug-resistant *E. coli*, including ESBL-producing *E. coli*, one of the most common Gram-negative pathogens from backyard poultry farm, farmers, farm environments and poultry meat products sold in open-air markets in Phitsanulok province, Northern Thailand. The results from this study may provide information related to ARB in backyard poultry farm and poultry meat products that may cause the spread of antimicrobial-resistant organism within Thai community.

Research hypothesis

Backyard poultry farms and poultry meat products may be reservoirs for multidrug-resistant and ESBL-producing *E. coli* which could disseminate among humans, food-producing animals and environments within community.

Objectives of the study

1. To investigate the occurrence of multidrug-resistant *Escherichia coli*, including extended-spectrum β-lactamase-producing (ESBL) *E. coli*, in backyard poultry farms and poultry meat products
2. To characterize, phenotypically and genotypically, *E. coli* isolates
3. To investigate the dissemination of ESBL-producing *E. coli* within the backyard farms, farm workers and the environment

Scope of the study

This study focused on multidrug-resistant and ESBL-producing *E. coli* in backyard poultry farms and poultry meat products. The dissemination of ESBL-producing *E. coli* within and between farms was investigated. Twenty-seven backyard poultry farms and poultry meat samples sold in 28 open-air markets in Phitsanulok province were included in this study. Disk diffusion method, microdilution broth method, extended-spectrum β-lactamase (ESBL) production were used for the determination of the antimicrobial-resistant phenotype of *E. coli*. Phylogenetic grouping and sequence typing (ST) were used for genotypic analysis. The presence of drug resistant-associated genes, focusing on ESBL- and AmpC-encoding genes was investigated. Conjugation and pulsed-filed gel electrophoresis (PFGE) were used to investigate the dissemination of ESBL-producing *E. coli* within community. Selected *E. coli* was performed by plasmid sequencing to study on the location and genetic context of $bla_{\text{CTX-M}}$.

Significance of the study

The high antimicrobial agent consumption in animal husbandry in Thailand is of concerned since this may contribute to the increase prevalence of ARB, especially *E. coli*, in Thai community. Many studies on antimicrobial-resistant *E. coli* in Thailand have been reported, however, those studies mainly focused on the isolates from intensive farm. Data on the antimicrobial-resistant *E. coli* in backyard farming are limited. The results in this study may provide insights into the understanding the dissemination of antimicrobial-resistant *E. coli* in backyard poultry farms and poultry meat products which may influence the policy-making process on the use of antimicrobial agents in food-producing animal and establish effective control measures to reduce the spread of ARB of potential health risk to humans.

CHAPTER II

LITERATURE REVIEWS

Antimicrobial agents are commonly used in farming in both developed and developing countries, especially Asia. The effect of drug usage causes selective pressure on commensal bacteria in animal to become resistance. Antimicrobial-resistant bacteria (ARB) could spread between animals and farm environment. Moreover, it could spread to human by direct contact or food consumption. This issue is a serious concern and has a huge impact on ARB research, especially among Gram-negative bacilli, in food-producing animals. Backyard farm is commonly found in Southeast Asia, including Thailand. Moreover, consumption of antimicrobial agent, including β-lactams, in backyard farm has been documented. However, the study on ARB from food-producing animal in backyard farm is limited. Researches involving ARB from farms are being carried out to understand the situation of ARB and finding effective solution to resolve the increase number of ARB in food-producing animal.

β-Lactams

β-Lactams are the most widely used antimicrobial agents for the treatment of bacterial infections. Most β-lactams possess broad spectrum activity against both Gram-positive and Gram-negative bacteria. They comprise various agents originating from natural and semisynthetic drugs. The structure of all β-lactams is basically composed of β-lactam ring which is a four-membered cyclic amide (Figure 1). The bacterial transpeptidase involving in cell wall synthesis by catalyzing the cross-link of peptidoglycan strands is the target of β-lactam. This target is well-known as penicillin-binding proteins (PBPs). PBPs are permanently bound to β-lactams which play roles as an irreversible inhibitor resulting in disruption of cell wall synthesis and bacterial cell lysis. Thus, β-lactams are bactericidal agents. β-Lactams are divided into 5 groups; penicillins, cephalosporins, carbapenems, monobactam, and β-lactamase inhibitors (Figure 1) (1).

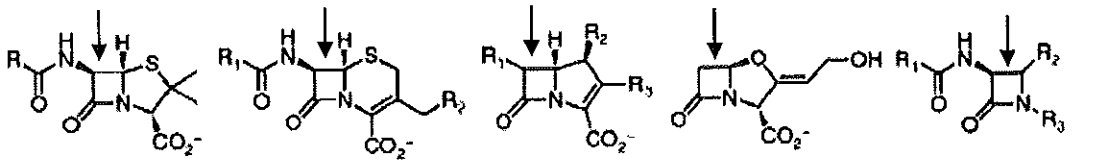


Figure 1 The structure of β-lactam groups (2)
β-Lactam rings are indicated by arrows.

1. Penicillins

Penicillins are highly active against many species of Gram-negative and Gram-positive bacteria. Ampicillin and amoxicillin are often used for the treatment of Enterobacteriaceae infections. Additionally, penicillin and amoxicillin are well-known as drugs used in food-producing animals in many countries, including Thailand (3–5). Besides, oxacillin and cloxacillin are used for the treatment of *Staphylococcus aureus* infection while the combination of penicillin with β-lactamase inhibitor, such as piperacillin with tazobactam, is widely used for the treatment of nosocomial infection (6). Nowadays, many species of Gram-negative bacilli, such as *Escherichia coli*, *Klebsiella* spp., and *Pseudomonas* spp., are resistant to these drugs.

2. Cephalosporins

Cephalosporins are common drug used in human for clinical treatment worldwide between 2000–2010 (7). Cephalonsposins comprises more than 20 drugs which are classified into 5 generations. The antibacterial activity of each drug varies greatly, from narrow to expanded spectrum. All 1st generation cephalosporins are narrow spectrum drugs, e.g. cepholothin and cefazolin. The 2nd generation cephalosporins, e.g. cefoxitin and cefuroxime, have an increased activity against Gram-negative bacteria. The antibacterial activities of 3rd and 4th generation cephalosporins are greater than that of previous generations, thus this generation is commonly called expanded-spectrum cephalosporin. Ceftiofur is common drug used in poultry farming (8, 9). Of these generations, drugs are frequently used for the treatment of infection caused by Gram-negative bacilli and 2nd generation cephalosporin–resistant bacteria, particularly many species of Enterobacteriaceae and *P. aeruginosa* (1). Cefotaxime, cefpodoxime, ceftazidime and ceftriaxone (3rd

generation cephalosporins) and cefepime (4^{th} generation cephalosporin) are widely used (6).

3. Carbapenems

Carbapenems, such as imipenem, meropenem and ertapenem, are broad-spectrum and the most effective β-lactams frequently used for treatment of bacterial infections, including those caused by antimicrobial-resistant bacteria. The drugs are able to capture PBPs, effectively penetrate into bacterial cell, especially Gram-negative bacteria, and resistant to many β-lactamases (1).

4. Monobactam

Aztreonam is only drug in monobactam group. The potent antibacterial activity of aztreonam is specific against aerobic Gram-negative bacteria, including β-lactamase producer. However, some Gram-negative pathogens, such as *P. aeruginosa*, and *Acinetobacter* spp., are slightly inhibited (1).

5. β-Lactamase inhibitors

Clavulanic acid, sulbactam, tazobactam are the members of β-lactam inhibitors which possess low level of antibacterial activity but they are able to inactivate β-lactamase. None of β-lactam inhibitors is used for a monotherapy (6). β-Lactamase inhibitors are covalently bound by β-lactamase resulting the rearrangement of their structure. β-Lactamase inhibitors in this irreversible mechanism are often called suicide substrates (1). In general, β-lactamase inhibitors are used in combination with β-lactam, such as amoxicillin/clavulanate and ampicillin/sulbactam, which are useful for the treatment of infections caused by β-lactamase-producing pathogens.

Extended-spectrum β-lactamases (ESBLs)

Extended-spectrum β-lactamases (ESBLs) are members of β-lactamases which are produced by a variety of β-lactam-resistant bacteria. These enzymes are named from their potentials of hydrolytic activity against expanded-spectrum cephalosporins, i.e. cefotaxime, ceftazidime, cefpodoxime or cefepime etc. Besides, ESBLs confer resistance to penicillins and aztreonam but are inhibited by β-lactamase-inhibitors. ESBL-encoding gene (bla_{ESBL}) is often located on conjugative plasmid that commonly carries other antimicrobial-resistant genes conferring aminoglycosides and

trimethoprim/sulphamethozaxole, thus ESBL-producing bacteria is often considered as multidrug-resistant bacteria. The enzyme is secreted into periplasm of Gram-negative bacteria or outside of Gram-positive bacteria. The occurrence of ESBL-producing bacteria has increasingly been reported worldwide and is a serious problem associated with significant treatment failure. More than 700 β-lactamases have been reported (10). β-Lactamases are classified into 4 classes: classes A, B, C, and D based on the homology of amino acid sequence (11). Classes A, C, and D are serine β-lactamases, while class B is a metallo β-lactamase. The active site of serine β-lactamase generally comprises 4 conserved amino acids (serine-threonine-phenylalanine-lysine). The free hydroxyl group on the side chain of serine plays an important role in breaking the amide bond (C-N) of β-lactam ring, then β-lactam ring is cleaved. Thus, the resulting β-lactam is inactive for transpeptidase, a target of drug (1). Unlike the serine β-lactamase, metallo β-lactamase hydrolyzes β-lactam using the coordination of hydroxyl group of water molecule and heavy-metal ion, mostly Zn^{2+}.

1. Class A β-lactamases

Class A β-lactamase is the largest class of β-lactamase (> 500 enzymes) (10) and has been reported as the most widespread enzymes in β-lactam-resistant bacteria. Generally, the enzymatic activity is inhibited by β-lactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam. The activity of individual enzyme against each drug is highly variant, however, it is comprehensively grouped according to its substrate profile, e.g. penicillinase, ESBL, inhibitor-resistant β-lactamase, and carbapenemase. The largest and most widespread enzymes are CTX-M, TEM, SHV, and KPC, respectively.

1.1 CTX-M

All CTX-M enzymes are ESBLs. CTX-M or cefotaximase is named according to its ability to hydrolyze cefotaxime. In addition, enzymes hydrolyze cefazidime and cefepime, although at a much lower activity, but carbapenems are not affected. The presence of $bla_{CTX\text{-}M}$ is reported in at least 26 species, predominantly *E. coli* and *Klebsiella pneumoniae* (12). The number of $bla_{CTX\text{-}M}$-positive bacteria isolated from human, animal and environment in many countries is continuously reported since $bla_{CTX\text{-}M}$ is frequently located on conjugative plasmid and closely associated with IS*Ecp1*, a mobile genetic element (12). CTX-M is the most

widespread of ESBL types detected in antimicrobial-resistant bacteria recovered poultry samples (13). CTX-M enzymes are grouped into 5 groups based on amino acid sequence as follow (14): CTX-M group 1 (CTX-M-1, -3, -15, -55 etc.), group 2 (CTX-M-2, -4, -5 etc.), group 8 (CTX-M-8, -40 and-63), group 9 (CTX-M-9, -14, -27 etc.) and group 25 (CTX-M-25, -26 and -41 etc.). CTX-M-14 and CTX-M-15 are reported as the most widespread enzymes worldwide and are mostly presented in *E. coli* and *K. pneumoniae* (12, 15).

1.2 TEM

The name "TEM" was derived from Temoneira, patient's name, and that enzyme is then called TEM-1. Recently, > 200 TEM enzymes have been reported. The enzymatic activities among TEM enzymes vary greatly. There are either non ESBL, ESBL or inhibitor-resistant TEM (IRT) (16). Moreover, some TEM enzymes are simultaneously resistant to expanded-spectrum cephalosporin and β-lactam inhibitor. Hence, they are called complex mutant of TEM β-lactamase (CMT), such as TEM-158 (17). $bla_{\text{TEM-52}}$ is the most common $bla_{\text{TEM ESBL}}$ found among ESBL-producing *E. coli* isolated from poultry (13).

1.3 SHV

SHV gets its name from sulfhydryl variable because the activity of SHV enzyme is specifically inhibited by sulfhydryl group of *p*-chloromercuribenzoate. The variation of the inhibitory activity of sulfhydryl group depends on each SHV enzyme. SHV-2, variant of SHV-1, has been documented as the first ESBL. Since the first ESEL SHV reported, > 40 ESBL SHV enzymes, e.g. SHV-5 and -12 etc., have been published (18). However, some SHV enzymes are not ESBL. More than 190 SHV enzymes have been reported and frequently found in member of Enterobacteriaceae (19). Especially, $bla_{\text{SHV-12}}$ is the most common $bla_{\text{SHV ESBL}}$ found among ESBL-producing Enterobacteriaceae isolated from poultry (13).

2. Class B β-lactamases

The structure of class B β-lactamase is different from other classes of β-lactamase because it individually comprises of two conserved metal-binding sites to capture heavy-metal ion, mostly Zn^{2+}, as an enzymatic cofactor. Generally, all class B β-lactamase is called metallo-β-lactamase (MBL) or carbapenemase, e.g. IMP, VIM and NDM enzymes (20). All β-lactams, e.g. penicillins, cephalosporins, and

carbapenems are hydrolyzed by metallo-β-lactamase but monobactam is poorly hydrolyzed. Almost enzyme activities are inhibited by EDTA. *P. aeruginosa* and *A. baumanii* have been reported as the most species harboring MBL-encoding gene. Enterobacteriaceae harboring MBL-encoding gene has increasingly been found (21).

3. Class C β-lactamases

Class C β-lactamase, well-known as AmpC β-lactamase, is the second most common enzyme produced by Enterobacteriaceae, such as *E. coli*, *K. pneumoniae*, *Enterobacter* spp., and *Citrobacter* spp. Many β-lactams including expanded-spectrum cephalosporins, except cefepime and carbapenems, are hydrolyzed by AmpC β-lactamases. Unlike class A β-lactamases, AmpC enzymes are poorly inhibited by common β-lactamase inhibitors. However, the enzymatic activity is greatly inhibited by cloxacillin, oxacillin and aztreonam (22). Genes encoding AmpC β-lactamase are found in both bacterial chromosome and plasmid. At present, AmpC β-lactamases can be divided into 10 groups based on their amino acid sequences. These are CMY, MIR, MOX, LAT, DHA, FOX, ACT, ACC, BIL, and CFE. Recently, > 70 enzymes are found in Gram-negative bacteria, CMY-2 being the most reported enzyme (23).

Normally, the expression of chromosomal AmpC β-lactamase (*ampC*) of many Gram-negative bacteria is repressed by AmpR protein (encoded from *ampR*) (24). However, increased expression is caused by inducible molecules, such as ampicillin, cefoxitin and clavulanic acid (25). Nevertheless, AmpC overexpression or constitutive hyperproduction can be achieved by the *ampR* mutation. The AmpC hyperproduction results from spontaneous mutation or inducible mechanism is a serious concern because this expression is often found in clinical isolates. Furthermore, most strains expressing AmpC hyperproduction are resistant to multiple β-lactams causing the treatment failure.

The origin of plasmid-mediated *ampC* gene (bla_{pAmpC}) is derived from chromosomal *ampC*. bla_{pAmpC} are increasingly found in *E. coli* and *K. pneumoniae* (23). The hyperproduction of bla_{pAmpC} can be caused by *ampC* on multiple copy numbers of plasmid. Moreover, some clinical isolates carrying both *ampC* or bla_{pAmpC} and bla_{ESBL} are reported. Consequently, these bacteria can not be confirmed either ESBL production or *ampC* production by antimicrobial susceptibility testing (26).

4. Class D β-lactamases

OXA enzyme, as a representative enzyme in class D β-lactamase, is named from its enzymatic activity because all enzymes in this class are able to hydrolyze oxacillin and cloxacillin. Individual class D β-lactamase varies in enzymatic activity against each drug. Most OXA enzymes poorly hydrolyze expanded-spectrum cephalosporin and are not inhibited by β-lactamase inhibitors but they are strongly inhibited by NaCl (27). Interestingly, some OXA enzymes, OXA-23 and -48, are able to hydrolyze carbapenems hence they are called carbapenem-hydrolyzing class D β-lactamase (28, 29). Similar to all classes of β-lactamases, bla_{OXA} is found on chromosome or transferable element, mainly plasmid.

Escherichia coli

Escherichia coli is a Gram-negative short rod bacterium in the Family Enterobacteriaceae. It is ubiquitous in the intestine of warm-blooded animals and in the environment. *E. coli* is an important human pathogen which causes various infections, such as urinary tract infection, intestinal infection, sepsis, and bacteremia. Many organs can be infected causing the high morbidity and mortality and increase health-care cost. Additionally, *E. coli* is the majority of ESBL-producing Gram-negative bacteria in poultry (13). Human infections are usually acquired by consumption of contaminated meat. Thus, *E. coli* is used to monitor ARB in food-producing animal and is used as an index microorganism in food safety program.

To understand and monitor the epidemiology of *E. coli* strains in non-clinical and clinical samples, *E. coli* strains are identified by two standard methods: phylogenetic grouping and multilocus sequence typing (MLST). *E. coli* strain can be classified into 4 phylogenetic groups (A, B1, B2, and D) based on the presence of *chuA* (heme transport gene), *yjaA* (unknown function), and TSPE4.C2 (anonymous DNA fragment) (30). This method distinguishes *E. coli* group A and B1 as commensal strains while group B2 and D are virulent extraintestinal strains. MLST is the most reliable method for bacterial characterization. This technique is based on analysis of the sequence of 7 housekeeping genes, including *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate dehydrogenase), *mdh* (malate dehydroge-nase), *purA* (adenylosuccinate synthetase), and *recA* (ATP/GTP binding

motif) (31). All 7 genes are analyzed to identify ST and clonal complex via database on website.

Among pathogenic *E. coli* group B2, *E. coli* ST131, belonging to serotype O25b (*E. coli* O25b-B2-ST131), is the most common pathogen globally (32). *E. coli* ST 131 exhibits resistance to multiple drugs including β-lactam and fluoroquinolone (33). The ESBL production is frequently found among β-lactam-resistant ST131 which often shows higher multiple drug resistance compared with non-ST131 (34). bla_{CTX-M} is the most common ESBL-encoding gene among ST131 while bla_{SHV} and bla_{TEM} are infrequently reported (34 36). Furthermore, ESBL-encoding genes found among ST131 are often located on conjugative plasmid that concurrently contains virulent genes and other antimicrobial-resistant gene, such as plasmid-mediated quinolone resistance (PMQR) genes. Thus, *E. coli* ST131 is becoming a serious concern by the public health organizations worldwide.

Multidrug resistance (MDR)

To define the degrees of antimicrobial resistance among bacteria, the universal terminology of antimicrobial resistance has generally been considered as 3 terms: multidrug resistance (MDR), extensive drug resistance (XDR) and pandrug resistance (PDR). MDR refers to "resistance to at least 1 agent in 3 or more antimicrobial categories". XDR is defined as "resistance to at least 1 agent in all but 1 or 2 antimicrobial categories". PDR is defined as "resistance to all agents in all antimicrobial categories" (37). To define MDR among Family Enterobacteriaceae, antimicrobial categories have been divided as penicillins, penicillins+β-lactamase inhibitors, carbapenems, 1st and 2nd generation cephalosporins, 3rd and 4th generation cephalosporins, cephamycins, monobactam, aminoglycosides, fluoroquinolones, folate pathway inhibitors and phenicols (38).

Inc compatibility groups

Bacterial plasmid is circular extrachromosomal element that often carries genes conferring antimicrobial and heavy metal resistance, including virulence factor. Plasmids also lead to the spread of antimicrobial-resistant genes and contribute genetic diversity among bacterial population. Plasmid type is classified as incompatibility

(Inc) according to two plasmids sharing similar replication and partition systems are unable to replicate and proliferate in the same host cell line (39). Nowadays, 27 known Inc groups (IncA to IncZ and the other groups) among Enterobacteriaceae have been noted. IncF, IncHI, IncI, IncK and IncL/M are narrow-host-range plasmids while IncA/C, IncN, IncP, IncQ, and IncW are broad-host-range plasmids (40, 41). Most Inc plasmid groups involve multidrug resistance. IncF, IncA/C, IncL/M, IncI1, IncHI2 and IncN are the majority of plasmid groups carrying antimicrobial-resistant gene among Enterobacteriaceae (40). ESBL-encoding genes, $bla_{\text{CTX-M}}$, bla_{AmpC} and bla_{SHV}, are commonly found on variety of Inc plasmid groups.

Backyard poultry farm and poultry meat product

Currently, there are > 8,000 registered poultry farms (intensive and organic farming) in Thailand, however, the number of backyard poultry farm is unclear (42). In 2017, poultry meat products, including frozen meat, was ranked the highest meat production (> 800,000 tons) compared to other types of meat samples (42). According to the data from the Office of Agricultural Economics in 2018, Phitsanulok province raises a high number of broilers (500,001–1,000,000) and laying hens (100,001–500,000) (43). Generally, backyard farm, non-intensive or household farm, is a small subsistence farm that is commonly found throughout the rural community of Asia. It is distinguished from commercial farm in four particular points: size, location, number of animal, and farm management. The particular characteristics of backyard poultry farm in Thailand are generally a small or medium farm comprising one or more types of poultry, such as chicken, duck, or goose. In addition, some farms concurrently raise other livestock, such as cattle or swine. Approximately 10–100 animals are raised in each house. Most farms are located in the farmer's house area, i.e. space under the house including the surrounding area. Therefore, farmer and family member have a close contact with their animals. Backyard poultry is usually raised in free-range or cage. Additionally, some farmers raise their animals outdoors (free-range) at daytime and in a cage during the night. Farm management is dependent on a local natural resource on or nearby their farms. The objectives of backyard farming are not only for a commerce but also for household consumption or a hobby and recreational sports, such as cockfighting, that is the common objectives of native chicken farming (44).

Occurrence of antimicrobial-resistant bacteria in farms and meat products

Soon after the first antimicrobial agents were used in clinical treatment, the antimicrobial-resistant bacteria have emerged. The spread of ARB from many parts of the world is unexpected. At least 80,000 patients were infected by ARB between 2009–2010 in Thailand. Of these, approximately 30,000 patients died. For economic loss, the cost of antimicrobial agents for clinical treatment was approximately 2,500–6,000 million Baht (45). In the past, the emergence of ARB is mainly reported from hospitals. However, infections caused by ARB have increasingly been documented from community settings. It is generally accepted that food-producing animals and its meat products are the main reservoirs for ARB (46–48).

The use of antimicrobial agents in food-producing animals is related to the emergence of ARB in poultry farming, including household or backyard farm (49). In addition, antimicrobial usage in food-producing animals may contribute to the emergence of ARB in human which is a global public health concern (50). Nowadays, many antimicrobial agents have been withdrawn in food animal production in many countries (51), however, the increased numbers of ARB in food-producing animals are continuously reported. Thailand and many countries in Asia highly consume antimicrobial agent in poultry farming (52). The occurrence of ARB from food-producing animal is found in both non-commercial and commercial farms of developed and developing countries (53–55). Gram-negative bacilli (especially Enterobacteriaceae) are reported as the most predominant ARB species. In addition, ARB isolates (mostly *E. coli*) from food-producing animal are reported as ESBL producer or MDR bacteria (56, 57).

Food-animal farming (especially non-commercial farm) may be a large reservoir for ARB causing the spread of ARB to human, animal and environment (Figure 2) because ARB could be accumulated in environmental sources by animal's excretion, antimicrobial agent residues in animal feed and animal waste, including wastewater from farm. Human may acquire ARB and antimicrobial-resistant genes via direct contact or food consumption. Consequently, farmer, food-producing animal and farm environment are considered as ARB carriers (58). Among food-producing animals, poultry is frequently reported as the highest contamination of ESBL-producing *E. coli* (13). Antimicrobial-resistant *E. coli* isolates recovered from poultry

were commonly resistant to ampicillin and amoxicillin and were resistant to ceftiofur, ceftriaxone, cefotaxime and cefpodoxime with moderate level of resistant rates (59). In addition, a moderate level of resistance rates to ampicillin and ceftriofur among chicken from farm and abattoir in Northern Thailand was also observed (60) while high resistant rates of penicillins and 3rd generation cephalosporin, cefotaxime and ceftriaxone, were found among *E. coli* isolated from farm animals in China (61). Among ESBL- and AmpC-encoding genes detected in *E. coli* from food-producing animal, $bla_{\text{CTX-M}}$, especially $bla_{\text{CTX-M group 1}}$ and $bla_{\text{CTX-M group 9}}$, and $bla_{\text{CMY-2}}$ have been reported as the most common genes (55, 62, 63). The clonal spread of ESBL-producing *E. coli* between poultry within and between farms was often reported (57, 64). Although transmission of ESBL-producing *E. coli* between farmer and chicken is uncommon, genetic relatedness of *E. coli* isolates recovered from those sources was reported (65) and farmer may be at high risk for contamination of ESBL-producing *E. coli* from poultry (66).

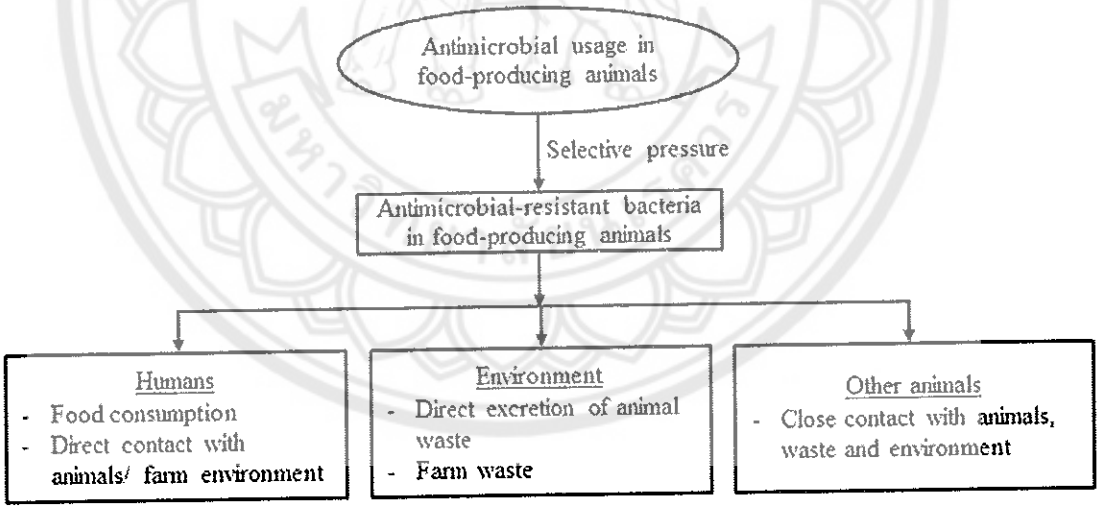


Figure 2 Transfer of antimicrobial-resistant bacteria between food-producing animals, humans and environment [modified from (67)]

Poultry farm environment may be contaminated ESBL-producing *E. coli* via animal waste, dust, air, even equipment in farm (68, 69). Antimicrobial residue in animal waste and feed possibly affects an increase number of ARB in environment (70). Studies revealed that the transmission of ARB between human, food-producing

animal, and the farm environment could occur (48, 71, 72). Moreover, ARB in food-producing animal could be distributed to wild animals by insects living in farm, such as flies, and shrews (73–75). Additionally, antimicrobial-resistant genes could remain in the farm environment, although the farm has been cleaned up (76).

Meat products have been introduced as a vehicle of ARB. Poultry meat was reported as having the highest rate of ESBL-producing *E. coli* contamination compared with other types of meat samples (77) and may be a reservoir of extraintestinal pathogenic *E. coli* (ExPEC) causing urinary tract infection in human (78). The transfer of ARB, ESBL-encoding genes and plasmids from poultry to human may be possible via consumption of poultry meat (79). In food processing, a slaughterhouse is reported as a place with high-level contamination of ARB (80). ARB contamination in meat product has frequently been reported worldwide, including Thailand (81–83). Dissemination of ARB may be the result of food transportation. For example, ceftiofur-resistant *E. coli* was obtained from imported poultry meat sold in Denmark was originally contaminated in meat imported from Brazil, Germany, and France (84) or ESBL-producing *E. coli* was found in imported chicken meat (from Brazil) sold in Japan (83). ARB obtained from food-producing animals, farm environments, meat products usually carry many antimicrobial-resistant genes. These genes are resided on mobile genetic elements and may cause the spread of antimicrobial-resistant organism within Thai community.

CHAPTER III

RESEARCH METHODOLOGY

Ethical statements

This study was approved by Naresuan University Institutional Review Board (COA No. 83/2013) and Naresuan University Institutional Biosafety Committee (5627).

Study area and sources of samples

The research was a cross-sectional study focusing on backyard poultry farms and open-air markets in Phitsanulok province, Lower Northern Thailand (Figure 3a), during the period of September 2013–August 2014. Phisanulok province covers approximately 10,000 km^2 comprising 9 districts: Bang Kratum, Bang Rakam, Chat Trakan, Muang, Nakhon Thai, Noen Maprang, Phrom Phiram, Wang Thong, and Wat Bot. This province is an agricultural city which has approximately 870,000 populations. Of these, there are nearly 200,000 people working in agricultural careers (85). According to the data from the Provincial Livestock Office, over 4.0 million poultry were raised in Phitsanulok province in the year 2017 (86). Additionally, Phitsanulok province, being a central area of lower northern Thailand, is also central to the distribution of agricultural and food-animal products throughout the north, central and north-east of Thailand. Scope of this study was shown in Figure 4.

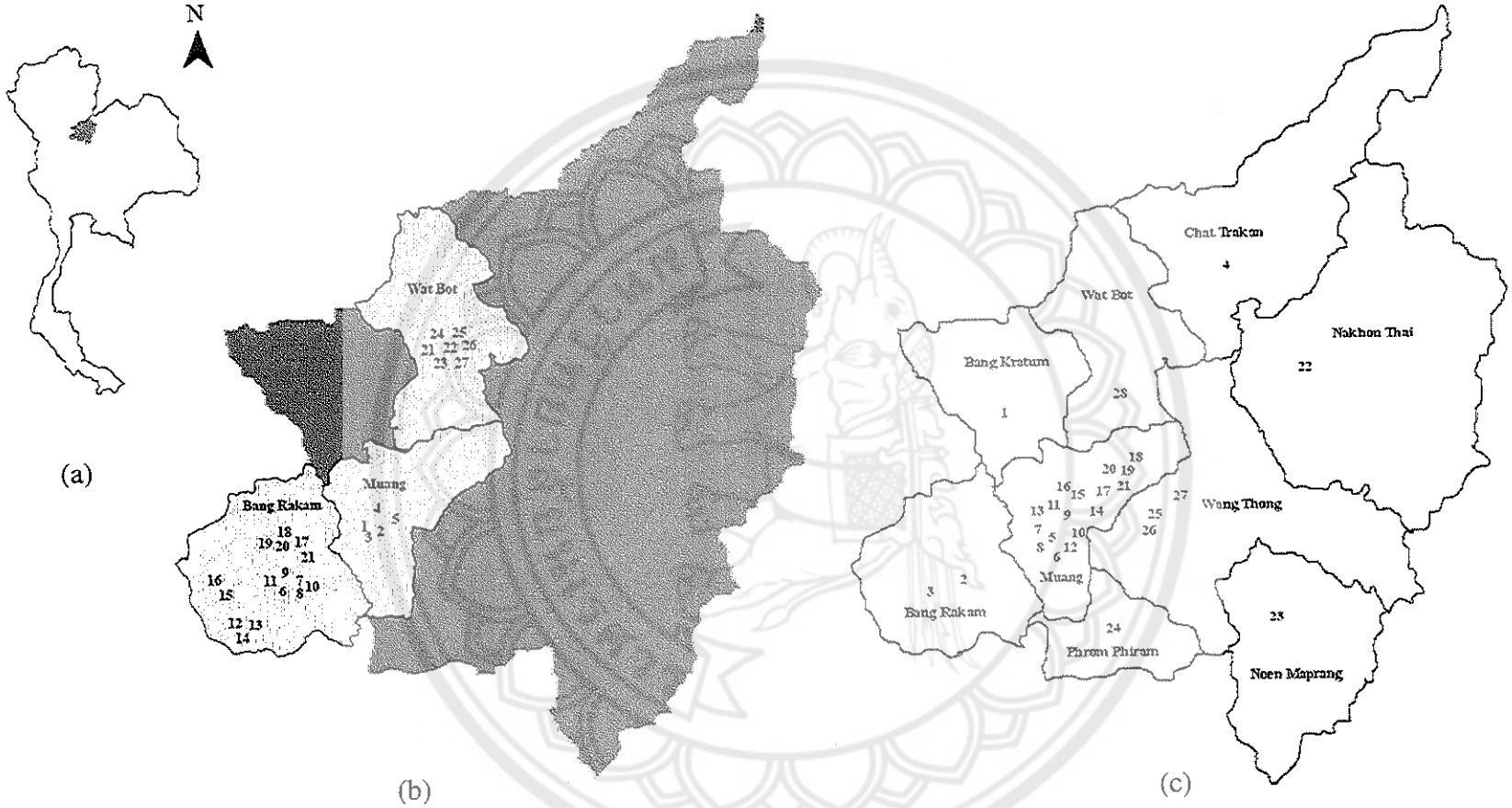


Figure 3 Sampling areas in this study (a) Location of Phitsanulok province (b) Location of 27 backyard poultry farms in 3 districts (c) Location of 28 open-air markets in 9 districts

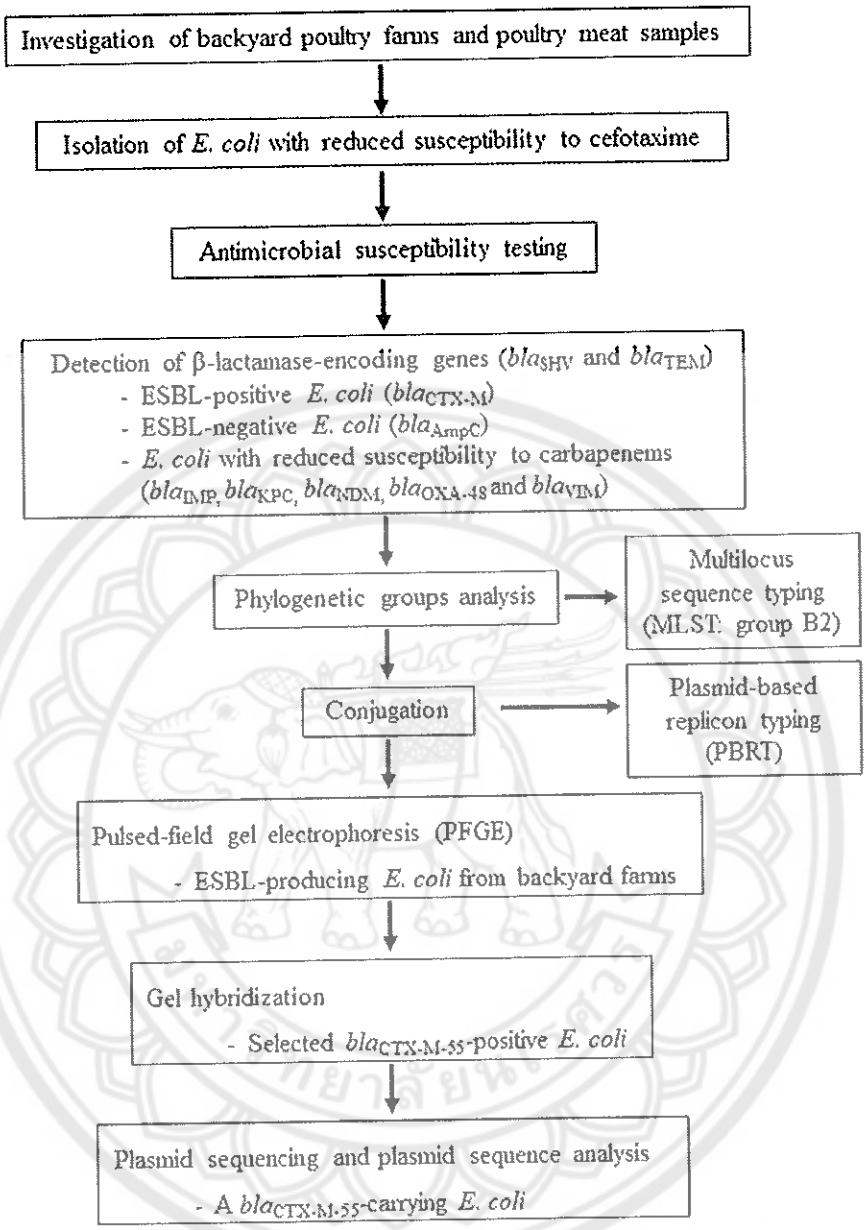


Figure 4 Scope of this study

1. Backyard poultry farms

Backyard poultry farm was defined as a small subsistence farm comprising poult at least 30 animals that were raised on farmer's house area. Chicken and duck were included. Written informed consent was obtained from all farmers prior to participate in this study. Farm sampling was performed in 3 districts: Muang, Bang Rakam, and Wat Bot. Three types of samples were collected including feces from

poultry, farmer and family member as well as farm environment. To avoid anxiety of owner and stress or pain in animals, samplings were carefully performed without contacting animals. Information on farm owner and backyard poultry farm management was carefully collected using questionnaires, visual observation and interview.

Occurrence of antimicrobial-resistant *E. coli* was carried out among 27 backyard poultry farms. They were located in Muang (n = 5, farms 1–5), Bang Rakam (n = 15, farms 6–20) and Wat Bot districts (n = 7, farms 21–27) (Figure 3b). Generally, all owners have raised their animals and managed farm by themselves. Poultry fed in cage was observed in 2 farms, (farms 9 and 10 in Bang Rakam district) while most animals were fed both in cage and free-range (Figure 5). Antimicrobial agent consumption in farm was confirmed in 19 farms (70.4%). The drugs were occasionally used for prophylactic and therapeutic treatment, however, the owners denied the use of drugs for promoting growth. During the visit, the majority of farms were cleaned, however, animal waste still appeared within the farms. The other descriptive information and farm management are shown in Appendix A: Table 15.

A total of 587 samples was obtained from 27 backyard poultry farms comprising 32 farmer feces, 88 environmental samples (35 water and 53 soil) and 467 poultry feces (41 ducks and 426 chicken) (Table 1). Famer feces were not obtained from 6 farms (farms 8, 10, 11 and 17 in Bang Rakam and farms 25 and 27 in Wat Bot districts). Environmental samples in farm 3 in Muang and farm 19 in Bang Rakam could not be obtained (Appendix A: Table 16).

(a) (b)

Figure 5 Types of backyard poultry farms in this study
(a) Raising poultry in cage only
(b) Raising poultry in cage and free-range

Table 1 Sources of backyard poultry farms and numbers of farm samples

Districts (No. of farms)	Sources of samples (n)					Total of samples
	Farmers	Poultry		Farm environment		
		Chicken	Ducks	Water	Soil	
Bang Rakam (15)	13	299	18	20	19	369
Muang (5)	11	60	9	12	18	110
Wat Bot (7)	8	67	14	3	16	108
Total (27)	**32**	**426**	**41**	**35**	**53**	**587**

2. Poultry meat samples

Poultry meat samples from 28 open-air markets or local markets (1 sample per a retailer) in 9 districts of Phitsanulok province were investigated (Figures 3c and 6; Table 2). Frozen meat product was not included. All samples were maintained in ice box and further proceeded within 18 h. In this study, 147 fresh poultry meat samples (3 ducks, 18 birds and 126 chicken) were obtained (Appendix A: Table 17).

Figure 6 Open-air markets in Phitsanulok province

Table 2 Sources and number of poultry meat samples sold in 28 open-air markets in Phitsanulok province

Districts (No. of market)	No. of meat samples			Total of samples
	Chicken	Duck	Bird	
Bang Kratum (1)	3	0	1	4
Bang Rakam (2)	9	0	1	10
Chat Trakan (1)	4	0	0	4
Muang (17)	58	1	11	70
Nakhon Thai (1)	19	0	0	19
Noen Maprang (1)	3	0	1	4
Phrom Phiram (1)	8	0	3	11
Wang Thong (3)	16	2	1	19
Wat Bot (1)	6	0	0	6
Total (28)	**126**	**3**	**18**	**147**

Isolation of *Escherichia coli* with reduced susceptibility to cefotaxime

E. coli with reduced susceptibility to cefotaxime (EC-CTX) was isolated as previously described with slightly modification (48, 56, 87).

1. Poultry fecal samples

Samples were randomly collected on different sites within farm. Only top side of fresh poultry feces was individually obtained to prevent contamination from soil. One gram of feces was collected and soaked in 9 ml of tryptone soy broth (TSB) (Oxoid LTD, Hampshire, England) for pre-enrichment, then sample was sent to the laboratory for investigation within 18 h. One milliliter of pre-enrichment broth was transferred to 9 ml of EE broth (Becton, Dickinson and Company, Sparks, USA), mixed by vortexing and incubated at 37°C for 24 h. For the isolation of EC-CTX, one loopful of enrichment culture was streaked on Eosin-Methylene Blue agar (EMB) (Oxoid LTD, Hampshire, England) supplemented with 2 mg/L cefotaxime (Sigma-Aldrich Pte Ltd, USA) and incubated at 37°C for 24 h. A typical morphology of *E. coli* presenting dark colony with metallic sheen (Appendix A: Figure 13) was selected and identified by standard biochemical tests (Gram stain, oxidase test and citrate test). Isolate showing gram-negative short rod-shaped bacteria, oxidase-negative and citrate-negative (one colony per samples) was selected for further study.

Isolates yielding doubtful results were biochemically identified by using RapID^{TM} ONE System (REMEL, KS, USA) according to the manufacturer's instruction (Apendix A: Figure 14) or using polymerase chain reaction (PCR) and DNA sequencing of 16S rRNA gene (88) as described in species identification.

2. Human fecal sample

Human fecal samples were obtanined from consenting farm owener, worker or family member. Only participants aged > 20 years old were included. Fecal sample was collected using a sterile transport medical cotton swab stick (DELTALAB, Barcelona, Spain), sent to the laboratory and kept in refrigerator until it was used (within 18 h). The stick was streaked on EMB agar supplemented with 2 mg/L cefotaxime and proceeded as described above.

3. Poultry farm environment

Soil and water samples from different sites within farm were randomly collected approximately 10 g at 10 cm depth and 10 ml at 30 cm depth from the soil

and water surface, respectively. Samples were homogenized and 1 gram of soil or 1 ml of water samples were transferred to 9 ml of tryptone soya broth (TSB), EC-CTX was isolated as described above.

4. Poultry meat product

Approximately 25 g of fresh poultry meat were homogenized in 225 ml TSB by stomacher. The 10 ml of homogenized broth was transferred to 90 ml EE broth. Then, the isolation of EC-CTX was performed as described above.

Species identification by PCR and sequencing of 16S rRNA gene

Single *E. coli* colony was suspended in 500 µl sterile deionized water and used as template in PCR. PCR mixture consisted of 1 µl of template, 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each primer and 1 U of platinum *Taq* DNA polymerase (Invitrogen, California, USA) in a final volume of 50 µl. Primers, conditions of PCR amplification, and sizes of PCR product were shown in Table 3. Amplification was carried out in a Veriti™ 96 well Thermal cycler (Applied Biosystems, California, USA). PCR product was electrophoresed on 0.8% (w/v) agarose gel (Vivantis Technologies Sdn. Bhd., Malaysia) in 0.5x TBE buffer for 45 min at 90 V compared with DNA marker (RBC Bioscience, New Taipei City, Taiwan) and observed under ultraviolet transilluminator (Gene genius, SYNGENE, Frederick, USA). The preparation of reagents and buffers used in this study were shown in Appendix B. PCR products were purified (RBC Bioscience, New Taipei City, Taiwan) and sent to a commercial facility for DNA sequencing (First Base Laboratories SDN, Seri Kembangan, Malaysia). The nucleotide sequence was compared with those available in the GenBank database using the BLAST algorithm available on the National Center for Biotechnology Information (NCBI) website: http://www.ncbi.nlm.nih.gov (89) (Appendix: Figure 15).

Antimicrobial susceptibility testing and minimum inhibitory concentration (MIC) of *E. coli*

Antimicrobial susceptibility testing was performed on all *E. coli* isolates (n = 250) according to the standard procedure of Clinical and Laboratory Standard Institute (CLSI, 2013) (90). *E. coli* DMST 4212 and extended-spectrum β-lactamase

(ESBL)-producing *E. coli* (91) were used as negative and positive controls, respectively.

1. Disk diffusion method

One pure *E. coli* colony was inoculated in TSB and incubated at 37°C with shaking at 150 rpm for 18 h. The turbidity of fresh culture was adjusted to 0.5 McFarland standard (1.5×10^8 CFU/ml) and spread on Mueller-Hinton agar (Oxoid LTD, Hampshire, England) by sterile cotton swab. The antimicrobial disks were placed on medium and incubated at 37°C for 18–24 h. The zone of complete inhibition was interpreted following the criteria of CLSL as a resistant (R), intermediate (I) and susceptible (S). Multidrug-resistant *E. coli* (MDR-EC) was defined as an isolate being resistant to at least 3 different classes of agents according to criteria of Magiorakos et al. (2012) (37). Twenty-four antimicrobial disks from 10 of antimicrobial classes (Oxoid LTD, Hampshire, England) were used. The β-lactams being main class of agents in this study comprised 5 groups.

Penicillin: ampicillin (10 μg)

Cephalosporins: cefazolin (30 μg), cefuroxime (30 μg), cefoxitin (30 μg), cefpodoxime (10 μg), ceftazidime (30 μg), cefotaxime (30 μg) and cefepime (30 μg)

Carbapemems: ertapenem (10 μg), imipenem (10 μg) and meropenem (10 μg)

Monobactam: aztreonam (30 μg)

β-Lactam/ β-lactamase inhibitor combinations: ampicillin/ sulbactam (10/10 μg) and amoxicillin/clavulanate (20/10 μg)

For non-β-lactam, the following antimcribial agents were used.

Aminoglycosides: amikacin (30 μg), gentamicin (10 μg) and streptomycin (30 μg)

Chloramphenicol: chloramphenicol (30 μg)

Folate inhibitor pathway: trimethroprim/sulfamethaxazole (1.25/23.75 μg)

Quinolone and fluoroquinolones: nalidixic acid, ciprofloxacin (5 μg) and levofloxacin (5 μg)

Tetracyclines: doxycycline (30 μg) and tetracycline (30 μg)

2. Extended-spectrum β-lactamase (ESBL) production

Production of ESBL (n = 250) was performed by combined disk method according to the standard procedure of CLSI, 2013 (90). The bacterial culture was prepared as previously described. The antimicrobial disks used in this method were cefazidime (30 μg), ceftazidime/clavulanate (30/10 μg), cefotaxime (30 μg) and cefotaxime/clavulanate (30/10 μg) (Becton, Dickinson and Company, Sparks, USA). The isolate producing ESBL was interpreted from a ≥ 5mm increase in a zone diameter of either ceftazidime/clavulanate or cefotaxime/clavulanate compared with a zone diameter of the antimicrobial agents without the combination of clavulanate.

3. Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent at which no growth could be detected using broth microdilution method. *E. coli* isolates expressing resistant to either ceftazidime (n = 153), imipenem (n = 2), meropenem (n = 6) or ciprofloxacin (n = 68) were determined for their MICs. The concentration range of ceftazidime and ciprofloxacin (M & H Manufacturing CO, LTD, Samutprokarn, Thailand) was 1–128 mg/L while those for imipenem and meropenem (JW Pharmaceutical Corporation, Chungcheongnam-do, Republic of Korea) was 0.5–64 mg/L. The final volume of 200 μl cation-adjusted Mueller-Hinton broth (Oxoid LTD, Hampshire, England) per well in a 96-well plate contained 5×10^5 CFU/ml of fresh *E. coli* and serial 2-fold dilution of antimicrobial agent. The plate was aerobically incubated at 37°C for 18–24 h following the criteria of CLSI, 2013 (90). The growth of bacteria was determined by measuring an OD at wavelength 600 nm by a microplate reader (Synergy™ HT, BioTek, USA).

Detection of β-lactamase-encoding genes by PCR

DNA template in PCR was prepared as described earlier. PCR reaction mixture were prepared as 1 μl of bacterial suspension as template, 0.5 μM of each primer, 200 μM dNTPs, 1x PCR buffer, 1.5 mM $MgCl_2$, and 1 U of *Taq* polymerase (Vivantis, Selangro Darul Ehsa, Malaysia) in a final volume of 25 μl. A sterile deionized water was used as a negative control in all experiments. Details of primer sequences, PCR amplifications, PCR product sizes, and reference methods were

shown in Table 3. PCR product was separated by 1.0% or 1.2% (w/v) agarose gel electrophoresis at 70□90 V for 30–60 min in 0.5x TBE buffer. The gel was observed under ultraviolet transilluminator. Selected amplicon was purified and sent to a commercial facility for sequencing. The nucleotide sequence was compared with those available in the GenBank database as described earlier. The following β-lactamase-encoding genes were investigated.

1. *bla*$_{SHV}$ and *bla*$_{TEM}$

The presence of *bla*$_{SHV}$ and *bla*$_{TEM}$ in all isolates (n = 250) were detected by duplex PCR. PCR reaction mixture was prepared as described earlier except the final concentration of each primer was 0.4 μM. *bla*$_{SHV-12}$ and *bla*$_{TEM-1}$-carrying *E. coli* from previous study (91) were used as positive controls.

2. *bla*$_{CTX-M}$

The detection of *bla*$_{CTX-M}$ (groups 1, 2, 8, 9, and 25) among ESBL-positive *E. coli* (n = 214) was carried out using multiplex PCR. PCR reaction mixture was prepared as described earlier. *bla*$_{CTX-M\ group\ 1}$ and *bla*$_{CTX-M\ group\ 9}$ (91) were used as positive controls.

3. *bla*$_{AmpC}$

E. coli isolates were ESBL-negative and/or with reduced susceptibility to cefoxitin (n = 36) was suggested as *bla*$_{AmpC}$-carrying strain. Multiplex PCR was used for detection of *bla*$_{AmpC}$. The final concentration of all reagents were prepared as described earlier except the final concentrations of six-pair primers in multiplex PCR reaction were used at 0.6 μM (MOXMF, MOXMR, CITMF, CITMR, DHAMF, and DHAMR), 0.5 μM (ACCMF, ACCMR, EBCMF, and EMBMR), and 0.4 μM (FOXMF, and FOXMR). *bla*$_{CMY-2}$ found in this study was ensured by DNA sequencing and was used as a positive control.

4. Carbapenemase-encoding genes

Five carbapenemase-encoding genes; *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, and *bla*$_{VIM}$, were detected among *E. coli* with reduced susceptibility to carbapenem (n = 6) using single PCRs which PCR reaction mixtures were prepared as described earlier. Four positive controls used in this study that were *bla*$_{IMP-1}$-carrying *Pseudomonas aeruginosa* (93) and *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ were kindly provided by Professor

Timothy Walsh, Department of Medical Microbiology and Infectious Disease, Division of Infection and Immunity, Cardiff University, United Kingdom.

Phylogenetic grouping

Phylogenetic groups (A, B1, B2 and D) were determined on all isolates (n = 250) using multiplex PCR in a final volume of 25 µl to amplify *chu*A, *yja*A and TSPE4.C2. PCR reaction mixture was prepared as described earlier. Primers, conditions of PCR amplification, and sizes of PCR product were shown in Table 3. *chu*A and *yja*A were heme transport-encoding gene and hypothetical protein, respectively, while TSPE4.C2 was a sequence within putative lipase esterase-encoding gene. Groups A and B1 were classified as commensal strains while groups B2 and D were virulent extra-intestinal strains.

Multilocus sequence typing (MLST)

E. coli belonging to phylogenetic group B2 (n = 14) was further analyzed with MLST to identify their sequence type (ST) based on PCR and DNA sequencing of 7 housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) according to *E. coli* MLST website (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search) (94). All genes were amplified in single PCR which was prepared as described earlier. Primers, conditions of PCR amplification, and sizes of PCR product were shown in Table 3. All PCR products were purified, sequenced and submitted to *E. coli* MLST website for ST identification.

Conjugation

For mating experiment, $bla_{\text{CTX-M group 1-, group 9-}}$ and $bla_{\text{CMY-2}}$-positive *E. coli* were randomly sampled as donors (n = 30) and a plasmid-free *E. coli* J53 strain (kindly provided from Prof. Timothy Walsh, Cardiff University) was used as recipient cell. Donor and recipient were individually cultured in TSB at 37°C with shaking at 200 rpm for 6 h. Then, the cultures were mixed at ratio 1: 3 (donor: recipient) and incubated at 37°C for 18 h without shaking. Ten-fold serial dilution was performed and cultured was spread on TSA supplemented with 150 mg/L sodium azide and 2 mg/L cefotaxime. The number of donor (CFU/ml) was concurrently performed. Plates

were incubated at 37°C for 24 h. Frequency of conjugative plasmid transfer was estimated as number of transconjugant divided by number of donor (CFU/ml). PCR in a final volume of 25 µl was used to confirm the presence of $bla_{\text{CTX-M group 1-, group 9-}}$ and $bla_{\text{CMY-2}}$ in transconjugant as described in earlier. Primers, conditions of PCR amplification, and sizes of PCR product were shown in Table 3. The transconjugant was further assessed for their MICs to ampicillin, cephalothin and cefotaxime using broth microdilution method (90).

Plasmid-based replicon typing (PBRT)

To trace plasmid in transconjugant (n = 27), PCR-based replicon typing (PBRT) was performed by 5 multiplex PCR (multiplex 1–5) and 3 simplex PCR using 18 pairs of primers. Plasmid incompatibility groups (Inc) were identified as FIA, FIC, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA. Five sets of multiplex PCR were multiplex 1 (Inc HI1, HI2, I1-Iγ), multiplex 2 (IncL/M, IncN, IncX), multiplex 3 (IncFIA, IncFIB, IncW), multiplex 4 (IncFIC, IncP, IncY) and multiplex 5 (IncA/C, IncT, repFIIA$_s$). Single PCR was used for amplication of IncB/O, IncF and IncK. PCR reaction mixtures were prepared as described earlier. Primers, conditions of PCR amplification, and sizes of PCR product were shown in Table 3.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed on ESBL-producing *E. coli* isolates. One hundred and thirty-two isolates were available for this study. The procedure used in this study was modified from Durmaz et al., 2009 (95). A single colony was inoculated in TSB and incubated at 37°C with shaking at 150 rpm for 18 h. Turbidity of a bacterial culture was adjusted to 1.3–1.4 at OD625 nm. One milliliter of culture was centrifuged at 13,000 rpm for 5 min to harvest bacterial cell. Pellet cell was washed twice using 1 ml phosphate buffer (pH 7.0) and 500 µl of cell suspension buffer I. After discard buffer I, pellet was resuspended in 1 ml of cell suspension buffer II, then 200 µl of cell suspension was transferred to new tube and incubated in water bath at 55°C for 3 min. Ten microliter of cell lysis solution I and 20 U of proteinase K (Amresco Inc., USA) were added in cell suspension. The solution was gently mixed with 200 µl of 2.0% (w/v) low-melting point agarose (Bio-Rad Laboratories, Hercules, USA) and added

into plug mold. Gel was set at room temperature for 20 min. Plug was removed and added to a new tube containing 500 µl of cell lysis solution II and 2,000 U of lysozyme (Bio Basic Canada Inc., Ontario, USA) and incubated 37°C for 1 h. The solution II was carefully removed, then plug was soaked with 500 µl of cell lysis solution I and incubated at 50°C for 24 h. Gel was soaked with 500 µl of 1x TE buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Thermo Fisher Scientific, USA) at room temperature for 4 h. The plug was washed twice with 10 ml of 1x TE buffer at 37°C for 20 min in shaking water bath. One third of plug was soaked in 1x restriction buffer at 37°C for 30 min and was further digested with 10 U *Xba*I (Fermentas, Hanover MD, USA) at 37°c for 16 h. After digestion, plug was put in 1% (w/v) PFGE gel (Bio-Rad Laboratories, Hercules, USA) with 0.5x TBE buffer, then gel was electrophoresed with parameter: gradient 6 v/cm, an included angle of 120° with initial switch time of 0.47 s, final switch time of 44.69 s at 14°C, 0.5x TBE buffer for 20.18 h using a CHEF Mapper system (Bio-Rad Laboratories, Hercules, USA). PFGE gel was observed under ultraviolet transilluminator.

DNA patterns of *E. coli* performed by PFGE were analyzed by Gelcompar II 6.6.11 software (Applied Maths, Belgium). The DICE metric and unweight-pair group average linkages (UPGMA) was used for assessment of the similarity coefficient which was compared from the calculation of band positions of sample pair. The optimization and tolerance of band matching were set at 1%. To interpret genetic relatedness, the parameter showing ≥ 85% similarity was used as a cut-off point. The software was kindly provided by Prof. Timothy Walsh, Cardiff University, UK.

S1 nuclease-pulsed-field gel electrophoresis (S1-PFGE), hybridization and plasmid sequencing

Genetic context of randomly selected bla_{CTX-M}-carrying *E. coli* isolates (n = 6) was performed by S1-PFGE, ^{32}P probe hybridization, plasmid sequencing and sequence analysis, respectively. The facilities and software were kindly provided from Prof. Timothy Walsh's laboratory and his staff in Department of Medical Microbiology and Infectious Disease, Division of Infection and Immunity, Cardiff University, UK.

1. S1-PFGE

One loopful of bacterial cell was harvested and suspended in 1 ml cell suspension buffer. A 400 µl of cell suspension was transferred to new 1.5 ml centrifuge tube and 20 µl of proteinase K (20 mg/ml) was added. A 400 µl of 1% (w/v) Seakem® Gold agarose (Lonza, ME, USA) was gently mixed in cell suspension solution and transferred into plug mold. Agarose plug was soaked in 5 ml of cell lysis buffer and 25 µl of proteinase K (20 mg/ml) at 50°C for 2 h with shaking. Plug was washed three times with 5 ml of preheated TE buffer at 50°C with shaking at 50°C for 15 min and 3 times with preheated sterile water. Plug was further washed in 1 ml of 1/10x TE buffer at room temperature for 20 min and soaked with 200 µl of 1x S1 nuclease buffer with previous condition. Enzyme solution was prepared using 3 ml of 1x S1 nuclease buffer containing 1.5 µl of S1 nuclease (100 U/µl) (Thermo Scientific, Lithuania), then 200 µl of solution per each plug was used for digestion of plasmid as previous description (96). Plug was incubated at 4°C for overnight. To perform S1-PFGE, 1/3 of plug was added to 0.9% (w/v) gel prepared with 0.5x TBE buffer and 10 µl of ethidium bromide (10 mg/ml), then gel was electrophoresed in 2 L of 0.5x TBE buffer supplemented with 20 ul of ethidium bromide with parameter at 6 v/cm^{-1}, 120° angle, initial switch time of 5 s, final switch time of 45 s, for 20 h on CHEF-DR III apparatus (Bio-rad, Hercules, CA, USA). Gel was visualized on UV transilluminator. The number of plasmid and sizes were considered by comparison with Lambda PFG ladder (New England BioLabs® Inc., UK).

2. Gel hybridization

S1-PFGE gel was placed between 2 sheets blotting paper, and dried in drying cabinet at 50°C for 24 h. The dried gel was placed in 200 ml of deionized water for 5 min and soaked in 200 ml of denaturing solution at room temperature for 45 min. Gel was transferred to 200 ml of neutralizing solution at room temperature for 45 min. Prior to probing, the gel was placed into a hybridization tube, then 20 ml of pre-hybridization solution was added into the tube and incubated in an incubation incubator at 65°C for 24 h. A *bla* probe labelled with radio-active ^{32}P on dCTP (Stratgene, Amsterdam, Netherlands) (kindly prepared by Prof. Timothy Walsh's staff) was added and incubated at 65°C for 24 h. The gel was washed by 100 ml washing solution I at 65°C for 1 h, and followed by 100 ml washing solution II at 65°C

for overnight. The gel was washed under warm tab water and wrapped in clingfilm. Then, the gel was placed against a film in a film cassette. The cassette was incubated at -80°C for overnight. *bla*-containing plasmid or chromosome on the film was illustrated by staining in staining solution, tap water and fix solution, respectively. The size of plasmid was determined by comparison with previous S1-PFGE gel.

3. Plasmid sequencing and sequence analysis

Selected 4 *E. coli* isolates were cultured in 2 ml TSB with shaking at 200 rpm, 37°C for 18 h. Bacterial cell was harvested by centrifugation at 10,000 rpm for 10 min. Pellet was further extracted to conduct whole genome using QIAcube automated system (Qiagen). Total genomic DNA (gDNA) was quantified using a Qubit (ThermoFisher Scienctific), then plasmid sequencing was performed by the Illumina MiSeq platform (MiSeq Reagent V3 kit; 2 x 300 cycles). Consequently, raw sequences were trimmed and de novo-assembled by Trim Galore and SPAdes (3.9.0), respectively. Genetic context of *bla* was analyzed by Geneious (10.0.9; Biomatters Ltd.). Characterization of *E. coli* sample, for examples, MLST, the presence of antimicrobial-resistant genes, virulence factor, was successful using program on Center for Genomic Epidemiology website: http://www.genomicepidemiology.org/ (97) and the sequences were compared with data on NCBI website: http://www.ncbi.nlm.nih.gov (89).

Table 3 Nucleotide sequences of primers, conditions of PCR amplification and PCR product sizes in this study

Objectives	Targets	Primers	Nucleotides (5'–3')[a]	PCR products (bp)	Conditions of PCR amplification (Temperature: Time)					References
					Initial denaturation	PCR cycles (30 cycles)			Final extension	
						Denaturation	Primer annealing	Primer extension		
16S rRNA gene	16S rRNA gene	27F 1492R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	1,487	94°C: 5 min	94°C: 0.45 min	50°C: 0.45 min	72°C: 2 min	72°C: 10 min	(88)
bla$_{ampC}$	*bla*$_{ACC}$	ACCMF ACCMR	AACAGCCTCAGCAGCCGGTTA TTCGCCGCAATCATCCCTAGC	346	94°C: 5 min	94°C: 0.30 min	64°C: 0.30 min	72°C: 1 min	72°C: 7 min	(98)
	bla$_{LAT-1\ to\ 4}$, *bla*$_{CMY-2\ to\ 7}$ and *bla*$_{BIL-1}$	CITMF CITMR	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462						
	bla$_{DHA-1\ to\ 2}$	DHAMF DHAMR	AACTTTCACAGGTGTGCTGGGT CCGTACGCATACTGGCTTTGC	405						
	bla$_{MIR-1T}$ and *bla*$_{ACT-1}$	EBCMF EBCMR	TCGGTAAAGCCGATGTTGCGG CTTCCACTGCGGCTGCCAGTT	302						
	bla$_{FOX-1\ to\ 5b}$	FOXMF FOXMR	AACATGGGGTATCAGGGAGATG CAAAGCGCGTAACCGGATTGG	190						
	bla$_{MOX-1\ to\ 2}$, *bla*$_{CMY-1}$ and *bla*$_{CMY-8\ to\ 11}$	MOXMF MOXMR	GCTGCTCAAGGAGCACAGGAT CACATTGACATAGGTGTGGTGC	520						
Full-length *bla*$_{CMY-2}$	*bla*$_{CMY-2}$	CMY-2-F CMY-2-R	ATGATGAAAAAATCGTTATGCT ATTCTTGAAAAGCTGCAAT	1,144	94°C: 5 min	94°C: 1 min	48°C: 0.40 min	72°C: 1.15 min	72°C: 5 min	(99)
bla$_{carbapenemase}$	*bla*$_{IMP}$	IMP-F IMP-R	CTACCGCAGCAGAGTCTTTG AACCAGTTTTTGCCTTACCAT	587	94°C: 10 min	94°C: 0.45 min	52°C: 0.45 min	72°C: 1 min	72°C: 7 min	(100)
	bla$_{KPC}$	KPC-F KPC-R	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	798	94°C: 10 min	94°C: 0.45 min	48°C: 0.45 min	72°C: 1 min	72°C: 7 min	(101)
	bla$_{OXA-48}$	OXA-48F OXA-48R	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438						

[a] M = A or C; H = A or C or T; Y = C or T

Table 3 (cont.)

Objectives	Targets	Primers	Nucleotides (5'–3')[a]	PCR products (bp)	Conditions of PCR amplification (Temperature: Time)					References
					Initial denaturation	PCR cycles (30 cycles)			Final extension	
						Denaturation	Primer annealing	Primer extension		
*bla*carbapenemase	*bla*NDM	NDM-F NDM-R	ATGGAATTGCCCAATATTATG TCAGCGCAGCTTGTCGGCC	813	94°C: 10 min	94°C: 0.45 min	52°C: 0.45 min	72°C: 1 min	72°C: 7 min	(102)
	*bla*VIM	VIM-F VIM-R	CCGACAGTCARCGAAATTCCG CTACTCRRCGACTGAGCGATT	700	94°C: 5 min	94°C: 1 min	55°C: 1 min	72°C: 1 min	72°C: 7 min	Kindly provided from Prof. Timothy Walsh, Cardiff University, UK
*bla*CTX-M-group	*bla*CTX-M-group1	CTX-1F CTX-1R	AAAAATCACTGCGCCAGTTC AGCTTATTCATCGCCACGTT	415	94°C: 5 min	94°C: 0.25 min	52°C: 0.40 min	72°C: 0.50 min	72°C: 6 min	(103)
	*bla*CTX-M-group2	CTX-2F CTX-2R	CGACGCTACCCTGCTATT CCAGCGTCAGATTTTTCAGG	552						
	*bla*CTX-M-group9	CTX-9F CTX-9R	CAAAGAGAGTGCAACGGATG ATTGGAAAGCGTTCATCACC	205						
	*bla*CTX-M-group8 and group26	CTX-8F CTX-25F CTX8/25R	TCGCGTTAAGCGGATGATGC GCACGATGACATTCGGG AACCCACGATGTGGGTAGC	666 and 327						
Full-length *bla*CTX group 1	*bla*CTX-M-group 1	CTX-M-15S-F CTX-M-15S-F	ATGGTTAAAAAATCACTGCG TTACAAACCGTCGGTGAC	876	94°C: 5 min	94°C: 1 min	48°C: 0.40 min	72°C: 1 min	72°C: 5 min	(99)
Full-length *bla*CTX group 9	*bla*CTX-M-group 9	M9 upper M9 lower	ATGGTGACAAAGAGAGTGCA CCCTTCGGCGATGATTCTC	850	94°C: 5 min	94°C: 1 min	48°C: 0.40 min	72°C: 1 min	72°C: 5 min	(104)
*bla*SHV and *bla*TEM	*bla*SHV	MultiTSO-S_for MultiTSO-S_rev	AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC	713	94°C: 10 min	94°C: 0.40 min	60°C: 0.40 min	72°C: 1 min	72°C: 7 min	(105)
	*bla*TEM	MultiTSO-T_for MultiTSO-T_rev	CATTTCCGTGTCGCCCTTATTC CGTTCATCCATAGTTGCCTGA C	800						
Full-length *bla*SHV	*bla*SHV	SHV-F SHV-R	GGGTTATTCTTATTTGTCGC TTAGCGTTGCCAGTGCTC	930	94°C: 5 min	94°C: 1 min	56°C: 1 min	72°C: 1 min	72°C: 10 min	(106)
Full-length *bla*TEM	*bla*TEM	*bla*TEM-F *bla*TEM-R	ATGAGTATTCAACATTTCCG ACCAATGCTTAATCAGTGAG	859	94°C: 5 min	94°C: 1 min	48°C: 0.40 min	72°C: 1 min	72°C: 5 min	(107)

Table 3 (cont.)

Objectives	Targets	Primers	Nucleotides (5'–3')[a]	PCR products (bp)	Condition of PCR amplifications (Temperature: Time)					References
					Initial denaturation	PCR cycles (30 cycles)			Final extension	
						Denaturation	Primer annealing	Primer extension		
Phylogenetic groups	*chuA*	ChuA.1 ChuA.2	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279	94°C: 5 min	94°C: 0.30 min	55°C: 0.30 min	72°C: 0.30 min	72°C: 7 min	(30)
	yjaA	YjaA.1 YjaA.2	TGAAGTGTCAGGAGACGCT ATGGAGAATGCGTTCCTCAAC	211						
	TSPE4.C2	TspE4C2.1 TspE4C2.2	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	152						
Multi Locus Sequence Type (MLST)	*adk*	adk-F adk-R	ATTCTGCTTGGCGCTCCGGG CCGTCAACTTTCGCGTATTT	583	94°C: 5 min	94°C: 1 min	54°C: 1 min	72°C: 2 min	72°C: 5 min	(108)
	fumC	fumC-F fumC-R	TCACAGGTCGCCAGCGCTTC GTACGCAGCGAAAAAGATTC	806	94°C: 5 min	94°C: 1 min	54°C: 1 min	72°C: 2 min	72°C: 5 min	
	gyrB	gyrB-F gyrB-R	TCGGCGACACGGATGACGGC ATCAGGCCTTCACGCGCATC	911	94°C: 5 min	94°C: 1 min	60°C: 1 min	72°C: 2 min	72°C: 5 min	
	icd	icd-F icd-R	ATGGAAAGTAAAGTAGTTGTT CCGGCACA GGACGCAGCAGGATCTGTT	878	94°C: 5 min	94°C: 1 min	54°C: 1 min	72°C: 2 min	72°C: 5 min	
	mdh	mdh-F mdh-R	ATGAAAGTCGCAGTCCTCGGC GCTGCTGGCGG TTAACGAACTCCTGCCCCAGA GCGATATCTTTCTT	932	94°C: 5 min	94°C: 1 min	60°C: 1 min	72°C: 2 min	72°C: 5 min	
	purA	purA-F purA-R	CGCGCTGATGAAAGAGATGA CATACGGTAAGCCACGCAGA	816	94°C: 5 min	94°C: 1 min	54°C: 1 min	72°C: 2 min	72°C: 5 min	
	recA	recA-F recA-R	CGCATTCGCTTTACCCTGACC TCGTCGAAATCTACGGACCGG A	708	94°C: 5 min	94°C: 1 min	58°C: 1 min	72°C: 2 min	72°C: 5 min	
PCR-based replicon typing	parA-parB	HI1 FW HI1 RV	GGAGCGATGGATTACTTCAGT AC TGCCGTTTCACCTCGTGAGTA	471	94°C: 5 min	94°C: 1 min	60°C: 0.30 min	72°C: 1 min	72°C: 5 min	(109)
	iterons	HI2 FW HI2 RV	TTTCTCCTGAGTCACCTGTTAA CAC GGCTCACTACCGTTGTCATCC T	644						

Table 3 (cont.)

Objectives	Targets	Primers	Nucleotides (5'–3')[a]	PCR products (bp)	Conditions of PCR amplification (Temperature: Time)					References
					Initial denaturation	PCR cycles (30 cycles)			Final extension	
						Denaturation	Primer annealing	Primer extension		
PCR-based replicon typing	RNAI	II FW II RV	CGAAAGCCGGACGGCAGAA TCGTCGTTCCGCCAAGTTCGT	139	94°C: 5 min	94°C: 1 min	60°C: 0.30 min	72°C: 1 min	72°C: 5 min	(109)
	ori γ	X FW X RV	AACCTTAGAGGCTATTTAAGT TGCTGAT TGAGAGTCAATTTTTATCTCAT GTTTTAGC	376						
	repA,B,C	L/M FW L/M RV	GGATGAAAACTATCAGCATCT GAAG CTGCAGGGGCGATTCTTTAGG	785						
	repA	N FW N RV	GTCTAACGAGCTTACCGAAG GTTTCAACTCTGCCAAGTTC	559						
	iterons	FIA FW FIA RV	CCATGCTGGTTCTAGAGAAGG TG GTATATCCTTACTGGCTTCCGC AG	462						
	repA	FIB FW FIB RV	GGAGTCCTGACACACGATTTT CTG CTCCCGTCGCTTCAGGGCATT	702						
	repA	W FW W RV	CCTAAGAACAACAAAGCCCCC G GGTGCGCGGCATAGAACCGT	242						
	repA	Y FW Y RV	AATTCAAACAACACTGTGCAG CCTG GCGAGAATGGACGATTACAA AACTTT	765						
	iterons	P FW P RV	CTATGGCCCTGCAAACGCGCC AGAAA TCACGCGCCAGGGCGCAGCC	534						
	repA2	FIC FW FIC RV	GTGAACTGGCAGATGAGGAA GG TTCTCCTCGTCGCCAAACTAG AT	262						
	repA	A/C FW A/C RV	GAGAACCAAAGACGGGGACT GGA ACGACAAACCTGAATTGCCTC CTT	465						

Table 3 (cont.)

Objectives	Targets	Primers	Nucleotides (5'–3')[a]	PCR products (bp)	Conditions of PCR amplification (Temperature: Time)					References
					Initial denaturation	PCR cycles (30 cycles)			Final extension	
						Denaturation	Primer annealing	Primer extension		
PCR-based replicon typing	repA	T FW T RV	TTGGCCTGTTGTGCCTAAACCAT CGTTGATTACACTTAGCTTTGGAC	750	94°C: 5 min	94°C: 1 min	60°C: 0.30 min	72°C: 1 min	72°C: 5 min	(109)
	repA	FII_S FW FII_S RV	CTGTCGTAAGCTGATGGC CTCTGCCACAAACTTCAGC	270						
	RNAI/repA	F_{repB} FW F_{repB} RV	TGATCGTTTAAGGAATTTTG GAAGATCAGTCACACCATCC	270	94°C: 5 min	94°C: 1 min	52°C: 0.30 min	72°C: 1 min	72°C: 5 min	
	RNAI	K/B FW K RV	GCGGTCCGGAAAGCCAGAAAAC TCTTTCACGAGCCCGCCAAA	160						
	RNAI	B/O RV	TCTGCGTTCCGCCAAGTTCGA	159						

CHAPTER IV

RESULTS

Prevalence of *Escherichia coli* with reduced susceptibility to cefotaxime in backyard farms and meat samples

A total of 734 samples comprising 587 samples from backyard farms and 147 meat samples from open-air markets was subjected for the isolation of *E. coli* with reduced susceptibility to cefotaxime (EC-CTX) (Appendix A: Figure 13). A total of 250 presumptive *E. coli* colonies (34.1%, 95% CI = 30.6–37.6%) was recovered (Table 4). One hundred and seventy-two (29.3%, 95% CI = 25.6–33.2%) EC-CTX isolates were obtained from backyard farms; 19 farmers, 27 environmental samples (12 soil and 15 water) and 126 poultry feces (15 duck and 111 chicken feces). The highest prevalence (19/32, 59.4%) of EC-CTX was found in farmer feces. The prevalence of EC-CTX from duck feces (36.6%) was higher than that in chicken feces (26.1%), however, this is not statistically different ($p > 0.05$). EC-CTX was also found in environmental samples (30.7%). The prevalence in water samples (44.1%) were significantly higher than that in soil (22.2%) ($p < 0.05$). Among poultry meat samples, 78 samples (53.1%, 95% CI = 44.7–61.3%) from chicken and birds yielded EC-CTX isolates. No EC-CTX was detected in duck meat (Table 4).

Table 4 Prevalence of EC-CTX and ESBL-EC isolated from backyard farms and meat samples

Sources of samples	No. of samples	No. of EC-CTX (%)	No. of ESBL-EC (%)
Poultry farms			
- Farmers	32	19 (59.4)	16 (50.0)
- Poultry	467	126 (27.0)	121 (25.9)
- Chickens	426	111 (26.1)	106 (24.9)
- Ducks	41	15 (36.6)	15 (36.6)
- Environment	88	27 (30.7)	22 (25.0)
- Water	34	15 (44.1)*	13 (38.2)
- Soil	54	12 (22.2)*	9 (16.7)
Subtotal	587	172 (29.3)	159 (27.1)
Poultry meat samples			
- Chickens	126	69 (54.8)	46 (36.5)
- Ducks	3	0	0
- Birds	18	9 (50.0)	9 (50.0)
Subtotal	147	78 (53.1)	55 (37.4)
Total	**734**	**250 (34.1)**	**214 (29.2)**

* Significant difference ($p < 0.05$)

Antimicrobial susceptibility testing and extended-spectrum β-lactamase (ESBL) production

Antimicrobial-resistant phenotypes of 250 EC-CTX were determined (Table 5 and Figure 7; Appendix A: Figure 16). All isolates were resistant to ampicillin, cefazolin, cefpodoxime and cefotaxime. In addition, > 60% of isolates were resistant to ceftazidime (61.2%), streptomycin (74.0%), tetracycline (77.2%), aztreonam (78.0%) and cefuroxime (98.0%). A half of EC-CTX isolates was resistant to chloramphenicol (51.6%), cefepime (52.4%), doxycycline (52.4%) and trimethroprime/sulfamethoxazole (56.0%). A small number of isolates obtained from

meat samples were resistant to imipenem (0.8%) and meropenem (1.6%). In contrast, all isolates from backyard farms were susceptible to carbapenems.

Antimicrobial resistant rates between *E. coli* obtained from 4 sources were comparatively analyzed (Table 5). EC-CTX from all sources showed similar rates of resistance. Interestingly, EC-CTX isolates recovered from meat samples apparently showed higher resistant rates to cefoxitin and amoxicillin/clavulanate than those from other sources.

EC-CTX isolates that showed resistance to imipenem, meropenem, ciprofloxaxin and ceftazidime were further determined for their MICs (Table 5; Appendix A: Figure 17). MIC range of ceftazidime was 4–>128 mg/L and MIC_{50} and MIC_{90} were 64 and 128 mg/L, respectively. MIC range of ciprofloxacin was 2–>128 and MIC_{50} and MIC_{90} were 16 and 128 mg/L, respectively. No imipenem- and meropenem-resistant EC-CTX was found. MIC range of both carbepenems was 0.5–1 mg/L.

In this study, 24 antimicrobial disks from 12 classes were used. A 30% of 250 EC-CTX were resistant to > 13 agents. Furthermore, 6 isolates (2.4%) from poultry feces (n = 3) and meat samples (n = 3) were resistant to ≥ 18 agents (Appendix A: Table 18). Overall, antimicrobial susceptibility testing performed by disk diffusion method revealed that all EC-CTX isolates were multidrug-resistant *E. coli* (MDR-EC).

The confirmatory test for ESBL production proved that 214 EC-CTX isolates (85.6%) were ESBL-producing *E. coli* (ESBL-EC) (Tables 5 and 6; Appendix A: Figure 18). Of these, the highest carriage rate of ESBL-EC was found in poultry (96.0%), followed by farmers (84.2%), environment (81.5%) and meat samples (70.5%). Thirty-six MDR-EC isolates recovered from 3 farmers, 5 poultry feces, 5 environment and 23 meat samples showed negative results on ESBL production. However, these isolates were resistant to cefoxitin, suggesting that they could be an AmpC-producing *E. coli* (AmpC-EC) (data not shown).

Table 5 Rates of antimicrobial resistance of EC-CTX isolated from backyard farms and meat samples

Antimicrobial agents / phenotypes	No. of EC-CTX (%)					
	Backyard poultry farm				Poultry meat samples (n = 78)	Total (n = 250)
	Farmers (n = 19)	Poultry (n = 126)	Environment (n = 27)	Subtotal (n = 172)		
Multridrug resistance	19 (100)	126 (100)	27 (100)	172 (100)	78 (100)	**250 (100)**
ESBL producer	16 (84.2)	121 (96.0)	22 (81.5)	159 (92.4)	55 (70.5)	**214 (85.6)**
ampicillin	19 (100)	126 (100)	27 (100)	172 (100)	78 (100)	**250 (100)**
cefazolin	19 (100)	126 (100)	27 (100)	172 (100)	78 (100)	**250 (100)**
cefuroxime	19 (100)	125 (99.2)	27 (100)	171 (99.4)	74 (94.9)	**245 (98.0)**
cefoxitin	3 (15.8)	7 (5.6)	5 (18.5)	15 (8.7)	28 (35.9)	**43 (17.2)**
cefpodoxime	19 (100)	126 (100)	27 (100)	171 (99.4)	78 (100)	**250 (100)**
cefotaxime	19 (100)	126 (100)	27 (100)	172 (100)	78 (100)	**250 (100)**
ceftazidime	10 (52.6)	65 (51.6)	19 (70.4)	94 (54.7)	59 (75.6)	**153 (61.2)**
cefepime	11 (57.9)	67 (53.2)	12 (44.4)	90 (52.3)	41 (52.6)	**131 (52.4)**
impenem	0	0	0	0	2 (2.6)	**2 (0.8)**
meropenem	0	0	0	0	4 (5.1)	**4 (1.6)**
ertapenem	0	0	0	0	0	**0**
aztreonam	12 (63.2)	95 (75.4)	22 (81.5)	129 (75.0)	66 (84.6)	**195 (78.0)**
ampicillin/sulbactam	12 (63.2)	57 (45.2)	12 (44.4)	81 (47.1)	45 (57.7)	**126 (50.4)**
amoxicillin/cluvulanate	4 (21.1)	8 (6.3)	5 (18.5)	17 (9.9)	32 (41.0)	**49 (19.6)**
amikacin	1 (5.3)	8 (6.3)	1 (3.7)	10 (5.8)	7 (9.0)	**17 (6.8)**
gentamicin	6 (31.6)	53 (42.1)	9 (33.3)	68 (39.5)	20 (25.6)	**88 (35.2)**
streptomycin	15 (78.9)	94 (74.6)	19 (70.4)	128 (74.4)	57 (73.1)	**185 (74.0)**
doxycycline	9 (47.4)	75 (59.5)	15 (55.6)	99 (57.6)	32 (41.0)	**131 (52.4)**
tetracycline	15 (78.9)	105 (83.3)	22 (81.5)	142 (82.6)	51 (65.4)	**193 (77.2)**
nalidixic acid	9 (47.40)	38 (30.2)	9 (33.3)	56 (32.6)	39 (50.0)	**95 (38.0)**
ciprofloxacin	5 (26.3)	35 (27.8)	9 (33.3)	49 (28.5)	19 (24.4)	**68 (27.2)**
levofloxacin	4 (21.1)	31 (24.6)	8 (29.6)	43 (25.0)	17 (21.8)	**60 (24.0)**
chloramphenicol	11 (57.9)	74 (58.7)	13 (48.1)	98 (57.0)	31 (39.7)	**129 (51.6)**
trimethroprim/sulfamethaxazole	14 (73.7)	79 (62.7)	11 (40.7)	104 (60.5)	36 (46.2)	**140 (56.0)**

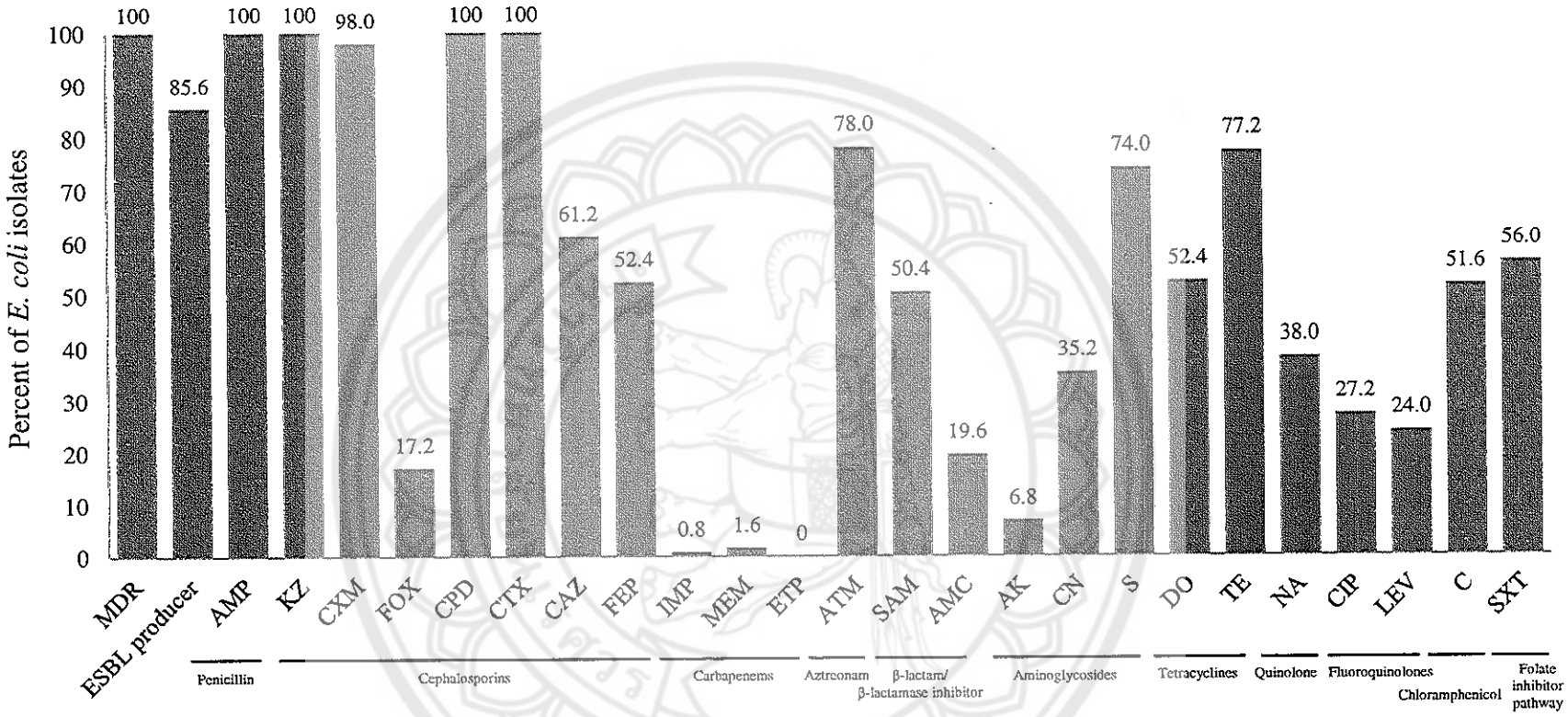


Figure 7 Percentages of antimicrobial resistance among EC-CTX isolates recovered from backyard farms and meat samples

Abbreviations: AMP; ampicillin, KZ, cefazolin; CXM, cefuroxime; FOX, cefoxitine; CPD, cefpodoxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MEM, meropenem; ETP, ertapenem; ATM, aztreonam; SAM, ampicillin/sulbactam; AMC, amoxicillin/clavulanate; AK, amikacin; CN, gentamicin; S, streptomycin; DO, doxycycline; TE, tetracycline; NA, nalidixic acid; CIP, ciprofloxacin; LEV, levofloxacin; C, chloramphenicol and SXT, trimethoprim/sulfamethoxazole

Table 6 Distribution of ESBL-positive and negative *E. coli*

Samples (n)	No. of *E. coli* isolates (%)	
	ESBL-positive isolates	ESBL-negative isolates
Backyard farms		
Farmers (19)	16 (84.2)	3 (15.8)
Poultry		
- Chickens (111)	106 (95.5)	5 (4.5)
- Ducks (15)	15 (100)	0
Subtotal (126)	121 (96.0)	5 (4.0)
Environment		
- Water (15)	13 (86.7)	2 (13.3)
- Soil (12)	9 (75.0)	3 (25.0)
Subtotal (27)	22 (81.5)	5 (18.5)
Subtotal (172)	159 (92.4)	13 (7.6)
Meat samples		
- Chicken (69)	46 (66.7)	23 (33.3)
- Birds (9)	9 (100)	0
Subtotal (78)	55 (70.5)	23 (29.5)
Total (250)	**214 (85.6)**	**36 (14.4)**

Prevalence of ESBL and AmpC-encoding gene among MDR-EC

Two-hundred and nineteen MDR-EC isolates (87.6%) carried either ESBL- or AmpC-encoding genes (Table 7; Appendix A: Figure 19). The highest prevalence of $bla_{ESBL/AmpC}$-positive isolates was found in poultry (94.4%) followed by environment (88.9%), farmers (78.9%) and meat samples (78.2%). Of these, 198 and 21 isolates were positive for bla_{ESBL} and bla_{AmpC}, respectively. $bla_{CTX\text{-}M\ group\ 1}$ was predominantly found among ESBL-positive *E. coli* (64.0%), followed by $bla_{CTX\text{-}M\ group\ 9}$ (27.6%). $bla_{SHV\text{-}2a}$ and $bla_{TEM\text{-}116}$ were found at very low frequencies (1 isolate each) in isolates recovered from meat samples. For the 36 presumptive *E. coli* isolates

(expressing negative results of ESBL production), 21 isolates (58.3%) were positive for bla_{CMY-2} (Table 7). Among $bla_{CTX-M\ group\ 1}$-positive isolates, 44 and 1 isolates, respectively, were positive for bla_{TEM-1} and bla_{TEM-1}+bla_{SHV-28}, the non ESBL genes. In addition, 24 and 2 of $bla_{CTX-M\ group\ 9}$ and bla_{CMY-2}-positive isolates, respectively, were positive for bla_{TEM-1}. Moreover, bla_{SHV-26} which is also considered as non ESBL-gene was found in 1 isolate (Appendix A: Table 20).

Table 7 Distribution of ESBL and AmpC-encoding genes (*bla*) among MDR-EC

Samples / ESBL production (n)	No. of isolates possessing ESBL and AmpC-encoding genes (%)					
	bla_{CTX-M} group 1	bla_{CTX-M} group 9	bla_{CMY-2}	bla_{SHV-2a}	$bla_{TEM-116}$	**Total**
Farmers (19)	8 (42.1)	7 (36.8)	0	0	0	15 (78.9)
Poultry (126)	78 (61.9)	39 (30.9)	2 (1.6)	0	0	119 (94.4)
Environment (27)	14 (51.9)	6 (22.2)	4 (14.8)	0	0	24 (88.9)
Meat samples (78)	37 (47.4)	7 (9.0)	15 (19.2)	1 (1.3)	1 (1.3)	61 (78.2)
Total (250)	**137[a] (54.8)**	**59[b] (23.6)**	**21[c] (8.4)**	**1 (0.4)**	**1 (0.4)**	**219 (87.6)**
ESBL-positive *E. coli* (214)	137 (64.0)	59 (27.6)	0	1 (0.5)	1 (0.5)	198 (92.5)
ESBL-negative *E. coli* (36)	0	0	21 (58.3)	0	0	21 (58.3)

[a] Of these, 44 and 1 isolates co-harbored bla_{TEM-1} and bla_{TEM-1}+ bla_{SHV-28}, respectively

[b] Of these, 24 isolates co-harbored bla_{TEM-1}.

[c] Of these, 2 isolates co-harbored bla_{TEM-1}.

Phylogenetic grouping of MDR-EC from backyard farms and meat samples

Phylogenetic grouping of 250 MDR-EC revealed that group A was the most common group (58.0%), followed by groups B1 (23.6%), D (12.8%) and B2 (5.6%) (Table 8; Appendix A: Figure 20). Prevalence of commensal strains (group A and B1) was obviously higher than that of pathogenic strains (group B2 and D), however, pathogen strains were found in all sources of samples. Group A was also the most common phylogenetic group of ESBL-EC (63.1%). Conversely, group D was the most common group of AmpC-EC (36.1%). In addition, group B2 was found in both ESBL-EC and AmpC-EC (4.2 and 13.9%, respectively) (Table 8).

Table 8 Distribution of phylogenetic groups among MDR-EC

Samples / ESBL production (n)	No. of *E. coli* isolates (%)			
	A	B1	B2	D
Backyard farms				
Farmers (19)	12 (63.2)	2 (10.5)	3 (15.8)	2 (10.5)
Poultry (126)	73 (57.9)	34 (27.0)	4 (3.2)	15 (11.9)
Environment (27)	18 (66.7)	5 (18.5)	2 (7.4)	2 (7.4)
Subtotal (172)	103 (59.9)	41 (23.8)	9 (5.2)	19 (11.1)
Meat samples (78)	42 (53.8)	18 (23.1)	5 (6.4)	13 (16.7)
Total (250)	**145 (58.0)**	**59 (23.6)**	**14 (5.6)**	**32 (12.8)**
ESBL-positive *E. coli* (214)	135 (63.1)	51 (23.8)	9 (4.2)	19 (8.9)
ESBL-negative *E. coli* (36)	10 (27.8)	8 (22.2)	5 (13.9)	13 (36.1)

The distribution of 219 bla_{ESBL} and bla_{CMY-2} versus phylogenetic groups was determined (Table 9). Most ESBL-EC possessing $bla_{CTX-M\ group\ 1}$ (68.6%) and $bla_{CTX-M\ group\ 9}$ (45.8%) were typed into group A. In contrast, most isolates possessing bla_{CMY-2} (52.4%) were classified into group D. Fourteen isolates belonging to B2 were found to carry $bla_{TEM-116}$ (n = 1), $bla_{CTX-M\ group\ 1}$ (n = 2), bla_{CMY-2} (n = 3) and $bla_{CTX-M\ group\ 9}$ (n = 6). No $bla_{ESBL/AmpC}$ gene was found in 2 isolates in group B2. Besides, bla_{SHV-2a}-positive isolate was typed into group A. Interestingly, among 46 isolates belonging to

groups B2 (n = 14) and D (n = 32) (Table 8), 13 isolates (group B2; n = 2 and D; n = 11) were isolated from chicken meat obtained from Nakhon Thai (data not shown). Of these isolates, 12 isolates carried bla_{CMY-2} while no gene was detected in 1 isolate.

Table 9 Distribution of ESBL and AmpC-encoding genes among phylogenetic groups

ESBL- and AmpC-encoding genes (n)	No. of *E. coli* isolates (%)			
	A	B1	B2	D
$bla_{CTX-M-group\ 1}$ (137)	94 (68.6)	27 (19.7)	2 (1.5)	14 (10.2)
$bla_{CTX-M-group\ 9}$ (59)	27 (45.8)	22 (37.3)	6 (10.1)	4 (6.8)
bla_{CMY-2} (21)	4 (19.0)	3 (14.3)	3 (14.3)	11 (52.4)
bla_{SHV-2a} (1)	1 (100)	0	0	0
$bla_{TEM-116}$ (1)	0	0	1 (100)	0
Total (219)	**126 (57.5)**	**52 (23.8)**	**12 (5.5)**	**29 (13.2)**

Antimicrobial-resistant rates of *E. coli* in each phylogenetic group were comparatively analyzed. In general, resistance rates of most antimicrobial agents among 4 groups were similar. However, pathogenic group B2 and D demonstrated higher resistance rates to cefoxitin and amoxicillin/clavulanate compared with commensal strains (A and B1). Furthermore, group B2 showed higher resistance rates to ampicillin/sulbactam, ciprofloxacin and levofloxacin compared with the other groups (Table 10).

Table 10 Antimicrobial susceptibility testing of 4 phylogenetic groups

ESBL production / Antimicrobial agents	No. of *E. coli* isolates* (%)			
	A (145)	B1 (59)	B2 (14)	D (32)
ESBL-positive	135 (93.1)	51 (86.4)	9 (64.3)	19 (59.4)
cefuroxime	141 (97.2)	59 (100)	14 (100)	31 (96.9)
cefoxitin	14 (9.7)	10 (16.9)	6 (42.9)	13 (40.6)
ceftazidime	82 (56.6)	37 (62.7)	7 (50.0)	27 (84.4)
cefepime	67 (46.2)	41 (69.5)	5 (35.7)	18 (56.3)
imipenem	2 (1.4)	0	0	0
meropenem	2 (1.4)	2 (3.4)	0	0
aztreonam	110 (75.9)	46 (78.0)	11 (78.6)	17 (53.1)
ampicillin/sulbactam	61 (42.1)	36 (61.0)	10 (71.4)	19 (59.4)
amoxicillin/cluvulanate	19 (13.1)	10 (16.9)	6 (42.9)	14 (43.8)
amikacin	12 (8.3)	3 (5.1)	0	2 (6.3)
gentamicin	48 (33.1)	23 (39.0)	3 (21.4)	14 (43.8)
streptomycin	113 (77.9)	41 (69.5)	8 (57.1)	23 (71.9)
doxycycline	80 (55.2)	36 (61.0)	2 (14.3)	13 (40.6)
tetracycline	124 (85.5)	44 (74.6)	7 (50.0)	18 (56.3)
nalidixic acid	46 (31.7)	20 (33.9)	10 (71.4)	19 (59.4)
ciprofloxacin	43 (29.7)	12 (20.3)	6 (42.9)	7 (21.9)
levofloxacin	34 (23.4)	12 (20.3)	6 (42.9)	8 (25.0)
chloramphenicol	90 (62.1)	29 (49.2)	3 (21.4)	7 (21.9)
trimethoprim/ sulfamethoxazole	89 (61.4)	31 (52.5)	6 (42.9)	14 (43.8)

* All isolates were resistant to ampicillin, cephazolin, cefotaxime and cefpodoxime and susceptible to ertapenem.

Characterization of *E. coli* belonging to group B2 from backyard farm and meat samples

To characterize B2 strains (n = 14; 2 soil, 3 farmer feces, 4 chicken feces and 5 meat samples), identification of *bla* alleles (Appendix A: Figures 27–31) and multilocus sequence typing (MLST) analysis were performed (Table 11). Eleven isolates were assigned to ST131 (n = 7), ST95 (n = 1), ST127 (n = 1), ST219 (n = 1) and ST8180 (n = 1) whereas 3 isolates were untypeable. Seven ST131 isolates were distributed in samples from all sources in 3 districts. Of these, 4 isolates were $bla_{\text{CTX-M-27}}$-positive ESBL-EC (1 farmer feces, 1 soil and 2 chicken feces) and showed similar antimicrobial-resistant patterns (AMP-CXM-KZ-CTX-CPD-ATM-CIP-LEV-NA-S-TE-SXT). The other 3 ST131 isolates were $bla_{\text{TEM-116}}$-positive ESBL-EC recovered from chicken meat (n = 1) and ESBL-negative *E. coli* (n = 2) which only 1 isolate carried $bla_{\text{CMY-2}}$ and other had no *bla* gene. Two ESBL-negative *E. coli* ST131, isolates recovered from chicken meat in Muang and Nakhon Thai districts and showed a similar basic antimicrobial-resistant profile (AMP-CXM-KZ-CTX-CPD-AK-AMC-ATM-CAZ-FOX-NA-S-SAM).

ST95, ST127 and ST219 isolates were recovered from soil, chicken feces and farmer feces, respectively from different farms. Of these, $bla_{\text{CTX-M-14}}$ was detected in ST95 and ST219 while $bla_{\text{CTX-M-15}}$ was found in ST127. Furthermore, $bla_{\text{CTX-M-15}}$ was found in ST8180. Among the 3 AmpC-EC isolates that were untypeable for their STs, 2 isolates carried $bla_{\text{CMY-2}}$.

Table 11 Distribution of *E. coli* phylogenetic group B2 in backyard farms and meat samples

Isolates	Sources	Location	STs	ESBL production	$bla_{ESBL/AmpC}$	Other antimicrobial resistance[b]
EC169	Soil	Farm 1 (Muang)	95	Positive	$bla_{CTX-M-14}$	ATM, CN, FEP, SAM
EC179	Farmer feces	Farm 4 (Muang)	219	Positive	$bla_{CTX-M-14}$	C, S, SAM, SXT, TE
EC223[a]	Chicken feces	Farm 15 (Bang Rakam)	131	Positive	$bla_{CTX-M-27}$	ATM, CAZ, CIP, LEV, NA, S, SXT, TE
EC224	Farmer feces	Farm 15 (Bang Rakam)	8180	Positive	$bla_{CTX-M-15}$	ATM, CAZ, CIP, CN, FEP, LEV, NA, S, SAM, SXT, TE
EC227[a]	Farmer feces	Farm 16 (Bang Rakam)	131	Positive	$bla_{CTX-M-27}$	ATM, CIP, LEV, NA, S, SXT, TE
EC234	Soil	Farm 24 (Wat Bot)	131	Positive	$bla_{CTX-M-27}$	ATM, CIP, LEV, NA, S, SXT, TE
EC241	Chicken feces	Farm 27 (Wat Bot)	untypeable	Negative	bla_{CMY-2}	CAZ, FOX, SAM
EC244	Chicken feces	Farm 27 (Wat Bot)	127	Positive	$bla_{CTX-M-15}$	ATM, C, CAZ, DO, FEP, NA, S, SAM, TE
EC245	Chicken feces	Farm 27 (Wat Bot)	131	Positive	$bla_{CTX-M-27}$	ATM, CIP, FEP, LEV, NA, S, SXT, TE

[a] Identical PFGE pattern

[b] All *E. coli* phylogenetic group B2 were resistant to ampicillin, cefuroxime, cefazolin, cefotaxime and cefpodoxime.

Abbreviations: AK, amikacin; ATM, aztreonam; AMC, amoxicillin/cluvalanate; C, chloramphenicol; CAZ, ceftazidime; CIP, ciprofloxacin; CN, gentamicin; DO, doxycycline; FEP, cefepime; FOX, cefoxitin; LEV, levofloxacin; NA nalidixic acid; S, streptomycin; SAM, ampicillin/sulbactam; STX, trimethoprim/sulfamethozaxole and TE, tetracycline

Table 11 (cont.)

Isolates	Sources	Location	STs	ESBL production	$bla_{ESBL/AmpC}$	Other antimicrobial resistance[b]
EC21	Chicken meat	Muang	untypeable	Negative	-	AMC, ATM, C, CAZ, CIP, FEP, FOX, NA, S, SAM
EC23	Chicken meat	Muang	131	Positive	$bla_{TEM-116}$	AMC, ATM, CAZ, DO, FOX, S, SAM
EC50	Chicken meat	Muang	131	Negative	-	AK, AMC, ATM, CAZ, CIP,CN, FOX, IMP, LEV, NA, S, SAM
EC133	Chicken meat	Nakhon Thai	131	Negative	bla_{CMY-2}	AK, AMC, ATM, CAZ, FOX, NA, S, SAM
EC147	Chicken meat	Nakhon Thai	untypeable	Negative	bla_{CMY-2}	AMC, ATM, CAZ, CIP, CN, DO, FOX, NA, S, SAM

Transferability of *bla*$_{CTX-M}$ and *bla*$_{CMY-2}$

One factor that contributes to a rapid spread of ESBL and AmpC-positive *E. coli* is the transfer of their genes. Thirty *E. coli* isolates from meat samples (n = 8) and backyard farms (n = 22) were randomly selected for conjugation experiment (Appendix A: Figure 34). Three *bla*$_{CMY-2}$-positive AmpC-EC, 9 *bla*$_{CTX-M\ group\ 9}$- and 15 *bla*$_{CTX-M\ group\ 1}$-positive ESBL-EC were used as donors. Sodium azide-resistant *E. coli* J53 was used as a recipient. Three isolates (1 and 2 isolates carried *bla*$_{CTX-M\ group\ 9}$ and *bla*$_{CMY-2}$, respectively) were not able to transfer their genes to recipient cell. Twenty-seven isolates successfully transferred their genes to *E. coli* J53 (Table 12). *bla*$_{CTX-M\ group\ 1}$ and *bla*$_{CTX-M\ group\ 9}$ could be transferred at frequencies of 1.8×10^{-7}–8.2×10^{-2} while *bla*$_{CMY-2}$ could be transferred at frequencies of 1.9×10^{-7}–1.5×10^{-5}. Cefotaxime MICs of respective transconjugants possessing *bla*$_{CTX-M}$ or *bla*$_{CMY-2}$ apparently increased from 8–64 folds and 8–16 folds, respectively, compared with recipient (cefotaxime MIC < 0.125 mg/L).

Plasmid replicon typing was performed in transconjugant using multiplex PCR. The replicons of 27 *bla*-positive plasmids were diverse, however, IncF (n = 4) and IncFIA (n = 4) were common replicon among plasmids possessing *bla*$_{CTX-M}$, followed by IncI1 (n = 3) (Table 12). The other replicon types were identified as IncFIB, IncX, IncY (2 isolates each) and IncHI1 (n = 1). The 3 *bla*$_{CMY-2}$ plasmids were typed into IncFIB (n = 2) and IncK (n = 1). Nevertheless, replicon types of 6 plasmids could not be specified.

Table 12 Frequency transfer of bla_{CTX-M} and bla_{CMY-2}, cefotaxime MICs of transconjugant and replicon types of conjugative plasmids

E. coli (EC) donors (sources) / recipient	Frequencies of transfer, genes and plasmid types in transconjugants				
	Transconjugants	Genes	Frequencies of transfer	cefotaxime MICs (mg/L)	Inc plasmid replicons
E. coli J53[a]	-	-	-	< 0.125	-
EC163 (waste water)	EC163_Tc[b]	$bla_{CTX-M\ group\ 1}$	6.4×10^{-2}	> 8	F
EC173 (chicken feces)	EC173_Tc	$bla_{CTX-M\ group\ 1}$	3.6×10^{-2}	> 8	untypeable
EC174 (duck feces)	EC174_Tc	$bla_{CTX-M\ group\ 1}$	2.5×10^{-4}	> 8	X
EC252 (chicken feces)	EC252_Tc	$bla_{CTX-M\ group\ 1}$	8.3×10^{-7}	4	X
EC278 (duck feces)	EC278_Tc	$bla_{CTX-M\ group\ 1}$	1.5×10^{-4}	2	F
EC291 (chicken feces)	EC291_Tc	$bla_{CTX-M\ group\ 1}$	4.3×10^{-4}	8	FIA
EC361 (chicken feces)	EC361_Tc	$bla_{CTX-M\ group\ 1}$	6.7×10^{-7}	1	HI1
EC379 (duck feces)	EC379_Tc	$bla_{CTX-M\ group\ 1}$	1.1×10^{-3}	4	FIA
EC393 (chicken feces)	EC393_Tc	$bla_{CTX-M\ group\ 1}$	1.8×10^{-7}	4	Y
EC401 (chicken feces)	EC401_Tc	$bla_{CTX-M\ group\ 1}$	1.1×10^{-2}	8	I1
EC427 (chicken feces)	EC427_Tc	$bla_{CTX-M\ group\ 1}$	4.8×10^{-5}	4	F
EC150 (chicken feces)	EC150_Tc	$bla_{CTX-M\ group\ 9}$	2.1×10^{-2}	2	F
EC218 (waste water)	EC218_Tc	$bla_{CTX-M\ group\ 9}$	2.0×10^{-7}	2	FIA
EC348 (chicken feces)	EC348_Tc	$bla_{CTX-M\ group\ 9}$	6.2×10^{-2}	8	untypeable

[a] Sodium azide-resistant *E. coli* J53 was used as a recipient. [b] Tc, transconjugant

Table 12 (cont.)

E. coli (EC) donors (sources) / recipient	Frequencies of transfer, genes and plasmid types in transconjugants				
	Transconjugants	Genes	Frequencies of transfer	cefotaxime MICs (mg/L)	Inc plasmid replicons
EC362 (chicken feces)	EC362_Tc	$bla_{\text{CTX-M group 9}}$	1.6×10^{-4}	2	untypeable
EC387 (chicken feces)	EC387_Tc	$bla_{\text{CTX-M group 9}}$	8.2×10^{-2}	4	FIB
EC388 (chicken feces)	EC388_Tc	$bla_{\text{CTX-M group 9}}$	1.2×10^{-2}	2	FIA
EC397 (human feces)	EC397_Tc	$bla_{\text{CTX-M group 9}}$	2.5×10^{-4}	> 8	FIB
EC414 (chicken feces)	EC414_Tc	$bla_{\text{CTX-M group 9}}$	4.5×10^{-7}	> 8	I1
EC2 (poultry meat)	EC2_Tc	$bla_{\text{CTX-M group 1}}$	4.2×10^{-3}	> 8	I1
EC121 (poultry meat)	EC121_Tc	$bla_{\text{CTX-M group 1}}$	2.5×10^{-5}	> 8	untypeable
EC129 (poultry meat)	EC129_Tc	$bla_{\text{CTX-M group 1}}$	8.1×10^{-2}	> 8	untypeable
EC146 (poultry meat)	EC146_Tc	$bla_{\text{CTX-M group 1}}$	7.5×10^{-2}	> 8	Y
EC119 (poultry meat)	EC119_Tc	$bla_{\text{CTX-M group 9}}$	4.7×10^{-4}	> 8	untypeable
EC125 (poultry meat)	EC125_Tc	$bla_{\text{CMY-2}}$	1.5×10^{-5}	2	K
EC128 (poultry meat)	EC128_Tc	$bla_{\text{CMY-2}}$	1.6×10^{-6}	1	FIB
EC132 (poultry meat)	EC132_Tc	$bla_{\text{CMY-2}}$	1.9×10^{-7}	1	FIB
EC153 (chicken feces) $bla_{\text{CTX-M group 9}}$	Not transfer	-	-	-	-
EC289 (chicken feces) $bla_{\text{CMY-2}}$	Not transfer	-	-	-	-
EC425 (waste water) $bla_{\text{CMY-2}}$	Not transfer	-	-	-	-

Transmission of ESBL-EC in backyard poultry farms

Backyard poultry farm could be a reservoir of ESBL-EC. To investigate the transmission of ESBL-EC between human, animal and environment, PFGE was performed (Appendix A: Figure 35). One-hundred and thirty-two ESBL-EC isolates (47 $bla_{\text{CTX-M group 9}}$ and 85 $bla_{\text{CTX-M group 1}}$) were available for analysis. Identification of full-length $bla_{\text{CTX-M}}$ alleles in these isolates was also determined (Table 13). $bla_{\text{CTX-M-55}}$ (56.8%) and $bla_{\text{CTX-M-14}}$ (30.3%) were the most frequently detected among $bla_{\text{CTX-M-group 1}}$- and $bla_{\text{CTX-M-group 9}}$-positive isolates, respectively. The other groups of $bla_{\text{CTX-M}}$, such as $bla_{\text{CTX-M-15}}$, $_{\text{-27}}$, and $_{\text{-65}}$, were found with lower prevalence (7.6, 4.5 and 0.8%, respectively). The similarity index of isolates was illustrated by showing in dendrogram. Isolates that showed at least 85% similarity, a cut-off point, suggested that they were clonal.

The PFGE profiles of 85 $bla_{\text{CTX-M-group 1}}$-positive isolates were shown in Figure 8. The fingerprints showed 66 distinct macrorestriction patterns. Of these, 13 identical patterns contained $bla_{\text{CTX-M-55}}$-positive isolates. Similarly, among 47 $bla_{\text{CTX-M-group 9}}$-positive isolates, DNA fingerprints were diverse and 42 PFGE patterns were found. Of these, clonal spreads were found in 4 patterns (3 $bla_{\text{CTX-M-14}}$ and 1 $bla_{\text{CTX-M-27}}$-positive isolates) (Figure 9).

Among 17 identical PFGE patterns, 14 patterns disseminating within the same farms were found within 12 farms. Of 14 identical patterns, 2 and 12 patterns were $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-55}}$-positive ESBL-EC, respectively (Table 14). Eight clonal disseminations were found among chicken (1 $bla_{\text{CTX-M-14}}$- and 7 $bla_{\text{CTX-M-55}}$-positive patterns) while 6 clonal patterns spread between poultry and environment within the same farm, i.e. chicken/duck and environmental water or chicken and soil. Of the 6 patterns, 1 and 5 patterns were of $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-55}}$-positive isolates, respectively.

Three identical patterns were cross-contamination between farms (Table 14). In the first case, the clonal spread was found between $bla_{\text{CTX-M-55}}$-positive ESBL-EC from farmer in farm 1 and soil in farm 5. Two farms are located within 1-kilometer radius. Identical patterns were found in $bla_{\text{CTX-M-27}}$-positive ESBL-EC from farm 15 (chicken) and farm 16 (farmer) in the second case. Both farms were located within the same district with 2 kilometers apart (Bang Rakam). Interestingly, these strains were

typed as group B2-ST131 that also had similar antimicrobial resistant patterns (Table 11). Lastly, clonal strains were detected between *bla*$_{CTX-M-14}$-positive ESBL-EC recovered from chicken in farm 10 in Bang Rakam and farm 24 in Wat Bot. The distance of two farms was approximately 98 kilometers.

Table 13 Distribution of *bla*$_{CTX-M}$ among 132 ESBL-EC isolated from backyard farm samples

Samples (n)	No. of *bla*$_{CTX-M}$-positive ESBL-EC (%)				
	bla$_{CTX-M\ group\ 1}$		*bla*$_{CTX-M\ group\ 9}$		
	bla$_{CTX-M-15}$	*bla*$_{CTX-M-55}$	*bla*$_{CTX-M-14}$	*bla*$_{CTX-M-27}$	*bla*$_{CTX-M-65}$
Farmers (12)	2 (16.7)	3 (25.0)	5 (41.6)	2 (16.7)	0
Poultry					
- Chickens (88)	4 (4.5)	55 (62.5)	26 (29.6)	2 (2.3)	1 (1.1)
- Ducks (13)	2 (15.4)	6 (46.2)	5 (38.4)	0	0
Subtotal (101)	6 (5.9)	61 (60.4)	31 (30.7)	2 (2.0)	1 (1.0)
Environment					
- Soil (8)	1 (12.5)	5 (62.5)	1 (12.5)	1 (12.5)	0
- Water (11)	1 (9.1)	6 (54.5)	3 (27.3)	1 (9.1)	0
Subtotal (19)	2 (10.5)	11 (57.9)	4 (21.1)	2 (10.5)	0
Total (132)	**10 (7.6)**	**75 (56.8)**	**40 (30.3)**	**6 (4.5)**	**1 (0.8)**

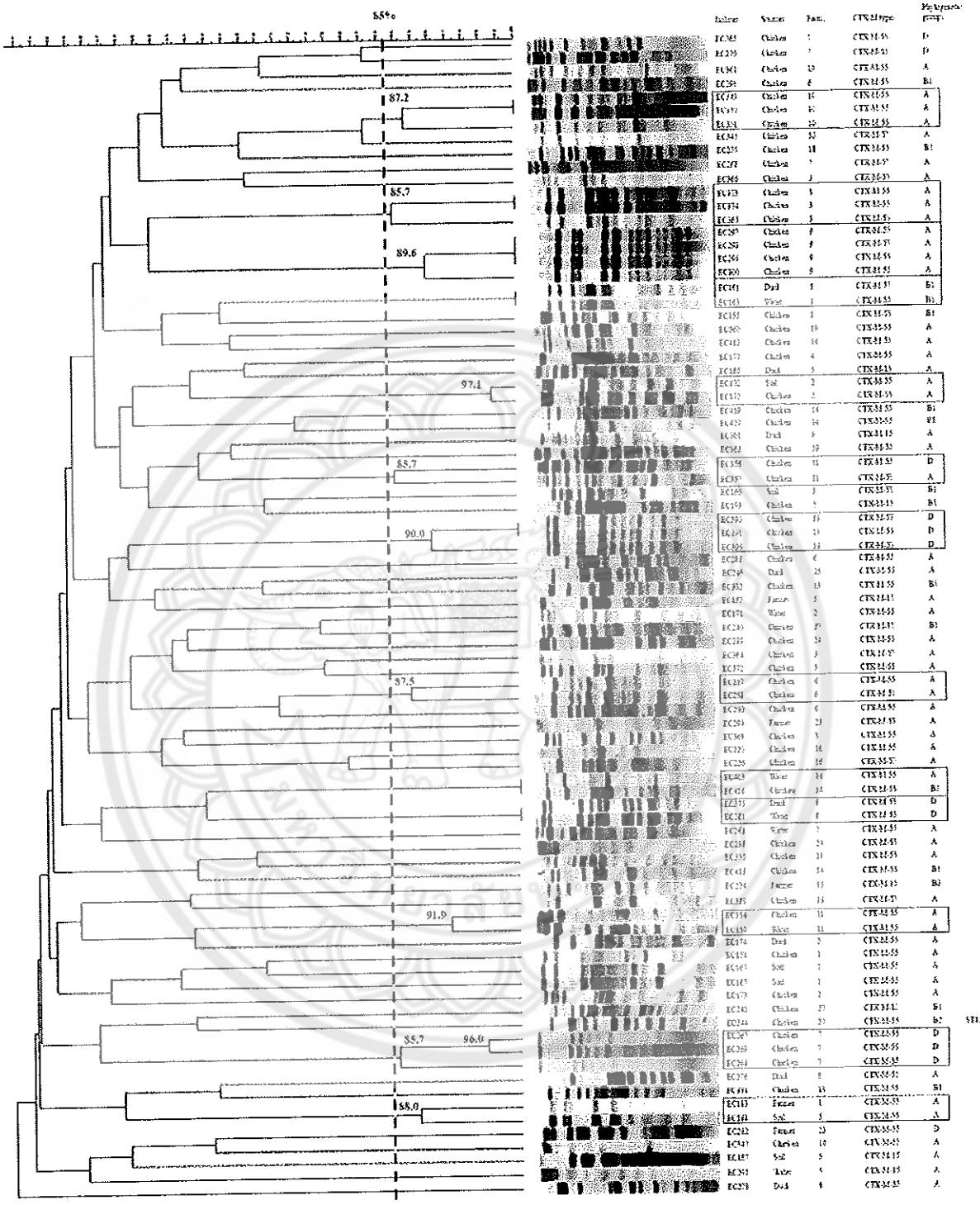
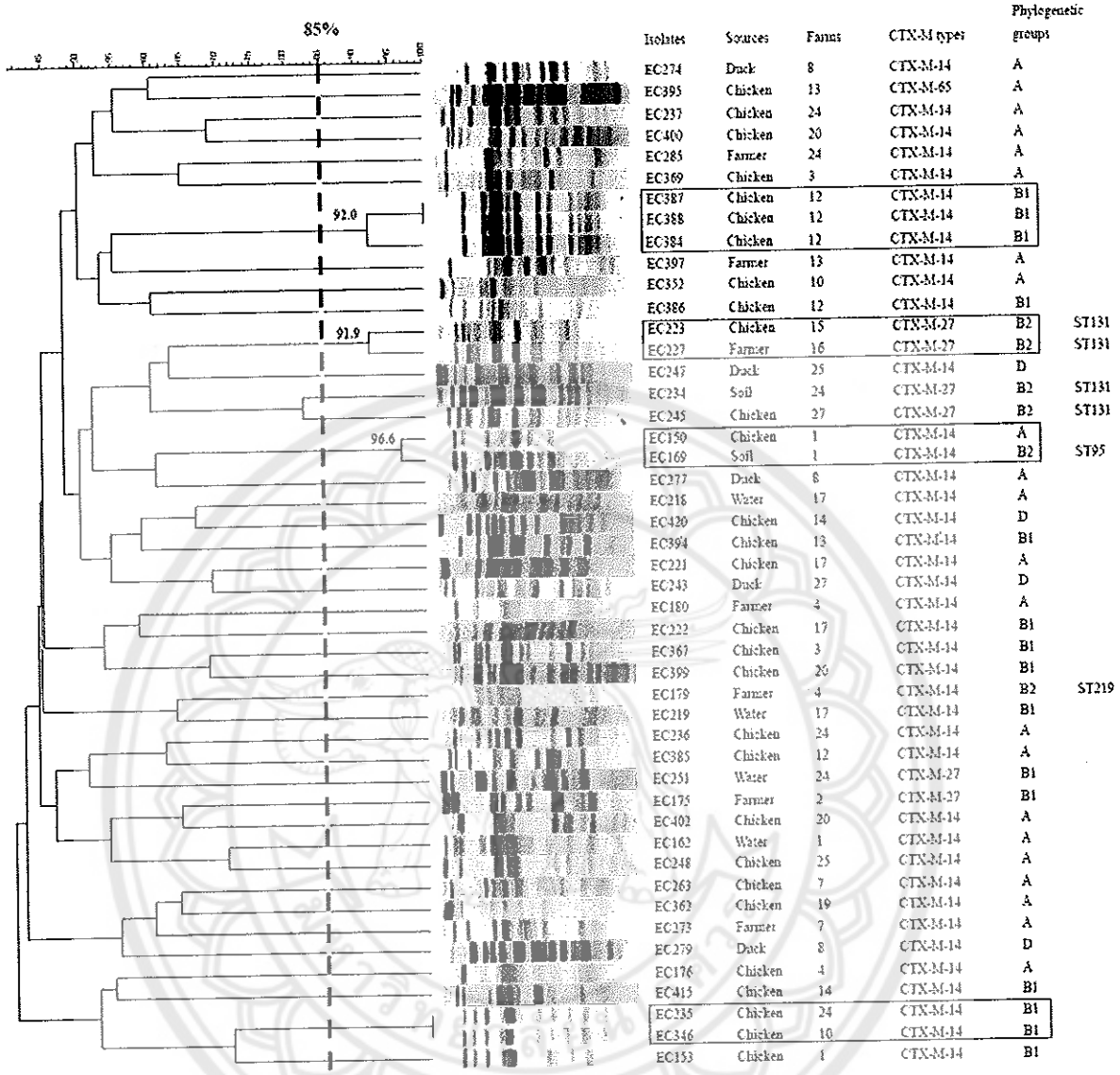


Figure 8 *XbaI*-PFGE dendogram of 85 $bla_{\text{CTX-M group 1}}$-positive ESBL-EC isolated from backyard farms

The scale bar shows the percent of similarity and the vertical dotted lines indicates ≥ 85% similarity. Isolates in the same rectangle are clonal strains.



Isolates	Sources	Farms	CTX-M types	Phylogenetic groups	
EC274	Duck	8	CTX-M-14	A	
EC395	Chicken	13	CTX-M-65	A	
EC237	Chicken	24	CTX-M-14	A	
EC400	Chicken	20	CTX-M-14	A	
EC285	Farmer	24	CTX-M-14	A	
EC369	Chicken	3	CTX-M-14	A	
EC387	Chicken	12	CTX-M-14	B1	
EC388	Chicken	12	CTX-M-14	B1	
EC384	Chicken	12	CTX-M-14	B1	
EC397	Farmer	13	CTX-M-14	A	
EC352	Chicken	10	CTX-M-14	A	
EC386	Chicken	12	CTX-M-14	B1	
EC223	Chicken	15	CTX-M-27	B2	ST131
EC227	Farmer	16	CTX-M-27	B2	ST131
EC247	Duck	25	CTX-M-14	D	
EC234	Soil	24	CTX-M-27	B2	ST131
EC245	Chicken	27	CTX-M-27	B2	ST131
EC150	Chicken	1	CTX-M-14	A	
EC169	Soil	1	CTX-M-14	B2	ST95
EC277	Duck	8	CTX-M-14	A	
EC218	Water	17	CTX-M-14	A	
EC420	Chicken	14	CTX-M-14	D	
EC394	Chicken	13	CTX-M-14	B1	
EC221	Chicken	17	CTX-M-14	A	
EC243	Duck	27	CTX-M-14	D	
EC180	Farmer	4	CTX-M-14	A	
EC222	Chicken	17	CTX-M-14	B1	
EC367	Chicken	3	CTX-M-14	B1	
EC399	Chicken	20	CTX-M-14	B1	
EC179	Farmer	4	CTX-M-14	B2	ST219
EC219	Water	17	CTX-M-14	B1	
EC236	Chicken	24	CTX-M-14	A	
EC385	Chicken	12	CTX-M-14	A	
EC251	Water	24	CTX-M-27	B1	
EC175	Farmer	2	CTX-M-27	B1	
EC402	Chicken	20	CTX-M-14	A	
EC162	Water	1	CTX-M-14	A	
EC248	Chicken	25	CTX-M-14	A	
EC263	Chicken	7	CTX-M-14	A	
EC362	Chicken	19	CTX-M-14	A	
EC273	Farmer	7	CTX-M-14	A	
EC279	Duck	8	CTX-M-14	D	
EC176	Chicken	4	CTX-M-14	A	
EC415	Chicken	14	CTX-M-14	B1	
EC335	Chicken	24	CTX-M-14	B1	
EC346	Chicken	10	CTX-M-14	B1	
EC353	Chicken	1	CTX-M-14	B1	

Figure 9 *XbaI*-PFGE dendogram of 47 *bla*$_{\text{CTX-M group 9}}$-positive ESBL-EC isolated from backyard farms

The scale bar shows the percent of similarity and the vertical dotted lines indicates ≥ 85% similarity. Isolates in the same rectangle are clonal strains.

Table 14 Intra-and Inter-dissemination of ESBL-EC in backyard farms

Districts	Location	Isolates	Origin of isolates	*bla*$_{CTX-M}$
Intra-farm dissemination				
Muang	Farm 1	EC150 EC169	Chicken Soil	*bla*$_{CTX-M-14}$
	Farm 1	EC161 EC163	Duck Water	*bla*$_{CTX-M-55}$
	Farm 2	EC170 EC172	Soil Chicken	*bla*$_{CTX-M-55}$
	Farm 3	EC365, EC373, EC374	Chicken	*bla*$_{CTX-M-55}$
Bang Rakam	Farm 6	EC257, EC258	Chicken	*bla*$_{CTX-M-55}$
	Farm 7	EC264, EC267, EC269	Chicken	*bla*$_{CTX-M-55}$
	Farm 8	EC275 EC281	Duck Water	*bla*$_{CTX-M-55}$
	Farm 9	EC297, EC298, EC299, EC300	Chicken	*bla*$_{CTX-M-55}$
	Farm 10	EC349, EC350, EC351	Chicken	*bla*$_{CTX-M-55}$
	Farm 11	EC356, EC357	Chicken	*bla*$_{CTX-M-55}$
	Farm 11	EC354 EC359	Chicken Water	*bla*$_{CTX-M-55}$
	Farm 12	EC387, EC388, EC384	Chicken	*bla*$_{CTX-M-14}$
	Farm 13	EC390, EC391, EC396	Chicken	*bla*$_{CTX-M-55}$
	Farm 14	EC403 EC416	Water Chicken	*bla*$_{CTX-M-55}$
Inter-farm dissemination				
Muang	Farm 1 Farm 5	EC183 EC188	Farmer Soil	*bla*$_{CTX-M-55}$
Bang Rakam	Farm 15 Farm 16	EC223 EC227	Chicken Farmer	*bla*$_{CTX-M-27}$
Bang Rakam Wat Bot	Farm 10 Farm 24	EC346 EC235	Chicken Chicken	*bla*$_{CTX-M-14}$

Characterization of *bla*$_{CTX-M-55}$-carrying plasmid

Six *bla*$_{CTX-M-55}$-positive ESBL-EC isolates (5 chicken feces, EC252, EC291, EC361, EC393, EC427 and 1 duck feces, EC278) were randomly chosen to study the location of gene by S1 nuclease-PFGE and gel hybridization. The results revealed that 5 isolates carried *bla*$_{CTX-M-55}$ on plasmid while EC361 carried gene on chromosome (Figure 10). Sizes of *bla*$_{CTX-M-55}$-carrying plasmid of EC252, EC278, EC291 and EC427 were approximately 50, 90, 150 and 100 kb in sizes, respectively. EC393 carried 2 *bla*$_{CTX-M-55}$ plasmids which were approximately 30 and 130 kb.

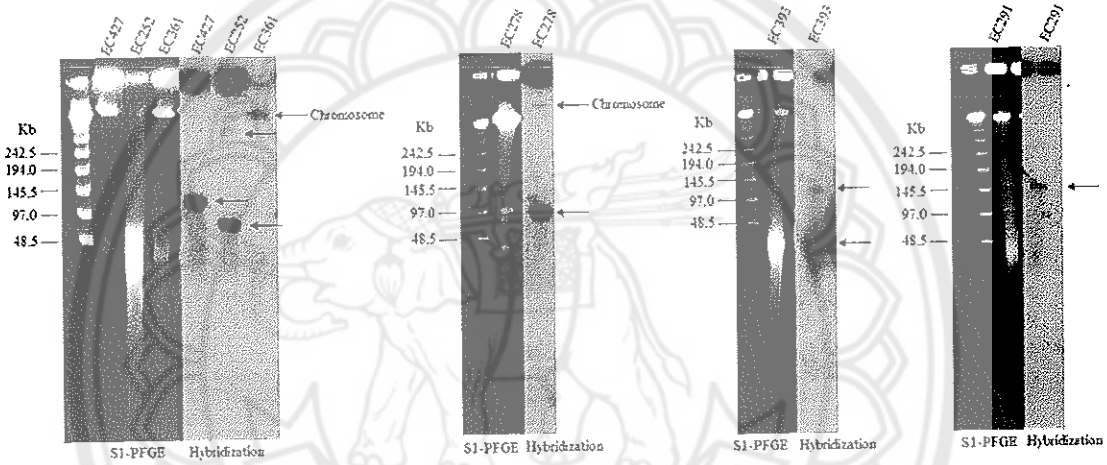


Figure 10 S1-PFGE and ^{32}P hybridization of *bla*$_{CTX-M-55}$ among 6 ESBL-EC isolates from backyard farms
Marker: Lambda PGE Ladder (New England Biolabs, Inc, UK)
Arrows indicated the location of *bla*$_{CTX-M-55}$

Plasmid sequencing was performed on 4 isolates (EC278, EC291, EC393 and EC427), however, full plasmid mapping was successfully generated on only 1 isolate, EC278, namely pEC278. Sequence analysis of pEC278 was analyzed by programs at Center for Genomic Epidemiology website (97).

pEC278 had a length of 97,426 bp with 51.8% G+C content. The plasmid replicon type was identified as IncFII. One hundred and two open reading frames (ORFs) were detected. Of these, 91 ORFs were known as functional genes. Plasmid sequence analysis revealed that pEC278 possessed multiple antimicrobial-resistant genes, including *aac(3)-IId* (aminoglycoside resistance), *bla*$_{CTX-M-55}$ (β-lactam

resistance), *qnrS1* (fluoroquinolone resistance) and *catA2* (phenicol resistance). Importantly, *mcr-3* causing colistin resistance was detected in pEC278.

BLAST search on pEC278 sequences demonstrated that pEC278 was closely related to pCHL50009T (accession no. CP032937), a 101,860-bp plasmid isolated from *E. coli* recovered from patient in New Zealand (unpublished). It shared 90.3% identity with > 97% query coverage. pEC278 was also related to pHNHN21 (accession no. KX246267), a 83,435 bp plasmid which was detected in *E. coli* isolated from pigeon in China (unpublished). To illustrate map of plasmid and its location of $bla_{\text{CTX-M-55}}$ in pEC278, BLAST ring image generator (BRIG v0.95555) was used for comparing plasmid background using pCHL50009T as a central reference plasmid (Figure 11). Plasmid backbone of pEC278 could be divided into 2 regions which were conserved region and cluster of antimicrobial-resistant genes (ARGs). Conserved regions commonly composed of genes related to conjugative (*tra*) and replication proteins (*rep*). Nucleotide sequences on this region highly shared > 99% similarity compared with pCHL50009T.

In contrast, cluster of ARGs comprised multiple genes linked with mobile genetic elements, such as transposon (Tn) and insertion sequence (IS) (Appendix A: Figure 36). pCHL50009T and pEC278 were highly homologous on this region (nearly 92% similarity). Nevertheless, *aac(3)-IId*, *qnrS1* and *mcr-3* were absent in pHNHN21 compared with pCHL50009T and pEC278. The genetic environment of $bla_{\text{CTX-M-55}}$ of pEC278 compared with pCHL50009T and pHNHN21 was sketched (Figure 12). Cluster of ARGs had approximately 20,000-bp length. Gene arrangement of pCHL50009T was **IS*6*-*catA2*-IS*6***-IS*Ecp1*(IS*1380* family)-$bla_{\text{CTX-M-55}}$-Tn*3*-**IS*3*-*qnrS1*-*res*-IS*6***-IS*4*-*aac(3)-IId*-IS*6*-Tn*3*-*mcr-3*-*dgkA*-**IS*3*-IS*6*** while 4 of IS*6* elements (bold) were replaced by IS*26* found in pEC278. IS*26*-IS*Ecp1* was found in upstream of $bla_{\text{CTX-M-55}}$ while functional gene-Tn*3*-IS*3* was found in downstream of gene. Furthermore, 1,327-bp and 166-bp insertion were observed on pEC278 sequence between IS*6*-Tn*3* and IS*3*-IS*26*, respectively.

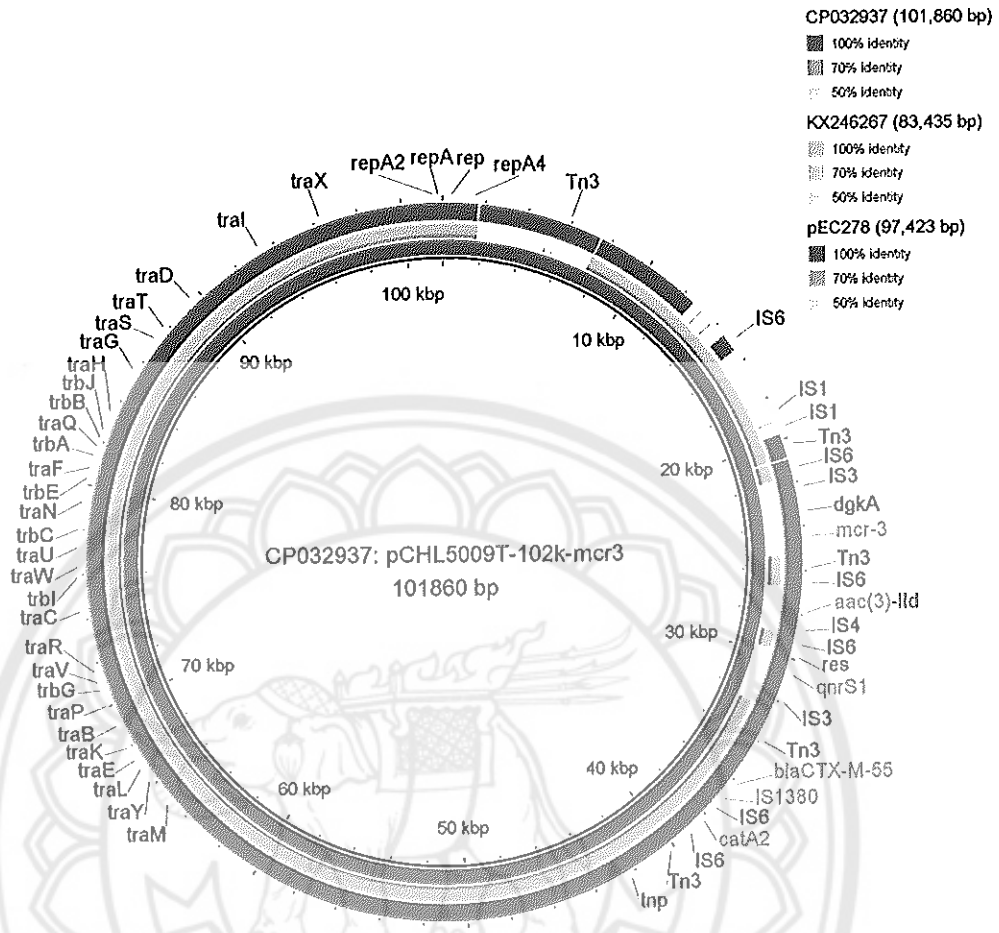


Figure 11 Alignment of *bla*$_{CTX-M-55}$ IncFII plasmid using BRIG (v0.9555) pCHL50009T (accession no. CP032937) is used as a reference sequence which is an inner ring. Middle ring is pHNHN21 (accession no. KX246267) and an outer ring is pEC278 isolated from duck feces. Gaps on the ring sequences indicate that nucleotide or genes are absent. Red, green and black letters are antimicrobial-resistant gene, mobile genetic element and functional gene, respectivly.

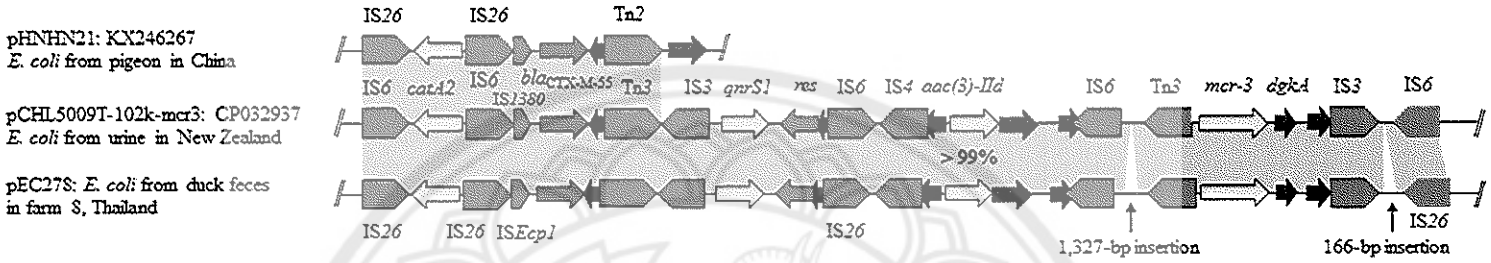


Figure 12 Comparison of genetic contexts of *bla*$_{\text{CTX-M-55}}$ on IncFII plasmid between sequences submitted on NCBI (CP032937 and KX246267) and pEC278 in this study

Red arrow is *bla*$_{\text{CTX-M-55}}$. Yellow and black arrows are antimicrobial-resistant gene and functional gene, respectively.

Blue long pentagon is mobile genetic element. Grey color painted between sequences indicates that nucleotide sequences show >99% similarity.

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Backyard poultry farm has commonly been found in local communities throughout Asia, including Thailand. Additionally, poultry meat is widely consumed by the majority of people worldwide (110). Meat has also been to act as mechanical vector that could deliver antimicrobial-resistant *E. coli* to human via food consumption. Although, a high prevalence of antimicrobial-resistant *E. coli* in Southeast Asia has been reported (111), limited data on the AR-EC from backyard farm and poultry meat in Thailand is available.

In this study, 734 samples from farmer feces, poultry feces, farm environment and poultry meat in Phitsanulok province, Northern Thailand, were obtained for the isolation of *E. coli* with reduced susceptibility to cefotaxime (EC-CTX). EC-CTX was found in all sources and the overall prevalence of EC-CTX was 34.1% (n = 250) (Table 4), consistent with previous publication reporting the ubiquitous antimicrobial-resistant *E. coli* in Thailand (83, 112, 113).

Antimicrobial susceptibility testing revealed that 250 *E. coli* isolates (Table 5) showed a resistant rate of 100% on ampicillin and 1st–3rd generation cephalosporins and were highly resistant (> 60%) to aztreonam, tetracycline, streptomycin, ceftazidime and cefuroxime. All EC-CTX isolates were identified as multidrug-resistant *E. coli* (MDR-EC), according to criteria of Magiorakos et al. (2012) (38). In addition, extended-spectrum β-lactamase-producing *E. coli* (ESBL-EC) was detected in 214 isolates (85.6%), consistent with those reported in various populations (human, animal and environment) worldwide, such as The Netherlands (55.0–81.0%), France (91.7%), China (96.7%) and Thailand (40.0–77.3%) (48, 68, 82, 114). ESBL-EC isolates showed cross-resistance to other antimicrobial classes, such as β-lactams with aminoglycosides, tetracyclines, fluoroquinolones and trimethroprim/sulfamethoxazole (Appendix A: Table 19), in agreement with the typical characteristics of ESBL-producing bacteria (115−117). Additionally, overexpression of efflux pump and

alteration of an outer membrane protein may associate with multiple drug resistance (118, 119).

Since the first report of ESBL- and AmpC-encoding genes, the increased incidence of these genes have markedly been found in *E. coli* recovered from both clinical and non-clinical samples worldwide, especially in food-producing animals (13, 62). Among Enterobacteriaceae, $bla_{\text{CTX-M}}$ and $bla_{\text{CMY-2}}$ have been recognized as the most common ESBL- and AmpC-encoding genes, respectively (15, 120). In this study, the overall prevalence of $bla_{\text{CTX-M}}$ was high (78.4%). $bla_{\text{CTX-M group 1}}$ was the most common group (54.8%) followed by $bla_{\text{CTX-M group 9}}$ (23.6%), consistent with the fact that they are the most widespread of $bla_{\text{CTX-M}}$ worldwide including Thailand (15, 113). These results differed from those reported from Asian countries, such as China and Vietnam where $bla_{\text{CTX-M group 9}}$ is predominant (121, 122). These differneces may, to some extent, reflect the epidemiology of ESBL-EC between regions. In this study, $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-14}}$ were the most common $bla_{\text{CTX-M-group 1}}$ and group 9, respectively, found among ESBL-EC recovered from backyard farms. The other genes, $bla_{\text{CTX-M-15, -27}}$ and $_{\text{-65}}$ were detected but with a much lower prevalence (Table 13). No other $bla_{\text{CTX-M}}$ groups, i.e. $bla_{\text{CTX-M group 2}}$ and $bla_{\text{CTX-M group 8}}$, was found which was different from a previous publication reporting the presence of these genes among poultry (13).

The occurrence of ESBL-EC among poultry in backyard farms was 25.9% (Table 4) which was higher than those in household and small-scale farms in Vietnam (0.2%) (49) while no ESBL-EC was found in backyard chicken or swine farms in India and Finland (123, 124). In contrast, it was apparently less than those reported from commercial farms in Germany (81.9%), Japan (99.4%) and Thailand (40.0%) (57, 114, 125). The differences in the prevalence of ESBL-EC may be due to the supplement of antimicrobial agents in animal feed with commercial farms in several countries including Thailand (5, 126, 127). In this study, the presence of ESBL-EC in backyard farms may also result from selective pressure from antimicrobial agents since antimicrobial usage (128) was reported in 19 farms (Appendix A: Table 15).

Among asymptomatic healthy farmers, the high prevalence of $bla_{\text{CTX-M}}$-positive *E. coli* (78.9%) (Table 7) was observed which was apparently higher than those found in farmers in non-intensive farms in Vietnam (31.1%) (65). This may not

be surprising since the prevalence of *bla*$_{CTX-M}$-positive *E. coli* among asymptomatic residents in Thailand, including Phitsanulok province, where this study was conducted, has been reported to be strikingly high (112, 129). The finding of *bla*$_{CTX-M}$-positive *E. coli* among farmers may be caused by a direct contact to animal and working time in farm (130) because all farmers raised their animals by themselves throughout period of production and some farmers concurrently consumed meat and/or egg from their animals. Nevertheless, antimicrobial usage, a history of hospitalization and consumption of undercooked meat may be the main reasons to cause the incidence of *bla*$_{CTX-M}$-positive *E. coli* among asymptomatic humans (112, 131, 132).

The prevalence of ESBL-EC in farm environment (25.0%) was similar to those in poultry (25.9%) (Table 4). The contaminated farm environment with ESBL-EC was often found in farm which has a high prevalence of ESBL-EC among poultry (68). The distribution of ESBL-EC in the environment was possibly excreted through feces and farm waste (69). Furthermore, commensal *E. coli* in the environment may be become resistance by selective pressure from the antimicrobial agent residues in animal feces (70, 133).

It has been suggested that poultry meat was an important reservoir for antimicrobial-resistant *E. coli* because the highest contamination of *E. coli* was found in poultry meat when compared with beef and pork (134). Among meat samples, the high prevalence (56.4%) of *bla*$_{CTX-M}$-positive *E. coli* was observed (Table 7). These results were hugely different from the report from India (15.0%) and Japan (39.3%) (83, 135) but was similar to the report from Cambodia (53.0%) (136). In addition, the prevalence of *bla*$_{CTX-M}$-positive *E. coli* was lower than those in previous findings in Europe, such as The Netherlands (76.0%) and United Kingdom (89.6%) (137, 138). The high prevalence of *bla*$_{CTX-M}$-positive *E. coli* in poultry meat in open-air markets may be associated with an antimicrobial usage in farms (5). Additionally, the distribution of ESBL-EC in poultry meat may associate with other factors, such as sanitation of the stall environment, source of cleaning water and type of cutting board in markets (139).

Thirty-six ESBL-negative isolates (14.4%) showing resistance to cefoxitin were isolated from backyard farms (n = 13) and meat samples (n = 23) (Table 6), suggesting that AmpC β-lactamase may be responsible for cefotaxime (AmpC

β-lactamase-producing *E. coli*; AmpC-EC) (23). AmpC-EC was reported worldwide but with lower prevalence, compared with ESBL-EC, such as China (1.0%) and France (3.9%) (61, 82). Within the bla_{AmpC}, bla_{CMY-2} has been noted as the most widespread of bla_{AmpC} (23). In this study, bla_{CMY-2} was the only bla_{AmpC} found among 21 AmpC-EC isolates (8.4%) (Table 7), consistent with previous reports in China (2.9%) and The Netherlands (5.2–12%) (140, 141). Studies on the bla_{AmpC}-positive bacteria in Thailand were limited, although a few studies reported the presence of bla_{CMY-2} in clinical and healthy human and environmental water samples (112, 142, 143). Additionally, no bla_{AmpC} was detected in 15 ESBL-negative isolates. Multidrug resistance phenotype of these isolates may result from the hyperproduction of bla_{TEM-1}, overexpression of efflux pump and alteration of an outer membrane protein (118, 119, 144).

Phylogenetic analysis of *E. coli* revealed that commensal (groups A and B1) and pathogenic strains (group B2 and D) were present in all sources of samples (Table 8). The predominant groups were commensal strains (groups A and B1, 81.6%), however, they could cause extraintestinal infections, such as bacteraemia, similar to the pathogenic strains (145). Pathogenic strains, especially group B2, are virulent and responsible for the majority of extraintestinal pathogenic *E. coli* (ExPEC) infections (146). Group B2 often carriers several virulence factors and exhibits resistance to multiple antimicrobial agents (147). In this study, all isolates belonging to group B2 showed MDR phenotype and expressed high resistance rates to 12 drugs, 9 of which were beta-lactams (Table 10). These results are not too surprising since most B2 isolates carried either bla_{CTX-M} or bla_{CMY-2} (Table 9). Moreover, groups B2 and D demonstrated higher resistance rates to cefoxitin and amoxicillin/clavulanate compared with commensal strains (Table 10). One possible explanation is that 14 of 46 isolates belonging to group B2 and D possessed bla_{CMY-2}. CMY-2 AmpC β-lactamase effectively confers resistance to cefoxitin and was poorly inhibited by β-lactamase inhibitors, clavulanate and sulbactam (23). For 32 isolates of group B2 and D, 27 isolates were positive for bla_{CTX-M} (n = 26) and $bla_{TEM-116}$ (n = 1) (Table 9), in addition, no bla_{ESBL} was found in 5 isolates. Of the 5 isolates, multidrug resistance may result from the hyperproduction of bla_{TEM-1}, overexpression of efflux pump and alterarion of an outer membrane as discussed earlier. Interestingly, among 14 bla_{CMY-2}-

positive isolates, 11 isolates (group B2, n = 1 and group D, n = 10) were recovered from meat samples in Nakhon Thai district, suggesting that the *bla*$_{CMY-2}$-positive isolate was highly endemic in this area.

In this study, 14 isolates were assigned to group B2 (Table 11). Of these, 10 isolates were typed as ST131 (n = 7), ST95 (n = 1), ST127 (n = 1) and ST219 (n = 1), all of them have been noted as the widespread STs of ExPEC causing UTI and bloodstream infection (148). *bla*$_{CTX-M}$-positive *E. coli* ST131 and ST95 were recently detected in healthy volunteer, chicken meat and water environment in Phitsanulok province and central of Thailand (81, 112, 143). ST131 has been noted as the worldwide pandemic type and frequently carries *bla*$_{CTX-M-15}$ or *bla*$_{CTX-M-27}$ (15, 149). Of the 7 ST131 isolates, 4 isolates were *bla*$_{CTX-M-27}$-positive ESBL-EC and had similar antimicrobial-resistant patterns, including resistance to fluoroquinolone, the typical characteristics of ST131 which were reported in other studies (150, 151). Furthermore, clonal spread of *bla*$_{CTX-M-27}$-positive ST131 between chicken and farmer (91.9% similarity) was found in Bang Rakam district (EC223 from farm 15 and EC227 from farm 16, Table 14 and Figure 9). Additionally, the other 2 *bla*$_{CTX-M-27}$-positive ST131 isolates showing different PFGE patterns were also found in Bang Rakam and Wat Bot districts. The finding of *bla*$_{CTX-M-27}$-positive ST131 suggest that this clade maybe sporadically disseminated in Phitsanulok province.

In addition, *bla*$_{CMY-2}$-positive AmpC-EC ST131 and *bla*$_{TEM-116}$-positive ESBL-EC ST131 were found among isolates in group B2. These strains have been found in clinical specimen from dog in Germany and patients in Norway (36, 152), however, the occurrence of these strains, especially *bla*$_{TEM-116}$-positive ST131, remains infrequent. Since *bla*$_{CMY-2}$ or *bla*$_{TEM-116}$ confer resistance to 3rd and 4th generation cephalosporins (23, 153), the finding of these genes in ST131 may contribute multidrug resistance.

ST95 and ST127 were also reported as causative agents of many extraintestinal infections (154), although with a comparatively lower prevalence compared with ST131 (155). However, ST95 and ST127 were responsible for the majority of STs causing community-acquired urinary tract infection. These STs were recovered from poultry and retail poultry meat (78). A single isolate was identified as ST127 which is of concerned because it apparently showed a pathogenic potential,

using *Galleria mellonella* larvae in vivo model, greater than ST95 and ST131 (156). This study, ST95 showed the lowest number of drug resistance compared with the other STs (Table 11).

It has been suggested that horizontal gene transfer may play a critical role in the spread of $bla_{CTX\text{-}M/CMY\text{-}2}$ within community (66, 157, 158). In this study, $bla_{CTX\text{-}M}$ and $bla_{CMY\text{-}2}$ were successfully transferred to a recipient at high transfer rates and apparently affected the rise in cefotaxime MICs in transconjugants. Nevertheless, the transfer of $bla_{CTX\text{-}M/CMY\text{-}2}$ in 3 isolates were unsuccessful. It is possible that the genes are located on chromosome (159). The plasmids were further identified for their Inc types. In the present study, the diversity of Inc types (IncF, IncI1 and IncK) of $bla_{CTX\text{-}M/CMY\text{-}2}$-positive plasmids among *E. coli* isolates was demonstrated, however, IncF was the most prevalent type (Table 12). These results were consistent with the fact that IncF (IncFIA and IncFIB) was frequently found in Enterobacteriaceae (65, 160). All Inc types found in this study were considered as narrow-host-range plasmids but commonly carried antimicrobial-resistant gene (40, 41). These plasmids may cause multidrug resistance and possibly share antimicrobial-resistant gene via conjugation among *E. coli* recovered from backyard farm and meat samples in this study.

Besides the horizontal gene transfer, the spread of clonal or closely related isolates, as judged by PFGE, existed among ESBL-EC isolates from backyard farms. Poultry meat samples were excluded from this experiment because most meat samples sold in open-air markets were not produced from backyard farm. Analysis of PFGE patterns illustrated the high genotypic diversities among ESBL-EC in backyard farms. However, 17 identical PFGE patterns, among poultry, farmers and environments, were observed in ESBL-EC within and between farms. Clonal spread of ESBL-EC among poultry farms has been observed in large commercial and backyard farm in China, Japan and Vietnam (48, 57, 65). Since, almost all backyard farms in this study raised poultry in free-range system, the dissemination of ESBL-EC may be due to the direct excretion of poultry feces to the environment (69). Not only within the farm but the clonal spread was also observed between the farms. There were 3 cases of identical genotypic patterns of ESBL-EC between farms. The first case involved $bla_{CTX\text{-}M\text{-}55}$-positive ESBL-EC from farmer in farm 1 and soil in farm 5. The second case was $bla_{CTX\text{-}M\text{-}27}$-positive ESBL-EC ST131 isolates from farmer and chicken, as discussed

earlier (farmer in farm 16 and chicken in farm 15). And the third case was $bla_{CTX\text{-}M\text{-}14}$-positive ESBL-EC from chicken in farms 10 and 24, which are approximately 98 kilometers away from each other. This case was uncommon because the transmission of *E. coli* between poultry has often been found within the same or nearby farms (57, 161). The spread of antimicrobial-resistant *E. coli* between farms, especially in different area, may be contributed by insects, such as blowfly (71, 162).

Six ESBL-EC isolates were randomly selected to study the location of $bla_{CTX\text{-}M\text{-}55}$. Five isolates carried $bla_{CTX\text{-}M\text{-}55}$ on plasmid with 30–150 kb in sizes whereas a single isolate (EC361) carried $bla_{CTX\text{-}M\text{-}55}$ on chromosome. Plasmid sequencing of EC278 was investigated. EC278 plasmid (pEC278) type was identified as IncFII, the type of plasmid that commonly possessed multiple antimicrobial-resistant genes (ARGs) (40). Therefore, it is not surprising that sequence of pEC278 showed multiple ARGs conferring resistance to both beta-lactam and non-beta-lactams, resulting in MDR phenotype. Upstream of $bla_{CTX\text{-}M\text{-}55}$ was IS*Ecp1*, the most common IS associated with $bla_{CTX\text{-}M}$ (12), including the $bla_{CTX\text{-}M\text{-}3,\ -14,\ -15,\ 27,\ 40}$ and $_{-55}$ in Thailand (163). Additionally, many IS*26* flanking ARGs were found on pEC278. IS*26* was the most common IS associated bla_{ESBL} ($bla_{CTX\text{-}M\text{-}1,\ 3,\ -14,\ \text{and}\ -15}$, $bla_{SHV\text{-}5}$, $bla_{SHV\text{-}12}$ and $bla_{TEM\text{-}52}$) found among ESBL-EC recovered from food products, farms, environment and human (164–166). It could suggest that IS*26* may contribute to the distribution of ARGs, including the distribution between different plasmids (167). Surprisingly, *mcr-3*, causing colistin resistance, was found. Several *mcr* genes have attracted global attention because colistin is last-line therapeutic drug against MDR bacteria. Since *mcr-1* was reported in 2016, *mcr* group is increasing worldwide (168). This is a serious concern because several documents reported that *mcr*-positive bacteria usually carry $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{KPC} or bla_{NDM} and are considered as extensively drug-resistant bacteria (169, 170).

Overall, studies on antimicrobial resistance in backyard farm in Thailand has rarely been done. Nevertheless, the high prevalence of bla_{CTX}-positive *E. coli* recovered various sources was common (112, 143, 171). One possilble explanation is that horizontal gene transfer of bla_{CTX}-carrying plasmid may contribute to the high dissemination of bla_{CTX}-positive *E. coli* in Thai communities.

Conclusion

This study determined the prevalence and dissemination of MDR-EC, including ESBL-EC and AmpC-EC, recovered from backyard poultry farms and poultry meat products in Phitsanulok province, Thailand. A total of 734 samples was obtained from backyard farms (farmer, poultry and farm environment, n = 587) and poultry meat samples (n = 147). Two-hundred and fifty (34.1%) EC-CTX isolates were recovered from all sources. Antimicrobial susceptibility testing using 24 drugs from 14 β-lactams and 10 non β-lactams revealed that all isolates were resistant to ampicillin, cephazolin, cefotaxime and cefpodoxime and were high resistant (> 60%) to many drugs in both β-lactam and non β-lactam categories. All isolates were confirmed as MDR-EC. In addition, 214 MDR-EC (85.6%) were ESBL-EC while 36 MDR-EC (14.4%) were AmpC-EC. A total of 219 MDR-EC (87.6%) was positive for the presence of $bla_{ESBL/AmpC}$. $bla_{CTX\text{-}M\ group\ 1}$ (54.8%) was the most common bla_{ESBL} followed by $bla_{CTX\text{-}M\ group\ 9}$ (23.6%), $bla_{SHV\text{-}2a}$ (0.4%) and $bla_{TEM\text{-}116}$ (0.4%) found in 198 ESBL-EC while $bla_{CMY\text{-}2}$ (58.3%) was the highest prevalence of bla_{AmpC} found in 21 AmpC-EC. $bla_{CTX\text{-}M}$ types were identified in 132 ESBL-EC from backyard farms. DNA sequencing revealed that $bla_{CTX\text{-}M\text{-}55}$ (56.8%) was the most common gene followed by $bla_{CTX\text{-}M\text{-}14}$ (30.3%), $bla_{CTX\text{-}M\text{-}15}$ (7.6%), $bla_{CTX\text{-}M\text{-}27}$ (4.5%) and $bla_{CTX\text{-}M\text{-}65}$ (0.8%). Phylogenetic grouping showed that group A and B1, commensal strains, were the most common groups (n = 204, 81.6%) while the prevalence of groups B2 and D, pathogenic strains, were 18.4% (46 isolates). Additionally, group B2 was found in all sources. Multi-locus sequence typing was performed on 14 isolates belonging to group B2. The results showed that sequence type (ST) 131, a highly virulent strain, was predominant (n = 7) followed by ST95, ST127, ST219 and ST8180 (1 isolate each) while 3 isolates were untypeable. ST95, ST127, ST219 and particularly ST131 were common ST causing extraintestinal infection reported worldwide. Randomly selected 30 isolates (5, 10 and 15 isolates possessed $bla_{CMY\text{-}2}$, $bla_{CTX\text{-}M\ group\ 1}$, and $bla_{CTX\text{-}M\ group\ 9}$, respectively) were used as donors in conjugation experiments. The results demonstrated that bla_{ESBL}/bla_{AmpC} plasmids from 27 donors could be transferred to recipient with high frequencies (1.8×10^{-7}–8.2×10^{-2}) and contributed the increase cefotaxime MICs in respective transconjugant (8–64 folds compare with recipient). This experiment could suggest that horizontal gene transfer may distribute $bla_{CTX\text{-}M}$

and *bla*$_{CMY-2}$ among MDR-EC. Moreover, identification of their plasmid replicon types revealed showed that IncF, including IncFIA and IncFIB, was the common Inc types. All replicon types found in this study have been known as narrow-host range plasmids, however, these types have commonly been known as antimicrobial-resistant gene-carrying plasmids. To study the dissemination of 132 ESBL-EC in backyard farms, pulsed-field gel electrophoresis (PFGE) was performed. A total of 132 ESBL-EC was available for analysis. The results showed 4 and 13 identical PFGE patterns of *bla*$_{CTX-M\ group\ 9}$- and $_{group\ 1}$-positive ESBL-EC, respectively, within the same and between farms, including cross-contamination between farmer and chicken as well as poultry and environment. Interestingly, clonal spread of *bla*$_{CTX-M-27}$-positive ST131 between human and chicken on different farms had occurred. Location of *bla*$_{CTX-M-55}$ was determined in 6 randomly selected *bla*$_{CTX-M-55}$-positive isolates by S1-PFGE and gel hybridization. Five isolates carried *bla*$_{CTX-M-55}$ on plasmid with 30–150 kb in sizes and 1 isolate carried gene on chromosome. Genetic context of *bla*$_{CTX-M-55}$ was determined by plasmid sequencing and the plasmid sequence (pEC278) was suscessfully contructed. The plasmid size was 97,423 bp which was similar to pCHL5009T recovered from patient in New Zealand. It shared 90.3% identity with > 97% query coverage. pEC278 carried multiple antimicrobial-resistant genes flanked by mobile genetic elements. Surprisingly, *mcr-3* conferring colistin resistance was also found on this plasmid. Genetic context of *bla*$_{CTX-M-55}$ was IS*26*-*catA2*-IS*26*-IS*Ecp1*-*bla*$_{CTX-M-55}$-Tn*3*-IS*3*-*qnrS1*-*res*-IS*26*-IS*4*-*aac(3)-IId*-IS*6*-Tn*3*-*mcr-3*-*dgkA*-IS*3*-IS*26*.

This study reported the high prevalence of MDR-EC, ESBL-EC and *bla*$_{CTX-M/CMY-2}$ among backyard poultry farms and poultry meat samples in Phitsanulok province. In addition, all isolates demonstrated the high resistant rates to many antimicrobial agents. Almost all isolates possessed *bla*$_{ESBL/AmpC}$, particularly *bla*$_{CTX-M}$ which could be transferred to other bacteria. Moreover, clonal spreads of ESBL-EC between human, poultry or environment within and between backyard farms were observed. Thus, backyard poultry farms and poultry meat samples may be an important reservoir of MDR-EC and may contribute to the spread of MDR-EC within Phitsanulok province.

Suggestion for further study

Although the restriction of antimicrobial consumption in food-producing animal has been admitted to resolve the increase of antimicrobial-resistant bacteria in food-producing animal and its products effectively, the decrease of those could not obviously be observed in short time (82). In fact, the high prevalence of antimicrobial-resistant bacteria in Thailand is not only *E. coli* but also other species, such as *K. pneumoniae*, *Salmonella* sp. and *Pseudomonas* sp., including other antimicrobial-resistant genes, such as carbepenemase-encoding genes and plasmid-mediated quinolone resistance (PMQR) genes, are infrequently studied. Thus, the investigation of antimicrobial-resistant bacteria in other species, genes and sources in Thailand is needed to prevent the spread of these organisms within communities.

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APPENDIX

APPENDIX A RESULTS

Table 15 General information of owners and 27 backyard poultry farms in this study

Characteristics of backyard poultry farms	Descriptions and numbers of farm
Owner information	
Age (median)	17–70 years (51.7)
Sex (male : female)	21 : 6
Educations (under : higher secondary school)	22 : 5
Main occupations (farmer : government officer)	24 : 3
Farm experience	6 months–> 10 years
Labors in farming (1 : >1 labor)	13 : 14
Farm management	
Location	3 Districts in Phitsanulok province, Northern Thailand: Bang Rakam, Muang, Wat Bot
Farm area	Within house area of owner
Objectives of farming	Commerce, consumption within household, hoppy, or recreational sports
Numbers of poultry	30–>300
Types of poultry	Native chicken, broiler, laying hen and duck
Farm systems (Cage only : cage and free range)	2 : 25
Types of animal (Poultry only: poultry with other animal)	17 : 10

Table 15 (cont.)

Characteristics of backyard poultry farms	Descriptions and numbers of farm
Animal feeds (Natural product : commercial feed : both)	15 : 3 : 9
Antimicrobial usage (No : use)	8 : 19
Farm cleanings (No : dry : wet cleaning)	3 : 19 : 5
Frequency of farm cleaning per month (No : 1 : > 1 time per month)	3 : 5 : 19

Table 16 Information of farm samples and numbers of *Escherichia coli* isolated from 27 backyard poultry farms

Farms	Districts	Date of collection	Samples						No. of EC-CTX (%)[a]	No. of ESBL-EC (%)[a]
			Farmers	Poultry		Environment		Total		
				Chicken	Ducks	Water	Soil			
1	Mueang	20/01/2014	3	17	3	5	7	35	14 (40.0)	13 (37.1)
2		20/01/2014	1	10	3	2	5	21	6 (28.6)	6 (28.6)
3		16/06/2014	1	24	0	0	0	25	12 (48.0)	12 (48.0)
4		20/01/2014	3	6	0	2	3	14	5 (35.7)	5 (35.7)
5		20/01/2014	3	3	3	3	3	15	11 (73.3)	11 (73.3)
6	Bang Rakam	22/02/2014	2	27	0	2	1	32	8 (25.0)	6 (18.8)
7		22/02/2014	1	11	0	2	3	17	12 (70.6)	10 (58.8)
8		22/02/2014	0	0	14	0	1	15	8 (53.3)	8 (53.3)
9		13/03/2014	1	19	4	0	1	25	6 (24.0)	6 (24.0)
10		14/06/2014	0	24	0	0	1	25	8 (32.0)	7 (28.0)
11		14/06/2014	0	20	0	1	1	22	6 (27.3)	5 (22.7)
12		24/08/2014	1	30	0	1	2	34	5 (14.7)	5 (14.7)

[a] EC-CTX: *E. coli* with reduced susceptibility to cefotaxime and ESBL-EC: extended-spectrum β-lactamase *E. coli*

Table 16 (cont.)

Farms	Districts	Date of collection	Samples					Total	No. of EC-CTX (%)[a]	No. of ESBL-EC (%)[a]
			Farmer	Poultry		Environment				
				Chicken	Ducks	Water	Soil			
13	Bang Rakam	24/08/2014	1	41	0	2	2	46	9 (19.6)	9 (19.6)
14		15/09/2014	1	31	0	3	0	35	17 (48.6)	15 (42.9)
15		26/01/2014	1	13	0	1	1	16	2 (12.5)	2 (12.5)
16		26/01/2014	1	15	0	2	3	21	3 (14.3)	3 (14.3)
17		26/01/2014	0	9	0	2	1	12	4 (33.3)	4 (33.3)
18		22/02/2014	1	23	0	1	2	27	7 (25.9)	7 (25.9)
19		14/06/2014	2	19	0	0	0	21	4 (19.0)	4 (19.0)
20		15/09/2014	1	17	0	2	1	21	5 (23.8)	3 (14.3)
21	Wat Bot	13/02/2014	1	8	0	1	4	14	0	0
22		13/02/2014	1	13	0	0	2	16	0	0
23		13/02/2014	2	8	2	1	2	15	2 (13.3)	2 (13.3)
24		13/02/2014	2	8	0	1	2	13	8 (61.5)	8 (61.5)
25		13/02/2014	0	6	5	0	2	13	3 (23.1)	3 (23.1)
26		13/02/2014	2	12	4	0	2	20	1 (5.0)	0
27		13/02/2014	0	12	3	0	2	17	6 (35.3)	5 (29.4)
Total			**32**	**426**	**41**	**34**	**54**	**587**	**172 (29.3)**	**159 (27.1)**

Table 17 Information of meat samples and number of *E. coli* isolated from 28 open-air markets

Open-air markets	Districts	Date of collection	Meat samples (n)				No. of EC-CTX (%)[a]	No. of ESBL-EC (%)[a]
			Chicken	Ducks	Birds	Total		
1	Bang Kratum	16/09/2013	3	0	1	4	2 (50.0)	2 (50.0)
2	Bang Rakam	22/09/2013	3	0	0	3	1 (33.3)	1 (33.3)
		13/10/2013	2	0	1	3	2 (66.7)	2 (66.7)
3		22/09/2013	4	0	0	4	2 (50.0)	1 (25.0)
4	Chat Trakan	21/10/2013	2	0	0	2	2 (100)	1 (50.0)
		27/10/2013	2	0	0	2	2 (100)	1 (50.0)
5	Mueang	22/09/2013	8	0	0	8	4 (50.0)	3 (37.5)
6		23/09/2013	6	0	0	6	3 (50.0)	2 (33.3)
7		23/09/2013	17	0	0	17	5 (29.4)	4 (23.5)
8		23/09/2013	3	0	0	3	1 (33.3)	1 (33.3)
9		28/09/2013	2	0	0	2	0	0
10		29/09/2013	3	0	0	3	2 (66.7)	2 (66.7)
11		29/09/2013	1	0	0	1	1 (100)	1 (100)
12		29/09/2013	2	0	0	2	1 (50.0)	1 (50.0)
13		29/09/2013	2	0	0	2	1 (50.0)	0
14		30/09/2013	1	0	0	1	1 (100)	1 (100)
15		30/09/2013	1	0	0	1	0	0
16		4/10/2013	3	1	1	5	3 (60.0)	3 (60.0)
17		4/10/2013	4	0	1	5	1 (20.0)	0
18		5/10/2013	2	0	3	5	1 (60.0)	1 (60.0)
19		5/10/2013	2	0	3	5	2 (40.0)	1 (20.0)
20		13/10/2013	1	0	0	1	1 (100)	1 (100)
21		15/10/2013	0	0	3	3	3 (100)	3 (100)
22	Nakhon Thai	7/11/2013	19	0	0	19	19 (100)	6 (31.6)
23	Noen Maprang	16/09/2013	3	0	1	4	2 (50.0)	1 (25.0)
24	Phrom Phiram	16/09/2013	3	0	0	3	1 (33.3)	1 (33.3)
		27/10/2013	5	0	3	8	3 (37.5)	3 (37.5)

[a] EC-CTX: *E. coli* with reduced susceptibility to cefotaxime and ESBL-EC: extended-spectrum β-lactamase *E. coli*

Table 17 (cont.)

Open-air markets	Districts	Date of collection	Meat samples (n)				No. of EC-CTX (%)*	No. of ESBL-EC (%)*
			Chicken	Ducks	Bird	Total		
25	Wang Thong	29/09/2013	6	1	1	8	3 (37.5)	3 (37.5)
26		29/09/2013	6	1	0	7	4 (51.7)	4 (51.7)
27		29/09/201	2	0	0	2	0	0
		27/10/2013	2	0	0	2	2 (100)	2 (100)
28	Wat Bot	16/09/2013	3	0	0	3	2 (66.7)	2 (66.7)
		23/09/2013	3	0	0	3	1 (33.3)	1 (33.3)
	Total		**126**	**3**	**18**	**147**	**78 (53.1)**	**55 (37.4)**

(a) **(b)**

Figure 13 Isolation of *Escherichia coli* with reduced susceptibility to cefotaxime on Eosin-Methylene Blue (EMB) agar
(a) antimicrobial agent free
(b) supplemented with 2 mg/L cefotaxime

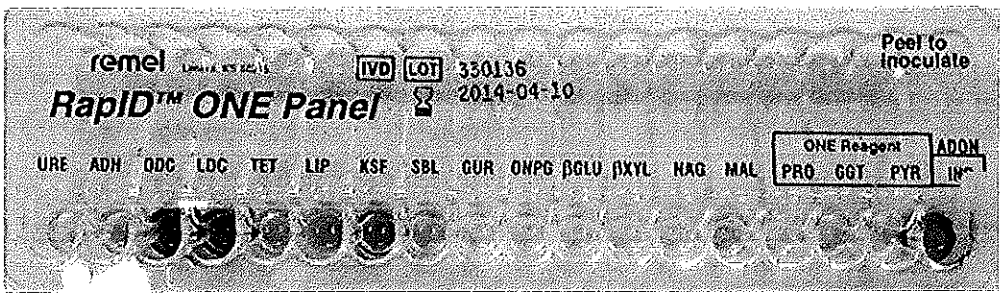


Figure 14 Biochemical test of *Escherichia coli* using RapID^{TM} ONE System (REMEL, KS, USA)

```
                10        20        30        40        50        60        70        80        90
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA GCTTCTTTAAGGTAAGGAGGTGATCCAACCGCAGGTTCCCCTACGGTTACCTTGTTACGACTTCACCCCAGTCATGAATCACAAAGTGGT
EC210    ------------------------------------------------------------------------------------------
                100       110       120       130       140       150       160       170       180
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA
         AAGCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTAT
EC210    ----------..........................................................................
                190       200       210       220       230       240       250       260       270
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA TCACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTAT
EC210    ..........................................................................................
                280       290       300       310       320       330       340       350       360
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA GAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTG
EC210    ..........................................................................................
                370       380       390       400       410       420       430       440       450
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA ACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAACAAAGGATAAGGGTTGCGCT
EC210    ..........................................................................................
                460       470       480       490       500       510       520       530       540
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA CGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCACAGTTCCCGAAGGCACCAATCCATC
EC210    ..........................................................................................
                550       560       570       580       590       600       610       620       630
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA TCTGGAAAGTTCTGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC
EC210    ..........................................................................................
                640       650       660       670       680       690       700       710       720
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA GTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACA
EC210    ..........................................................................................
                730       740       750       760       770       780       790       800       810
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA ACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTC
EC210    ..........................................................................................
                820       830       840       850       860       870       880       890       900
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA GTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACT
EC210    ..........................................................................................
                910       920       930       940       950       960       970       980       990
                |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA CAAGCTTGCCAGTATCAGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAAACCGCCTGCGTGCGCTTTACGCCCA
EC210    ..........................................................................................
```

Figure 15 Comparison of nucleotide sequences of 16S rRNA gene between *Escherichia coli* (Accession number: CP009859) and *E. coli* EC150 in this study

Dot (.) is identical nucleotide and dash line (-) denotes that nucleotides are not performed.

```
                1000      1010      1020      1030      1040      1050      1060      1070      1080
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA GTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATGAG
EC210    ..................................................................................................

                1090      1100      1110      1120      1130      1140      1150      1160      1170
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA CAAAGGTATTAACTTTACTCCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGG
EC210    ..................................................................................................

                1180      1190      1200      1210      1220      1230      1240      1250      1260
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA CTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAG
EC210    ..................................................................................................

                1270      1280      1290      1300      1310      1320      1330      1340      1350
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16s rRNA ACCAGCTAGGGATCGTCGCCTAGGTGAGCCTTTACCCCACCTACTAGCTAATCCCATCTGGGCACATCCGATGGCAAGAGGCCCGAAGGT
EC210    ..................................................................................................

                1360      1370      1380      1390      1400      1410      1420      1430      1440
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA CCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTC
EC210    ..................................................................................................

                1450      1460      1470      1480      1490      1500      1510      1520      1530
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
16S rRNA ACCCGTCCGCCACTCGTCAGCAAAGAAGCAAGCTTCTTCCTGTTACCGTTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTG
EC210    ..................................------------------------------------------------------

                1540      1550
                ....|....|....|....|.
16S rRNA AGCCATGATCAAACTCTTCAATTTAA
EC210    -------------------------
```

Figure 15 (cont.)

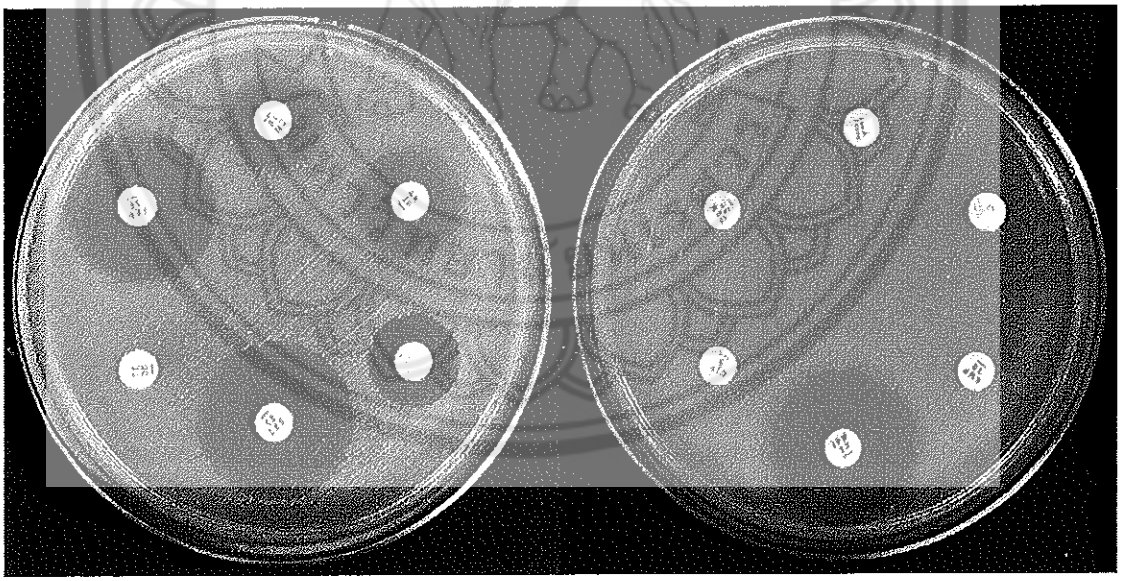


Figure 16 Antimicrobial susceptibility testing by disc diffusion method

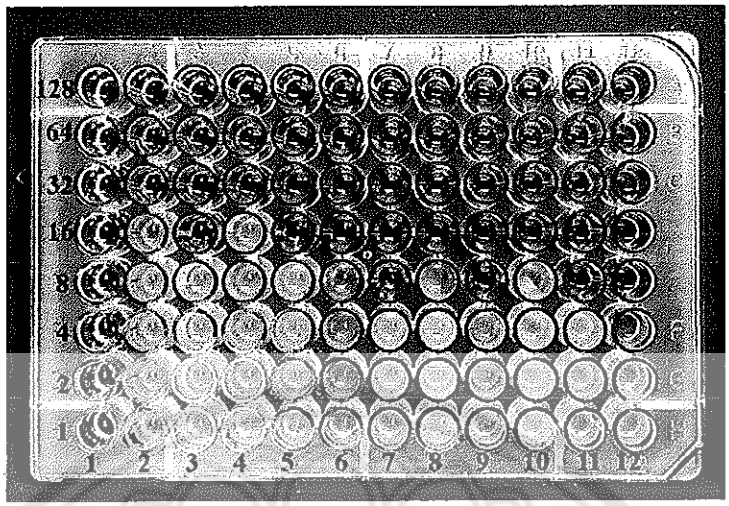
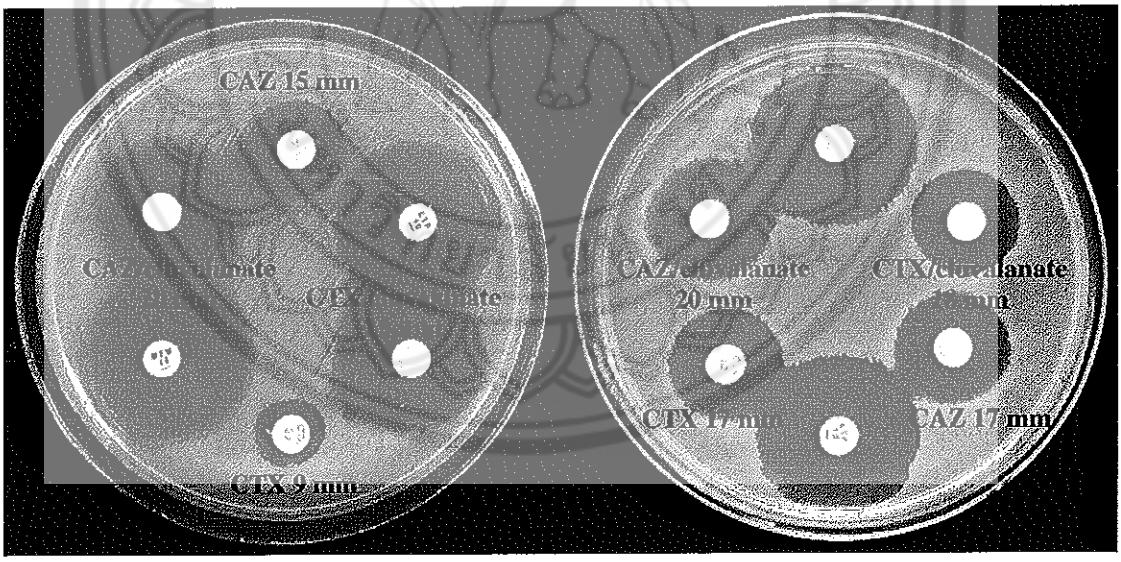


Figure 17 Minimum inhibitory concentration (MIC) of ceftazidime ranging 1–128 mg/L using broth microdilution
Lane 1 is a negative control. Lane 2–12 is EC155, 254, 265, 268, 276, 296, 297, 299, 354, 363, 259, respectively.



(a) (b)

Figure 18 Detection of ESBL production using combined-disc method
(a) ESBL-producing *E. coli*, (b) ESBL-negative *E. coli*
The isolate producing ESBL was interpreted from a ≥ 5 mm increase in a zone diameter of either CAZ/clavulanate or CTX/clavulanate compared with a zone diameter of the antimicrobial agents without the combination of clavulanate

Table 18 Numbers of antimicrobial agent resistance of MDR-EC

Samples	No. of *E. coli* isolates (%)					
	≤ 13	**14**	**15**	**16**	**17**	**≥ 18**
Backyard farms						
Farmers (19)	12 (63.1)	3 (15.8)	1 (5.3)	2 (10.5)	1 (5.30	0
Poultry (126)	88 (69.8)	10 (7.9)	8 (6.4)	11 (8.7)	6 (4.8)	3 (2.4)
- Chickens (111)	74	9	6	9	5	3
- Ducks (15)	9	1	2	2	1	0
Environment (27)	20 (74.1)	2 (7.4)	1 (3.7)	3 (11.1)	1 (3.7)	0
- Water (15)	9	1	1	2	0	0
- Soil (12)	8	1	0	0	0	0
Subtotal (172)	120 (69.8)	15 (8.7)	10 (5.8)	16 (9.3)	8 (4.7)	3 (1.7)
Meat samples						
Chicken meat (69)	29	6	2	5	2	2
Bird meat (9)	5	2	1	1	0	0
Subtotal (78)	55 (70.5)	9 (11.5)	3 (3.9)	6 (7.7)	2 (2.5)	3 (3.9)
Total (250)	**175 (70.0)**	**24 (9.6)**	**13 (5.2)**	**22 (8.8)**	**10 (4.0)**	**6 (2.4)**

Table 19 Rates of antimicrobial resistance of ESBL-EC isolated from backyard farms and meat samples

Antimicrobial agents / phenotypes	No. of ESBL-EC (%)					
	Backyard poultry farm				Poultry meat samples (n = 55)	Total (n = 214)
	Farmers (n = 16)	Poultry (n = 121)	Environment (n = 22)	Subtotal (n = 159)		
ampicillin	16 (100)	121 (100)	22 (100)	159 (100)	55 (100)	**214 (100)**
cefazolin	16 (100)	121 (100)	22 (100)	159 (100)	55 (100)	**214 (100)**
cefuroxime	16 (100)	121 (100)	22 (100)	121 (100)	53 (96.4)	**174 (81.3)**
cefoxitin	0	2 (1.7)	0	2 (1.3)	6 (10.9)	**8 (3.7)**
cefpodoxime	16 (100)	121 (100)	22 (100)	159 (100)	55 (100)	**214 (100)**
cefotaxime	16 (100)	121 (100)	22 (100)	159 (100)	55 (100)	**214 (100)**
ceftazidime	9 (56.3)	60 (49.6)	14 (63.6)	83 (52.2)	40 (72.7)	**123 (57.3)**
cefepime	11 (68.8)	67 (55.4)	12 (54.5)	90 (56.6)	39 (70.9)	**129 (60.3)**
imipenem	0	0	0	0	2 (3.6)	**2 (0.9)**
meropenem	0	0	0	0	2 (3.6)	**2 (0.9)**
ertapenem	0	0	0	0	0	**0**
aztreonam	12 (75.0)	91 (75.2)	19 (86.4)	122 (76.7)	50 (90.9)	**172 (80.4)**
ampicillin/sulbactam	9(56.3)	53 (43.8)	7 (31.8)	69 (43.4)	23 (41.8)	**92 (43.0)**
amoxicillin/cluvulanate	1 (6.3)	3 (2.5)	0	4 (2.5)	9 (16.4)	**13 (6.1)**
amikacin	1 (6.3)	8 (6.6)	1 (4.5)	10 (6.3)	6 (10.9)	**16 (7.5)**
gentamicin	6 (37.5)	52 (43.0)	7 (31.8)	65 (40.9)	18 (32.7)	**83 (38.8)**
streptomycin	12 (75.0)	91 (75.2)	14 (63.6)	117 (73.6)	44 (80.0)	**161 (75.2)**
doxycycline	7 (43.8)	74 (61.2)	12 (54.5)	93 (58.5)	29 (52.7)	**122 (57.0)**
tetracycline	13 (81.9)	103 (85.1)	17 (77.3)	133 (83.6)	47 (85.5)	**180 (84.1)**
nalidixic acid	8 (50.0)	36 (29.8)	7 (31.8)	51 (32.1)	17 (30.9)	**68 (31.8)**
ciprofloxacin	5 (31.3)	35 (28.9)	7 (31.8)	47 (29.6)	15 (27.3)	**62 (29.0)**
levofloxacin	4 (25.0)	31 (25.6)	6 (27.3)	41 (25.8)	16 (29.1)	**57 (26.6)**
chloramphenicol	10 (62.5)	73 (60.3)	11 (50.0)	94 (59.1)	29 (52.7)	**123 (57.5)**
trimethroprim/sulfamethaxazole	12 (75.0)	78 (64.5)	10 (45.5)	100 (62.9)	36 (65.5)	**136 (63.6)**

Table 20 Distribution of β-lactamase encoding genes (*bla*) of *E. coli* isolated from backyard farms and meat samples

Genes (n)	No. of *E. coli* isolates						
	Farmers	Poultry		Environment		Poultry meat	
		Chickens	Ducks	Water	Soil	Chicken	Birds
$bla_{\text{CTX-M group 1}}$ (92)	6	44	7	5	5	23	2
$bla_{\text{CTX-M group 9}}$ (35)	2	17	5	4	2	2	3
$bla_{\text{CMY-2}}$ (19)	0	2	0	2	1	14	0
$bla_{\text{TEM-1}}$ (10)	1	4	0	0	1	4	0
$bla_{\text{SHV-2a}}$ (1)	0	0	0	0	0	1	0
$bla_{\text{TEM-116}}$ (1)	0	0	0	0	0	1	0
$bla_{\text{SHV-26}}$ (1)	0	0	0	0	0	0	1
$bla_{\text{CTX-M-group 1}}$ + $bla_{\text{TEM-1}}$ (44)	2	25	2	3	1	10	1
$bla_{\text{CTX-M-group 9}}$ + $bla_{\text{TEM-1}}$ (24)	5	16	1	0	0	1	1
$bla_{\text{CMY-2}}$ + $bla_{\text{TEM-1}}$ (2)	0	0	0	0	1	1	0
$bla_{\text{SHV-28}}$ + $bla_{\text{CTX-M group 1}}$ + $bla_{\text{TEM-1}}$ (1)	0	0	0	0	0	1	0
No gene (20)	3	3	0	1	1	11	1

Table 21 Distribution of β-lactamase encoding genes (*bla*) among ESBL-positive and -negative *E. coli*

Genes	No. of *E. coli* isolates (n)	
	ESBL-positive *E. coli*	ESBL-negative *E. coli*
$bla_{\text{CTX-M group 1}}$ (92)	92	0
$bla_{\text{CTX-M group 9}}$ (35)	35	0
$bla_{\text{CMY-2}}$ (19)	0	19
$bla_{\text{TEM-1}}$ (10)	6	4
$bla_{\text{SHV-2a}}$ (1)	1	0
$bla_{\text{TEM-116}}$ (1)	1	0
$bla_{\text{SHV-26}}$ (1)	1	0
$bla_{\text{CTX-M-group 1}}$ + $bla_{\text{TEM-1}}$ (44)	44	0
$bla_{\text{CTX-M-group 9}}$ + $bla_{\text{TEM-1}}$ (24)	24	0
$bla_{\text{CMY-2}}$ + $bla_{\text{TEM-1}}$ (2)	0	2
$bla_{\text{SHV-28}}$ + $bla_{\text{CTX-M group 1}}$ + $bla_{\text{TEM-1}}$ (1)	1	0
No gene (20)	9	11

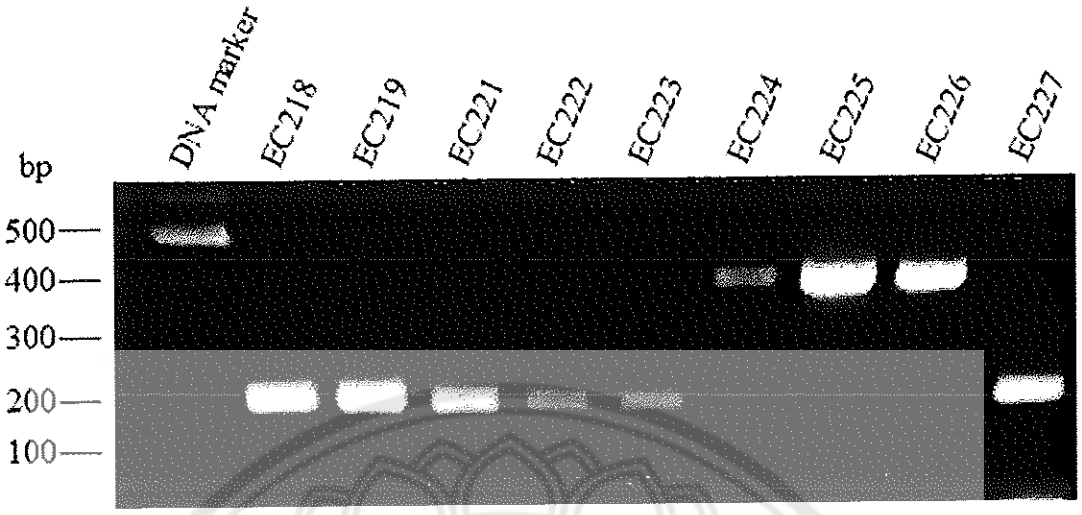


Figure 19 Multiplex PCR detection of *bla*$_{\text{CTX-M}}$
DNA marker: 100 bp Sharp Ladder (RBC Bioscience, Taiwan)
EC218, 219, 221, 222, 223 and 227 represent positive results of *bla*$_{\text{CTX-M group 9}}$. EC224, 225 and 226 represent positive results of *bla*$_{\text{CTX-M group 9}}$.

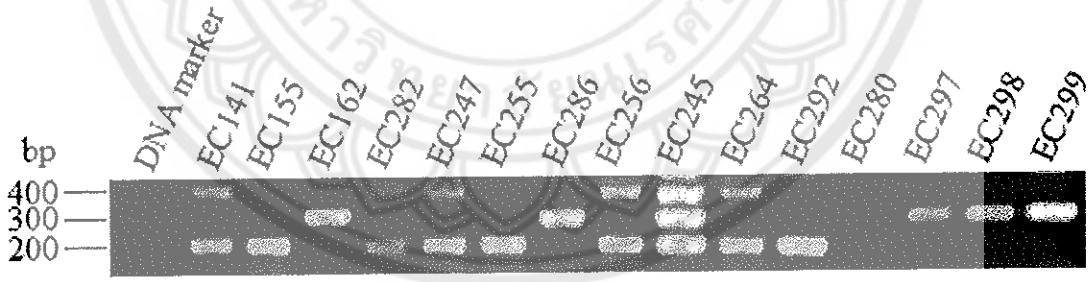


Figure 20 Multiplex-PCR of phylogenetic groups of MDR-EC
DNA marker: 100 bp Sharp Ladder (RBC Bioscience, Taiwan)
EC162, 286, 297, 298 and 299 represent group A.
EC155, 255, 292 and 280 represent group B1.
EC245 represents group B2.
EC141, 282, 247, 256 and 264 represent group D.

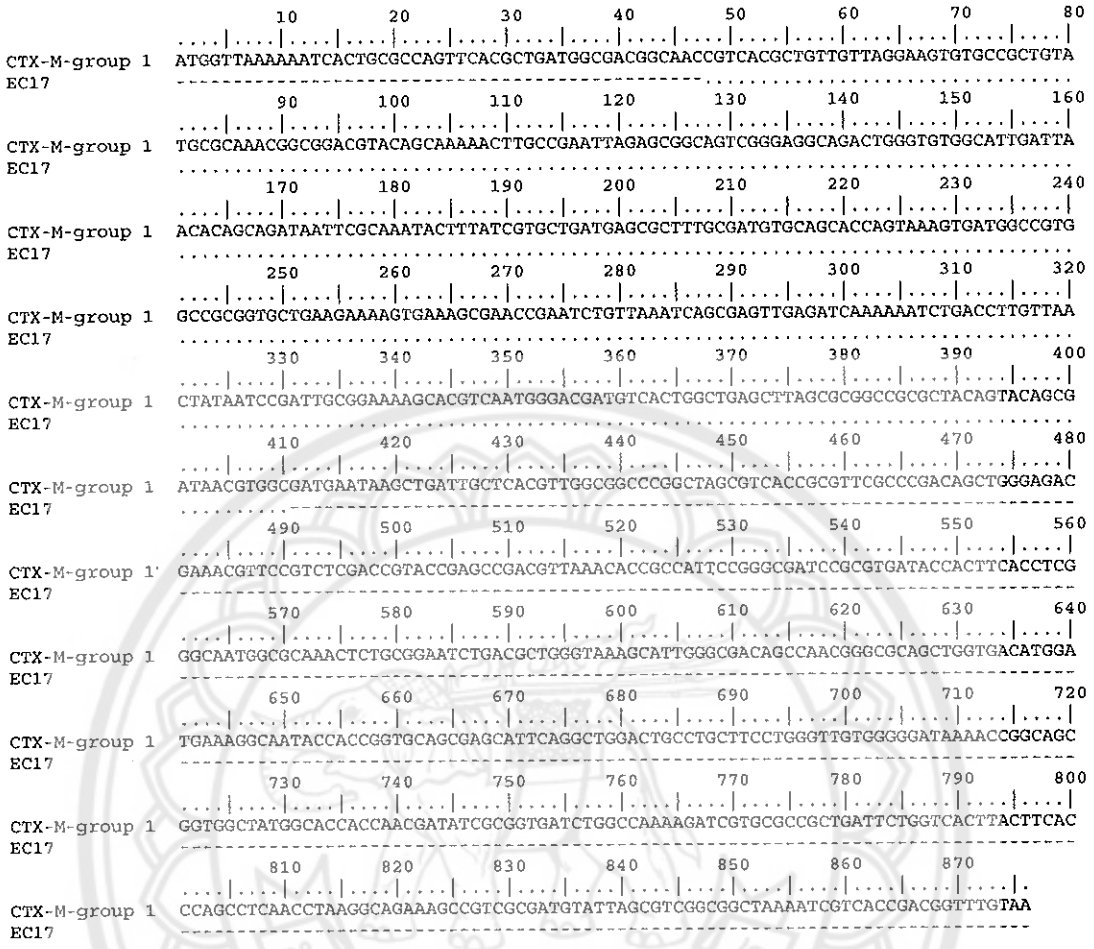


Figure 21 Comparison of nucleotide and amino acid sequences between *bla*$_{\text{CTX-M group 1}}$ (Accession number: DQ885477) and *bla*$_{\text{CTX-M group 1}}$ detected in *E. coli* (EC17)

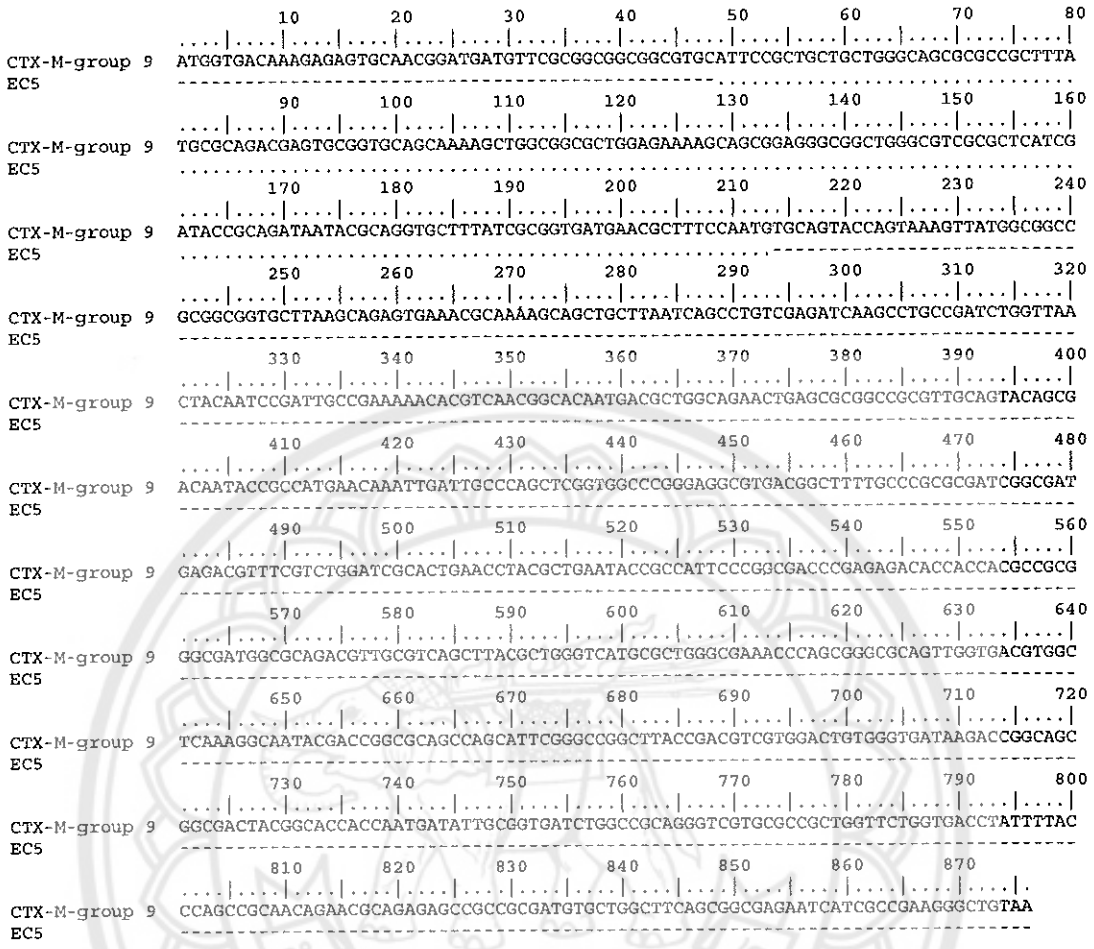


Figure 22 Comparison of nucleotide and amino acid sequences between *bla*$_{\text{CTX-M group 9}}$ (Accession number: AF252622) and *bla*$_{\text{CTX-M group 9}}$ detected in *E. coli* isolates (EC5)

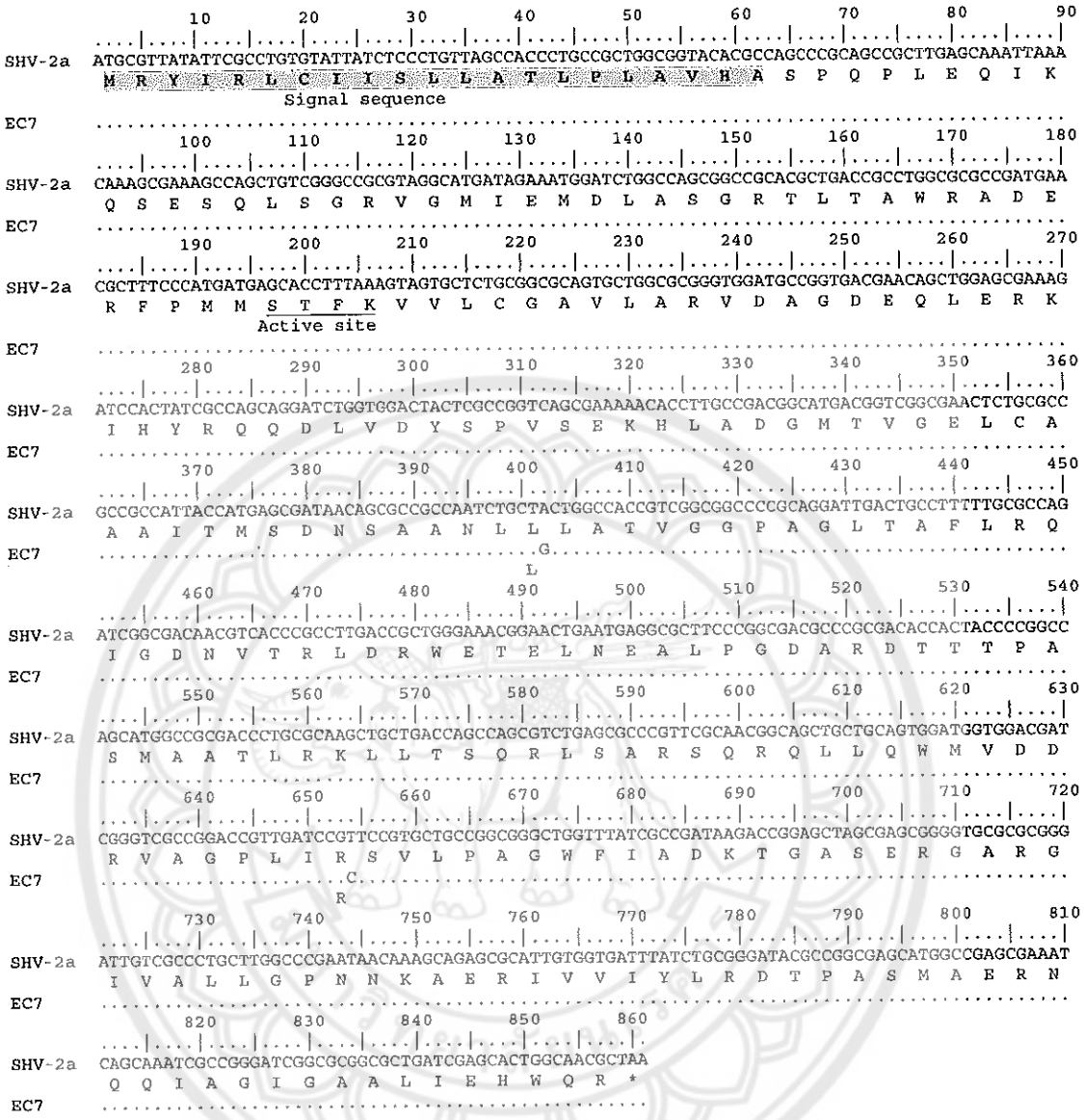


Figure 23 Comparison of nucleotide and amino acid sequences between *bla*$_{SHV-2a}$ (Accession number: X98102) and *bla*$_{SHV-2a}$ detected in *E. coli* (EC7)

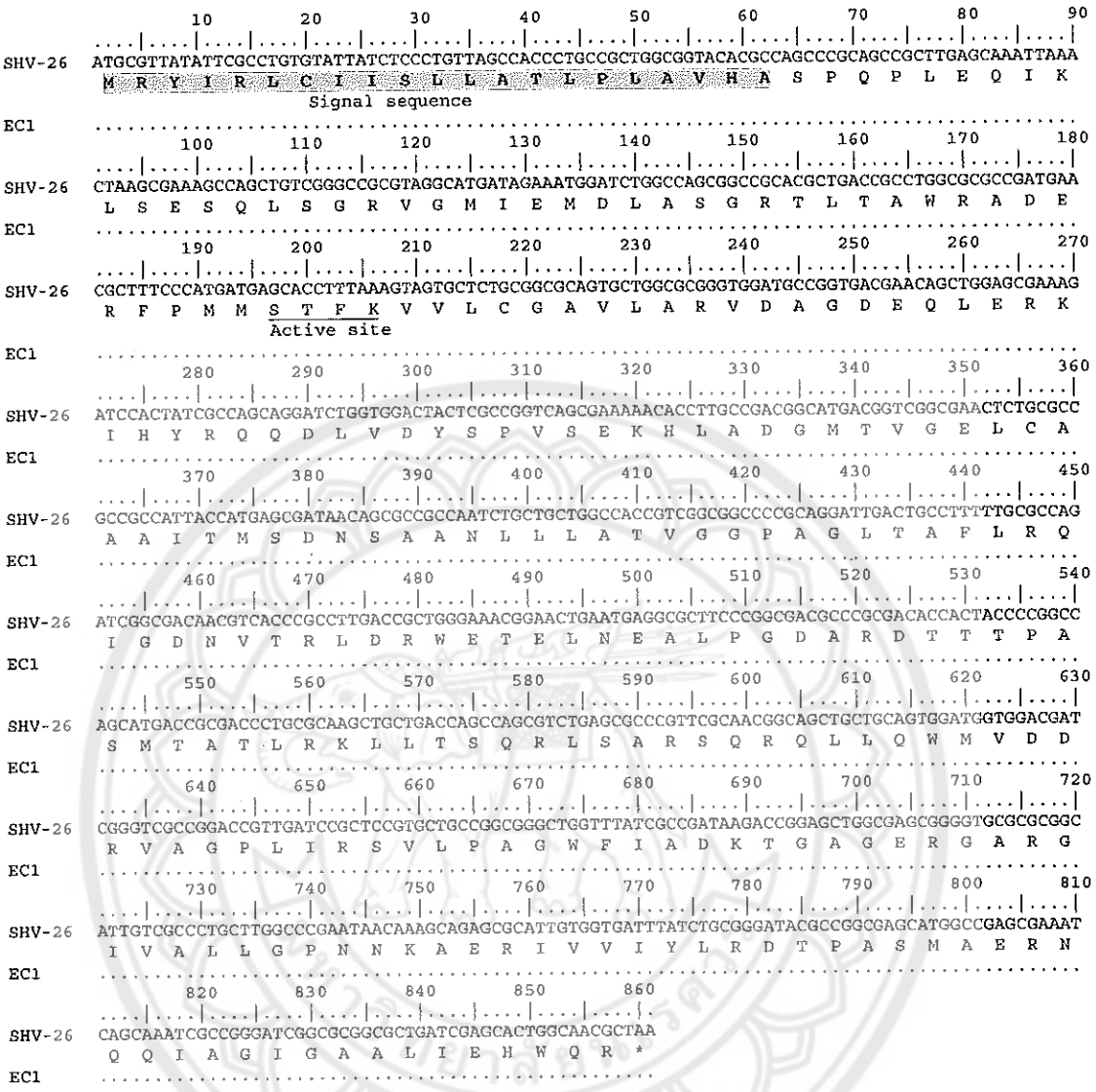


Figure 24 Comparison of nucleotide and amino acid sequences between *bla*$_{SHV-26}$ (Accession number: KF585144) and *bla*$_{SHV-26}$ detected in *E. coli* (EC1)

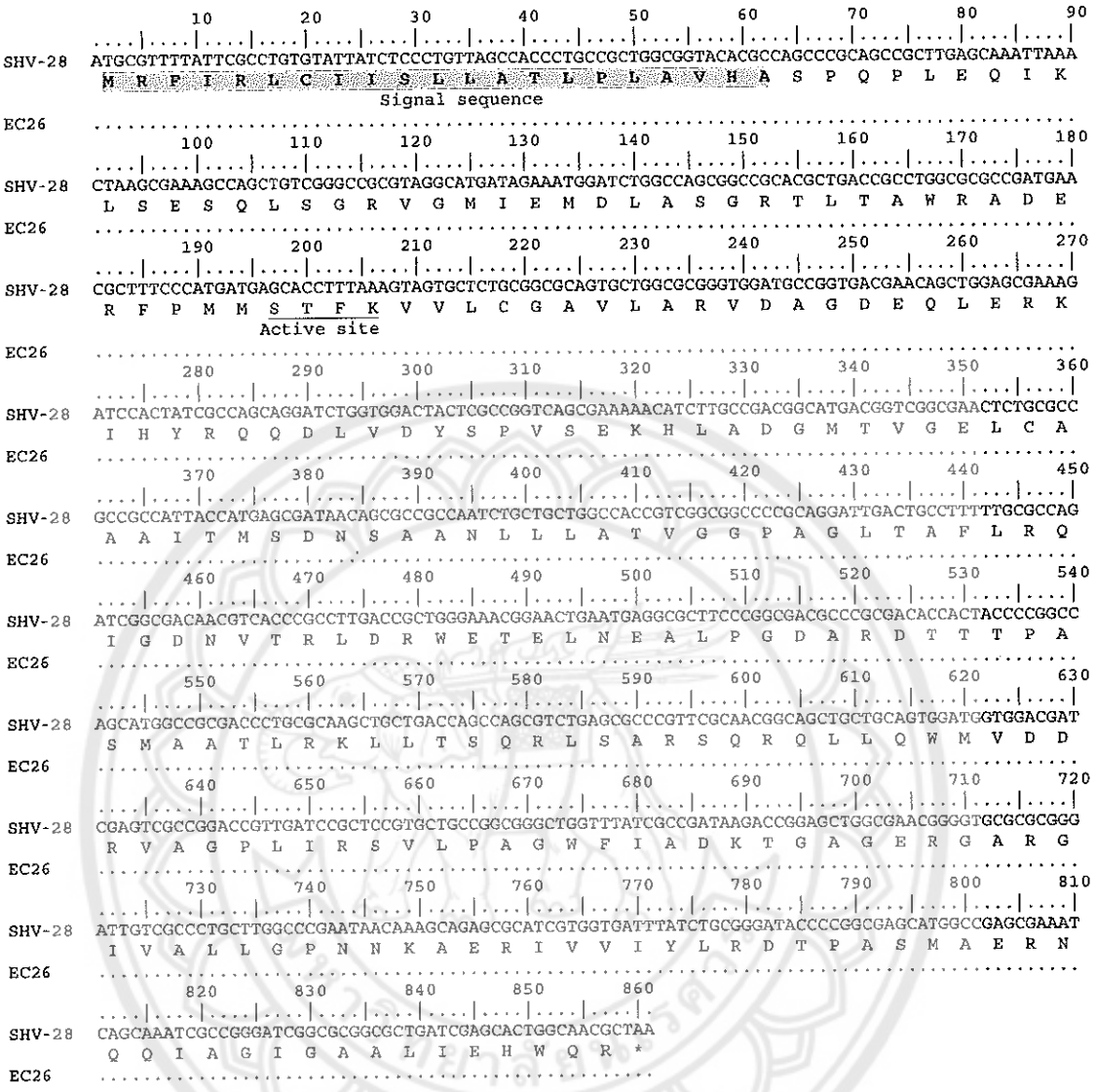


Figure 25 Comparison of nucleotide and amino acid sequences between *bla*$_{SHV-28}$ (Accession number: EU418912) and *bla*$_{SHV-28}$ detected in *E. coli* (EC26)

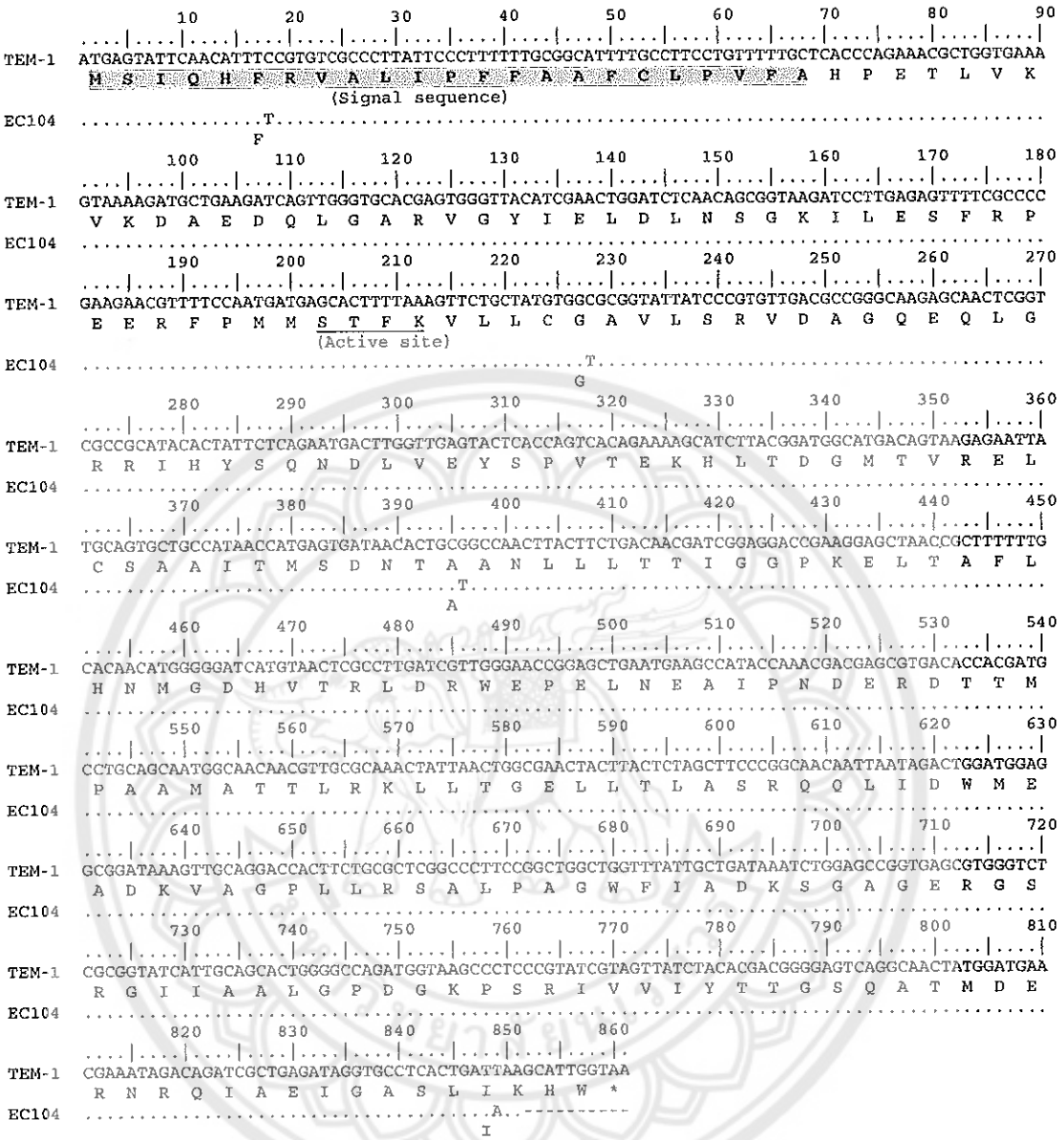


Figure 26 Comparison of nucleotide and amino acid sequences between *bla*$_{TEM-1}$ (Accession number: J01749) and *bla*$_{TEM-1}$ detected in *E. coli* (EC104)

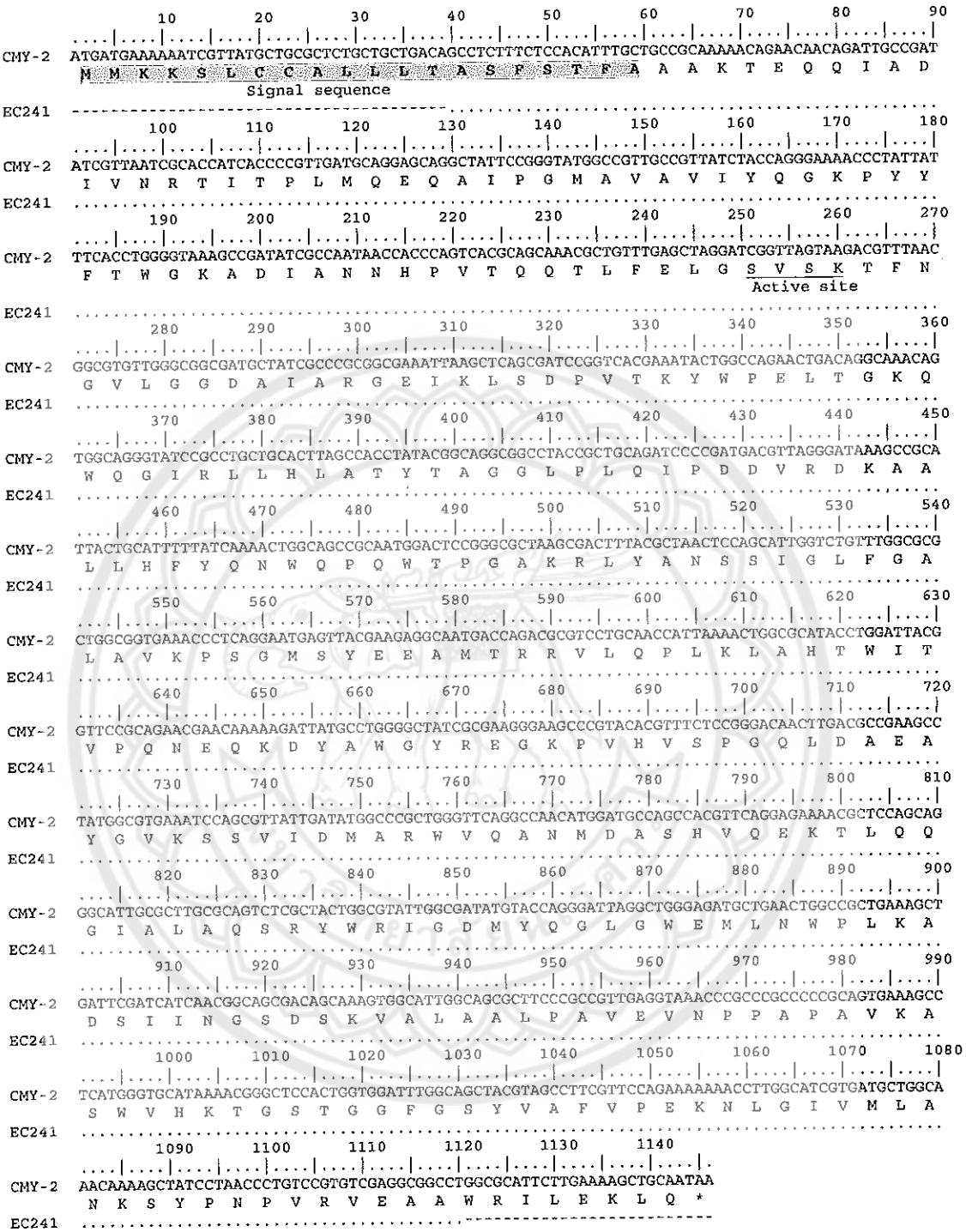


Figure 27 Comparison of nucleotide and amino acid sequences between *bla*$_{CMY-2}$ (Accession number: X91840) and *bla*$_{CMY-2}$ detected in *E. coli* (EC241)

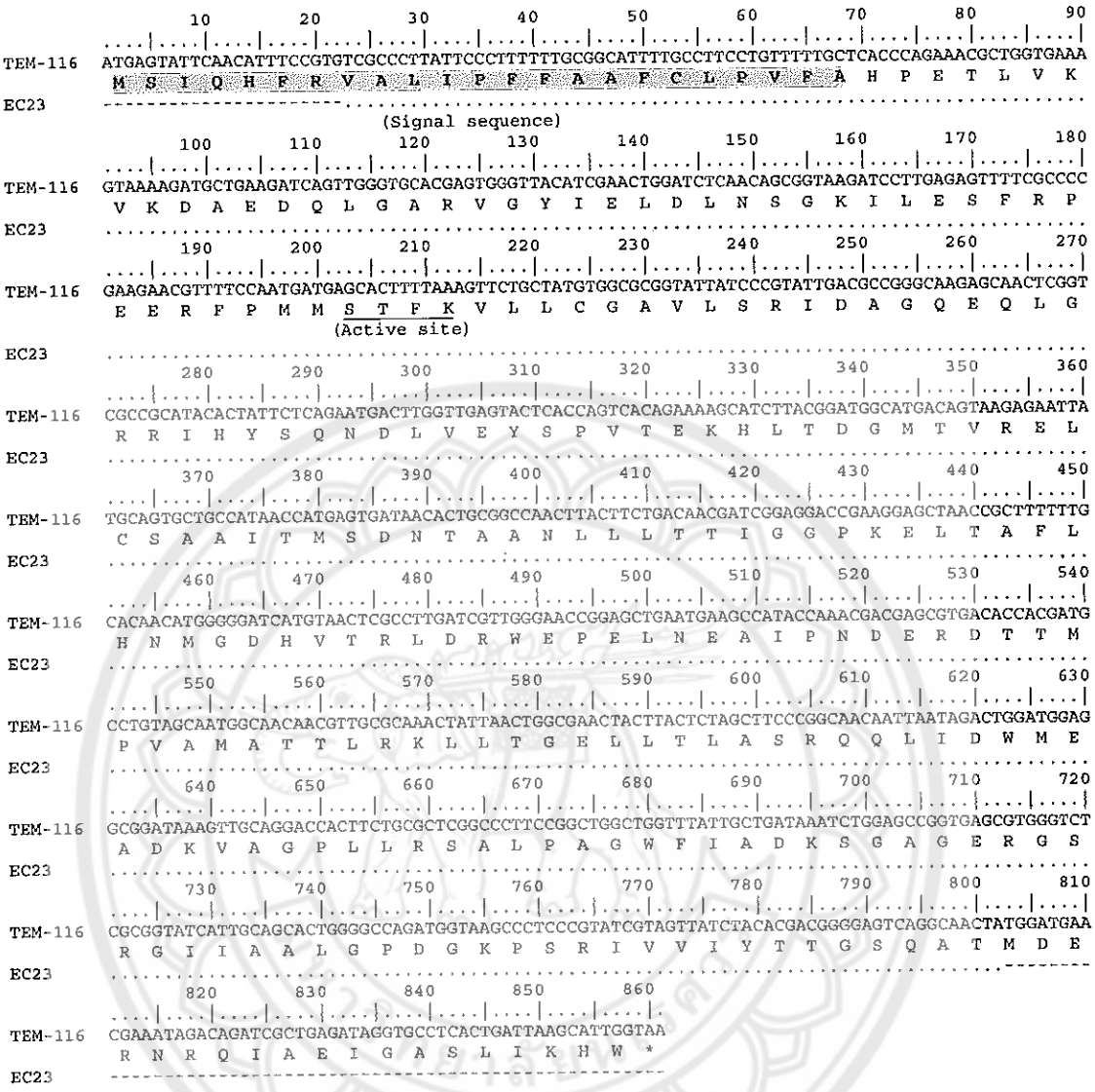


Figure 28 Comparison of nucleotide and amino acid sequences between *bla*$_{TEM-116}$ (Accession number: U36911) and *bla*$_{TEM-116}$ detected in *E. coli* (EC23)

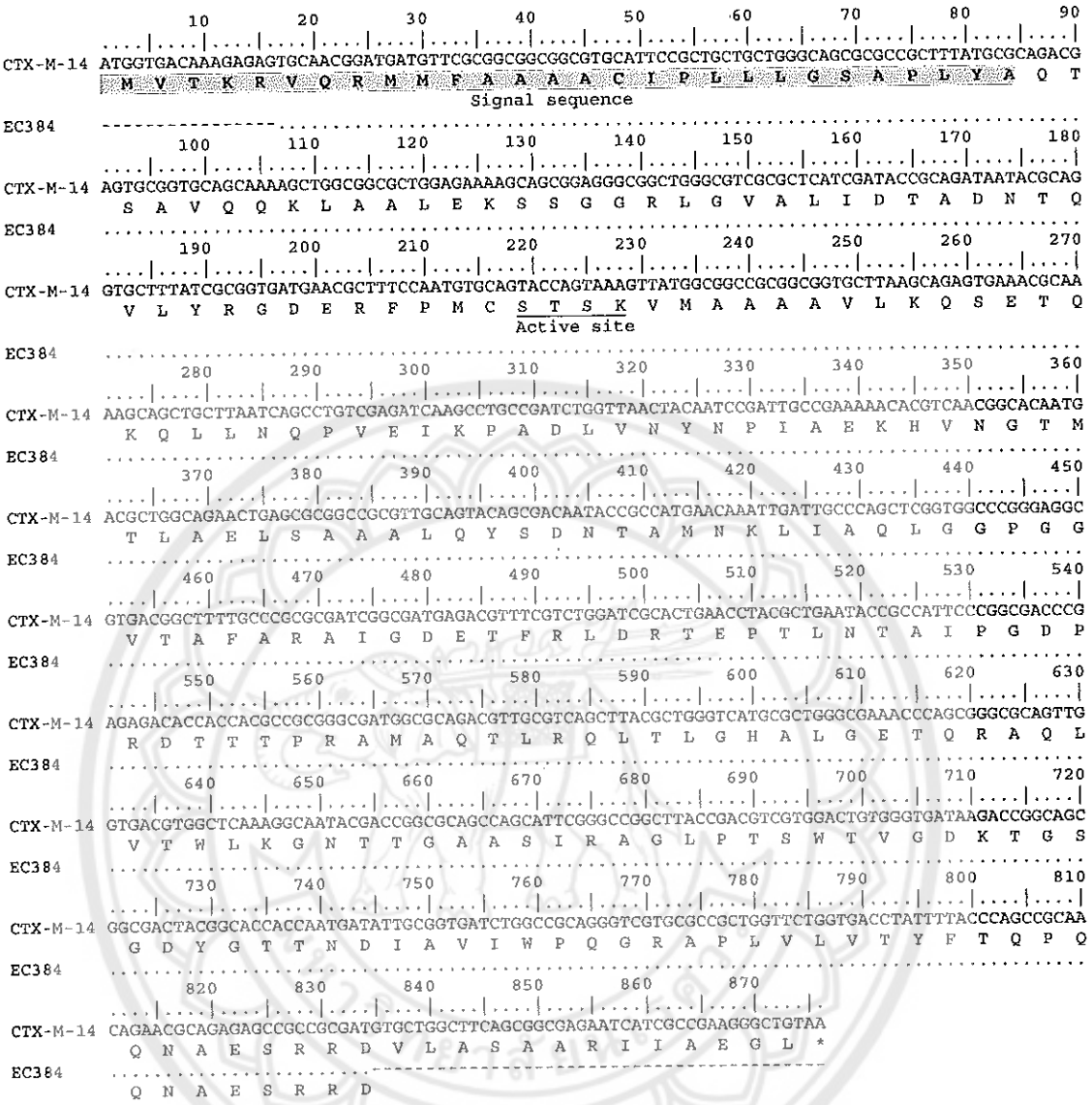


Figure 29 Comparison of nucleotide and amino acid sequences between *bla*$_{\text{CTX-M-14}}$ (Accession number: AF252622) and *bla*$_{\text{CTX-M}}$ detected in *E. coli* isolates (EC384)

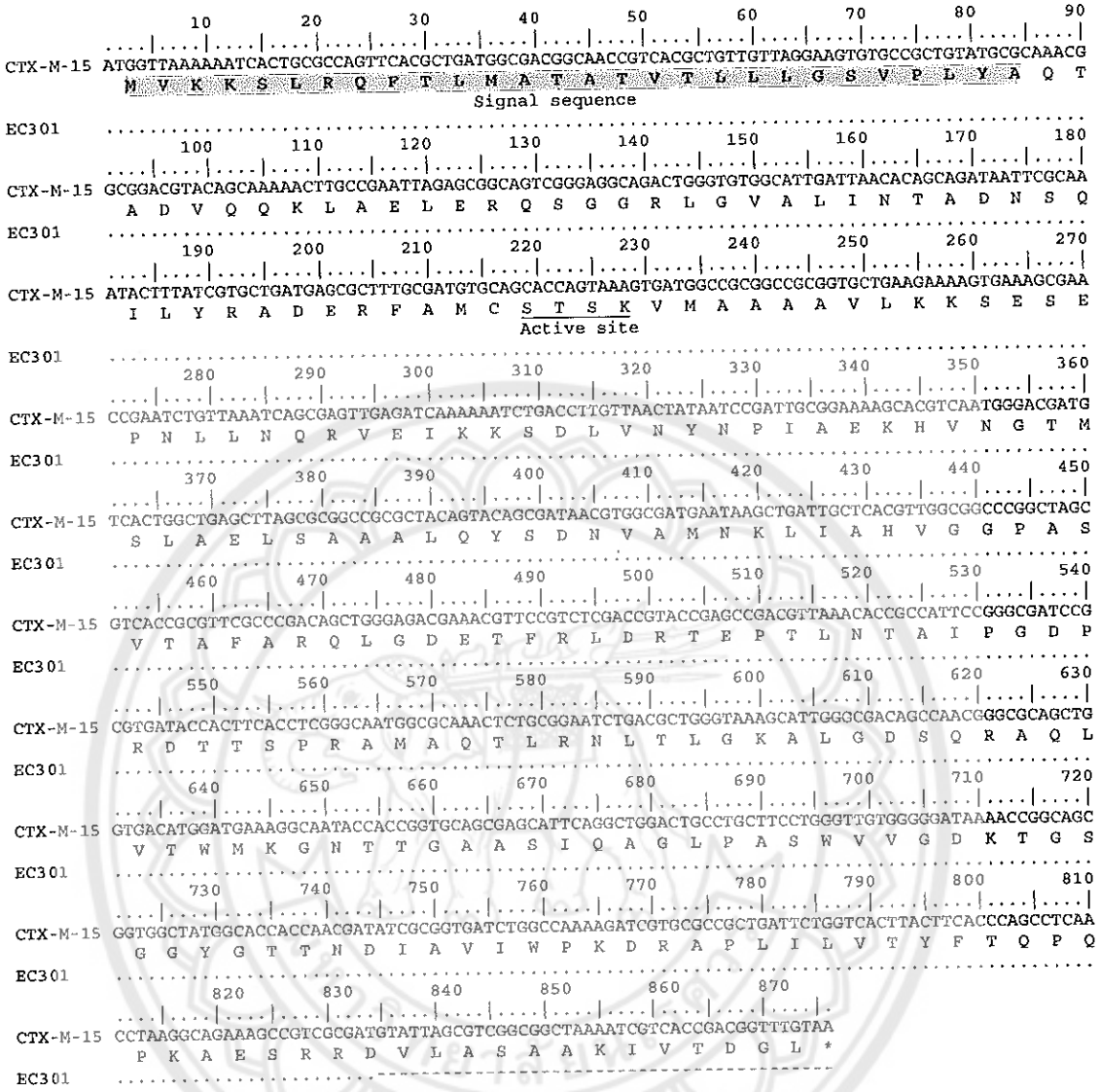


Figure 30 Comparison of nucleotide and amino acid sequences between *bla*$_{CTX-M-15}$ (Accession number: AY044436) and *bla*$_{CTX-M}$ detected in *E. coli* isolates (EC301)

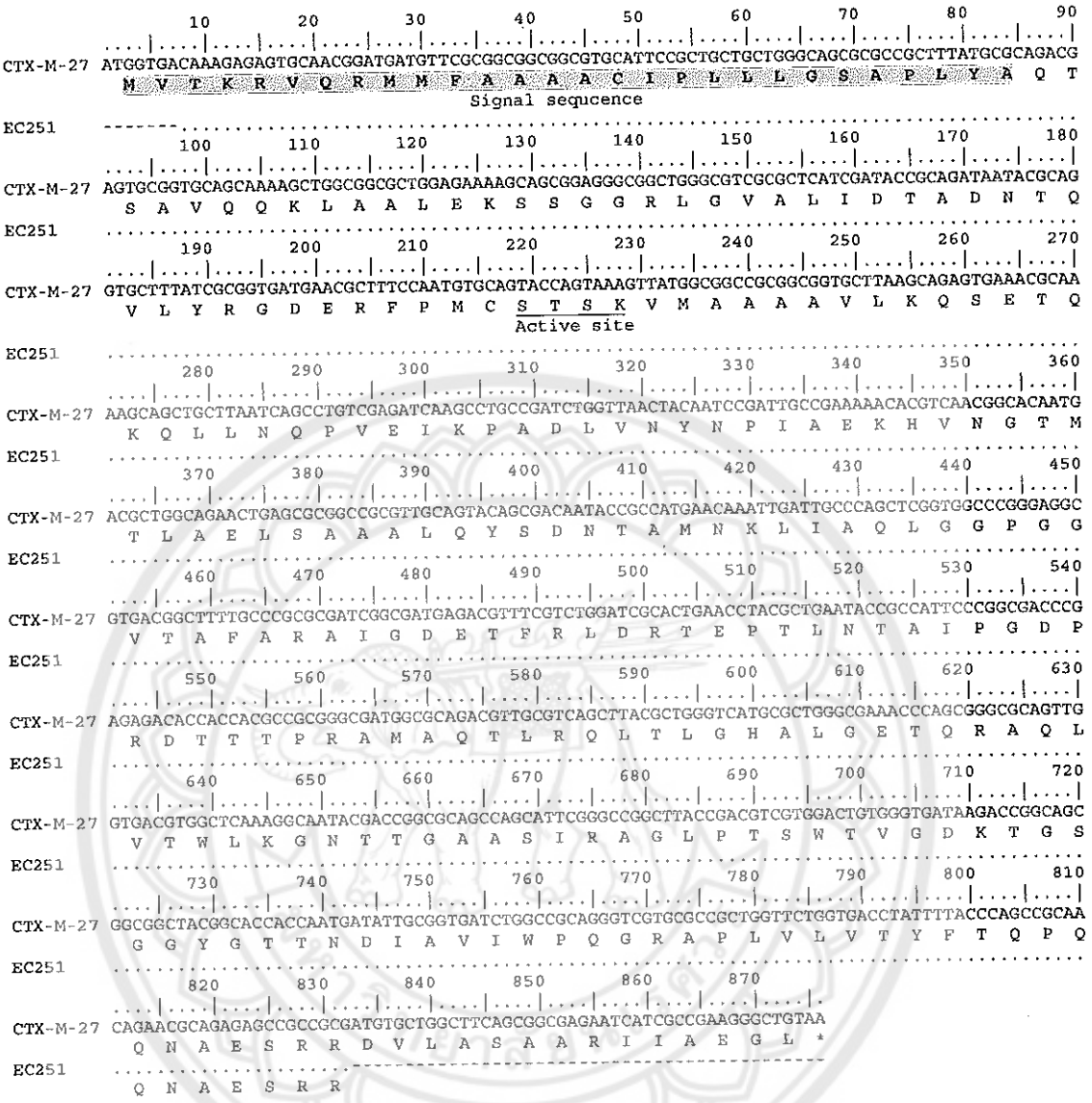


Figure 31 Comparison of nucleotide and amino acid sequences between *bla*$_{CTX-M-27}$ (Accession number: AY156923) and *bla*$_{CTX-M}$ detected in *E. coli* isolates (EC251)

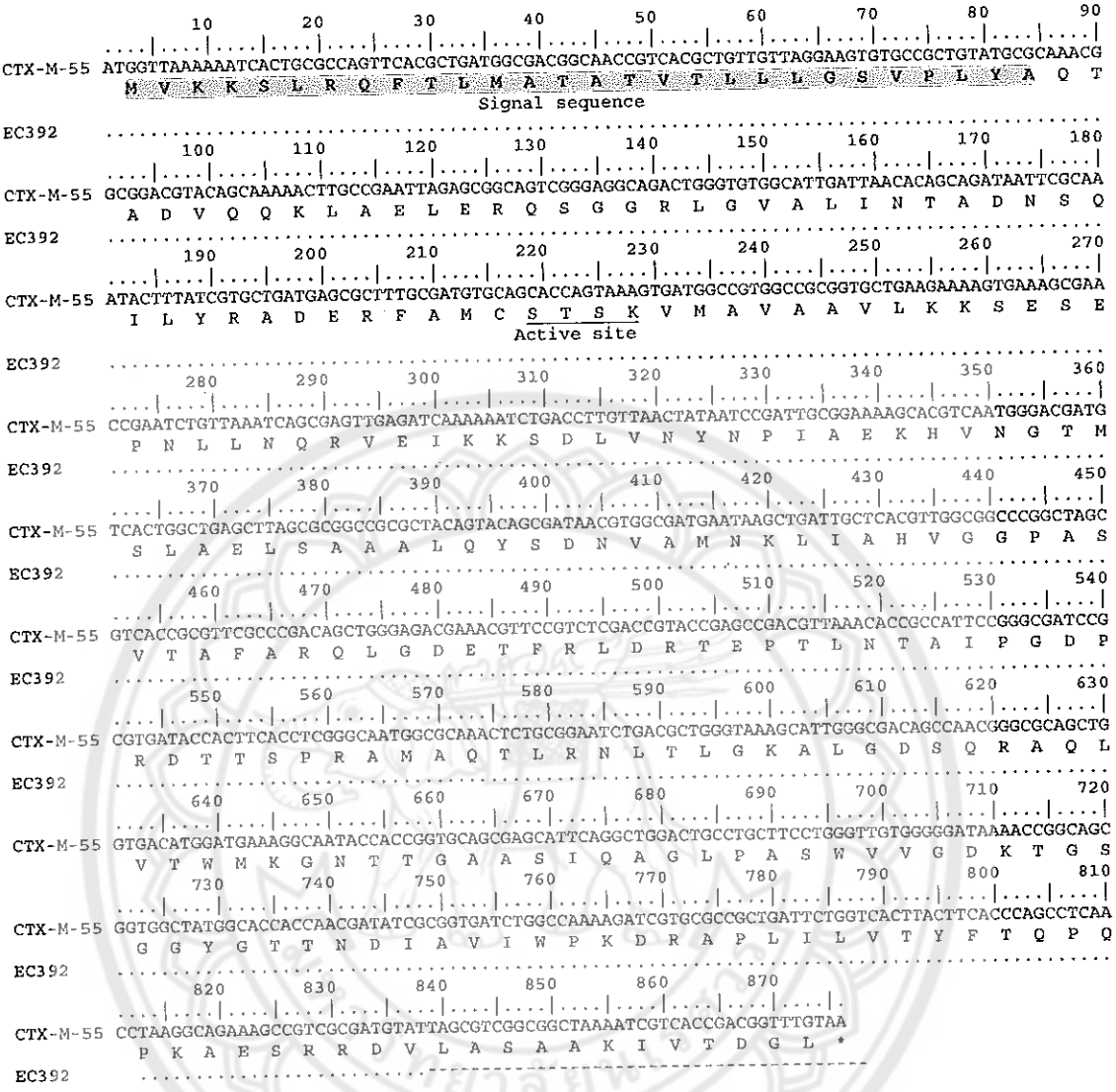


Figure 32 Comparison of nucleotide and amino acid sequences between *bla*$_{CTX-M-55}$ (Accession number: DQ885477) and *bla*$_{CTX-M}$ detected in *E. coli* isolates (EC392)

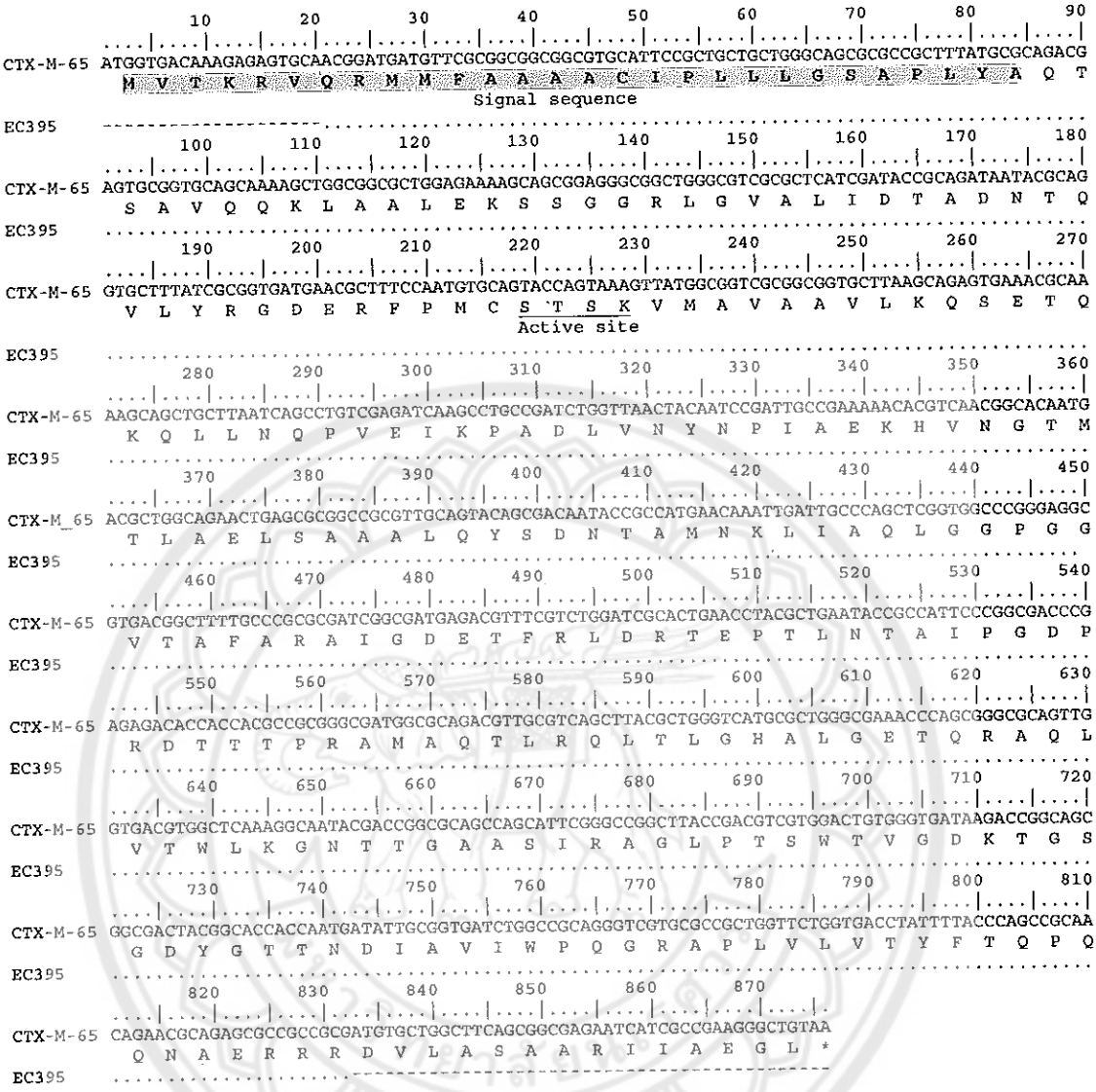


Figure 33 Comparison of nucleotide and amino acid sequences between *bla*$_{\text{CTX-M-65}}$ (Accession number: EF418608) and *bla*$_{\text{CTX-M}}$ detected in *E. coli* (EC395)

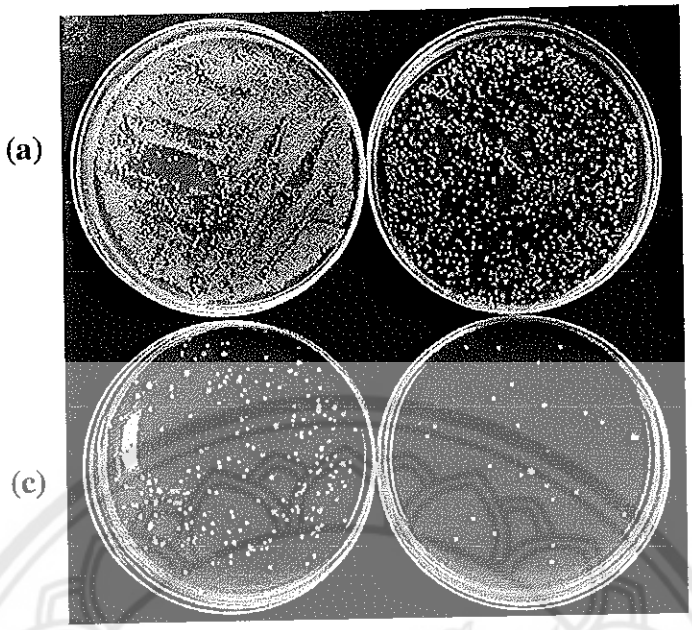


Figure 34 Serial dilution of isolation of transconjugant on TSA agar supplemented with 2 mg/L cefotaxime and 150 mg/L sodium azide (a) 10^{0} dilution, (b) 10^{-1}, (c) 10^{-2} and (d) 10^{-3}

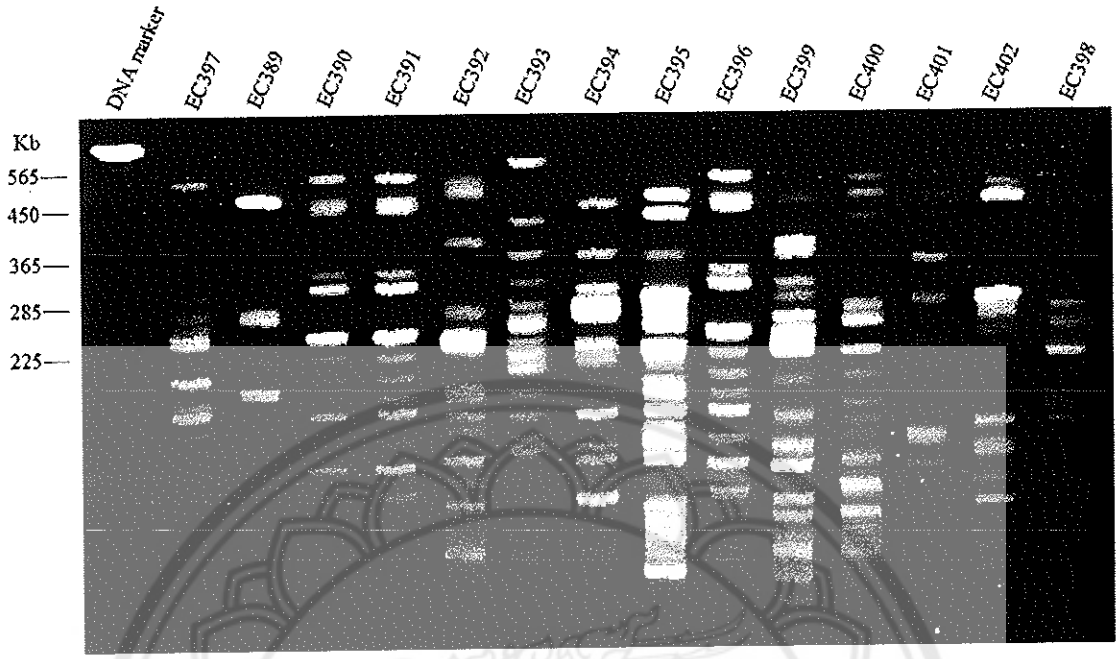


Figure 35 PFGE patterns of *E. coli* isolated from 2 backyard farms in Bang Rakam district, Phitsanulok province (farm 13: EC397–EC396; farm 20: EC399–EC398)

M: DNA size marker *S. cerevisiae* chromosomes (Bio-Rad, Hercules, USA)

EC397 and 398 isolated from farmer and soil, respectively

EC389-EC402 from chicken (EC390, 391 and 396 showing identical PFGE patterns)

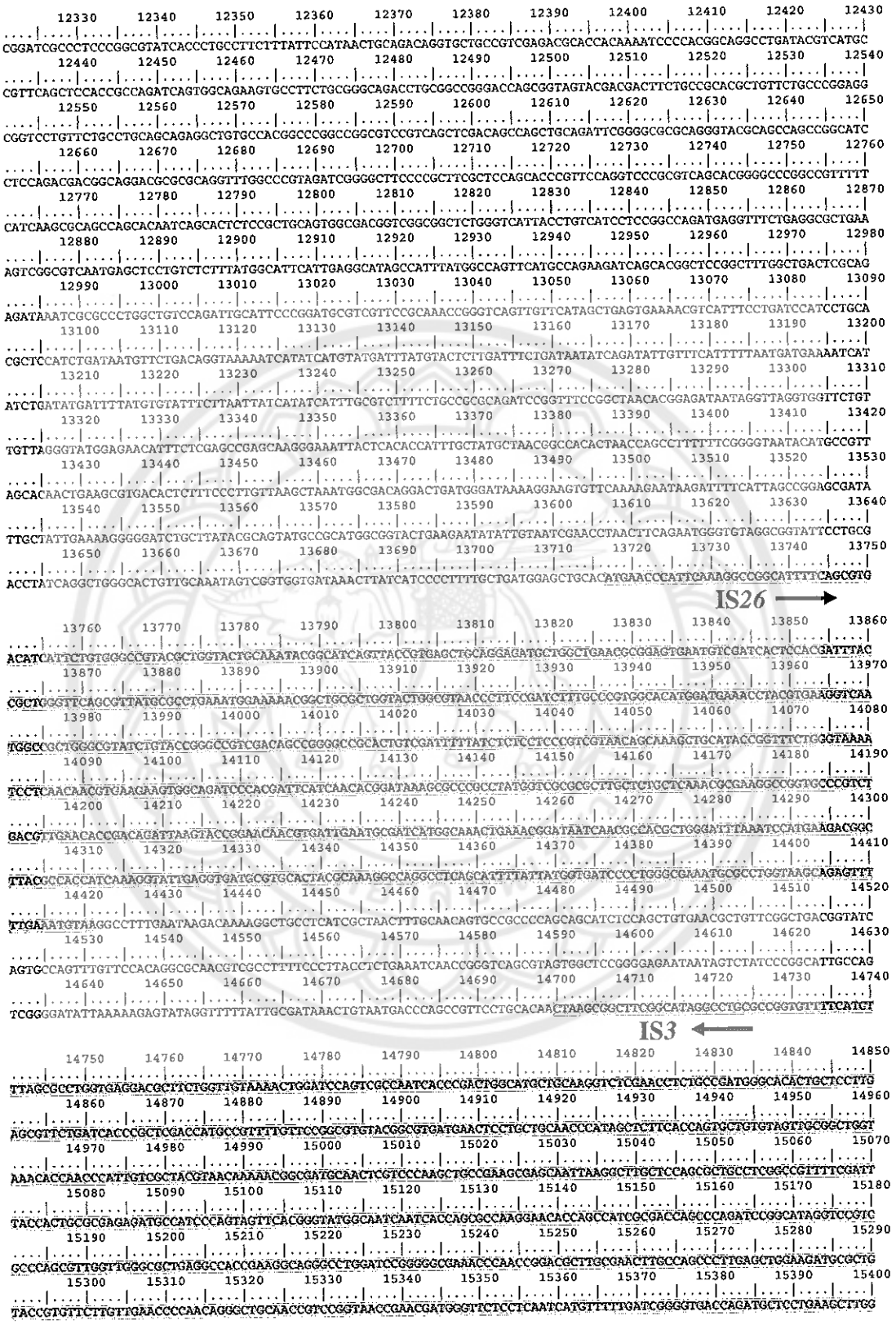


Figure 36 Nucleotide and gene sequences of genetic context of *bla*$_{CTX-M-55}$ on pEC278 (Position 12,320–33,660)

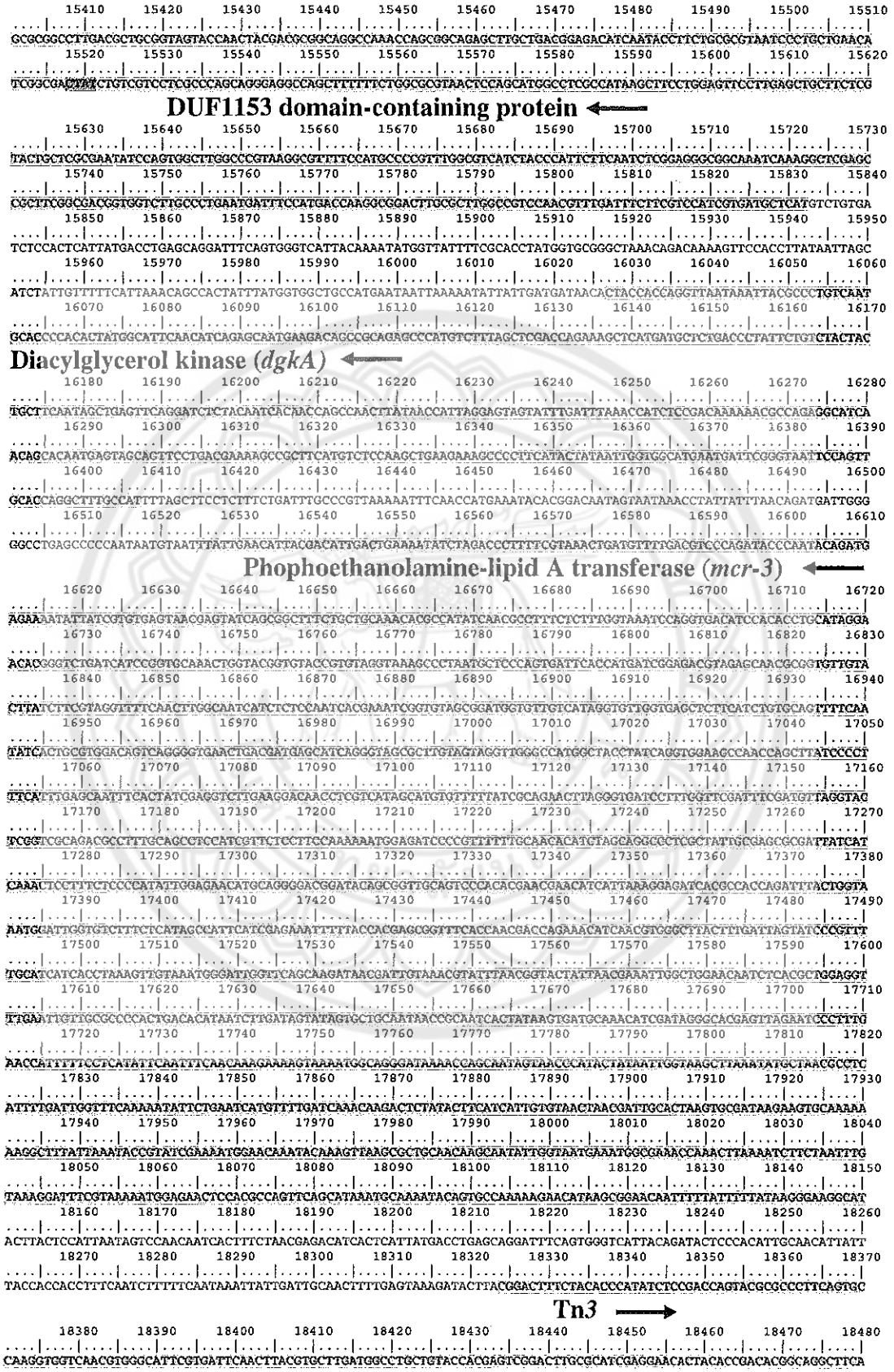


Figure 36 (cont.)

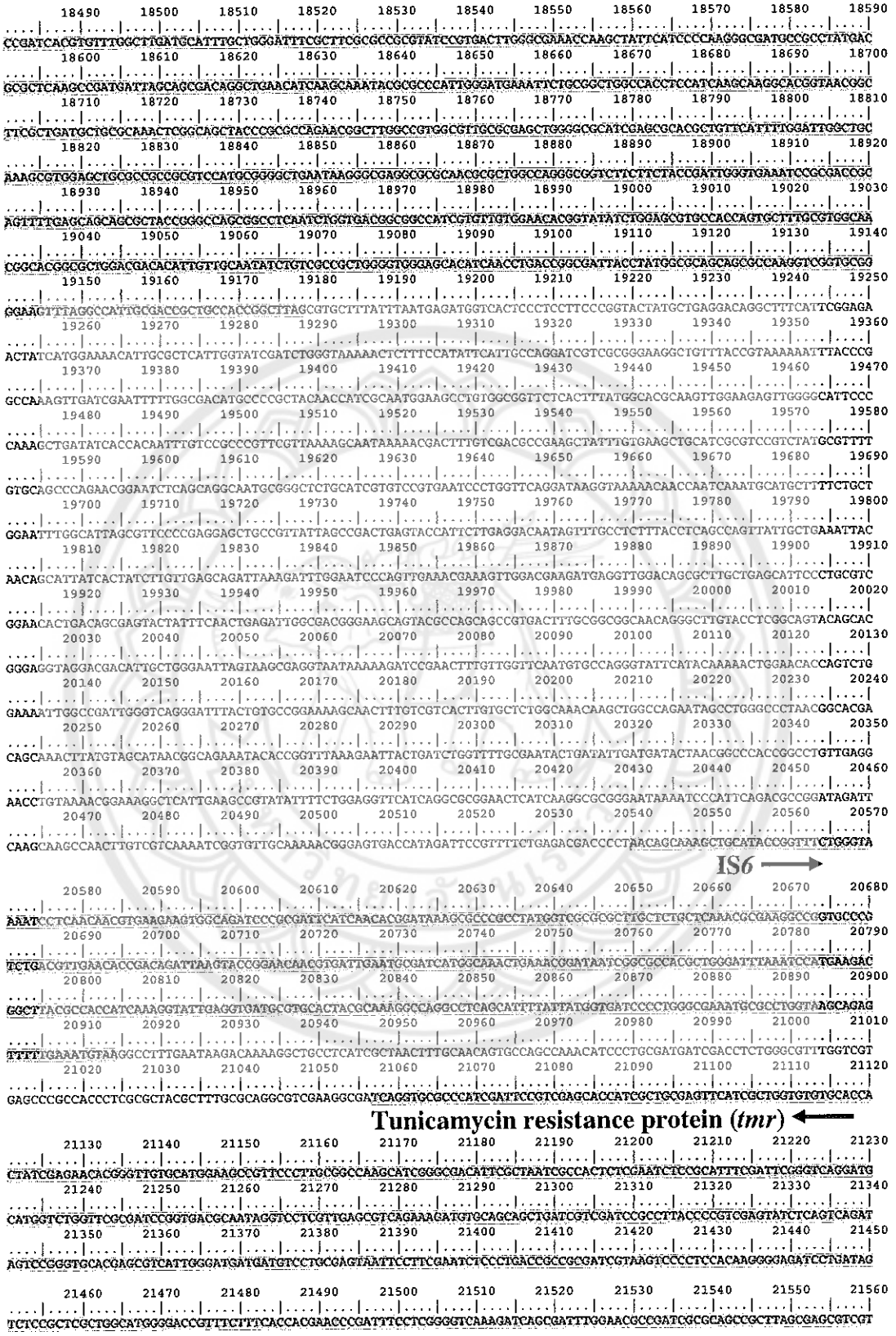


Figure 36 (cont.)

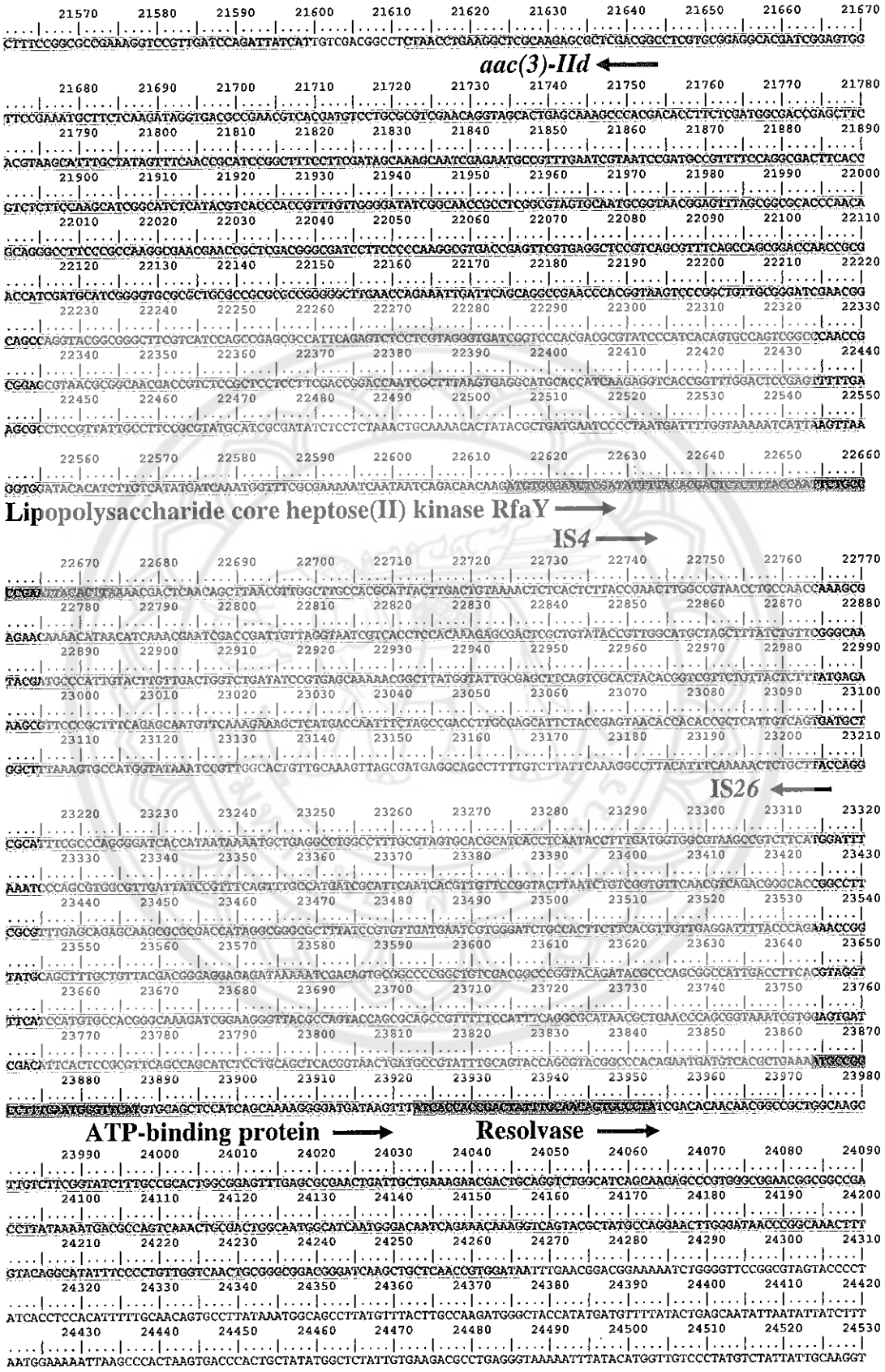


Figure 36 (cont.)

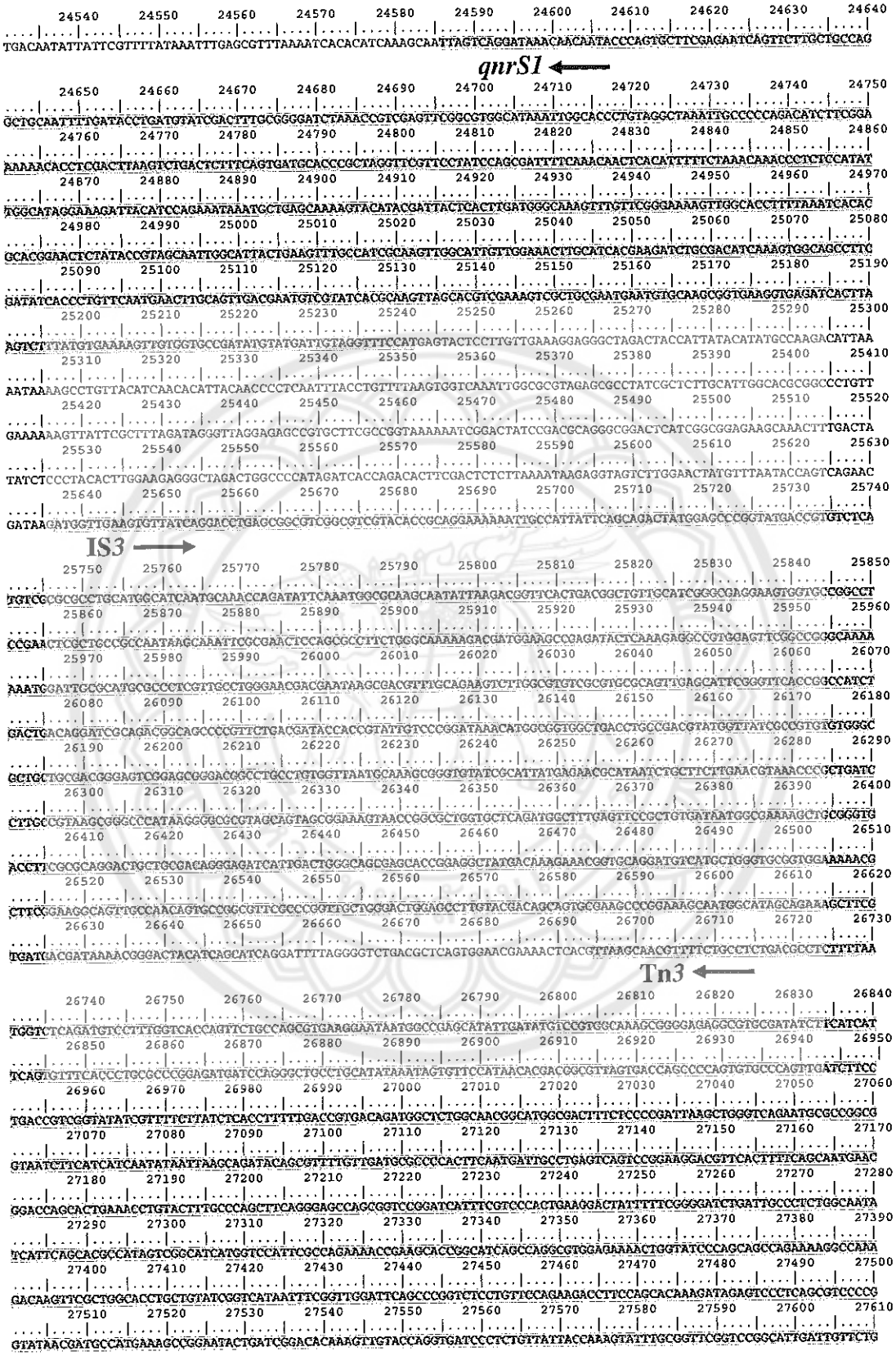


Figure 36 (cont.)

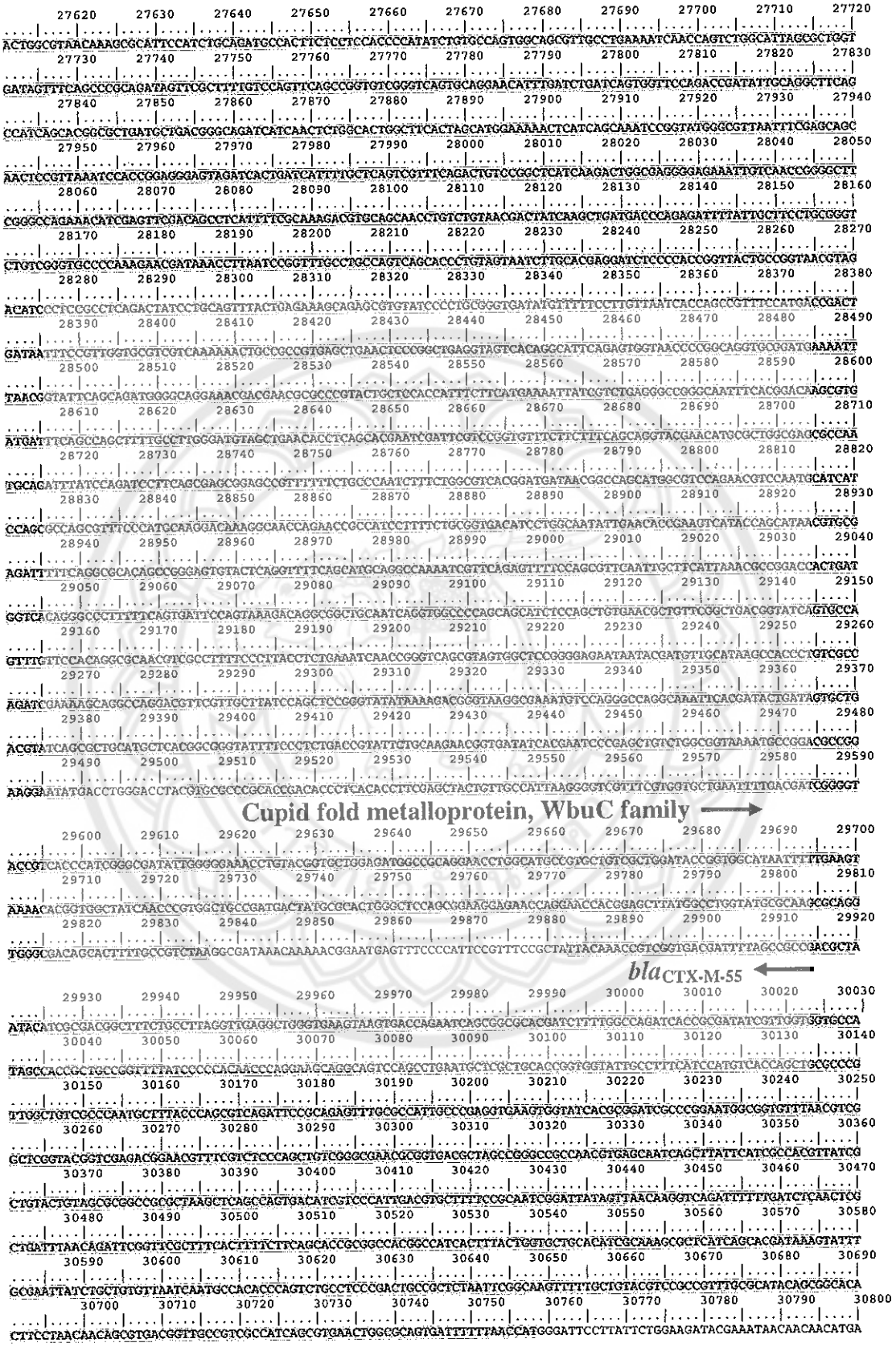


Figure 36 (cont.)

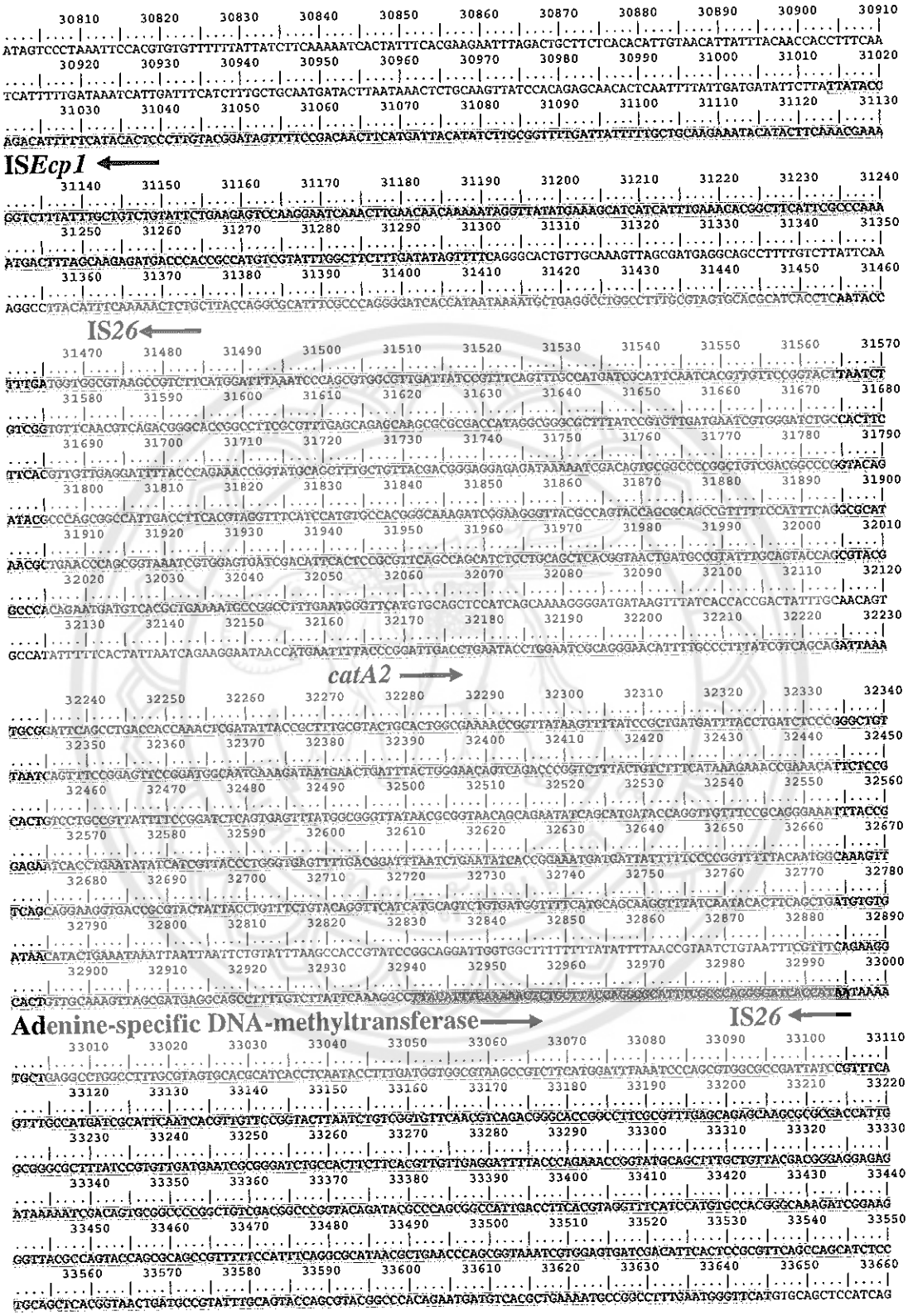


Figure 36 (cont.)

APPENDIX B REAGENTS AND BUFFERS

The preparation of reagents and buffers was followed by Sambrook & Russell, 2001 (172).

Gel electrophoresis

Stock solution

1. 5x TBE buffer

Dissolve 54 g of Tris base, 27.5 g of boric acid and 20 ml of 0.5 M EDTA (pH 8.0) in a final volume of 1 liter of deionized water. Solution is sterilized by autoclaving.

2. 0.5 M EDTA (pH 8.0)

Dissolve 186.1 g of disodium EDTA.$2H_2O$ to 800 ml of distilled water. Adjust pH to 8.0 with approximately 20 of NaOH pellet. Adjust the final volume to 1 liter with distilled water. Solution is sterilized by autoclaving.

Working solution

3. 0.5X TBE

Dilute 100 ml of 5X TBE buffer in a final volume of 1 liter of deionized water. Solution is sterilized by autoclaving.

Pulsed-Field Gel Electrophoresis (PFGE)

Stock solution

1. 1 M Na_2HPO_4

Dissolve 70.89 g of Na_2HPO_4 in 420 ml of distilled water. Adjust the final volume to 1 liter with distilled water. Solution is sterilized by autoclaving.

2. 1 M $NaH_2PO_4.H_2O$

Dissolve 69.0 g of $NaH_2PO_4.H_2O$ in 420 ml of distilled water. Adjust the final volume to 1 liter with distilled water. Solution is sterilized by autoclaving.

3. 5 M NaCl

Dissolve 292 g of NaCl in 800 ml of distilled water. Adjust the final

volume to 1 liter with distilled water. Solution is sterilized by autoclaving.

4. 1 M Tris-HCl (pH 8.0)

Dissolve 121.1 g of Tris base in 800 ml of distilled water. Adjust the pH 8.0 by adding approximately 42 ml of concentrated HCl. Adjust the final volume to 1 liter with distilled water. Solution is sterilized by autoclaving.

5. 10X TE buffer (pH 8.0) (100mM Tris-Cl [pH 8.0], 10 mM EDTA [pH 8.0])

Add 100 ml of 1 M Tris-Cl and 20 ml of 0.5 M EDTA in a final volume of 1 liter of distilled water. Solution is sterilized by autoclaving.

Working solution

1. 0.1 M phosphate buffer (pH 7.0)

Add 57.7 ml of 1 M Na_2HPO_4 and 42.3 ml of 1 M NaH_2PO_4 in a final volume of 1 liter of distilled water. Solution is sterilized by autoclaving.

2. Cell suspension buffer I (0.15 M NaCl, 10 mM EDTA [pH 8.0])

Add 30 ml of 5 M NaCl and 20 ml of 0.5 M EDTA in a final volume of 1 liter of distilled water. Solution is sterilized by autoclaving.

3. Cell suspension buffer II (1 M NaCl, 10 mM EDTA [pH 8.0])

Add 200 ml of 5 M NaCl and 20 ml of 0.5 M EDTA in a final volume of 1 liter of distilled water. Solution is sterilized by autoclaving.

4. Cell lysis solution I (0.25 M EDTA [pH8.0], 1% (w/v) sarkosyl)

Dissolve sarkosyl 0.1 g and 5 ml of 0.5 M EDTA in a final volume of 10 ml of sterilized water.

5. Cell lysis solution II (1 M NaCl, 0.1 M EDTA [pH8.0], 10 mM Tris-HCl [pH 8.0], 0.2% (w/v) sodium deoxycholate, 0.5% (w/v) sarkosyl)

Dissolve sodium deoxycholate 0.04 g, sarkosyl 0.1, 4 ml of 5 M NaCl, 4 ml of 0.5 M EDTA, 0.2 ml of 1 M Tris-Cl in a final volume of 20 ml of sterilized water.

6. 1X TE buffer

Dilute 100 ml of 10X TE buffer in a final volume of 1 liter of deionized water. Solution is sterilized by autoclaving.

S1 nuclease-Pulsed-Field Gel Electrophoresis (S1-PFGE) and ^{32}P hybridization

Stock solution

1. SDS (20% w/v)

Dissolve 200 g of sodium lauryl sulfate in 900 ml of distill water. Heat to 65°C and stir with a magnetic stirrer and adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the final volume to 1 liter with distilled water. Solution is sterilized by autoclaving.

2. 20X SSC

Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml distilled water. Adjust the pH to 7.0 with 14 N of HCl. Adjust the final volume to 1 liter with distilled water. Solution is sterilized by autoclaving.

3. 10% (w/v) Sarcosyl (N-Lauroylsarcosine, sodium salt)

Dissolve 50 g of N-Lauroylsarcosine, sodium salt in a final volume 500 ml with sterilized water. Solution is sterilized by autoclaving.

Working Solution

1. Cell suspension buffer (100 mM Tris-Cl [pH 8.0], 100 mM EDTA, [pH 8.0])

Add 100 ml of 1 M Tris-Cl and 200 ml of 0.5 M EDTA in a final volume of 1 liter of distilled water. Solution is sterilized by autoclaving.

2. Cell lysis buffer (50 mM Tris, 50 mM EDTA [pH 8.0], 1% sarcosyl)

Add 50 ml of 1 M Tris-Cl, 100 ml of 0.5 M EDTA and 10 ml of 10% (w/v) sarkcosyl in a final volume of 1 liter of distilled water. Solution is sterilized by autoclaving.

3. Denaturing solution (0.5 M NaOH, 1.5 M NaCl)

Dissolve 20 of NaOH and 87.66 g of NaCl in a final volume of 1 liter of sterilized water.

4. Neutralizing solution (0.5 M Tris-HCl [pH 7.5], 1.5 M NaCl)

Dissolve 60.5 g of Tris base and 87.6 g of NaCl in 800 ml of sterilized water. Adjust to pH 7.6 with concentrated HCl and adjust the final volume to 1 liter of sterilized water.

5. Pre-hybridization solution (6X SSC, 0.1% (W/V) polyvinylpyrrolidone, 0.1% Ficoll, 0.5% SDS, 150 μg/ml Herring testes DNA, 1 ml

UHT full cream milk)

Add 6 ml of 20x SSC, 0.02 g of polyvinylpyrrolidone, 0.02 ml of Ficoll, 0.1 ml of 20% (w/v) SDS, 150 μg/ml Herring testes DNA and 1 ml UHT full cream milk in a final volume to 20 ml of sterilized water.

6. Washing solution I (2x SSC, 0.1% SDS)

Add 100 ml of 20x SSC and 5 ml of 20% SDS in a final volume of 1 liter of distilled water.

7. Washing solution II (0.1x SSC, 0.1% SDS)

Add 50 ml of 20x SSC and 5 ml of 20% SDS in a final volume of 1 liter of distilled water.