ลักษณะของเชื้อเอสเชอริเชีย โคไลที่ดื้อยาหลายชนิดพร้อมกันที่แยกได้จากสุกรขุน

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CHARACTERISTICS OF MULTIDRUG-RESISTANT *ESCHERICHIA COLI* ISOLATED

FROM FATTENING PIGS

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	ESCHERICHIA COLI ISOLATED FROM FATTENING PIGS
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คิน คิน เล   : ลักษณะของเชื้อเอสเชอริเชีย โคไลที่ดื้อยาหลายชนิดพร้อมกันที่แยกได้จาก สุกรขุน. (CHARACTERISTICS OF MULTIDRUG-RESISTANT *ESCHERICHIA COLI* ISOLATED FROM FATTENING PIGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.สพ.ญ.รุ่งทิพย์ ชวนชื่น, 96 หน้า.

ทำการศึกษาใน *Escherichia coli* จำนวน 344 เชื้อ ที่แยกได้จากสุกรสุขภาพดี โดยศึกษาความไวต่อยาปฏิชีวนะ จำนวน 8 ชนิด ลักษณะทางพันธุกรรมของ class 1 integrons ยีนดื้อยาการกลายพันธุ์ในส่วน Quinolone Resistance-Determining Regions (QRDRs) ยีนที่ก่อให้เกิดความรุนแรงของโรค และ phylogenetic group พบว่าในการศึกษาความไวต่อยาปฏิชีวนะ เชื้อดื้อต่อยาเตตร้าซัยคลิน (92.6%) และแอมพิซิลิน (91.6%) การศึกษา class 1 integrons พบการปรากฎของยีน *intI1* ร้อยละ 73 ซึ่งมี gene cassette ร้อยละ 22.3 โดย gene cassette array ที่พบคือยีน *aadA22 aadA1 dfrA12-aadA2* และ *sat-psp-aadA2* ซึ่ง gene cassette ที่พบมากที่สุดคือยีน *aadA2* (42.9%) และ *aadA1* (26.8%) พบการถ่ายทอดของ class 1 integrons ใน 8 isolates โดย class 1 integrons ที่ถ่ายทอดได้ทั้งหมดมี gene cassette array แบบ *dfrA12-aadA2* พบยีนดื้อยาทั้งหมด 16 ชนิดซึ่งการปรากฎของยีนเป็นไปในทิศทางเดียวกับรูปแบบการดื้อยา การศึกษาการกลายพันธุ์ในส่วน Quinolone Resistance-Determining Regions (QRDRs) พบการเปลี่ยนแปลงกรดอะมิโนของยีน   *gyrA* ได้แก่ Ser-83-Leu Asp-87-Asn และ Gln-94-Pro ในส่วนของยีน *parC* พบการเปลี่ยนแปลงของ Ser-58-Ile ตรวจพบยีนที่ก่อให้เกิดความรุนแรง 10 ยีน ได้แก่ *elt estA estB astA faeGn fasA fedA eseA paa* และ *sepA* โดยยีนที่พบมากที่สุดคือ *fasA* (98.3%) เมื่อจัด phylogenetic group พบว่าร้อยละ 82 ของเชื้อที่ทำการศึกษาจัดอยู่ใน group B1 รองลงมาคือ group A (8%) group B2 (7%) และ group D (3%) การปรากฎของยีนที่ก่อให้เกิดความรุนแรง ยีนดื้อยาและการแสดงออกของการดื้อยามีความเกี่ยวข้องกันอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ผลการศึกษาพบว่าเชื้อเอสเชอริเชีย โคไลที่ พบในสุกรสุขภาพดีมีความสำคัญในการกระจายของยีนดื้อยาและยีนควบคุมที่ก่อให้เกิดความรุนแรงของโรค

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Three hundred and forty-four *Escherichia coli* isolates from clinically healthy fattening pigs were evaluated for susceptibilies to 8 antimicrobials, class1 integrons, antimicrobial resistance genes, mutation in Quinolone Resistance-Determining Regions (QRDRs), virulence genes and phylogenetic groups. *E. coli* isolates were resistant to tetracycline (96.2%) and followed by ampicillin (91.6%). Seventy-three percent contained *intI1* gene, of which 22.3% carried inserted gene cassettes, i.e., incomple *sat*, *aadA22*, *aadA1*, *dfrA12-aadA2,* and *sat-psp-aadA2*. Two most commonly observed gene cassettes were *aadA2* (42.9%) and *aadA1* (26.8%). Horizontal transfer of class 1 integrons was detected in 8 *E. coli* isolates with class1 integrons carrying *dfrA12-aadA2* gene cassettes. Sixteen resistance genes were detected in *E. coli* isolates and their presence was correlated to resistant phenotype. The amino acid substitutions Ser-83-Leu, Asp-87-Asn and Gln-94-Pro were observed in GyrA and Ser-58-Ile in ParC. Ten virulence genes including *elt, estA, estB, astA, faeG, fasA, fedA, eseA, paa* and *sepA* were detected, of which *fasA* (98.3%) was most commonly observed. Eighty-two percent of the *E. coli* isolates were assigned to group B1 followed by group A (8%), B2 (7%) and D (3%). Statistically-significant associations were found among the specific virulence genes, the specific resistance phenotypes and genotypes ($P<0.05$). The results supported the significant role of commensal *E. coli* as reservoirs for antimicrobial resistance determinants and virulence factors.

Department : Veterinary Public Health   Student's Signature ....

Field of Study : Veterinary Public Health   Advisor's Signature ....

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LIST OF ABBEREVIATIONS

bp	base pair
CFU	colony-forming unit
ºC	degree Celsius
DNA	deoxyribonucleic acid
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
e.g.	exampla gratia, for example
et al.	et alii, and others
g	gram (s)
g	gravity
h	hour (s)
i.e.	id est, that is
M	molar
mg	milligram (s)
ml	milliliter (s)
mM	millimolar
mPCR	multiplex Polymerase Chain Reaction
µg	microgram (s)
µl	microliter (s)
µM	micromolar
NSS	normal saline solution
PCR	Polymerase Chain Reaction
pH	the negative logarithm of hydrogen ion concentration
rpm	round per minutes
sec	second (s)
TAE	Tris-Acetate -EDTA
U	unit

CHAPTER I

INTRODUCTION

Swine production in Thailand has started since 1960, when the exotic breeds were introduced into the country by the Department of Livestock Development from the United Kingdom. Industralized pig farming was gradually developed and rapidly grew after year 1980s (FAO, 2002). Estimation of pig population in Thailand was 4.6 million in 1991 and reached 11.6 million in 2007, reflecting the increased demand of pork and pork products. Pork has become the second most important meat in Thailand in late 1990s, of which per capital consumption was 4.7 Kg and increased to 13.7 Kg per person per year in 2007. Swine production significantly contributes to the country income, of which the total value of about 2, 000 million baht in 2007 (DLD, 2007).

In general, pigs carry several types of bacteria including both pathogenic and non pathogenic strains. Among these bacteria, *Escherichia coli* are very common. Most *E. coli* strains are harmless to hosts and considered commensal in pig's intestinal tract. Instead, these harmless *E. coli* strains provide benefits to their hosts, not only in food digestion but also in prevention of threats from pathogenic bacteria that may enter gastrointestinal tract. However, commensal *E. coli* have been shown to serve as potential reservoirs for antimicrobial-resistance encoding determinants and virulence factors. Of particular concern is that, the presence of *E. coli* strains in clinically healthy pigs may act as a health hazard to humans and contribute to cross-contamination among carcasses during slaughtering and post slaughtering process.

As diarrhea in growing pigs is the most common problem in pig farming, *E. coli* infection is one of the major causes of the disease. This is attributed to the ability of the pathogens to colonize intestinal tract quickly after birth and produce toxins causing acute enteritis and diarrhea (Qadri et al., 2005). It was previously reported that

pathogenic *E. coli* caused up to forty-eight percent of diarrhea in pigs in Thailand (Punyarat and Thongwai, 2008).

In humans, many *E. coli* strains are considered common foodborne pathogens. Cases of *E. coli*-foodborne diseases associated with consumption of pork and pork products have been increasingly reported (Conedera et al., 2007). Noticely, pathogenic *E. coli* strains could be derived from commensal strains by obtaining virulence determinants through mobile genetic elements (Finlay and Falkow, 1997; Ochman et al., 2000). In this case, clinically healthy pigs could serve as a major reservoir of pathogenic *E. coli* strains that could enter food chain eventually.

Antimicrobial agents have been widely applied in both human and veterinary medicine for a quite long time. In food-producing animals, the main purposes of antimicrobial uses include treatment of diseases, prevention of infections and promotion of growth. While most antimicrobial agents are generally mixed into feed or water and dispensed to pigs in large portions, some are also administered to individual animals. It has been well known that imprudent and overuse of antimicrobial substances is responsible for widespread of multiple drug resistance among bacteria of animal origins including *E. coli* in pigs (van Den Bogaard et al., 2000). These resistant *E. coli* could be transferred to humans via direct contact and consumption of contaminated food products resulting in reduced efficacy of antibiotic treatment, prolonged hospitalization and increased treatment expenses.

Antimicrobial resistance has been extensively studied in *E. coli* and several resistance mechanisms exist (Hooper, 1999; Okusu et al., 1996). In the last decade, it has been shown that multidrug resistance in the pathogens is mainly associated with integrons, of which the most common type is class 1 integrons. Integrons are mobile genetic elements that have the ability to capture, integrate and express resistance gene cassettes located in their variable region (Leverstein-van Hall et al., 2003). Due to these

particular characteristics, the presence of class 1 integrons raises particular concern that using a single antibiotic may co-select for different-resistance gene cassettes leading to multidrug resistance phenotypes in bacteria. Besides, variable resistance-encoding genes that are not class 1 integrons have been identified in *E. coli* (Lu et al., 2010). The major concern has been paid to localization of class 1 integrons and non-borne class 1 integrons resistance genes on transferable genetic elements, in particular, conjugative plasmids, contributing to wide dissemination of antimicrobial resistance determinants among *E. coli* and also other bacterial species.

Antimicrobial resistance among bacteria could be concerned with not only horizontal transfer but also clonal expansion. Based on phylogenetic analyses, the *E. coli* strains exist in four phylogenetic-groups, i.e., group A, B1, B2 and D (Herzer et al., 1990; Picard et al., 1999). The *E. coli* isolates in each group differ in the presence of virulence factors, antibiotic resistance profiles, life history and ecological niches (Gordon and Cowling, 2003). Commensal *E. coli* strains are involved phylogenetic groups A and B1 (Johnson et al., 2001), while pathogenic *E. coli* strains are usually classified into group B2 and D (Picard et al., 1999). As seen in other bacteria, the *E. coli* strains are usually phylogenetic-grouped by multilocus enzyme electrophoresis, ribotyping, and multilocus sequence typing (Bingen et al., 1998; Enright and Spratt, 1999; Herzer et al., 1990). However, the drawbacks of these techniques include complicated protocol, time consuming, and necessities of expertise. Recently, a simple and rapid method, a triplex PCR-based method, has been developed and currently well recognized for assigning *E. coli* strains into those four distinct phylogenetic groups (Clermont et al., 2000). The accuracy with triplex-PCR was recently confirmed by MLST (Gordon et al., 2008). This method additionally provides better understanding for acquisition of virulence factors in *E. coli*.

The pathogenicity of *E. coli* usually requires various virulence factors that could be present on plasmids. Since antimicrobial resistance genes and virulence genes can

be located on the plasmids, particular concern has been raised for emergence of newly virulence-resistance plasmids. Cointegration of resistance plasmid and virulence plasmid has been previously reported (Franklin and Mollby, 1983) and the presence of new plasmids carrying both resistance and virulence genes could lead to more resistant and virulent *E. coli* strains.

Up to date, data on genetic characteristics including mechanisms underlying antimicrobial resistance, virulence factors and genetic relatedness has been widely reported but mostly originated from diseased pigs and humans. Even though clinically healthy pigs are closed to markets and considered a major source of food, such data from commensal *E. coli* of clinically healthy pigs is still limited and have never been reported in Thailand. Therefore, we examined antimicrobial resistance including class 1 integrons and also their transferability and the presence of non-class 1 integrons borne resistance genes, virulence genes and phylogenetic groups among the *E. coli* isolates from clinically-healthy pigs in Thailand.

Objectives of study

1. To study antimicrobial resistance including class1 integrons and other antimicrobial resistance genes in *E. coli* isolated from swine
2. To determine the presence of virulence genes in *E. coli* isolated from swine
3. To examine the phylogenetic groups in *E. coli* isolated from swine
4. To examine the possible associations among phylogenetic groups, virulence genes and antimicrobial resistance in *E. coli* isolated from swine

Questions of study

1. What are the genetic characteristics of class1 integrons and their transferability in *E. coli* isolated from swine in Thailand?

2. Which antibiotic resistance genes are distributed in *E. coli* isolated from swine in Thailand?

3. Which type of virulence genes can be detected in *E. coli* isolated from swine in Thailand?

4. What are phylogenetic groups of *E. coli* isolated from swine in Thailand?

5. Is there the relationship among phylogenetic groups, virulence factors and resistance determinants among *E. coli* isolated from swine?

CHAPTER II

LITERATURE REVIEW

1. General characteristics and pathogenesis of *E. coli*

 E. coli was discovered by a German pediatrician and bacteriologist Theodor Escherich in 1885. This bacterium belongs to the family *Enterobacteriaceae*. The enteric bacteria are facultatively anaerobic Gram-negative, rod shaped and non-sporulating. Cells are about 2 μm long and 0.5 μm in diameter and cell volume is 0.46-0.7 μm^3 (Kubitschek, 1990). The optimal temperature for growth is 37°C and optimum pH is 6-7. *E. coli* can grow on MacConkey or eosin methylene- blue agar, which selectively grows gram negative bacteria and differentiates organisms that ferment lactose with nucleated colonies.

 According to the modified Kauffmann scheme, *E. coli* is serotyped depend on surface antigens such as somatic (O), flagella (H) and capsular (K). Currently, more than 180 different O-serogroups and at least 60 H-serogroups have been identified (Robins-Browne and Hartland, 2002).

 E. coli is commonly found in the gastrointestinal tract of humans and warm-blooded animals. Most strains of *E. coli* are harmless. Infection due to pathogenic *E. coli* may be restricted to colonize mucosal surface or can spread throughout the body. The clinical symptoms include gastroenteritis, urinary tract infection and neonatal meningitis. Collibacillosis caused by *E. coli* is one of the common enteric diseases in newborn livestocks. The disease usually occurs during the suckling and post-weaning period in pigs. Diarrheagenic *E. coli* may be caused by six major categories of *E. coli* pathotypes as follows:

 1. Enterotoxigenic *E. coli* (ETEC)
 2. Enteropathogenic *E. coli* (EPEC)

3. Enteroinvasive *E. coli* (EIEC)

4. Enterohemorrhagic *E. coli* (EHEC)

5. Enteroaggregative *E. coli* (EAEC)

6. Diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004).

Enterotoxigenic *E. coli* is the major pathogen causing diarrhea in neonatal and post-weaning piglets. EHEC may produce shiga like toxin that can cause severe foodborne diseases. It can be transmitted to humans via consumption of contaminated foods, such as raw or undercooked ground meat products, raw milk and water. *E. coli* O157:H7, an EHEC strain, causes hemorrhagic colitis and hemolytic uremic syndrome in humans. The US Centers for Disease Control and Prevention estimated that approximately 73,400 illness and 60 deaths occurring each year in the United States was due to *E. coli* O157: H7 (Mead et al., 1999).

2. Occurrence and epidemiology of antimicrobial resistance in *E. coli*

Antimicrobial agents are used in human and livestock systems to treat disease, keep in good health and improve animal productivity. Such uses of antimicrobial agents in both livestock production and human medicine are selection pressures that select for resistant pathogens. These are important factors in the emergence of antibiotic-resistant bacteria that are transferred to humans via the food chain (Tollefson et al., 1997).

E. coli from the pig farms applying conventional production methods and antimicrobial free farms were examined for the antimicrobial susceptibility to 14 antimicrobial agents. It was found that *E. coli* isolates from conventional pig farms were significantly higher level of resistant to ampicillin, sulfamethoxazole, tetracycline and chloramphenicol than those from antimicrobial free farms. The commonly found antimicrobial resistance patterns were streptomycin-tetracycline, sulfamethoxazole-

tetracycline, and kanamycin-streptomycin-sulfamethoxazole-tetracycline (Bunner et al., 2007).

In Canada, *E. coli* isolates from fecal samples of grow-finish pigs in 20 herds in Alberta and Saskatchewan were examined for antimicrobial susceptibility test to 16 antibiotics. It was found that 66.8%, 46 %and 33.4% of isolates were resistant to tetracycline, sulfamethoxazole and streptomycin, respectively. Fifty-seven percent of isolates were resistant to two or more antimicrobial and 21 % were susceptible to all drugs (Rosengren et al., 2008).

In Argentina, 69 *E. coli* isolates from swine farms were tested for antimicrobial susceptibility to antibiotics frequently used in veterinary and human medicine. It was showed that 62% were multidrug resistance. In addition, high resistance percentage to ampicillin, streptomycin and tetracycline, the common antibiotic used in swine farms, was also observed. Likewise, these *E. coli* isolates were also resistant to trimethoprim, sulfamethoxazole and chloramphenicol although these groups of antibiotic agents have not been used in the farm for several years ago (Moredo et al., 2007).

E. coli strains from Swiss weaned pigs and sows were resistant to streptomycin (60.6% and 64.3% of the isolates from weaners and sows), sulfonamide (51.5% and 26.9%), tetracycline (35.2% and 22.0%) and trimethoprim (27.5% and 11.1%) (Stannarius et al., 2009).

In Northen part of Thailand, antimicrobial resistance in *E. coli* strains from swine, broiler chickens, farm workers and abattoirs was 91.5% for tetracycline, 67.4% for nalidxic acid, 61.6% for ampicillin, 51.8% for florfenicol, 28.7% for enrofloxacin, 12.5% for ciprofloxacin, 4.9% for ceftiofur, 1.5% for ceftriaxone (Hanson et al., 2002). In addition, one study from Southern Thailand demonstrated that *E. coli* isolates from pigs and pig farmers were resistant to sulfamethoxazole, tetracycline, ampicillin,

streptomycin, trimethoprim-sulfamethoxazole, naldixic acid, chloramphenicol, kanamycin, cephalothin, gentamicin, ciprofloxacin, cefoxitin, amoxicillin-clavulanic and amikacin at the rate of 100%, 97.1%, 92.8%, 89.9%, 88.1%, 60.9%, 58.0%, 55.1%, 44.9%, 39.1%, 33.3%, 8.7%, 5.8%, and 2.9%, respectively (Phongpaichit et al., 2007).

3.   Class 1 integrons in *E. coli*

Integrons are mobile genetic elements and involved in dissemination of antimicrobial resistance determinants in Gram-negative bacteria including *E. coli*. It can be divided into two groups such as super integrons and resistance integrons. The super integrons are found on bacterial chromosomes while resistance integrons are located on plasmids, transposons and chromosomes. More than 100 gene cassettes have been detected in super integrons and less than 10 cassettes are present in resistance integrons. Most of the gene cassettes from resistance integrons encode resistance to antibiotics or disinfectants (Fluit and Schmitz, 2004). The essential components of   integrons are integrase gene (*intI*) encoding a site-specific recombinase, an adjacent site (*attI*) which is recognized by the integrase and the promoter responsible for inserted gene cassette expression (Recchia and Hall, 1995). There are 9 classes of integrons have been identified according to the sequence of their integrase genes (Hochhut et al., 2001; Nield et al., 2001) and most commonly found integrons are class1 integrons. The structure of class 1 integrons (Figure 1) consists of 5' conserved segment, variable region and 3' conserved segment (Liebert et al., 1999; Stokes and Hall, 1989).The 5' conserved segment contains the *intI* gene, an *attI* site where gene cassettes are integrated and a promoter region (Stokes and Hall, 1989). The 3' conserved segment consists of *qacEΔ 1*, which encodes resistance to quaternary ammonium compound and the sulfonamides resistance gene *sul1* (Paulsen et al., 1993). The variable region locate between these two conserved segments and contains antibiotic resistance gene cassettes and recombination site *attC* (59- based element) (Stokes et al., 1997). Gene cassette contains a promoterless gene and *attC* site.

The presence of class 1 integrons is strongly related with multiple resistance in *Enterobacteriaceae* (Leverstein-van Hall et al., 2003). The horizontal transfer of integrons may contribute to wide spread of resistance genes and emergence of multi-resistance strains (Rowe-Magnus and Mazel, 2001).

The prevalence of class 1 integrons in *Enterobacteriaceae* was 22-59% (Maguire et al., 2001; Sallen et al., 1995). Integrons were also detected in resistant *Enterobacteriaceae* in the community (Leverstein-Van Hall et al., 2002).

In Taiwan, 436 *E. coli* isolates from clinical specimens from hospitals showed that 64% carried class 1 integrons and 15 different gene cassettes which encode resistance to aminoglycosides (*aadA1*, *aadA2*, *aadA5*, *aadB*, and *aacA4*), chloramphenicol (*cmlA*, *catB8*), trimethoprim (*dfrA1*, *dfrA5*, *dfrA7*, *dfrA12*, and *dfrA17*), lincosamide (*linf*) and unknown genes (*orfD*,*orfF*) were detected (Chang et al., 2007).

In Japan, tetracycline-resistant *E. coli* strains of healthy swine from an abattoir in Osaka were examined and showed that 48% of isolates were positive for integrase 1 gene and 24 strains carried resistance gene cassettes including *dhfrI, dhfrXII, dfr17, aadA, aadA2, aadA5, aadA21, aacA4, and catb3.* Antimicrobial resistance was most common to chloramphenicol (56.9%), ampicillin (43.1%), kanamycin (22.2%), trimethoprim-sulfamethoxazole (15.4%), ofloxacin (2.6%) and gentamicin (1.5%) (Kumai et al., 2005).

In Eastern China, class 1 integrons with nine gene cassettes were identified in *E. coli* isolates from farm animals including swine, cattle and chicken. The three most frequent gene cassettes were *yheS Δ-yheR-kefB*, *dfrA12-orfF-aadA2* and *aadA* (Lu et al., 2010). Beside, class1 integrons with gene cassette arrays including *dfrA17-aadA5*, *dfrA12-orfF-aadA2*, and *aadA1* were detected from *E. coli* isolated from animals and humans in Korea (Kang et al., 2005).

In Thailand, it has been reported that 63.5 % of class 1 integrons were detected in commensal *E. coli* from pigs and pig farmers. It was described that all integrons positive samples were resistant to at least 3 antimicrobial agents (Phongpaichit et al., 2007).
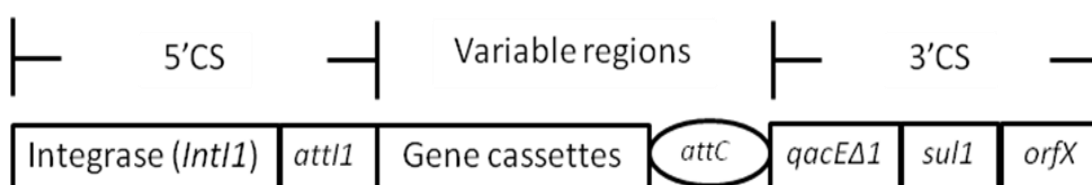
Figure1: Structure of class 1 integrons. *intI1*, integrase gene; *attI1*, attachment site; *attC*, recombination site; *qacE**Δ**1*, quaternary ammonium compound resistance gene; *sul1*, sulfonamide resistance gene; *orfX*, open reading frame of unknown function.

4.   Occurrence of antimicrobial resistance genes in *E. coli*

Antimicrobial resistance phenotypes in *E. coli* may be originated from many genetic resistance determinants. For example, more than 30 different tetracycline resistance genes have been identified at the present. The most common tetracycline resistance genes found in *E. coli* include *tetA*, *tetB*, *tetC*, *tetD* and *tetE* (Chopra and Roberts, 2001). The prevalence of *tetA* gene in commensal *E coli* isolates from healthy-pigs was around 70% (Sabarinath et al., 2011). Eighty-three percent of ampicillin-resistant *E. coli* isolates from foods, humans and healthy animals carried bla_{TEM}. Similarly, *strA-strB* and/or *aadA* genes were also present in 81% in *E. coli* isolates from meat and meat products (Sunde and Norstrom, 2005). The presence of *cmlA* gene in chloramphenicol resistance in commensal porcine *E. coli* was >80% while the presence of *catA* was <15% (Harada et al., 2006). Sulphonamide resistance genes such as *sul1* (55%), *sul2* (81%) and *sul3* (11%) were prevalent in sulphonamide resistant *E. coli* isolates from pigs in Denmark (Hammerum et al., 2006). Among the trimethoprim resistance determinants, the most frequently found *dfrA* genes in *E. coli* isolates from

cattle, swine and poultry in Germany were *dfrA1* (77%), *dfrA12* (7%) and *dfrA17* (13%) (Guerra et al., 2003).

5. Fluoroquinolones resistance in *E. coli*

Fluoroquinolones, synthetic antimicrobial agents, are used in human and veterinary medicine to treat bacterial infection caused by *E. coli* and other gram-negative bacteria. The major mechanism of fluroquinolone resistance is alteration of target enzymes, DNA gyrase and topoisomerase IV. The DNA gyrase enzyme is composed of two GyrA subunits and two GyrB subunits encoded by *gyrA* and *gyrB*, respectively. The topoisomerase IV enzyme is made up of two ParC subunits and two ParE subunits encoded by *parC* and *parE* respectively. The region in N-termini of GyrA and ParC is related with quinolone resistance known as quinolone resistance determining regions (QRDRs). The major target for fluroquinolones in *E. coli* has been described to be DNA gyrase, specifically the GyrA subunit. Single or double mutation in QRDR of GyrA and further mutation in the QRDR of ParC leads to increase the level of resistance to fluroquinolones (Ruiz et al., 2002). Mutations in *gyrB* and *parE* have also been detected but they are not common like mutations in *gyrA* or *parC*. Amino acid changes at alanine 83, leucine 83, serine 83, aspartate 87, asparagines 87, glycine 87 and tyrosine 87 occurred in GyrA and amino acid alters at arginine 80, isoleucine 80 and serine 80 found in ParC were previously reported from nalidixic acid resistant *E. coli* strains of cattle, swine and poultry (Guerra et al., 2003).

6. Virulence genes in *E. coli*

E. coli infections are associated with virulence factors that are located on mobile genetic elements including chromosome, plasmid, transposons and bacteriophages. Virulence factors in *E. coli* contain adhesins, invasins, toxins and capsule. Virulence genes in pathogenic *E. coli* strains can cause diarrhea and edema disease in swine. Among pathogenic *E.coli* strains, Enterotoxigenic *E. coli* (ETEC) and shiga toxin

producing *E. coli* (STEC) strains are most commomnly found in pigs. ETEC possesses *elt*, *estA* and *estB* genes encoding for heat labile and heat stable enterotoxins that stimulate small intestine to secrete electrolytes and water. ETEC produce fimbriae encoded by *faeG, fanA, fedA, fasA, fim41* that helps to colonize the small intestine and resulting in diarrhea (Nagy and Fekete, 1999). These strains also express the presence of enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1), adhesin involved in diffuse adherence (AIDA-I) and porcine attaching and effacing-associated factor (*paa*) (Zhang et al., 2007).

STEC strains carrying *stx2e* gene and also produce fimbriae to attach and colonize small intestine. The intestine absorb shiga toxin that can cause systemic vascular damage via the blood and resulting edema disease in pig (Gannon et al., 1989).

7. Cointegration of resistance plasmid and virulence plasmid

Many antimicrobial resistance genes in *E. coli* are carried on resistance plasmids (Sherley et al., 2004). Similarly, virulence determinants encoding for heat stable enterotoxin, heat labile enterotoxin in *E. coli* (Gyles et al., 1974) and shigatoxin producing *E. coli* reside on plasmids called virulence plasmids (Brunder et al., 1999). The *E. coli* strains with virulence plasmid could carry additional plasmids such as resistance plasmids. For example, genes coding for *E. coli* enterotoxin and antibiotic resistance including streptomycin, tetracycline and sulfonamides are located on the same plasmid. This virulence-resistance plasmid could transfer horizontally among *E. coli* strains (Gyles et al., 1977).

8. Phylogenetic groups of *E. coli*

Bases on phylogenetic analyses, *E. coli* have been classified into four phylogenetic groups including groups A, B1, B2 and D (Herzer et al., 1990; Picard et al.,

1999). The phylogenetic grouping of *E. coli* could be performed by using multilocus enzyme electrophoresis, ribotyping, and multilocus sequence typing. These reference methods are evidently complicated and time-consuming as well as require an accumulation of typed strains. Therefore, a simple and rapid method, named a triplex PCR method was established to determine phylogenetic groups of *E. coli* (Clermont et al., 2000). The phylo-grouping by the triplex PCR method has been well recognized for screening a large population of *E. coli* strains. The triplex PCR method is based on the presence of three-specific genetic markers for phylogenetic grouping, including *chuA*, the outer-membrane hemin receptor gene, and *yjaA* encoding an uncharacterized protein and, a DNA fragment TSPE4.C2. These three genes were selected based on the libraries of *E. coli* from different phylogenetic groups. They were found to be specific for certain phylogenetic groups and their combinations could be used to designate *E. coli* into four existing-phylogenetic groups. The presence of *chuA* gene identifies two sister groups that are group B2 and D and the others group A and B1. The *yjaA* gene separates group B2 and D and TSPE4.C2 discriminates group A and B1. The commensal *E. coli* isolates are usually assigned in phylogeneic group A and B1 while virulence strains of *E. coli* belong to group B2 and D (Duriez et al., 2001). The Triplex-PCR technique is claimed to be appropriate for pathogenic, non pathogenic strains and unclassified *E. coli* reference strains.

CHAPTER III

MATERIALS AND METHODS

The experiment was divided into 4 phases including phase 1, Characterization of antimicrobial resistance; phase 2, Detection of virulence genes; phase 3, Determination of phylogenetic groups; and phase 4, Determination of plasmid profiles (Figure 2).
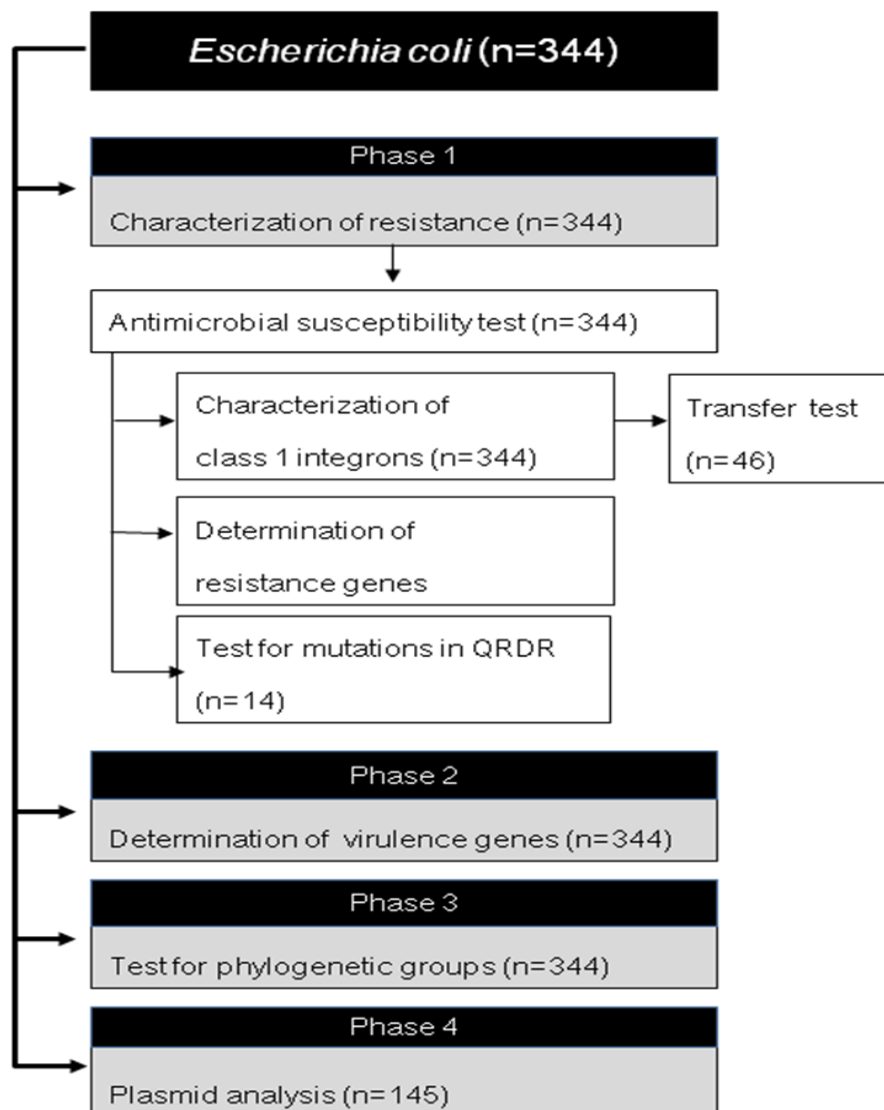
Figure 2: Flow of experiments performed in this study

Escherichial coli isolates

Three-hundred and forty-four *E. coli* isolates were examined in this study. All of the *E. coli* isolates were obtained from the strain collection of Veterinary Diagnostic Laboratory (VDL), Faculty of Veterinary Science, Chulalongkorn University. They were isolated from faecal samples directly collected per rectum from pigs in Ratchaburi, Chonburi, Buriram, Nakhonratchasrima and Udon Thani. These pigs were clinically healthy, confirmed by farm veterinarians. The use of antimicrobial agents in most pig farms was not recorded systematically. However, those well recorded were not allowed to disclose.

The *E. coli* strains were isolated by using the standard method (Quinn et al., 1994) and biochemically confirmed according to the previously-published protocol (Carter and Cole, 1990). The *E. coli* isolates were purified to get single colonies. One colony from each positive sample was collected. All the isolates were stored in 20% glycerol stocks at -80°C. All the *E. coli* isolates were sent to Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University for further investigations.

Phase 1: Characterization of antimicrobial resistance

1.1 Antimicrobial susceptibility test

All the *E. coli* isolates (*n*=344) were examined for their susceptibilities to eight antimicrobials including ampicillin (AMP), chloramphenicol (CHP), ciprofloxacin (CIP), gentamicin (GEN), streptomycin (STR), sulfamethoxazole (SUL), tetracycline (TET) and trimethoprim (TRI) by determination of Minimum Inhibitory Concentration (MIC) using two-fold agar dilution technique according to the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2006). All antimicrobial agents were purchased from Sigma-Aldrich[®] (Steinheim, Germany). Antimicrobial agents were prepared in appropriate

diluents at various concentrations. The diluents and antimicrobial concentrations used are shown in Table 1.

Table 1: Solvents, concentrations and breakpoint of antimicrobial agents used in this study

Antimicrobial	Solvent	Concentration range (µg/ml)	Breakpoint (µg/ml)
ampicillin	sterile distilled water	0, 1-512	32
chloramphenicol	95% ethanol	0, 1-512	32
ciprofloxacin	0.1MNaOH and sterile distilled water	0, 0.125-256	4
gentamicin	sterile distilled water	0, 0.25-256	8
streptomycin	sterile distilled water	0, 1-256	32
sulfamethoxazole	0.1MNaOH and sterile distilled water	0, 1-1024	512
tetracycline	70% ethanol	0, 1-512	16
trimethoprim	dimethyl acetamide	0, 1-512	16

The *E. coli* isolates were grown overnight at 37°C in Mueller-Hinton agar (MHA, Difco®, MD, USA). Single colony was picked and resuspended in 0.85% NaCl solution (NSS) and the cell density was adjusted to 0.5 McFarland (~10^8 CFU/ml). The suspension was ten fold diluted to 10^7 CFU/ml in NSS. Then, the suspensions were transferred into the microtiter plates and inoculated onto the MHA plates that contain suitable concentrations of antibiotic using multipoint-inoculator. After incubation for 18-24 hr at 37°C, the MIC value designated as the lowest concentration of an antimicrobial that inhibits the visible growth of bacteria was observed. Breakpoints that are the antimicrobial concentrations used for determining the isolates as susceptible or resistant are those established by CLSI. Multidrug resistance (MDR) was defined as resistance to at least three or more different classes of antimicrobials. *E. coli* ATCC 25922 was used as quality control organism.

1.2 Characterization of class 1 integrons

For all PCR reactions, template DNA was prepared from all the *E. coli* isolates (*n*=344) by the whole cell boiled lysate procedure as previously described (Levesque et al., 1995). *E. coli* were grown on Luria- Bertani (LB) agar (Difco) at 37°C for overnight. A single colony was picked and suspended in 50 µL of sterile distilled water. Then, the suspension was heated in a boiling water bath for 10 minutes and immediately placed on ice. The suspension was centrifuged at 12,000x*g* for 5 minutes. The supernatant was placed into a new 1.5 ml Eppendorf tube and stored at -20°C. All primers used for characterization of antimicrobial resistance are listed in Table 2.

All *E. coli* isolates were screened for the presence of *intI1* gene by PCR with specific primers *int1*F and *int1*R (Chuanchuen et al., 2007). The PCR reactions consisted of 12.5 µl of Fermentas MasterMix (Fermentas®, Burlington, Canada), 5.5 µl of sterile-distilled water, 1 µl of each primer at 10 µM and 5µl DNA template. PCR thermal cycling conditions were as follows: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 40 sec, 50 °C for 40 sec and 72°C for 1 minute, and one cycle of final extension at 72°C for 5 minutes. PCR products obtained were separated on 1-1.2 % agarose gel electrophoresis (Sigma-Aldrish®) in 1xTris-acetate/EDTA (TAE) buffer. The gels were stained in ethidium bromide solution (Sigma-Aldrish®) and visualized using the Bio-Rad Gel-Documentation System (Bio-Rad Laboratories, Ventura, CA, USA).

All the isolates carrying *intI1* gene were examined for the presence of gene cassettes with PCR using 5´CS and 3´CS primers. PCR conditions included initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 45 sec, 54 °C for 1 minute, and 72°C for 3 minutes and the final extension step for 5 minutes at 72°C. The PCR products were purified using Nucleospin Gel Extraction Kit (Nucleospin®, Gutenberg, France) and submitted for DNA sequencing (Selangor, Malaysia). Nucleotide sequences were analyzed by comparison to the published sequence using

NCBI blast search available at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). The PCR products with the same size were further examined by restriction endonuclease digestion using at least two different restriction endonuclease enzymes including *Alu*I, *Eco*RI, *Mse*I and *Bam*HI. The PCR amplicons generating the same restriction pattern were considered identical.

Table 2: Primers used for characterization of antimicrobial resistance

Primer	Sequence (5'-3')	Gene	Amplicon size (bp)	Reference
Class1 integrons				
Int1F	CCT GCA CGG TTC GAA TG	*intI 1*	497	Chuanchuen et al., 2007
Int1R	TCG TTT GTT CGC CCA GC		497	
5'CS	GGC ATC CAA GCA GCA AG	Variable	Variable	Levesque et al., 1995
3'CS	AAG CAG ACT TGA CCT GA	regions	Variable	
Antimicrobial resistance genes				
bla$_{PSE1}$F	GCA AGT AGG GCA GGC AAT CA	*bla$_{PSE1}$*	422	Chuanchuen et al., 2008
bla$_{PSE}$ R	GAG CTA GAT AGA TGC TCA CAA		422	
bla$_{TEM}$F	ATC AGT TGG GTG CAC GAG TG	*bla$_{TEM}$*	608	Chuanchuen et al., 2008
bla$_{TEM}$R	ACG CTC ACC GGC TCC AGA		608	
catAF	CCA GAC CGT TCA GCT GGA TA	*catA*	452	Chuanchuen et al., 2008
catAR	CAT CAG CAC CTT GTC GCC T		452	
catBF	CGG ATT CAG CCT GAC CAC C	*catB*	461	Chuanchuen et al., 2008
catBR	ATA CGC GGT CAC CTT CCT G		461	
cmlAF	TGG ACC GCT ATC GGA CCG	*cmlA*	641	Chuanchuen et al., 2008
cmlAR	CGC AAG ACA CTT GGG CTG C		641	
aadA1F	CTC CGC AGT GGA TGG CGG	*aadA1*	631	Chuanchuen et al., 2008
aadA1R	GAT CTG CGC GCG AGG CCA		631	
aadA2F	CAT TGA GCG CCA TCT GGA AT	*aadA2*	500	Chuanchuen et al., 2008
aadA2R	ACA TTT CGC TCA TCG CCG GC		500	
aadBF	CTA GCT GCG GCA GAT GAG C	*aadB*	300	Chuanchuen et al., 2008
aadBR	CTC AGC CGC CTC TGG GCA		300	
strAF	TGG CAG GAG GAA CAG GAG G	*strA*	405	Chuanchuen et al., 2008
strAR	AGG TCG ATC AGA CCC GTG C		405	
strBF	GCG GAC ACC TTT TCC AGC CT	*strB*	621	Chuanchuen et al., 2008
strBR	TCC GCC ATC TGT GCA ATG CG		621	
sul1F	CGG ACG CGA GGC CTG TAT C	*sul1*	600	Chuanchuen et al., 2008

Continued Table 2

Primer	Sequence (5'-3')	Gene	Amplicon size (bp)	Reference
sul1R	GGG TGC GGA CGT AGT CAG C		600	
sul2F	GCG CAG GCG CGT AAG CTG AT	*sul2*	514	Chuanchuen et al., 2008
sul2R	CGA AGC GCA GCC GCA ATT C		514	
sul3F	GGG AGC CGC TTC CAG TAA T	*sul3*	500	Chuanchuen et al., 2008
sul3R	TCC GTG ACA CTG CAA TCA TTA		500	
tetAF	GCT GTC GGA TCG TTT CGG	*tetA*	658	Chuanchuen et al., 2008
tetAR	CAT TCC GAG CAT GAG TGC C		658	
tetB F	CTG TCG CGG CAT CGG TCA T	*tetB*	615	Chuanchuen et al., 2008
tetBR	CAG GTA AAG CGA TCC CAC C		615	
dfrA1F	CAA TGG CTG TTG GTT GGA C	*dfrA1*	254	Chuanchuen et al., 2008
dfrA1R	CCG GCT CGA TGT CTA TTG T		254	
dfrA10F	TCA AGG CAA ATT ACC TTG GC	*dfrA10*	432	Chuanchuen et al., 2008
dfrA10R	ATC TAT TGG ATC ACC TAC CC		432	
dfrA12F	TTC GCA GAC TCA CTG AGG G	*dfrA12*	330	Chuanchuen et al., 2008
dfrA12R	CGG TTG AGA CAA GCT CGA AT		330	

QRDRs in *gyrA* and *parC*

Primer	Sequence (5'-3')	Gene	Amplicon size (bp)	Reference
gyrA SALF	GCT GAA GAG CTC CTA TCT GG	*gyrA*	436	Chuanchuen et al., 2010
gyrA SALR	GGT CGG CAT GAC GTC CGG		436	
parCF	GAT CGT GAT CAT GGA TCG TG	*parC*	391	Chuanchuen et al., 2010
parCR	TTC CTG CAT GGT GCC GTC G		391	

1.3 Transferability test of class 1 integrons

All the *E. coli* isolates carrying class 1 integrons with resistance gene cassettes (*n*=46) were tested for the possession of plasmids by using alkaline lysis method (Liou et al., 1999). Transferability test of class 1 integrons were conducted by biparental mating method (Woodall, 2003). All the *E. coli* isolates carrying class 1 integrons with the resistance gene cassettes were used as donors and the spontaneous rifampicin-resistant derivatives of *Salmonella* Enteritidis strain SE12 (rif^r SE12, rifampicin MIC=256 µg/ml) were recipients. SE12 is susceptible to all antimicrobials tested and does not harbor class 1 integrons and plasmid.

To generate the rif^r SE12 strain, the purified SE12 was streaked to get single colonies on LB agar containing rifampicin at different concentrations (i.e. 8, 16, 32, 64, 128 and 256 µg/ml). The inoculated plates were incubated at 37°C overnight. The rifampicin-resistant colonies were collected from each plate and grown on LB agar without rifampicin for 10 consecutive days. At day 11, single colonies of the spontaneous rifampicin-resistant SE12 (MIC=256 µg/ml) were collected and the MICs for other antimicrobial agents were examined.

For biparental mating, the donors and recipients were grown in 4 ml Luria-Bertani (LB) broth at 37°C for overnight in a shaking incubator. Eighty-µl aliquots of each culture of the donors and recipients were separately added into 4 ml of fresh LB broth and incubated at 37°C for 3-4 hours in a shaking incubator to reach log phase of growth. Seven-hundred µl of each pair of donor and recipient was gently mixed in an Eppendorf tube and centrifuged at 8,000 rpm for 1 minute. The supernatant was discarded and the pellets were resuspended in 30 µl LB broth warmed at 37°C. The mixture was gently dropped on a sterile 0.45 µm membrane filter (Millipore, Massachusetts, USA) that was placed on a LB agar plate without antibiotics and incubated at 37°C for 18-24 hours. The inoculated filter paper was taken and placed into

1 ml of NSS in a new Eppendorf tube. The tube was vortexed to dislodge the cells and then the filter paper was removed. The suspension was centrifuged at 13,000x*g* for 1 minute and the supernatant was discarded. A hundred-µl of fresh LB broth was added into the bacterial pellets. The conjugation mixture was spreaded on LB agar supplemented with 32 µg/ml of rifampicin and one of the following antibiotics: streptomycin (50µg /ml), and trimethoprim (10 µg/ ml). Then the mixture was incubated at 37°C for overnight. The colonies were picked and grown on Brilliant Green (BG) agar (Difco) with antibiotics. Transconjugants that were rif^r SE12 with resistance plasmid appeared as pink colonies on BGA. Transconjugants were confirmed to be *Salmonella* by growing on Xylose Lysine Deoxycholate (XLD) agar (Difco) with corresponding antibiotics and appeared black colonies on XLD agar. Plasmid DNA was extracted from each transconjugant using alkaline lysis method and tested for the presence of *intI1* with gene cassettes corresponding to those in the donors.

1.4 Detection of antimicrobial-resistance encoding genes

All the *E. coli* isolates were examined for the presence of 17 resistance genes based on their resistance phenotypes (Table 3) by using multiplex Polymerase Chain Reaction (mPCR) (Chuanchuen et al., 2008b). PCR assays were conducted in a final volume of 25 µL using PCR Master Mix (Fermentas$^®$) according to the manufacturer's instructions. Each multiplex PCR reaction contained 12.5 µl of master mix, 3.5 µl of sterile distilled water, 1 µl of each primer at 10µM and 5 µl of DNA template. PCR amplifications were performed on a PCR T-personal combi model$^®$ (Biometra$^®$, Germany). PCR thermal cycling condition consisted of an initial denaturation at 94°C for 5 minutes, and 30 cycles each of which consisting of denaturation for 45 seconds at 94°C, primer annealing for 45 seconds at 54°C, and DNA extension for 1 minute at 72°C and one cycle of a final extension at 72°C for 5 minutes. The PCR products were separated on 1.2 % agarose gel electrophoresis in 1x TAE buffer.

Table 3: Antimicrobial resistance phenotypes and their respective resistance encoding genes tested

Resistance phenotype	Resistance genes
Ampicillin	bla_{PSE1}, bla_{TEM}
Chloramphenicol	*catA*, *catB*, *cmlA*
Gentamicin	*aadA1*, *aadA2*, *aadB*
Streptomycin	*aadA1, aadA2, strA-strB*
Sulfamethoxazole	*sul1*, *sul2*, *sul3*
Tetracycline	*tetA*, *tetB*
Trimethoprim	*dfrA1*, *dfrA10*, *dfrA12*

1.5 Examination of mutations of the QRDRs in *gyrA* and *parC* genes

The *E. coli* strains exhibiting resistance to ciprofloxacin were examined for mutation (s) in Quinolone Resistance Determining Regions (QRDRs) of *gyrA* and *parC* using PCR and DNA sequencing. Amplifications of the QRDRs of *gyrA* and *parC* were performed using primer pairs gyrA SALF/gyrA SALR and parCF/parCR (Chuanchuen et al., 2010). PCR conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 45 sec, 54 °C for 45 sec, and 72°C for 1 minute and the final extension step for 5 minutes at 72°C. PCR products were purified and submitted for DNA sequencing. The nucleotide sequences were analyzed by comparison to the published *gyrA* (GenBank accession number X06373) and *parC* genes (M58408) using Edit seq and Seq man programs.

Phase 2: Detection of virulence genes

All the *E. coli* isolates were screened for the presence of 14 virulence genes (Table 4) using multiplex PCR with specific primer pairs (Boerlin et al., 2005; Bosworth and Casey, 1997; Chapman et al., 2006; Ngeleka et al., 2003). The primers used in phase 2 are listed in Table 5. All PCR assays were carried out in the final volume of 25 µl containing 12.5 µl of Fermentas MasterMix, 3.5 µl of sterile-distilled water, 1 µl of each primer at 10 µM and 5 µl of DNA template. PCR amplication for *elt*, *estA*, *estB*, *faeG*, *fanA*, *fasA*, *fedA*, *fimF41* and *stx2e* genes were performed using the following cycling: 1 cycle of an initial denaturation at 95°C for 15 minutes; 30 cycles of which each consisting of 1 minute at 95°C, 63 sec at 55°C, and 2 minutes at 72°C; and 1 cycle for 10 minutes at 72°C. PCR thermal cycling conditions for *astA*, *eaeA, paa, aidA* and *sepA* genes included an initial denaturation at 94°C for 15 minutes; followed by 35 cycles of 1 minute at 94°C, 90 sec at 62°C, 1 minute at 72°C and a final step of 72°C for 10 minutes. The PCR products were separated on 1.2 % agarose gel electrophoresis in 1x TAE buffer.

Table 4: Virulence genes that were determined in this study

Virulence factor	Virulence gene
Heat labile enterotoxin	*elt*
Heat stable enterotoxin	*estA*, *estB*
Enteroaggregative heat-stable enterotoxin	*astA*
Shiga toxin	*stx2e*
Fimbriae (F4, F5, F6, F18, F41)	*faeG*, *fanA*, *fasA*, *fedA*, *fimF41*
Attaching / effacing activity	*eaeA*, *paa*, *aidA, sepA*

Table 5: Primers used for determination of virulence genes

Primer	Sequence (5'-3')	Gene	Amplicon size (bp)	Reference
LTF	GGC GTT ACT ATC CTC TCT AT	*elt*	272	Bosworth and Casey, 1997
LTR	TGG TCT CGG TCA GAT ATG T		272	
STaF	CAA CTG AAT CAC TTG ACT CTT	*estA*	158	Bosworth and Casey, 1997
STaR	TTA ATA ACA TCC AGC ACA GG		158	
STbF	TGC CTA TGC ATC TAC ACA AT	*estB*	113	Bosworth and Casey, 1997
STbR	CTC CAG CAG TAC CAT CTC TA		113	
EAST1F	TCG GAT GCC ATC AAC ACA GT	*astA*	125	Ngeleka et al., 2003
EAST1R	GTC GCG AGT GAC GGC TTT GTA AG		125	
Stx2eF	AAT AGT ATA CGG ACA GCG AT	*stx2e*	733	Bosworth and Casey, 1997
Stx2eR	TCT GAC ATT CTG GTT GAC GC		733	
F4F	GAA TCT GTC CGA GAA TAT CA	*faeG*	499	Bosworth and Casey, 1997
F4R	GTT GGT ACA GGT CTT AAT GG		499	
F5F	AAT ACT TGT TCA GGG AGA AA	*fanA*	230	Bosworth and Casey, 1997
F5R	AAC TTT GTG GTT AAC TTC CT		230	
F6F	GTA ACT CCA CCG TTT GTA TC	*fasA*	409	Bosworth and Casey, 1997
F6R	AAG TTA CTG CCA GTC TAT GC		409	
F18F	TGG TAA CGT ATC AGC AAC TA	*fedA*	313	Bosworth and Casey, 1997
F18R	ACT TAC AGT GCT ATT CGA CG		313	
F41F	GAG GGA CTT TCA TCT TTT AG	*fimF41*	431	Chapman et al., 2006
F41R	AGT CCA TTC CAT TTA TAG GC		431	
eaeAF	GAC CCG GCA CAA GCA TAA GC	*eaeA*	384	Chapman et al., 2006
eaeAR	CCA CCT GCA GCA ACA AGA GG		384	
PAAF	GGC CCG CAT ACA GCC TTG	*paa*	282	Boerlin et al., 2005
PAAR	TCT GGT CAG GTC GTC AAT ACT C		282	
AIDAF	ACA GTA TCA TAT GGA GCC A	*aidA*	585	Ngeleka et al., 2003
AIDAR	TGT GCG CCA GAA CTA TTA		585	
SEPAF	TAA AAC CCG CCG CCT GAG TA	*sepA*	611	Boerlin et al., 2005
SEPAR	TGC CGG TGA ACA GGA GGT TT		611	

Phase 3: Determination of phylogenetic groups

The phylogenetic groups were determined in all the *E. coli* isolates by using a triplex PCR-based method as previously described (Clermont et al., 2000). This examination was performed using three genetic markers including *chuA*, the outer membrane hemin receptor gene; *yjaA*, an uncharacterized protein encoding gene and TSPE4.C2, a part of a putative lipase esterase gene. The primers used in phase 3 are listed in Table 6. All triplex PCR assays were conducted in the final volume of 25 μl as described above. PCR thermal cycling conditions included 1 cycle of an initial denaturation at 94°C for 5 minutes; 30 cycles of which each consisting of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C; and 1 cycle for 7 minutes at 72°C. The outcome PCR products were separated on 2 % agarose gel electrophoresis. All the isolates tested were classified into one of four phylogenetic groups based on the PCR products obtained from triplex PCR as shown in Figure 3 and Table 7.

Table 6: Primers for identification of phylogenetic groups

Primer	Sequence (5'-3')	Gene	Amplicon size (bp)	Reference
ChuAF	GAC GAA CCA ACG GTC AGG AT	*chuA*	279	Clermont et al., 2000
ChuAR	TGC CGC CAG TAC CAA AGA CA		279	
YjaAF	TGA AGT GTC AGG AGA CGC TG	*yjaA*	211	Clermont et al., 2000
YjaAR	ATG GAG AAT GCG TTC CTC AAC		211	
TspE4C2F	GAG TAA TGT CGG GGC ATT CA	TspE4C2	152	Clermont et al., 2000
TspE4C2R	CGC GCC AAC AAA GTA TTA CG		152	
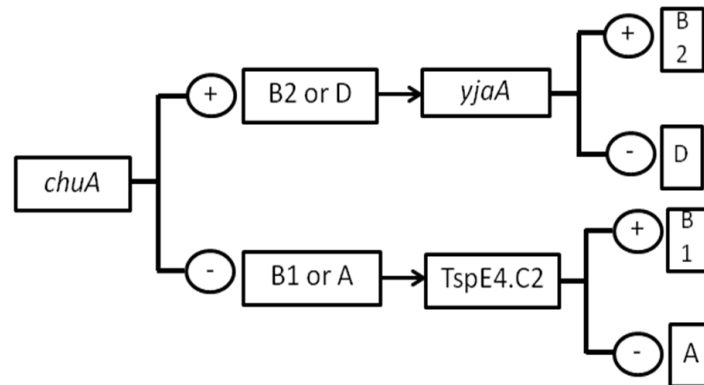
Figure 3. Phylogenetic groups classification by using triplex PCR amplification results

Table 7: Interpretation for phylgenetic groups

Phylogenetic groups	The presence of three genetic markers		
	ChuA	*YjaA*	TSPE4.C2
A	-	-/+	-
B1	-	-/+	+
B2	+	+	-/+
D	+	-	-/+

Phase 4: Determination of plasmid profiles

A total of 145 *E. coli* isolates were randomly selected to detect the presence of plasmid DNA. Plasmid DNA was extracted by using the alkaline lysis method (Liou et al., 1999).

All the *E. coli* isolates were grown in LB broth and incubated at 37°C for overnight. The 1.5 ml of bacteria culture was removed into a new Eppendorf tube and

centrifuged at 16,000x*g* for 5 minutes and the supernatant was discarded. Then, the cell pellets were washed with 1 ml of phosphate buffer saline (PBS, Diagxotics®, Wilton, USA). A hundred-µl of 10 mg/ml lysozyme (Biobasic Inc®, Canada) was added and the cell pellets were resuspended by pipetting. The suspension was incubated for 5-10 minutes at 37°C. Then, two hundred-µl of lysis solution containing 0.2 N NaOH and 1% sodium dodecyl sulfate (SDS, Amresco®, Ohio, USA) was added. The mixture was mixed by inverting and incubated on ice for 5 minutes. One hundred-fifty-µl of cold potassium acetate, pH 4.8 was added. The mixture was again mixed by vortexing, incubated on ice for 5 minutes and centrifuged at 16,000x*g* for 5 minutes. The supernatant was pipetted to a new eppendorf tube. Two-µl of 10 mg/ml Ribonuclease A (RNaseA, Fermetas®) was added and the mixture was incubated at 37°C for 30 minutes. Four hundred-µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The mixture was mixed by vortexing, and centrifuged at 16,000x*g* for 2 minutes. The aqueous phase was transferred to a new eppendorf tube and 1 ml of cold absolute ethanol was added. The mixture was incubated at -20°C for 24 hours and centrifuged at 16,000x*g* for 5 minutes. The supernatant was removed. The pellet was washed with 1 ml of cold 70% ethanol for one time. The DNA pellet was air-dried at room temperature for 10-15 minutes and dissolved in 15 µl sterile distilled water. The plasmid DNA was stored at -20°C. Purified plasmids were separated on 0.5-1% agarose gels using the horizontal gel electrophoeresis system. The experiment was repeated in three separate occasions for each *E. coli* isolate.

The molecular weights of plasmids were estimated using the standard curve produced by plotting $\log_{10}$ molecular weights (kb) of known DNA sizes versus their migration distance (mm). The relative mobilities on agarose gel were used to estimate sizes of unknown plasmids. Plasmid profiles (PPs) were assigned based on the sizes and numbers of plasmids (Kado and Liu, 1981).

Statistical analysis

The associations between antimicrobial-resistance phenotypes, resistance-encoding genes, virulence genes and phylogenetic groups were analyzed by using Pearson's chi-square test (SPSS, version 17.0). A P value of <0.05 was considered statistically significant. Odd ratios and their 95% confidence intervals (CI) were calculated.

CHAPTER IV

RESULTS

1. Characterization of antimicrobial resistance

1.1 Antimicrobial susceptibility

The minimum inhibitory concentrations of antimicrobial agents required to inhibit 50% (MIC $_{50}$), 90% (MIC $_{90}$) and MIC range of *E. coli* strains tested are shown in Table 8.

Table 8: Antimicrobial susceptibilities of *E. coli* isolates (*n*=344)

Antimicrobial agents	MIC (µg/ml)		
	MIC$_{50}$	MIC$_{90}$	MIC range
ampicillin	1024	1024	1-1024
chloramphenicol	128	1024	1-1024
ciprofloxacin	8	128	0.125-256
gentamicin	32	512	0.25-512
streptomycin	128	512	1-1024
sulfamethoxazole	512	1024	2-2048
tetracycline	256	512	2-512
trimethoprim	1024	1024	1-1024

Distribution of antimicrobial resistance is shown in Figure 4. Most of the isolates were resistant to tetracycline (96.2%), followed by ampicillin (91.6%), streptomycin (82.6%), chloramphenicol (79.4%), trimethoprim (79.4%), sulfamethoxazole (67.4%), gentamicin (63.4%) and ciprofloxacin (52.3%).

Ninety-nine percent of the isolates were resistant to at least one antimicrobial agent. Five isolates were susceptible to all antimicrobial agents tested. Ninety-eight percent of the isolates were resistant to at least 3 different classes of antimicrobial agents and were defined as MDR. Sixteen percent of the isolates were resistant to all antimicrobial agents tested. A total of 41 different antibiotic resistance patterns were observed of which the most common pattern was AMP-CHP-CIP-GEN-STR-SUL-TET-TRI (Table 9).
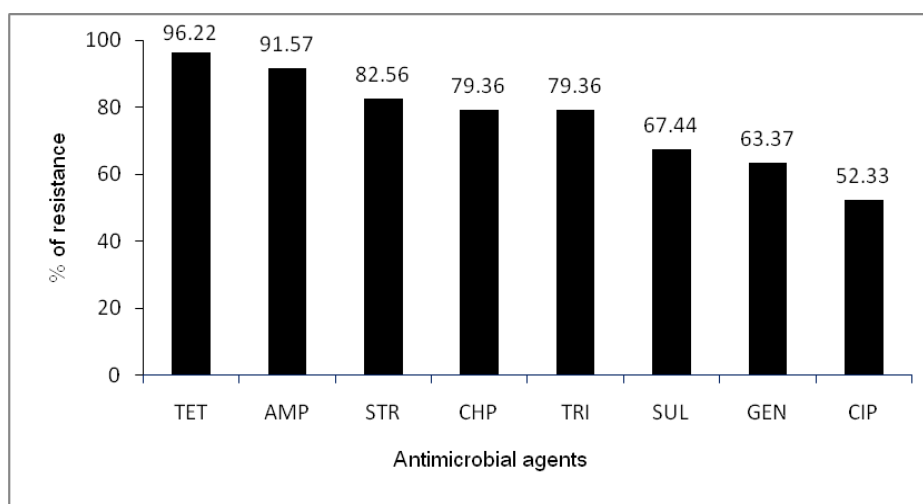
Figure 4: Distribution of antimicrobial resistance in *E. coli* (*n*=344). Abbreviations: AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; GEN, Gentamicin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim

Table 9: Antibiotic resistance patterns of *E. coli* isolates (*n*=339)

Antibiotic resistance patterns	No. of isolates (%)
CIP	1 (0.3)
CIP-GEN	2 (0.6)
AMP-CHP-TET	8 (2.3)
AMP-CIP-STR	1 (0.3)
AMP-STR-TET	1 (0.3)
CHP-TET-TRI	11(3.2)
AMP-CHP-CIP-TET	2 (0.6)
AMP-CHP-TET-TRI	6 (1.7)
AMP-GEN-SUL-TET	1 (0.3)
AMP-STR-SUL-TET	5 (1.5)
CHP-CIP-TET-TRI	1 (0.3)
CHP-STR-TET-TRI	6 (1.7)
AMP-CHP-CIP-TET-TRI	7 (2.0)
AMP-CHP-GEN-STR-TET	3 (0.9)
AMP-CHP-GEN-STR-TRI	1 (0.3)
AMP-CHP-SUL-TET-TRI	7 (2.0)
AMP-CIP-GEN-STR-TET	4 (1.2)
AMP-GEN-STR-SUL-TET	17 (4.9)
AMP-GEN-STR-TET-TRI	3 (0.9)
AMP-GEN-SUL-TET-TRI	1 (0.3)
AMP-STR-SUL-TET-TRI	1 (0.3)
CHP-GEN-STR-TET-TRI	1 (0.3)
CHP-STR-SUL-TET-TRI	1 (0.3)
AMP-CHP-CIP-GEN-STR-TRI	3 (0.9)
AMP-CHP-CIP-STR-TET-TRI	1 (0.3)

Continued Table 9:

Antibiotic resistance patterns	No. of isolates (%)
AMP-CHP-CIP-SUL-TET-TRI	4 (1.2)
AMP-CHP-GEN-STR-TET-TRI	9 (2.6)
AMP-CHP-GEN-STR-SUL-TET	10 (2.9)
AMP-CHP-STR-SUL-TET-TRI	25 (7.3)
AMP-CIP-GEN-STR-SUL-TET	2 (0.6)
AMP-CIP-GEN-STR-TET-TRI	2 (0.6)
AMP-CIP-GEN-SUL-TET-TRI	2 (0.6)
AMP-GEN-STR-SUL-TET-TRI	7 (2.0)
CHP-CIP-GEN-STR-TET-TRI	1 (0.3)
AMP-CHP-CIP-GEN-STR-SUL-TET	9 (2.6)
AMP-CHP-CIP-GEN-STR-TET-TRI	33 (9.6)
AMP-CHP-CIP-GEN-SUL-TET-TRI	2 (0.6)
AMP-CHP-CIP-STR-SUL-TET-TRI	33 (9.6)
AMP-CHP-GEN-STR-SUL-TET-TRI	35 (10.2)
AMP-CIP-GEN-STR-SUL-TET-TRI	16 (4.7)
AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	54 (15.7)
Total	339

Abbreviations: AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; GEN, Gentamicin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim

1.2. Characterization of class 1 integrons

1.2.1 The presence of class 1 integrons

Of all isolates, 73% of the *E. coli* isolates were positive for *intI1*. The DNA representative was also submitted for nucleotide sequencing and the sequencing result confirmed the specificity of primers. The PCR amplicons of *intI1* are shown in Figure 5.
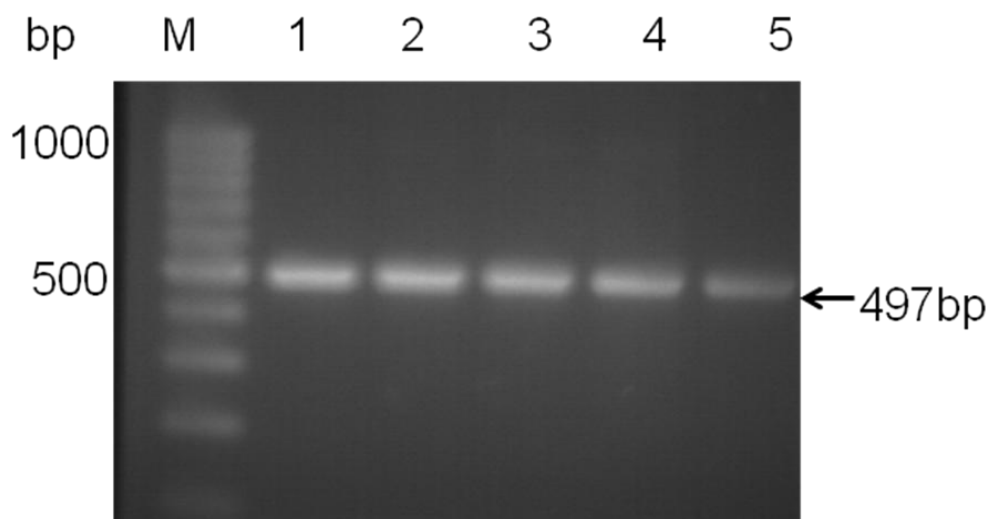
Figure 5. PCR amplicons of *intI1* gene. PCR amplifications were carried out using primers int1F and int1R and yield a 497 bp amplicon. M, 100bp marker; lanes 1-5, *intI1* gene

1.2.2 Antimicrobial resistance rates of the *intI1*-positive and negative *E. coli* isolates

Resistance rates of antimicrobial agents including chloramphenicol, ciprofloxacin, sulfamethoxazole, tetracycline and trimethoprim in the *intI1*-positive *E. coli* isolates were significantly higher than those in the *intI1*-negative isolates (P<0.05). In contrast, the resistance rates of gentamicin and streptomycin in the *intI1*-negative isolates were significantly higher than those in the *intI1*-positive *E. coli* isolates (P<0.05). Ampicillin resistance rate was not significantly different between these two groups of *E. coli* strains (P≥ 0.05) (Appendix B). Distribution of antimicrobial resistance based on the presence of calss 1 integrons is shown in Figure 6.
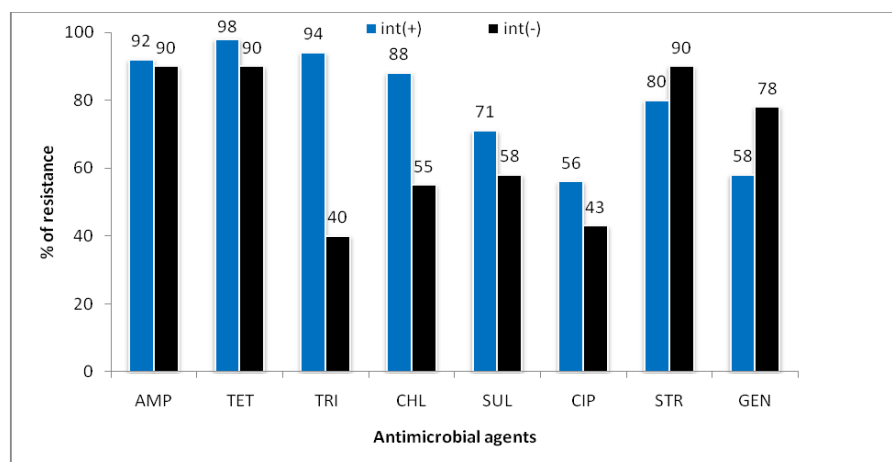
Figure 6: A comparison of antimicrobial resistance rates in the *intI1*-positive and *intI1*-negative *E. coli* isolates. Abbreviations: AMP, ampicillin; TET, tetracycline; TRI, trimethoprim; CHP, chloramphenicol; SUL, sulfamethoxazole; CIP, ciprofloxacin; STR, streptomycin; GEN, gentamicin, GEN.

1.2.3 Gene cassettes in class 1 integrons-variable region

All the *intI1*-positive *E. coli* isolates were detected for the presence of gene cassettes in variable regions. Among these isolates, 56 isolates (22.3%) carried gene cassettes in variable regions with the size ranging from 650 bp to 2700 bp (Table 10). Nucleotide sequencing revealed five gene cassette arrays including incomplete *sat*, *aadA22*, *aadA1*, *dfrA12-aadA2*, and *sat-psp-aadA2* (Figure 7). Among these gene cassette arrays, *aadA1* was most commonly observed (26.8%) followed by *dfrA12-aadA2* (25%), incomplete *sat* (17.9%), *sat-psp-aadA2* (17.9%) and *aadA2* (12.5%). All the *E. coli* isolates carrying class 1 integrons with gene cassette isolates were MDR. Restriction patterns of variable region with gene cassette arrays are shown in Figure 8.
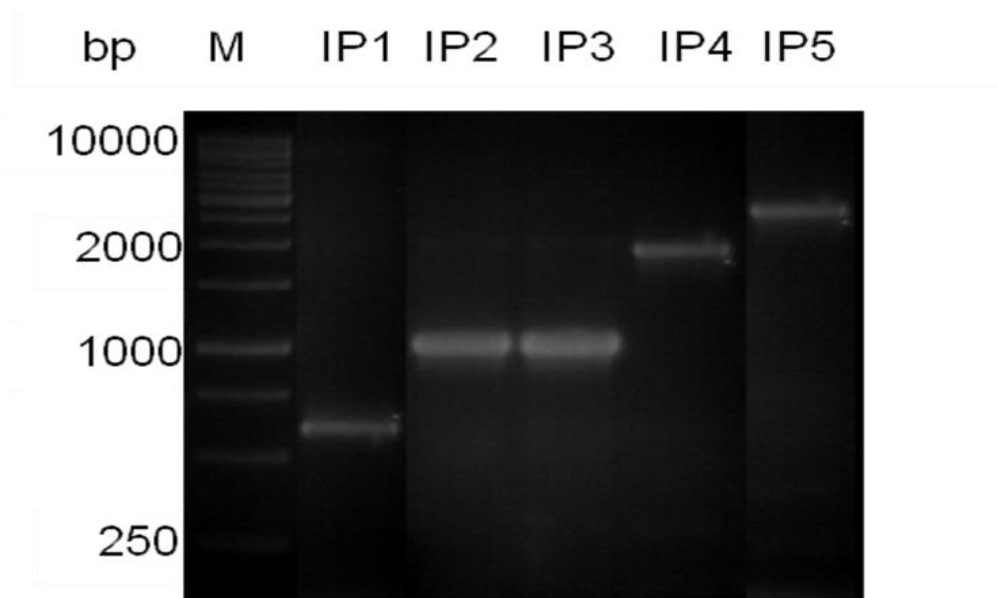
Figure 7: PCR amplicons of class 1 integrons variable regions. Lane M, 1kb marker, Lane 1; IP 1, incomplete *sat*, lane 2; IP 2*, aadA22*, lane 3; IP 3, *aadA1*, lane 4; IP 4, *dfrA12-aadA2* and lane 5; IP 5, *sat-psp-aadA2*.
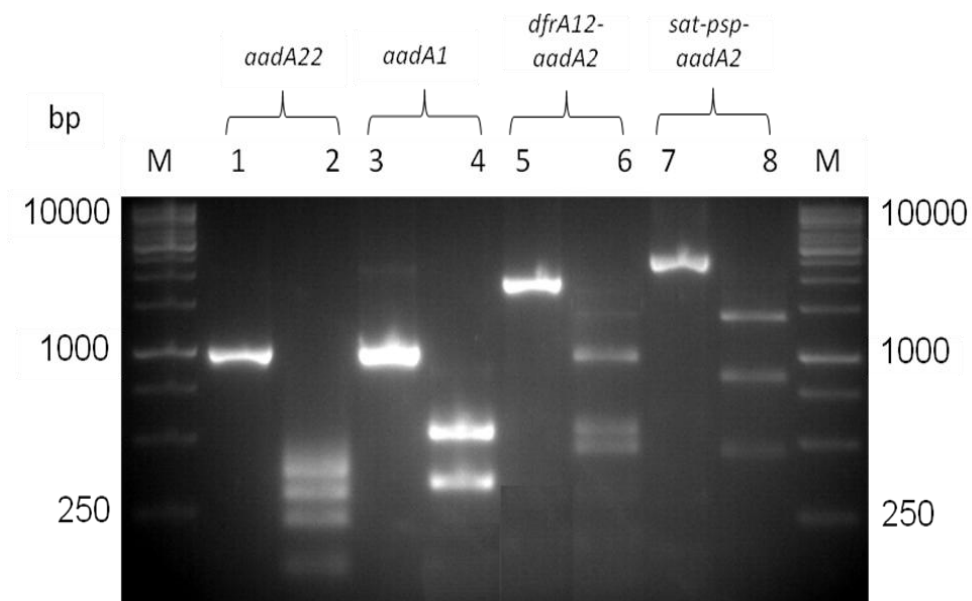
Figure 8: Restriction patterns of gene cassette arrays. M, 1kb marker

Lane 1, 3, 5, 7, uncut PCR products containing resistanc gene cassettes, Lane 2, *Alu*I digested *aadA22*; Lane 4, *Mse*Idigested *aadA1*; Lane 6, *Eco*RI digested *dfrA12-aadA2*, and Lane 8, *Bam*HI and *Eco*RI digested *sat-psp-aadA2*.

Table 10: Class 1 integrons in *E. coli* with gene cassettes (*n*=56)

Integron profile	Amp. Size (bp)	Gene cassette	No. of isolates (%)	Antimicrobial resistance pattern (no. of isolate)	Virulence gene profile
I	650	Incomplete *sat*	10 (17.9)	AMP-CHP-CIP-STR-SUL-TET-TRI (10)	*astA*, *elt*, *estB*, *fasA* (10)
II	1000	*aadA1*	15 (26.8)	CHP-STR-TET-TRI (3)	*elt*, *fasA* (4)
				AMP-GEN-STR-TET-TRI (3)	*astA*, *elt*, *estB*, *faeG*, *fasA*, *sepA* (10)
				AMP-GEN-SUL-TET-TRI (1)	*eaeA*, *elt*, *estA*, *fasA*, *fedA*, *paa* (1)
				CHP-GEN-STR-TET-TRI (1)	
				AMP-GEN-STR-SUL-TET-TRI (4)	
				AMP-CHP-GEN-STR-SUL-TET-TRI (1)	
				AMP-CIP-GEN-STR-SUL-TET-TRI (1)	
				AMP-CHP-CIP-GEN-STR-SUL-TET-TRI (1)	
III	1000	*aadA22*	7 (12.5)	AMP-CHP-GEN-STR-TRI (1)	*elt*, *fasA* (7)
				AMP-CHP-GEN-STR-TET-TRI (3)	
				AMP-CHP-GEN-STR-SUL-TET-TRI (3)	
IV	2000	*dfrA12-aadA2*	14[a] (25.0)	AMP-CIP-GEN-STR-TET-TRI (1)	*elt*, *fasA* (5)
				AMP-CHP-CIP-GEN-STR-TET-TRI (4)	*astA*, *estA*, *fasA* (1)
				AMP-CHP-CIP-GEN-STR-SUL-TET-TRI (9)	*astA*, *fasA* (6)
					astA, *faeG*, *fasA* (2)
V	2700	*sat-psp-aadA2*	10 (17.9)	AMP-CHP-GEN-STR-SUL-TET-TRI (10)	*elt*, *fasA* (10)

[a] Eight out of fourteen isolates could horizontally transfer class 1 integrons.

1.3 Transfer of class 1 integrons with resistance gene cassettes

All the *E. coli* isolates carrying class 1 integrons with antibiotic resistance gene cassettes inserted in variable regions (n=46) were detected for the possession of plasmid DNA and found to carry plasmids with size ranging from 1 kb to 24.5 kb.

The conjugation experiments showed that eight *E. coli* isolates carrying class 1 integrons *dfrA12-aadA2* gene cassette array could horizontally transfer their plasmids to the *Salmonella* recipients. The transconjugants were obtained in LB agar containing 32 µg/ml of rifampicin in combination 50 µg/ml streptomycin or 10 µg/ml and trimethoprim. These horizontally-transferred plasmids were confirmed to contain class 1 integrons with *dfrA12-aadA2* array.

1.4 The presence of antibiotic resistance genes

Sixteen antimicrobial resistance genes except bla_{PSE} were detected in *E. coli* isolates (Table 11). Only bla_{TEM} was identified in ampicillin-resistant strains (94%). The *cmlA* gene was found in the majority of chloramphenicol-resistant strains (89%) and *aadA1* was most prevalent among gentamicin-resistant strains (92%). Most of streptomycin-resistant isolates (89%) were positive to *aadA1* and followed by *strA-strB* (82%). The *sul3* gene was the most commonly-found sulfonamide-resistance encoding gene in sulfonamide-resistant strains (82%). The *tetA* and *dfrA12* genes were most prevalent among the tetracycline- (79%) and trimethoprim- (70%) resistant isolates, respectively. Most resistant *E. coli* strains carried at least one resistance gene matching up with their resistance phenotypes. Some resistant *E. coli* isolates were positive to multiple genes encoding for resistance to a single antimicrobial agent.

Table 11: Antibiotic resistance genes in *E. coli* isolates from pigs

Resistance phenotype	Resistance genes	Number (%)
Ampicillin (315)	bla_{PSE1}	0 (0)
	bla_{TEM}	297 (94.3)
Chloramphenicol (273)	*catA*	10 (3.7)
	catB	9 (3.3)
	cml1	167 (61.2)
	catB, cml1	76 (27.8)
	At least one	262 (95.9)
Gentamycin (218)	*aadB*	13 (5.9)
	aadA1	14 (6.4)
	aadA2	4 (1.8)
	aadB, aadA1	10 (4.6)
	aadA1, aadA2	114 (52.3)
	aadB, aadA1, aadA2	63 (28.9)
	At least one	218 (100)
Streptomycin (284)	*aadA1*	7 (2.5)
	aadA2	3 (1.1)
	strA-strB	21 (7.4)
	aadA1, aadA2	41 (14.4)
	strA-strB, aadA1	34 (11.9)
	strA-strB, aadA2	5 (1.8)
	strA-strB, aadA1, aadA2	173 (60.9)
	At least one	284 (100)
Sulfamethoxazole (232)	*sul2*	10 (4.31)
	sul3	129 (55.6)
	sul1, sul2	11 (4.7)
	sul2, sul3	62 (26.7)
	At least one	212 (91.4)
Tetracycline (331)	*tetA*	184 (55.6)
	tetB	46 (13.9)
	tetA, tetB	77 (23.3)
	At least one	307 (92.8)
Trimethoprim (273)	*dfrA1*	12 (4.4)
	dfrA10	32 (11.7)
	dfrA12	119 (43.6)
	dfrA1, dfrA12	17 (6.2)
	dfrA10, dfrA12	45 (16.5)
	dfrA1, dfrA10, dfrA12	10 (3.7)
	At least one	235 (86.1)

1.5 Mutations of the QRDRs in *gyrA* and *parC* genes

Among 344 *E. coli* isolates, 180 (52.3%) isolates were resistant to ciprofloxacin. From these resistant isolates, 14 isolates (MIC ranged 8-128 µg/ml) were randomly picked for examination of mutations in QRDRs of *gyrA* and *parC.* Eleven strains were detected at least a single point mutation in *gyrA* (10 isolates) and/or *parC* (11 isolates) (Table 12 and Appendix A). Three point mutations identified in *gyrA* consisted of C-248-T leading to Ser-83-Leu, G-259-A leading to Asp-87-Asn and A-281-C leading to Gln-94-Pro. Among 10 isolates carrying mutations in GyrA, only one isolate (MIC 32 µg/ml) carried all three point mutations in GyrA, seven isolates (MIC ranged 8-128 µg/ml) possessed double mutations in GyrA, and two isolates (MIC 16 and 64 µg/ml) carried single point mutation GyrA. Nine *E. coli* isolates with a point mutation in *gyrA* additionally possessed mutations in *parC*. A single point mutation in ParC in 11 *E. coli* isolates was G-173-T leading to Ser-58-Ile. Two isolates carrying point mutation in ParC (MIC 32 and 128 µg/ ml) did not contain GyrA mutation.

Table 12: Mutations in QRDRs of *gyrA* and *parC*

Isolate	MIC	*gyrA*		*parC*	
No.	µg/ml	Nucleotide change	Amino acid change	Nucleotide change	Amino acid change
92.3	8	C-248-T, G-259-A	Ser-83-Leu, Asp-87-Asn	G-173-T	Ser-58-Ile
84.2	8	C-248-T, G-259-A	Ser-83-Leu, Asp-87-Asn	G-173-T	Ser-58-Ile
92.5	8	C-248-T, G-259-A	Ser-83-Leu, Asp-87-Asn	G-173-T	Ser-58-Ile
106.2	8	C-248-T, G-259-A	Ser-83-Leu, Asp-87-Asn	Silent mutation	Silent mutation
33.3	16	Silent mutation	Silent mutation	Silent mutation	Silent mutation
34.3	16	Silent mutation	Silent mutation	Silent mutation	Silent mutation
55.6	16	C-248-T	Ser-83-Leu	G-173-T	Ser-58-Ile
57.1	32	Silent mutation	Silent mutati on	G-173-T	Ser-58-Ile
56.5	32	C-248-T, G-259-A, A-281-C	Ser-83-Leu, Asp-87-Asn, Gln-94-Pro	G-173-T	Ser-58-Ile
83.1	32	C-248-T, G-259-A	Ser-83-Leu, Asp-87-Asn	G-173-T	Ser-58-Ile
55.4	64	G-259-A	Asp-87-Asn	G-173-T	Ser-58-Ile
54.7	128	C-248-T, G-259-A	Ser-83-Leu, Asp-87-Asn	G-173-T	Ser-58-Ile
55.1	128	Silent mutation	Silent mutation	G-173-T	Ser-58-Ile
55.9	128	C-248-T, G-259-A	Ser-83-Leu, Asp-87-Asn	G-173-T	Ser-58-Ile

2. The presence of virulence genes

Distribution of virulence genes is shown in (Figure 9). Ten virulence genes were detected in all 344 *E. coli* isolates (Figure 10). The virulence genes most commonly found were *fasA* (98.3%), followed by *elt* (57.9%), *astA* (34.9%), *estB* (25%), *paa* (22.7%), *fedA* (16.9%), *sepA* (14.5%), *eaeA* (13.1%), *faeG* (4.7%) and *estA* (0.6%). The *sxtx2e*, *fanA*, *aidA* and F41 genes were not found among the isolates in this study. All virulence genes observed were arranged into 30 virulence gene profiles of which the predominant virulence gene profile was *elt, fasA* (28.2%). At least one virulence gene was detected in all isolates and at least two virulence genes were detected in 271 isolates (78.8%). Four to eight virulence genes were detected in 95 isolates (27.6%).
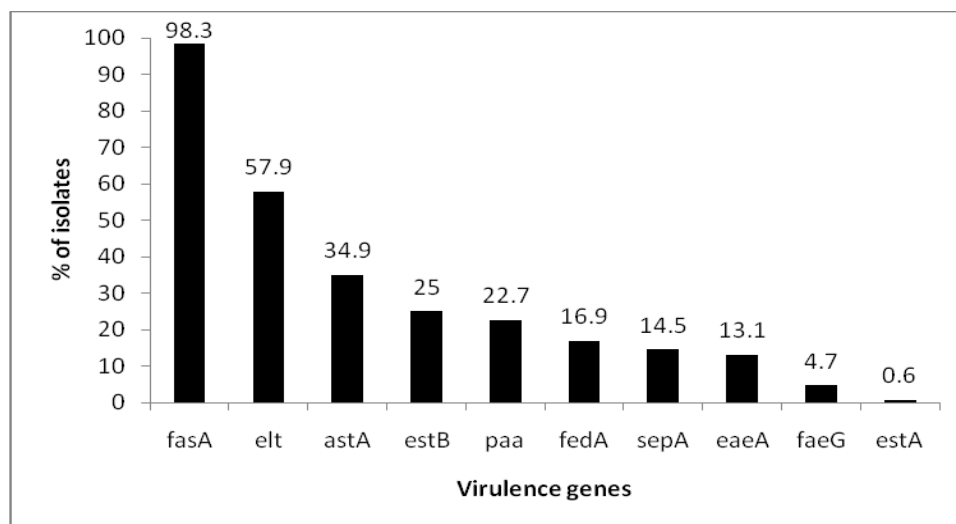
Figure 9: Distribution of virulence genes in *E. coli* isolates (n=344).
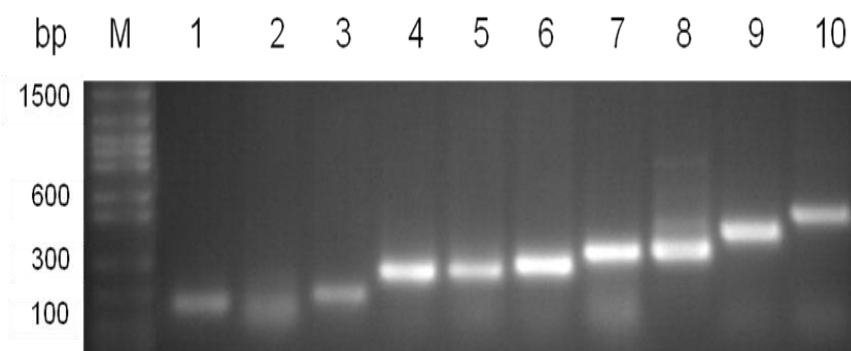
Figure 10: PCR amplicons of virulence genes in *E. coli* isolates*.* Lane M, 100-bp marker; lane 1 *estB* (113bp)*;* lane 2*, astA* (125 bp); lane 3*, estA* (158 bp); lane 4*, elt* (272 bp); lane 5, *paa* (282 bp); lane 6*, fedA* (313 bp); lane 7, *eaeA* (384 bp), lane 8, *fasA* (409 bp), lane 9, *faeG* (499 bp), lane 10, *sepA* (611 bp).

2.1 Association between virulence genes in *E. coli* isolates

The association between eight virulence genes including *faeG*, *paa*, *astA*, *fedA*, *sepA*, *estB*, *elt* and *eaeA* were observed (Table 13). Among eight virulence genes, six genes including *faeG, astA, fedA, sepA, estB* and *eaeA* revealed positive associations to certain virulence genes. Among these, the strongest association was between *fedA* and *paa* (OR=143.4). Negative association was identified only between *paa* and *elt*, while these two genes were positively associated with other virulence genes.

Table 13: The association between virulence genes of *E. coli* isolates (*n*=344) [a]

	faeG	paa	astA	fedA	sepA	estB	elt	eaeA
faeG		-	31.9 (4.2-244.4)	-	56.8(12.4-260.0)	10.3 (3.2-32.9)	5.4 (1.2-24.2)	-
paa	-		5.0 (2.9-8.6)	143.4(49.1-442.0)	2.7 (1.4-5.1)	16.5 (9.0-30.2)	0.4 (0.3-0.7)	17.9 (8.5-38.0)
astA	31.9 (4.2-244.4)	5.0 (2.9-8.6)		16.49 (7.7-35.2)	3.8 (2.0-7.0)	59.7(25.7-138.6)	1.8 (1.1-2.8)	-
fedA	-	143.4 (49.1-442.0)	16.5 (7.7-35.2)		-	64.2(25.7-160.8)	-	-
sepA	56.8(12.4-260.0)	2.7 (1.4-5.1)	3.8 (2.0-7.0)	-		2.8 (1.5-5.3)	-	7.2 (3.6-14.4)
estB	10.3 (3.2-32.9)	16.5 (9.0-30.2)	59.7(25.7-138.6)	64.2(25.7-160.8)	2.8 (1.5-5.3)		-	-
elt	5.4 (1.2-24.2)	0.4 (0.3-0.7)	1.8 (1.1-2.8)	-	-	-		-
eaeA	-	17.9 (8.5-38.0)	-	-	7.2 (3.6-14.4)	-	-	

[a] The number represent odds ratio (OR) for the associations between virulence genes (95% confidence intervals are in parenthesis);

 - indicates no statistically significant association ($P \geq 0.05$); OR >1 represent positive association and OR <1 represent negative association.

2.2 Association between antimicrobial resistance phenotypes and virulence genes in *E. coli* isolates

The association between antimicrobial resistance phenotypes and virulence genes were observed (Table 14). Overall, negative associations were more frequently detected than positive associations. Resistance to some antimicrobial agents including ampicillin, ciprofloxacin and trimethoprim were negatively associated with certain virulence genes. Trimethoprim resistance was negatively associated with *estB, astA, fedA, eaeA, paa* and *sepA* (P<0.05). In contrast, resistance to other antimicrobial agents (i.e. chloramphenicol, gentamicin, sulfamethoxazole and tetracycline) was positively associated with particular virulence genes and additionally negatively associated with others. Among these, chloramphenicol resistance isolates were negatively associated with 6 virulence genes including *estB*, *faeG*, *fedA, eaeA, paa* and *sepA*, while positively associated with *elt*. In addition, streptomycin resistance exhibited only positive association to *estB*, *astA* and *fedA*. The strongest association was found between *fedA* and gentamicin resistance (OR=44.3).

Table 14: The association between resistance phenotypes and virulence genes in *E. coli* isolates (*n*=344)

Virulence genes	AMP		CHP		CIP		GEN		STR		SUL		TET		TRI	
	No[a]	Assos.[b]	No[a]	Assos.[b]	No[a]	Assos.[b]	No[a]	Assos.[b]	No[a]	Assos.[b]	No[a]	Assos.[b]	No[a]	Assos.[b]	No[a]	Assos.[b]
elt (199)	-	-	172	2.8 (1.6-4.8)	87	0.4(0.3-0.7)	-	-	-	-	123	0.5 (0.3-0.9)	195	3.2 (1.0-10.7)	-	-
estA (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
estB (86)	-	-	55	0.4 (0.2-0.6)	-	-	63	1.9(1.1-3.3)	82	7.7 (2.4-25.3)	67	2.1 (1.2-3.8)	-	-	46	0.2 (0.1-0.3)
astA (120)	-	-	-	-	-	-	-	-	107	2.2 (1.1-4.2)	93	2.1 (1.3-3.5)	-	-	83	0.4 (0.2-0.7)
faeG (16)	-	-	7	0.2 (0.1-0.5)	-	-	-	-	-	-	-	-	-	-	-	-
fasA (338)	-	-	-	-	-	-	-	-	-	-	231	10.8 (1.3-93.6)	-	-	-	-
fedA (58)	-	-	38	0.4 (0.2-0.8)	-	-	57	44.3 (6.0-324)	57	14.8(2.0-109.2)	-	-	-	-	19	0.1(0.03-0.1)
eaeA (45)	34	0.2 (0.09-0.5)	27	0.3 (0.2-0.6)	-	-	18	0.33 (0.2-0.6)	-	-	-	-	36	0.1 (0.02-0.2)	19	0.1 (0.07-0.3)
paa (78)	-	-	40	0.2 (0.1-03)	49	0.5(0.3-0.8)	59	2.1 (1.2-3.7)	-	-	-	-	69	0.1 (0.04-0.4)	22	0.02 (0.01-0.1)
sepA (50)	40	0.28(0.12-0.64)	26	0.2(0,1-0.4)	-	-	-	-	-	-	-	-	-	-	33	0.4 (0.2-0.8)

Abbreviations: AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim

Only antimicrobial resistance phenotypes with a statistically significant association (*P*<0.05) with virulence genes are described.

[a]No. Number of isolates resistant to corresponding antimicrobial agents and carrying the relevance virulence genes

[b] Odds ratio (OR) for associations between antimicrobial resistance phenotype and virulence gene (95% confidence interval in parenthesis)

OR>1 represent positive associations and OR<1 represent negative associations

[c]- indicates no statistically significant associations (*P* ≥ 0.05)

2.3 Association between antimicrobial resistance-encoding and virulence genes in *E. coli* isolates

The associations between resistance genes and virulence genes were investigated (Table 15). The *bla*$_{TEM}$, *catA, tetA, dfrA10* and *dfrA12* genes were negatively associated with all the corresponding virulence genes. In contrast, *the catB, strA-strB* and *sul3* revealed only positive associations. Other antimicrobial resistance genes including the *cmlA*, *aadB*, *aadA1*, *aadA2*, *tetB* and *dfrA1* were positively associated with certain virulence genes and also negatively associated with others. The *sul1* gene was not associated with any virulence genes while *sul2* and *sul3* showed positive associations with many virulence genes. The strongest positive associations were between gene pairs *fedA/aadA2* and *fedA/strA-strB*.

Table 15: The association between antimicrobial resistance-encoding and virulence genes in *E. coli* isolates (*n*=344)

gene	Association between the following antimicrobial resistance-encoding genes and virulence genes (OR, 95% confidence interval)[a]															
	bla_{TEM}	cmlA	catA	catB	aadB	aadA1	aadA2	strA-strB	sul1	sul2	sul3	tetA	tetB	dfrA1	dfrA10	dfrA12
elt	0.4	3.1	0.1	-	-	-	-	-	nd	0.6	-	-	-	3.2	-	-
	(0.2-0.9)	(1.9-5.0)	(0.01-0.6)							(0.4-1.0)				(1.4-7.1)		
estA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	-
estB	-	-	-	2.7	1.8	2.5	1.8	4.2	-	8.3	4.1	0.5	1.7	-	nd	0.12
				(1.6-4.6)	(1.1-3.1)	(1.5-4.3)	(1.1-3.0)	(2.1-8.4)		(4.8-14.5)	(2.3-7.2)	(0.3-0.9)	(1.0-2.			(0.1-0.2)
astA	-	-	-	2.3	-	1.7	-	2.9	nd	2.94	3.04	-	-	-	-	0.4
				(1.4-3.8)		(1.1-2.7)		(1.7-5.0)		(1.8-4.9)	(1.9-4.9)					(0.2-0.6)
faeG	-	0.3	-	-	5.5	-	-	0.1	-	nd	-	0.2	3.2	-	-	-
		(0.1-0.8)			(1.9-15.7)			(0.05-0.4)				(0.1-0.6)	(1.1-9.0)			
fasA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
fedA	-	-	-	2.37	-	27.23	36.1	35.6	-	4.7	2.0	-	0.2	0.2	0.04	0.03
				(1.3-4.3)		(6.5-113.7)	(8.6-150.7)	(4.9-261.0)		(2.6-8.6)	(1.1-3.6)		(0.1-0.5)	(0.1-1.0)	(0.01-0.3)	(0.01-0.1)
eaeA	0.1	-	-	nd	0.3	0.2	0.3	0.4	-	0.06	1.9	-	0.1	-	-	0.4
	(0.04-0.2)				(0.1-8.8)	(0.1-0.4)	(0.2-0.6)	(0.2-0.8)		(0.01-0.4)	(1.0-3.76)		(0.03-0.4)			(0.2-0.8)
paa	0.3	0.3	-	-	-	-	2.3	2.9	-	2.5	1.7	-	0.2	0.2	0.2	0.05
	(0.1-0.5)	(0.2-0.5)					(1.3-3.9)	(1.5-5.6)		(1.5-4.3)	(1.0-2.9)		(0.1-0.4)	(0.04-0.7)	(0.1-0.5)	(0.02-0.1)
sepA	0.15	0.4	-	nd	2.1	-	-	-	-	0.1	2.6	-	-	-	-	-
	(0.1-0.3)	(0.2-0.7)			(1.1-3.9)					(0.01-0.4)	(1.3-5.1)					

[a]Odds ratio (OR) for associations between virulence gene and antimicrobial resistance (95% confidence interval in parenthesis).

OR>1 represent positive associations and OR<1 represent negative associations; - indicates no significant associations ($P{\geq}0.05$); nd means not determined (OR could not be calculated because none of the isolates carried one of the combinations of virulence genes and antimicrobial resistance genes.

3. Phylogenetic groups

All the isolates in this collection were examined for their phylogenetic group. According to triplex PCR results, the *E. coli* isolates in group A, B1, B2 and D were 8%, 82%, 7% and 3% respectively (Figure 11). The PCR amplifications of *chuA*, *yjaA* and TspE4.C2 are shown in Figure 12.
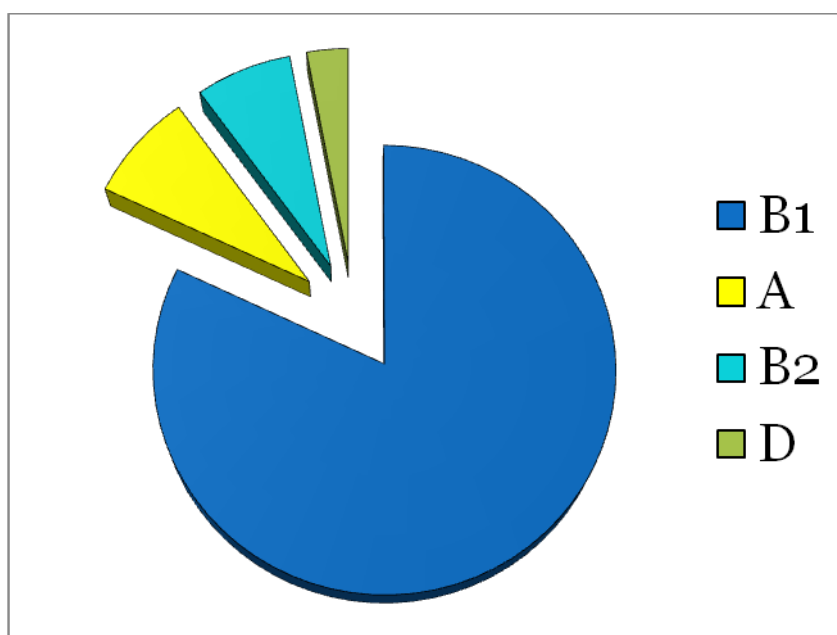

Figure 11: Distribution of 4 phylogenetic groups among *E. coli* isolates (*n*=344)
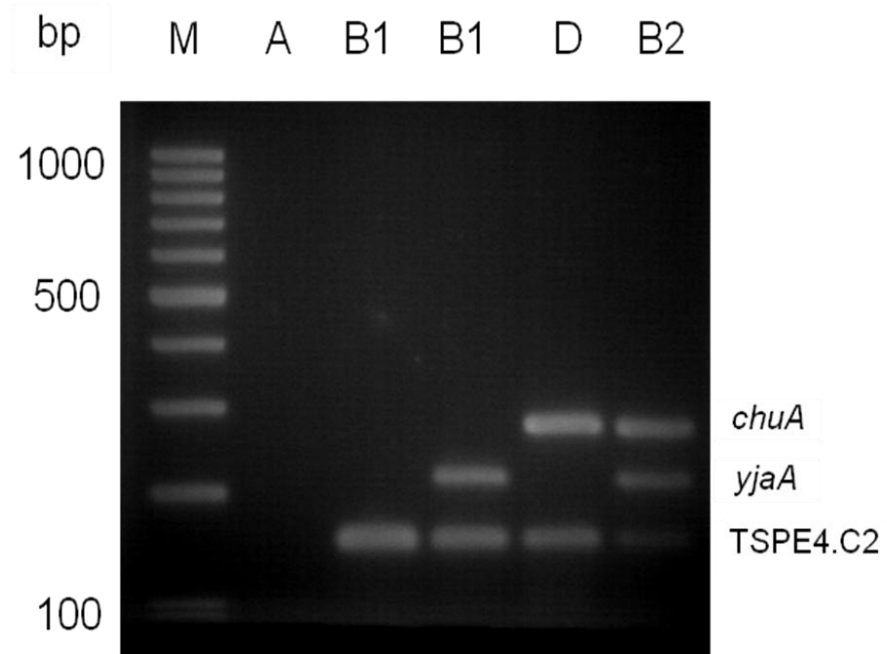
Figure 12: PCR patterns for identification of phylogenetic groups of *E. coli*. Lane M, 100bp-marker

Table 16: Virulence gene profiles and phylogenetic groups *E. coli* isolates (*n*=344)

Virulence gene profiles		Phylogenetic group (No. of isolates)			
Gene	No. (%)	A (*n*=28)	B1 (*n*=282)	B2 (*n*=24)	D (*n*=10)
elt	5 (1.5)	-	-	-	5 (50.0)
fasA	68 (19.8)	6 (21.4)	62(22.0)	-	-
astA, estB	1 (0.3)	-	1 (0.4)	-	-
astA, fasA	7 (2.0)	-	7 (2.5)	-	-
eaeA, fasA	2 (0.6)	2 (7.1)	-	-	-
elt, fasA	97 (28.2)	9 (32.1)	72 (25.5)	15 (62.5)	1 (10.0)
fasA, paa	1 (0.3)	-	1 (0.4)	-	-
fasA, sepA	9 (2.6)	-	9 (3.2)	-	-
astA, elt, fasA	20 (5.8)	-	20 (7.1)	-	-
astA, estA, fasA	1 (0.3)	-	1 (0.4)	-	-
astA, estB, fasA	9 (2.6)	-	9 (3.2)	-	-
astA, faeG, fasA	2 (0.6)	-	2 (0.7)	-	-
eaeA, elt, fasA	9 (2.6)	-	-	9 (37.5)	-
eaeA, fasA, paa	13 (3.8)	1 (3.6)	12(4.3)	-	-
elt, estB, fasA	1 (0.3)	-	1 (0.4)	-	-
elt, *fasA, fedA*	4 (1.2)	-	-	-	4 (40.0)
astA, elt, estB, fasA	10 (2.9)	10 (35.7)	-	-	-
astA, elt, fasA, sepA	8 (2.3)	-	8 (2.8)	-	-
eaeA, fasA, paa, sepA	8 (2.3)	-	8 (2.8)	-	-
estB, fasA, fedA, paa	4 (1.2)	-	4 (1.4)	-	-
astA, elt, faeG, fasA, sepA	2 (0.6)	-	2 (0.7)	-	-
astA, estB, fasA, fedA, paa	20 (5.8)	-	20 (7.1)	-	-
elt, estB, faeG, fasA, sepA	1 (0.3)	-	1 (0.4)	-	-
astA, elt, estB, faeG, fasA, sepA	10 (2.9)	-	10 (3.5)	-	-
astA, elt, estB, fasA, fedA, paa	19 (5.5)	-	19 (6.7)	-	-
eaeA, elt, estA, fasA, fedA, paa	1 (0.3)	-	1 (0.4)	-	-
eaeA, elt, estB, fasA, paa, sepA	1 (0.3)	-	1 (0.4)	-	-
astA, eaeA, elt, fasA, fedA, paa, sepA	1 (0.3)	-	1 (0.4)	-	-
astA, eaeA, elt, estB, faeG, fasA, paa, sepA	1 (0.3)	-	1 (0.4)	-	-
astA, eaeA, elt, estB, fasA, fedA, paa, sepA	9 (2.6)	-	9 (3.2)	-	-

4. Plasmid profiles

Plasmids were detected in 99.3% of the total *E. coli* isolates with size ranging from 1.0 kb to 24.5 kb (Table 19). The copies of plasmid were found to vary one to nine. One hundred twenty seven isolates contained more than one plasmid, while 10 isolates carried up to nine plasmids. Fifty-six different plasmid profiles (PP-1 to PP-56) were observed, of which the prevalent plasmid profiles was PP-5 (7%) and PP-21 (7%). All pictures of plasmid profiles were included in Appendix D. The predominant plasmids in this study had molecular weight of 2 kb (40 %), 4 kb (31 %) and 5 kb (31 %).The only *E. coli* strain without plasmid exhibited resistance to AMP-CHP-TET-TRI. Fifty-six *E. coli* isolates (39%) in PP-11, 14, 20, 21, 27, 32, 39, 40 and 50-56 carried class 1 integrons with gene cassettes.

Table 17: Plasmid profiles and antimicrobial resistance patterns of *E. coli* isolates (*n*=145)

pp[a]	Antimicrobial resistance pattern	Total No.	Class 1 integrons		Plasmid profile (kb)
			empty[b]	Gene cassette[c]	
1	SUS	5	-	-	8, 4, 2
	CIP	1	-	-	8, 4, 2
	CIP-GEN	2	-	-	8, 4, 2
	AMP-CIP-STR	1	-	-	8, 4, 2
2	AMP-CHP-TET	8	8	-	3, 2
3	AMP-STR-TET	1	-	-	12, 8
4	CHP-TET-TRI	1	1	-	19
	AMP-GEN-SUL-TET	1	-	-	19
5	CHP-TET-TRI	10	10	-	23.9, 6, 5, 2.5, 2
6	AMP-CHP-CIP-TET	2	2	-	1.75
7	AMP-CHP-TET-TRI	5	5	-	8, 6, 5, 4, 3, 1.5, 1
8	AMP-STR-SUL-TET	5	-	-	24.5, 19, 4, 2
9	CHP-CIP-TET-TRI	1	1	-	23.9
	AMP-CHP-GEN-STR-SUL-TET-TRI	1	1	-	23.9
10	CHP-STR-TET-TRI	3	3	-	19, 15, 8, 4
11	CHP-STR-TET-TRI	3	-	3	17
12	AMP-CHP-CIP-GEN-STR-SUL-TET	3	-	-	23.9, 12, 6
13	AMP-CHP-CIP-GEN-STR-TET-TRI	1	1	-	6
14	AMP-CHP-CIP-GEN-STR-TET-TRI	4	-	4	24.5, 17, 8.6
15	AMP-CHP-CIP-GEN-STR-TET-TRI	1	1	-	23.9, 1.5, 1
16	AMP-CHP-CIP-GEN-STR-TET-TRI	1	-	-	23.9, 2.5
17	AMP-CHP-CIP-GEN-STR-TET-TRI	1	-	-	24.5, 8.6, 5
18	AMP-CHP-CIP-GEN-SUL-TET-TRI	2	2	-	10, 5, 3.5, 3, 2
19	AMP-CHP-CIP-STR-SUL-TET-TRI	1	1	-	6, 4
20	AMP-CHP-CIP-STR-SUL-TET-TRI	1	1	-	24.5
	AMP-CHP-GEN-STR-SUL-TET-TRI	3	-	3	24.5
	CHP-ERY-GEN-STR-TET-TRI	1	-	1	24.5
21	AMP-CHP-CIP-STR-SUL-TET-TRI	10	-	10	24.5, 23.9, 6, 5, 3.5, 2.5, 2, 1.5, 1
22	AMP-CHP-CIP-STR-SUL-TET-TRI	1	1	-	15, 8, 7, 3.5
23	AMP-CHP-CIP-ERY-STR-SUL-TET-TRI	1	1	-	8, 3.5
24	AMP-CHP-GEN-STR-SUL-TET-TRI	1	1	-	24.5, 12, 3.5
25	AMP-CHP-GEN-STR-SUL-TET-TRI	4	3	-	17, 8, 4, 2.5
26	AMP-CHP-GEN-STR-SUL-TET-TRI	1	1	-	24.5, 6, 4, 3, 1
27	AMP-CHP-GEN-STR-SUL-TET-TRI	10	-	10	15, 10, 6, 3.5
28	AMP-CHP-GEN-STR-SUL-TET-TRI	1	1	-	6, 3.5, 3, 1.5

[a]Plasmid profile

[b]The *E. coli* isolates carrying empty class 1 integrons without gene cassettes.

[c]The *E. coli* isolates carrying class 1 integrons with gene cassettes.

SUS indicates susceptible to all antimicrobial agents

Continued Table 19:

pp[a]	Antimicrobial resistance pattern	Total No.	Class 1 integrons		Plasmid profile (kb)
			empty[b]	Gene cassette [c]	
29	AMP-CHP-GEN-STR-SUL-TET-TRI	1	1	-	12, 8, 5
30	AMP-CHP-GEN-STR-SUL-TET-TRI	2	2	-	12
31	AMP-CHP-GEN-STR-SUL-TET-TRI	3	3	-	23.9, 4, 2.5
32	AMP-CIP-GEN-STR-SUL-TET-TRI	1	-	1	24.5, 6, 5, 2.5
33	AMP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	15, 5, 2, 1.5, 1
34	AMP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	17, 6
35	AMP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	24.5, 15, 6, 5
36	AMP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	5, 3
37	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	4, 2.5, 1
38	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	6, 3.5, 2, 1.5
39	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	10, 5, 2, 1.5
40	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	9	-	9[d]	10, 4, 2, 1.5, 1
41	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	-	1	24.5, 5, 3, 2.5
42	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	24.5, 12, 6, 5
43	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	23.9, 6
44	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	-	-	17, 10, 5, 4.5, 3, 2.5
45	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	15, 6, 3.5
46	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	6, 3.5
47	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	10, 5
48	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	10, 8.6
49	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	1	1	-	4, 2.5
50	AMP-GEN-STR-TET-TRI	3	-	3	24.5, 8, 5, 2.5
51	AMP-GEN-SUL-TET-TRI	1	-	1	8, 5
52	AMP-GEN-STR-SUL-TET-TRI	4	-	4	8, 5, 4
53	AMP-CHP-GEN-STR-SUL-TET-TRI	1	-	1	12, 6
54	AMP-CHP-GEN-STR-TRI	1	-	1	8, 7, 5, 4, 2.5, 2
55	AMP-CHP-GEN-STR-TET-TRI	3	-	3	24.5, 5, 2.5
56	AMP-CIP-GEN-STR-TET-TRI	1	-	1	24.5, 8, 2

[a]Plasmid profile

[b]The *E. coli* isolates carrying empty class 1 integrons without gene cassettes.

[c]The *E. coli* isolates carrying class 1 integrons with gene cassettes.

[d] The *E. coli* isolates carrying class 1 integrons with *dfrA12-aadA2* that could be horizontal transfer.

CHAPTER V

DISCUSSION

Antimicrobial agents have been used in pig production to treat clinical diseases, keep good health, improve feed efficiency and increase growth rate. It is evident that the use of antimicrobials is the major cause of the increasing rate of antimicrobial resistance among bacteria. Emergence of multidrug-resistant *E. coli* has been increasingly reported in many parts of the world and previous studies showed that multidrug resistance in bacteria is usually related to integrons, in particular class 1 integrons (Lapierre et al., 2008; Literak et al., 2009; Mathai et al., 2004; Phongpaichit et al., 2007). Several non-class 1 integrons resistance encoding genes have been additionally reported and virulence genes have been shown to widely distribute among *E. coli*. Particular concern has been raised regarding coexistence of antimicrobial and virulence determinants on the same plasmids, resulting in an emergence of more resistant and virulent *E. coli* strains.

In this study, high resistance rates of *E. coli* to the clinically-important antimicrobials including tetracycline, ampicillin, streptomycin, chloramphenicol, trimethoprim, sulfamethoxazole, gentamicin and ciprofloxacin were observed. Most isolates were resistant to multiple drugs, sometimes up to 8 antimicrobial agents. This finding was similar to a previous study in healthy swine farm reporting in *E. coli* isolates resistant to eight to 11 antimicrobial agents (Lu et al., 2010), which was more likely resulted from the long term use of these antimicrobial agents in pig farms in Thailand.

The application of chloramphenicol in livestock production had been banned in Thailand since 1999, but chloramphenicol resistance rate was still high in this study. Similar finding was previously observed in *Salmonella* isolates from swine and poultry (Chuanchuen et al., 2008a). Several explanations for this phenomenon could be made, including coexistence of chloramphenicol resistance genes with other antimicrobial

resistance genes on the same plasmid(s), expression of multidrug resistance mechanism(s), e.g. active efflux and use of other antimicrobial agents that are chemically related to chloramphenicol and property to be coselected for chloramphenicol resistance (Okusu et al., 1996). Furthermore, ciprofloxacin resistance rate in *E. coli* isolates was high up to 52.3%. However, this occurrence of ciprofloxacin resistance rate in our study was less than that reported in a previous study from China (Yang et al., 2004). Although use of ciprofloxacin in pig production is uncommon, other fluoroquinolone, for example, enrofloxacin has been widely applied. This could be a possible reason for such high ciprofloxacin resistance rate observed.

In this study, most of the *E. coli* isolates contained *intI1* gene. This finding was similar to previous studies showing high prevalence of *intI1* in *E. coli* from swine in southern Thailand (74.5%) (Phongpaichit et al., 2007) and Taiwan (93.4 %) (Hsu et al., 2006). The high prevalence of *intI 1* in *E. coli* isolates was suggested to be related to its localization on plasmid that could be horizontally transferred (Hsu et al., 2006).

In the present study, up to 22% of *intI1* positive isolates possessed resistance gene cassettes in variable region. This was consistent with previous studies in Eastern China reporting that 93.3% of *E. coli* from swine were *intI1* positive, of which 20% carried class 1 integrons with gene cassettes (Lu et al., 2010). In contrast, much higher rate of class 1 integrons with gene cassette was detected in the *intI1* positive *E. coli* strains in other studies, e.g 98% in *E. coli* from humans and cattle (Guerra et al., 2006) and 100% in Shiga toxin-producing *E. coli* from humans and food animals (Singh et al., 2005). However, up to 78% of class 1 integrons in this study did not carry gene cassettes in variable regions. Such high rate of empty integrons might be related to different antimicrobial use in different geographic locations, the weak promoter that could not express gene cassettes in variable region and excision of gene cassettes that were readily moved to other integrons (Partridge et al., 2002). Regardless, class 1

integrons without gene cassette inserts are available for new-comig gene cassettes, leading to spreading of resistance determinants (Bissonnette and Roy, 1992).

In this study, five gene cassettes were identified. Among these gene cassettes, *aadA2* encoding resistance to aminoglycosides was most commonly found and followed by *dfrA12* encoding resistance to trimethoprim. These gene cassettes were formerly observed in *E. coli* from humans, swine and poultry and *S.* Choleraesuis from swine (Hsu et al., 2006; Kang et al., 2005), *E. coli* isolates from humans and cattle (Guerra et al., 2006), *Salmonella* from swine and poultry (Khemtong and Chuanchuen, 2008), and *Salmonella* isolates from pork and humans (Wannaprasat et al., 2011). The observation of the same resistance gene cassettes in the *E. coli* isolates from different sources and/or different bacterial species from different sources implied that the major route for dissemination of these antibiotic resistance gene cassettes could be horizontal transfer.

Bilateral mating experiments showed that plasmid containing class 1 integrons with *dfrA12-aadA2* gene cassettes were conjugatively transferred to *S.* Enteritidis recipients. This is similar to our previous study in *Salmonella* isolates from poultry and swine where class 1 integrons with *dfrA12-aadA2* gene cassettes could horizontally transfer to *E. coli* K12 strain MG1655 (Khemtong and Chuanchuen, 2008). Even though transferability rate observed in this study was lower than previous studies from Korea and the Netherlands (Kang et al., 2005; van Essen-Zandbergen et al., 2007), conjugative transfer appears to be an important route to accomplish the horizontal spread of class 1 integrons with gene cassettes between and within species (Kang et al., 2005).

Moreover, class 1 integrons with incomplete *sat* was also observed. Horizontal transfer of this gene cassette was not examined in this study due to lack of resistance-selection marker. This non-functional gene cassette was previously found in *E. coli* isolated from poultry and human in Greece (Vasilakopoulou et al., 2009). Additionally, it

was formely identified in *Salmonella* isolates from poultry and swine in Thailand (Khemtong and Chuanchuen, 2008), *S.* Kentucky isolated from poultry in Ireland (Boyle et al., 2010), *S.* Choleraesuis isolates from humans and animals in Taiwan (Lee et al., 2009). The wide distribution of this incomplete-gene cassette suggested its colocallization with other resistance genes on plasmid(s) that could be horizontally transferred.

Moreover, resistance rates of most antimicrobial agents in the *intI1*-positive *E. coli* isolates were significantly higher than those in the *intI1*-negative isolates, consistent with other reports and suggesting role of class1 integrons in multidrug resistance among bacteria (Lu et al., 2010; Shaheen et al., 2010). However, resistance rates of some antimicrobial agents in *intI 1*-negative isolates were higher than those in *intI 1*-positive *E. coli* isolates. It is likely due to the presence of non-class1 integrons resistance genes. These additionally resistance mechanisms could be chromosomal mutation, multidrug efflux systems and antimicrobial resistance determinants that are not located on class 1 integrons (Nakamura et al., 1989; Ruiz and Levy, 2010; Swick et al., 2011).

In this study, many resistance genes were identified in *E. coli* isolates from clinically healthy pigs. Eighty-six to a hundred percent of the resistance isolates carried at least one resistance gene tested and their presence was well corresponded to their resistance phenotypes. Most *E. coli* isolates carried multiple genes encoding the same resistance phenotypes e.g. *aadB*, *aadA1*, *aadA2* for gentamicin resistance, *strA-strB*, *aadA1, aadA2* for streptomycin resistance, *dfrA1*, *dfrA10*, *dfrA12* for trimethoprim resistance. Several resistance genes mediating the same resistance phenotype could be defined by their different location i.e one gene on chromosome and the others on genetic elements including integrons, plasmid and transposons. This is in agreement with the notion that class 1 integrons-carrying isolates usually carry additional resistance genes located outside the integron structure (Seputiene et al., 2010).

In our study, *tetA* was most prevalent among the tetracycline-resistant isolates, in aggrement with former studies (Chuanchuen and Padungtod, 2009; Sabarinath et al., 2011). In contrast, some previous studies demonstrated that *tetB* was predominant among the tetracycline resistant commensal *E. coli* (Delsol et al., 2005; Diarrassouba et al., 2007). However, some studies found that both *tetA* and *tetB* genes were prevalent in *E. coli* from swine (Boerlin et al., 2005; Guerra et al., 2003). Regardless of the type of *tet* genes, these observations indicate that normal microflora of pigs' intestinal tract play a role as an accumulator of tetracycline-resistance genes.

In this collection, bla_{TEM} gene was detected in most of ampicillin-resistant isolates, in consistent with previous studies (Ahmed et al., 2010; Brinas et al., 2002; Guerra et al., 2003). It is still unclear why this ampicillin-resistance encoding gene is predominant and is most probably related to the type of β-lactams used.

Most trimethoprim-resistant *E. coli* isolates carried *dfrA12* followed by *dfrA1* and *dfrA10*. Some *dfrA12* genes were carried on class 1 integrons but it could not cover the whole prevalence of this gene, suggesting existence of non-class 1 integrons borne *dfrA12* gene. Similarly, *sul3* gene was most frequently detected in sulfonamide-resistant isolates, in aggrement with previous reports in *E. coli* isolates from healthy grow-finish pigs in Canada (Rosengren et al., 2009). However, some study showed that *sul1* and *sul2* genes were prevalent in *E. coli* isolates from healthy pigs (Wu et al., 2010). Therefore *sul1*, *sul2* and *sul3* genes are widely distributed in *E. coli* isolates from pigs. However, the reason underlying different types of *sul* genes observed is still unclear.

In this strain collection, most gentamicin-resistant isolates carried *aadA1* followed by *aadA2* and *aadB*. Some isolates carried class 1 integrons with *aadA* gene cassettes. Once again, these antimicrobial resistance genes should be located on different genetic elements. In addition, most streptomycin-resistant isolates is this study

carried *aadA1*, *aadA2* and *strA-strB* genes. Similar finding was reported from commensal *E. coli* in pig from other countries (Martin et al., 2005).

Analysis from mutation in QRDR of *gyrA* and *ParC* showed three mutations in GyrA and one mutation in ParC. The two amino acids chages including Ser-83-Leu and Asp-87-Asn found in GyrA were previously described (Guerra et al., 2003) and is like to be located on DNA-interaction region of GyrA (Vashist et al., 2009). However, the other amino acid change Gln-94-Pro has never been reported in ciprofloxacin-resistant *E. coli* and its contribution to fluroquinolone resistance is unknown. A single aminoacid changes Ser-58-Ile was found in ParC. From our knowledge, this amino acid substitution is novel as well.

As several virulence genes are widely distributed in this study, all isolates carried at least one virulence gene tested, in agreement with previous studies in clinically healthy pigs (Schierack et al., 2006). Even though clinically healthy pigs carry several virulence genes, they do not show any clinical signs of illness. This is likely related to the weak expression of virulence genes of *E. coli* in the intestinal tract. Another explanation is that the *E. coli* isolates occupy only a very little portion of the total intestinal microorganisms (Schierack et al., 2006). However, clinically healthy pigs carrying virulence genes may be a risk to humans. Additionally, previous studies reported that these virulence genes are located on plasmid and could transfer horizontally among bacteria (Echeverria et al., 1985; Wittig et al., 1994).

A previous report showed that *E coli* isolates from pigs with postweaning diarrhoea and edema disease carried a variety of virulence genes and all pathogenic *E. coli* isolates carried critical fimbrial genes *faeG* or *fedA* (Frydendahl, 2002). In this study, only 21% of *E. coli* isolates from clinically healthy pigs carried either *faeG* or *fedA*. If any appropriate pressure exists, commensal *E. coli* may become pathogenic (Gordon, 1992).

Associations between eight virulence genes of *E. coli* isolates were observed. The *elt* gene was positive association with *faeG* and *astA*. The findings were consistent with other studies reported that the presence of enterotoxin genes (*estB, estA, elt, astA*) is associated with fimbrial genes (*faeG* and *fedA*) (Vu-Khac et al., 2007; Wang et al., 2010; Zhang et al., 2007). This could be explained by the coexistence of these virulence genes on the same plasmid. The strong positive association was found in three virulence genes i.e *eaeA*, *sepA* and *paa*. The results were agreement with other studies reported that the presence of *paa* is associated with *eaeA* and *sepA* (Leclerc et al., 2007; Wang et al., 2010). The reason could be the co-localization of *paa* and *sepA* on same the plasmid.

Furthermore, the association between antimicrobial resistance phenotype and virulence genes was identified. Positive associations were observed between phenotypic resistance to gentamicin, streptomycin, sulfamethoxazole and certain virulence genes, i.e *fedA* , *estB* and *astA* suggesting that these resistance and virulence genes may be located on the same plasmid (Gyles et al., 1977). The results indicate that restricting antimicrobial use in pig production may also restrict the emergence of resistance *E. coli* with increasing virulence genes. The strongest association was observed between gentamicin resistance and *fedA*, while the strong positive association between *fedA* and *aadA1* and *aadA2* genes was observed. This suggested that co-existence of gentamicin resistance encoding genes and *fedA* on the same plasmid.

Some antimicrobial resistance phenotypes including ampicillin, ciprofloxacin, and trimethoprim were negatively associated with several virulence genes. Such phenomenon suggests that *E. coli* strains resistant to these antimicrobials may be less virulent than those susceptible strains (Wang et al., 2010). Some antimicrobial agents were positively associated with certain virulence genes and also negatively associated with others. The possible explanation could be that resistance genes and their positively-associated virulence genes were located on the same plasmid or other mobile

genetic elements, facilitating co-selection of both determinants. The resistance genes and their negatively-associated virulence genes may be located on plasmids in the same incompatibility group (Boerlin et al., 2005).

For deeper analysis, association between resistance and virulence gene was determined. The strong positive associations were observed among the resistance and virulence genes pairs including *aadA1, fedA*; *aadA2, fedA; strA-strB, fedA; sul2, fedA; sul3, fedA,* indicating that antimicrobial administration in pig farm could serve as selection pressure for coselection of virulence genes *in E. coli* from healthy pigs. In contrast, a previous study found that association did not occur between resistance and virulence genes in *E. coli* isolates from healthy grow-finish pigs (Rosengren et al., 2009). This discrepancy might be because the association between resistance genes and virulence determinants tended to be strain specific and could be different effect of various antimicrobial uses in various geographical areas (Boerlin et al., 2005). The findings from our study supported that *E. coli* strains harboring both resistance and virulence genes can stay in gastrointestinal tracts of clinically healthy pigs, possibly contaminate pork and pork products, easily pass into food chain and causes diseases in humans.

Most of *E. coli* isolates in this collection belonged to phylogenetic group B1. This is not surprising because the commensal *E. coli* are mainly assigned to phylogenetic group A and B1 that are sister groups (Johnson et al., 2001). However, the *E. coli* isolates from diseased pigs in China belonged to phylogenetic group A and B1 (Wang et al., 2010). Therefore, it cannot be concluded that *E. coli* isolates in nonpathogenic phylogenetic groups A and B1 do not confer adverse health effects on human at all. This was additionally supported by the current findings that a range of virulence genes were detected among the *E. coli* strains of group A and B1. It is possible that these virulence genes may express and these *E. coli* can cause disease in immuonosuppressive conditions and in the presence enough bacterial loads.

Plasmid analysis showed that the sizes and numbers of plasmid varied, in agreement with previous studies (Malkawi and Youssef, 1998). However, it should be noted that some plasmids that are very large or very small may not be extracted by the plasmid extraction protocol used in this study.

Two largest plasmids with size of 24.5 kb and 23.9 kb were found in 26 % and 21% of the isolates, respectively. This is similar to previous reports in clinical isolates of *E. coli,* where plasmids with size ranging from 2 kb to 3 kb and 26 kb were commonly found (Jan et al., 2009). The widespread of these particular plasmids implied their ability to horizontally transfer. However, transmission of these particular plasmids was not examined in this study.

It has been well known that the *E. coli* clinical isolates usually possess multiple plasmids with different sizes and this is due to exposure to various antibiotics in treatment process (Jan et al., 2009). The presence of multiple plasmids in a single bacterial strain has been linked to prolonged use of antibiotics (Levy et al., 1976). In this study, most *E. coli* isolates were multiresistant and carried multiple plasmids. Since the *E. coli* isolates in this collection were normal flora, such wide distribution of plasmids was more likely associated with use of antibiotic-growth promoters.

Some isolates carrying class 1 integrons with resistance cassette inserts possessed common plasmids, of which two large plasmids with the size of 24.5 kb and 23.9 kb were frequently found. However, the size of plasmids was diverse, indicating different combinations of genes on these plasmids. Further studies are warranted to analyze plasmid structure.

All but 8 isolates chosen for plasmid analysis were multidrug resistant. Still, there was no significant correlation between plasmid profiles and antimicrobial resistance patterns observed among the isolates in this collection, in accordance to a previous

report in the human clinical isolates (Jan et al., 2009). This is also supported by our observation that some strains in the same plasmid profile (i.e. PP-1, 9 and 20) exhibited different resistance phenotypes. The data support the notion that the acquisition of resistance has an impact on a change of the distribution of plasmids (Platt et al., 1984).

Only an *E. coli* isolate in the present study did not harbor plasmid and was resistant up to 4 antimicrobials (i.e. AMP-CHP-TET-TRI), suggesting existence of non-plasmid borne resistance determinants. In this particular strain, antimicrobial resistance might be chromosomally encoded or medicated by other mobile genetic elements. However, such very low occurrence supported that plasmids play a crucial role in mediating of antimicrobial resistance among the *E. coli* isolates.

The results in this study confirm the significant role of commensal *E. coli* as reservoirs of multidrug-resistance plasmids. Antimicrobial use has a serious implication for the possible co-selection of multidrug resistance mediated by plasmids. While plasmid profile analysis has been extensively used for assessing of epidemiological information, future studies are needed to study molecular characteristics of resistance plasmids for deep understanding of their role in dissemination of antimicrobial resistance.

Conclusion and suggestions

The main findings of this study were the wide distribution of class 1 integrons, antimicrobial resistance genes and virulence genes among *E. coli* from clinically healthy pigs. Horizontal-transfer of class 1 integrons carrying resistance gene cassettes confirmed the important role of these mobile genetic elements in wide dissemination of antimicrobial resistance genes among bacteria. The data enlightens the significant roles of commensal *E. coli* as reservoirs of resistance and virulence determinants. In this case, commensal *E. coli* carrying both resistance and virulence genes can potentially contaminate pork and pork products, and finally humans get disease. The strongly-positive relationship between resistance and virulence genes confirmed that on farm antimicrobial use can co-select for virulence traits in bacterial strains. Therefore, the restrictive policies on the use of antimicrobial in food animal production are mandatory and the surveillance program to monitor resistance situation and virulence features need to be regularly performed among not only diseased *E. coli* but also commensal *E. coli* isolates.

The data, bacterial isolates and genetic materials obtained from this study could be used for further studies as follows:

1. The data on antimicrobial resistance could be included in antimicrobial resistance monitoring program of the country.

2. These data could be partly used in risk analysis of antimicrobial resistance, particularly when *E. coli* from food animals is a part of the experimental design.

3. When combined the data from humans and food products from animal origins, the results of this study could also be applied to explain the link of antimicrobial resistance and virulence factors in *E. coli* along the food chain.

4. The *E. coli* strains and plasmids obtained can be applied for further studies for examples:

4.1 In this study, we found that non-class1 integrons borne *E. coli* isolates were resistance to multiple antimicrobial agents, supporting the existence of non-class1integrons–mediating resistance. These resistance mechanisms could be multidrug efflux systems, chromosomal mutation, antimicrobial resistance determinants that are not present on class1 integrons. Further investigation should be conducted to explore such resistance mechanisms.

4.2 Some antimicrobial resistance determinants and virulence genes are co-located on the same plasmid, from which resistance and virulence factors could be distributed among pathogens. Plasmid analysis worth further investigations since the data will help to better understand emergence of multidrug resistance and virulence in *E. coli* strains.

4.3 The pathogenicity of *E. coli* isolates was not determined in this study. To determine pathogenicity and epidemiology of *E. coli* strains, serotyping is needed to perform. Therefore, the further studies are recommended to investigate the relationship between serotypes and virulence factors in *E. coli* strains from both animals and humans.

4.4 The study of *E. coli* and other food-borne pathogens along food chain should be conducted.

REFERENCES

Ahmed, M.O., Clegg, P.D., Williams, N.J., Baptiste, K.E. and Bennett, M. 2010. Antimicrobial resistance in equine faecal *Escherichia coli* isolates from North West England. Ann. Clin. Microbiol. Antimicrob. 9.

Bingen, E., Picard, B., Brahimi, N., Mathy, S., Desjardins, P., Elion, J. and Denamur, E. 1998. Phylogenetic analysis of *Escherichia coli* strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains. J. Infect. Dis. 177(3): 642-650.

Bissonnette, L. and Roy, P.H. 1992. Characterization of In*0* of *Pseudomonas aeruginosa* plasmid pVS1, an ancestor of integrons of multiresistance plasmids and transposons of gram-negative bacteria. J. Bacteriol. 174(4): 1248-1257.

Boerlin, P., Travis, R., Gyles, C.L., Reid-Smith, R., Janecko, N., Lim, H., Nicholson, V., McEwen, S.A., Friendship, R. and Archambault, M. 2005. Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. Appl.Environ. Microbiol. 71(11): 6753-6761.

Bosworth, B.T. and Casey, T.A. 1997. Identification of toxin and pilus genes in porcine *E. coli* using polymerase chain reaction (PCR) with multiple primer pairs. In: Proceedings of the 97th Annual General Meeting, abstract B-509. Am. Soc. Microbiol., Miami, Florida.

Boyle, F., Morris, D., O'Connor, J., Delappe, N., Ward, J. and Cormican, M. 2010. First report of extended-spectrum-beta-lactamase-producing Salmonella enterica serovar Kentucky isolated from poultry in Ireland. Antimicrob. Agents Chemother. 54(1): 551-553.

Brinas, L., Zarazaga, M., Saenz, Y., Ruiz-Larrea, F. and Torres, C. 2002. Beta-lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. Antimicrob. Agents Chemother. 46(10): 3156-3163.

Brunder, W., Schmidt, H., Frosch, M. and Karch, H. 1999. The large plasmids of Shiga-toxin-producing *Escherichia coli* (STEC) are highly variable genetic elements. Microbiol. 145(5): 1005-1014.

Bunner, C.A., Norby, B., Bartlett, P.C., Erskine, R.J., Downes, F.P. and Kaneene, J.B. 2007. Prevalence and pattern of antimicrobial susceptibility in *Escherichia coli* isolated from pigs reared under antimicrobial-free and conventional production methods. J. Am. Vet. Med. Assoc. 231(2): 275-283.

Carter, G.R. and Cole, J.R. 1990. Enterobacteria. In: Diagnostic Procedures in Bacteriology and Mycology. 5th ed. CA: USA. 107-128.

Chang, L.L., Chang, T.M. and Chang, C.Y. 2007. Variable gene cassette patterns of class 1 integron-associated drug-resistant *Escherichia coli* in Taiwan. Kaohsiung J. Med. Sci. 23(6): 273-280.

Chapman, T.A., Wu, X.Y., Barchia, I., Bettelheim, K.A., Driesen, S., Trott, D., Wilson, M. and Chin, J.J. 2006. Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. Appl. Environ. Microbiol. 72(7): 4782-4795.

Chopra, I. and Roberts, M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 65(2): 232-260.

Chuanchuen, R., Ajariyakhajorn, K., Koowatananukul, C., Wannaprasat, W., Khemtong, S. and Samngamnim, S. 2010. Antimicrobial resistance and virulence genes in *Salmonella enterica* isolates from dairy cows. Foodborne Pathog. Dis. 7(1): 63-69.

Chuanchuen, R., Khemtong, S. and Padungtod, P. 2007. Occurrence of qacE/qacEDelta1 genes and their correlation with class 1 integrons in salmonella enterica isolates from poultry and swine. Southeast Asian J. Trop. Med. Public Health. 38(5): 855-862.

Chuanchuen, R., Koowatananukul, C. and Khemtong, S. 2008a. Characterization of class 1 integrons with unusual 3' conserved region from *Salmonella enterica* isolates. Southeast Asian J. Tro. Med. Public Health. 39(3): 419-424.

Chuanchuen, R. and Padungtod, P. 2009. Antimicrobial resistance genes in *Salmonella enterica* isolates from poultry and swine in Thailand. J. Vet. Med. Sci. 71(10): 1349-1355.

Chuanchuen, R., Pathanasophon, P., Khemtong, S., Wannaprasat, W. and Padungtod, P. 2008b. Susceptibilities to antimicrobials and disinfectants in *Salmonella* isolates obtained from poultry and swine in Thailand. J. Vet. Med. Sci. 70(6): 595-601.

Clermont, O., Bonacorsi, S. and Bingen, E. 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl. Environ. Microbiol. 66(10): 4555-4558.

CLSI. 2006. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria, Approved Guideline. In : CLSI document M45-A. Pennsylvania, Clinical and Laboratory Standards Institute 12-13.

Conedera, G., Mattiazzi, E., Russo, F., Chiesa, E., Scorzato, I., Grandesso, S., Bessegato, A., Fioravanti, A. and Caprioli, A. 2007. A family outbreak of *Escherichia coli* O157 haemorrhagic colitis caused by pork meat salami. Epidemiol. Infect. 135(2): 311-314.

Delsol, A.A., Randall, L., Cooles, S., Woodward, M.J., Sunderland, J. and Roe, J.M. 2005. Effect of the growth promoter avilamycin on emergence and persistence of antimicrobial resistance in enteric bacteria in the pig. J. Appl. Microbiol. 98(3): 564-571.

Diarrassouba, F., Diarra, M.S., Bach, S., Delaquis, P., Pritcrard, J., Topp, E. and Skura, B.J. 2007. Antibiotic resistance and virulence genes in commensal *Escherichia coli* and *Salmonella* isolates from commercial broiler chicken farmst. J. Food Prot. 70(6): 1316-1327.

DLD. 2007. The office of Agriculture Economics, the Department of Livestock Development, the swine Producers and Processors for Exporting Association, the swine Raisers Association of Thailand.

Duriez, P., Clermont, O., Bonacorsi, S., Bingen, E., Chaventre, A., Elion, J., Picard, B. and Denamur, E. 2001. Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. Microbiology. 147(Pt 6): 1671-1676.

Echeverria, P., Seriwatana, J., Taylor, D.N., Tirapat, C. and Rowe, B. 1985. *Escherichia coli* contains plasmids coding for heat-stable b, other enterotoxins, and antibiotic resistance. Infect. Immun. 48(3): 843-846.

Enright, M.C. and Spratt, B.G. 1999. Multilocus sequence typing. Trends Microbiol. 7(12): 482-487.

FAO. 2002. "The Livestock industries of Thailand". [Online]. Available:http://www.fao.org/docrep/004/ac797e07.htm.

Finlay, B.B. and Falkow, S. 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. 61(2): 136-169.

Fluit, A.C. and Schmitz, F.J. 2004. Resistance integrons and super-integrons. Clin. Microbiol. Infect. 10(4): 272-288.

Franklin, A. and Mollby, R. 1983. Concurrent transfer and recombination between plasmids encoding for heat-stable enterotoxin and drug resistance in porcine enterotoxigenic *Escherichia coli*. Med. Microbiol. Immunol. 172(3): 137-147.

Frydendahl, K. 2002. Prevalence of serogroups and virulence genes in *Escherichia coli* associated with postweaning diarrhoea and edema disease in pigs and a comparison of diagnostic approaches. Vet. Microbiol. 85(2): 169-182.

Gannon, V.P., Gyles, C.L. and Wilcock, B.P. 1989. Effects of *Escherichia coli* Shiga-like toxins (verotoxins) in pigs. Can. J. Vet. Res. 53(3): 306-312.

Gordon, D.M. 1992. Rate of plasmid transfer among *Escherichia coli* strains isolated from natural populations. J. Gen. Microbiol. 138(1): 17-21.

Gordon, D.M., Clermont, O., Tolley, H. and Denamur, E. 2008. Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. Environ. Microbiol. 10(10): 2484-2496.

Gordon, D.M. and Cowling, A. 2003. The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. Microbiol. 149(Pt 12): 3575-3586.

Guerra, B., Junker, E., Schroeter, A., Helmuth, R., Guth, B.E. and Beutin, L. 2006. Phenotypic and genotypic characterization of antimicrobial resistance in *Escherichia coli* O111 isolates. J. Antimicrob. Chemother. 57(6): 1210-1214.

Guerra, B., Junker, E., Schroeter, A., Malorny, B., Lehmann, S. and Helmuth, R. 2003. Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. J. Antimicrob. Chemother. 52(3): 489-492.

Gyles, C., So, M. and Falkow, S. 1974. The enterotoxin plasmids of *Escherichia coli*. J. Infect. Dis. 130(1): 40-49.

Gyles, C.L., Palchaudhuri, S. and Maas, W.K. 1977. Naturally occurring plasmid carrying genes for enterotoxin production and drug resistance. Science. 198(4313): 198-199.

Hammerum, A.M., Sandvang, D., Andersen, S.R., Seyfarth, A.M., Porsbo, L.J., Frimodt-Moller, N. and Heuer, O.E. 2006. Detection of *sul1*, *sul2* and *sul3* in sulphonamide resistant *Escherichia coli* isolates obtained from healthy humans, pork and pigs in Denmark. Int. J. Food Microbiol. 106(2): 235-237.

Hanson, R., Kaneene, J.B., Padungtod, P., Hirokawa, K. and Zeno, C. 2002. Prevalence of *Salmonella* and *E. coli*, and their resistance to antimicrobial agents, in farming communities in northern Thailand. Southeast Asian J. Trop. Med. Public Health. 33(3): 120-126.

Harada, K., Asai, T., Kojima, A., Ishihara, K. and Takahashi, T. 2006. Role of coresistance in the development of resistance to chloramphenicol in *Escherichia coli* isolated from sick cattle and pigs. Am. J. Vet. Res. 67(2): 230-235.

Herzer, P.J., Inouye, S., Inouye, M. and Whittam, T.S. 1990. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of Escherichia coli. J. Bacteriol. 172(11): 6175-6181.

Hochhut, B., Lotfi, Y., Mazel, D., Faruque, S.M., Woodgate, R. and Waldor, M.K. 2001. Molecular analysis of antibiotic resistance gene clusters in vibrio cholerae O139 and O1 SXT constins. Antimicrob. Agents Chemother. 45(11): 2991-3000.

Hooper, D.C. 1999. Mechanisms of fluoroquinolone resistance. Drug Resist. Updat. 2(1): 38-55.

Hsu, S.C., Chiu, T.H., Pang, J.C., Hsuan-Yuan, C.H., Chang, G.N. and Tsen, H.Y. 2006. Characterisation of antimicrobial resistance patterns and class 1 integrons among *Escherichia coli* and *Salmonella enterica* serovar Choleraesuis strains isolated from humans and swine in Taiwan. Int. J. Antimicrob. Agents. 27(5): 383-391.

Jan, N., Meshram, S.U. and Kulkarni, A. 2009. Plasmid profile analysis of multidrug resistant *E. coli* isolated from UTI patients of Nagpur City, India. Rom. Biotechnol. Lett. 14(5): 4635-4640.

Johnson, J.R., O'Bryan, T.T., Kuskowski, M. and Maslow, J.N. 2001. Ongoing horizontal and vertical transmission of virulence genes and papA alleles among *Escherichia coli* blood isolates from patients with diverse-source bacteremia. Infect. Immun. 69(9): 5363-5374.

Kado, C.I. and Liu, S.T. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145(3): 1365-1373.

Kang, H.Y., Jeong, Y.S., Oh, J.Y., Tae, S.H., Choi, C.H., Moon, D.C., Lee, W.K., Lee, Y.C., Seol, S.Y., Cho, D.T. and Lee, J.C. 2005. Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. J. Antimicrob. Chemother. 55(5): 639-644.

Kaper, J.B., Nataro, J.P. and Mobley, H.L. 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2(2): 123-140.

Khemtong, S. and Chuanchuen, R. 2008. Class 1 integrons and *Salmonella* genomic island 1 among *Salmonella enterica* isolated from poultry and swine. Microb. Drug Resist. 14(1): 65-70.

Kubitschek, H.E. 1990. Cell volume increase in *Escherichia coli* after shifts to richer media. J. Bacteriol. 172(1): 94-101.

Kumai, Y., Suzuki, Y., Tanaka, Y., Shima, K., Bhadra, R.K., Yamasaki, S., Kuroda, K. and Endo, G. 2005. Characterization of multidrug-resistance phenotypes and genotypes of *Escherichia coli* strains isolated from swine from an abattoir in Osaka, Japan. Epidemiol. Infect. 133(1): 59-70.

Lapierre, L., Cornejo, J., Borie, C., Toro, C. and San Martin, B. 2008. Genetic characterization of antibiotic resistance genes linked to class 1 and class 2 integrons in commensal strains of *Escherichia coli* isolated from poultry and swine. Microb. Drug Resist. 14(4): 265-272.

Leclerc, S., Boerlin, P., Gyles, C., Dubreuil, J.D., Mourez, M., Fairbrother, J.M. and Harel, J. 2007. *paa*, originally identified in attaching and effacing *Escherichia coli*, is also associated with enterotoxigenic E. coli. Res. Microbiol. 158(1): 97-104.

Lee, M.F., Chen, Y.H. and Peng, C.F. 2009. Molecular characterisation of class 1 integrons in *Salmonella enterica* serovar Choleraesuis isolates from southern Taiwan. Int. J. Antimicrob. Agents. 33(3): 216-222.

Leverstein-van Hall, M.A., Blok, H.E.M., Donders, A.R.T., Paauw, A., Fluit, A.C. and Verhoef, J. 2003. Multidrug resistance among Enterobacteriaceae is strongly associated with the presence of integrons and is independent of species or isolate origin. J. Infect. Dis. 187(2): 251-259.

Leverstein-Van Hall, M.A., Paauw, A., Box, A.T., Blok, H.E.M., Verhoef, J. and Fluit, A.C. 2002. Presence of integron-associated resistance in the community is widespread and contributes to multidrug resistance in the hospital. J. Clin.Microbiol. 40(8): 3038-3040.

Levesque, C., Piche, L., Larose, C. and Roy, P.H. 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. 39(1): 185-191.

Levy, S.B., FitzGerald, G.B. and Macone, A.B. 1976. Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. N. Engl. J. Med. 295(11): 583-588.

Liebert, C.A., Hall, R.M. and Summers, A.O. 1999. Transposon Tn21, flagship of the floating genome. Microbiol. Mol. Biol. Rev. 63(3): 507-522.

Liou, J.T., Shieh, B.H., Chen, S.W. and Li, C. 1999. An improved alkaline lysis method for minipreparation of plasmid DNA. Prep. Biochem. Biotechnol. 29(1): 49-54.

Literak, I., Dolejska, M., Rybarikova, J., Cizek, A., Strejckova, P., Vyskocilova, M., Friedman, M. and Klimes, J. 2009. Highly variable patterns of antimicrobial resistance in commensal *Escherichia coli* isolates from pigs, sympatric rodents, and flies. Microb. Drug Resist. 15(3): 229-237.

Lu, L., Dai, L., Wang, Y., Wu, C., Chen, X., Li, L., Qi, Y., Xia, L. and Shen, J. 2010. Characterization of antimicrobial resistance and integrons among *Escherichia coli* isolated from animal farms in Eastern China. Acta Trop. 113(1): 20-25.

Maguire, A.J., Brown, D.F., Gray, J.J. and Desselberger, U. 2001. Rapid screening technique for class 1 integrons in Enterobacteriaceae and nonfermenting gram-negative bacteria and its use in molecular epidemiology. Antimicrob. Agents Chemother. 45(4): 1022-1029.

Malkawi, H.I. and Youssef, M.T. 1998. Antibiotic susceptibility testing and plasmid profiles of *Escherichia coli* isolated from diarrhoeal patients. J. Trop. Pediatr. 44:128-132.

Martin, B.S., Campos, L., Bravo, V., Adasne, M. and Borie, C. 2005. Evaluation of antimicrobial resistance using indicator bacteria isolated from pigs and poultry in Chile. Int. J. Appl. Res. Vet. Med. 3(2): 171-178.

Mathai, E., Grape, M. and Kronvall, G. 2004. Integrons and multidrug resistance among *Escherichia coli* causing community-acquired urinary tract infection in southern India. APMIS. 112(3): 159-164.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5(5): 607-625.

Moredo, F.A., Vigo, G.B., Cappuccio, J.A., Pineyro, P., Perfumo, C.J. and Giacoboni, G.I. 2007. [Antimicrobial resistance of *Escherichia coli* isolated from pigs in Argentina]. Rev. Argent. Microbiol. 39(4): 227-229.

Nagy, B. and Fekete, P.Z. 1999. Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. Vet. Res. 30(2-3): 259-284.

Nakamura, S., Nakamura, M., Kojima, T. and Yoshida, H. 1989. gyrA and gyrB mutations in quinolone-resistant strains of *Escherichia coli*. Antimicrob. Agents Chemother. 33(2): 254-255.

Ngeleka, M., Pritchard, J., Appleyard, G., Middleton, D.M. and Fairbrother, J.M. 2003. Isolation and association of *Escherichia coli* AIDA-I/STb, rather than EAST1 pathotype, with diarrhea in piglets and antibiotic sensitivity of isolates. J. Vet. Diagn. Invest. 15(3): 242-252.

Nield, B.S., Holmes, A.J., Gillings, M.R., Recchia, G.D., Mabbutt, B.C., Nevalainen, K.M. and Stokes, H.W. 2001. Recovery of new integron classes from environmental DNA. FEMS Microbiol. Lett. 195(1): 59-65.

Ochman, H., Lawrence, J.G. and Groisman, E.A. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature. 405(6784): 299-304.

Okusu, H., Ma, D. and Nikaido, H. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. 178(1): 306-308.

Partridge, S.R., Brown, H.J. and Hall, R.M. 2002. Characterization and movement of the class 1 integron known as Tn2521 and Tn*1405*. Antimicrob. Agents Chemother. 46(5): 1288-1294.

Paulsen, I.T., Littlejohn, T.G., Radstrom, P., Sundstrom, L., Skold, O., Swedberg, G. and Skurray, R.A. 1993. The 3' conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob. Agents Chemother. 37(4): 761-768.

Phongpaichit, S., Liamthong, S., Mathew, A.G. and Chethanond, U. 2007. Prevalence of class 1 integrons in commensal *Escherichia coli* from pigs and pig farmers in Thailand. J. Food. Prot. 70(2): 292-299.

Picard, B., Garcia, J.S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., J, E. and Denamur, E. 1999. The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infect. Immun. 67(2): 546-553.

Platt, D.J., Sommerville, J.S., Kraft, C.A. and Timbury, M.C. 1984. Antimicrobial resistance and the ecology of *Escherichia coli* plasmids. J. Hyg. Camb. 93:181-188.

Punyarat, M. and Thongwai, N. 2008. Isolation and screening of lactic acid bacteria capable of inhibiting growth of some *Escherichia coli* isolated from swine and studying their properties used as probiotics. KMITL Sci. J. 8(2): 46-51.

Qadri, F., Svennerholm, A.M., Faruque, A.S. and Sack, R.B. 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin. Microbiol. Rev. 18(3): 465-483.

Quinn, P.J., Carter, M.E., Markey, B.K. and Carter, G.B. 1994. Enterobacteriaceae. In: Bacteriology. Clinical Veterinary Microbiology. Grafos, S. A. (ed.).Spain : Mosby-Year Book Europe.209-236.

Recchia, G.D. and Hall, R.M. 1995. Gene cassettes: a new class of mobile element. Microbiology. 141 (Pt 12): 3015-3027.

Robins-Browne, R.M. and Hartland, E.L. 2002. *Escherichia coli* as a cause of diarrhea. J. Gastroenterol. Hepatol. 17(4): 467-475.

Rosengren, L.B., Waldner, C.L. and Reid-Smith, R.J. 2009. Associations between antimicrobial resistance phenotypes, antimicrobial resistance genes, and

virulence genes of fecal *Escherichia coli* isolates from healthy grow-finish pigs. Appl. Environ. Microbiol. 75(5): 1373-1380.

Rosengren, L.B., Waldner, C.L., Reid-Smith, R.J., Checkley, S.L., McFall, M.E. and Rajic, A. 2008. Antimicrobial resistance of fecal *Escherichia coli* isolated from grow-finish pigs in 20 herds in Alberta and Saskatchewan. Can. J. Vet. Res. 72(2): 160-167.

Rowe-Magnus, D.A. and Mazel, D. 2001. Integrons: natural tools for bacterial genome evolution. Curr. Opin. Microbiol. 4(5): 565-569.

Ruiz, C. and Levy, S.B. 2010. Many chromosomal genes modulate MarA-mediated multidrug resistance in *Escherichia coli*. Antimicrob. Agents Chemother. 54(5).

Ruiz, J., Gomez, J., Navia, M.M., Ribera, A., Sierra, J.M., Marco, F., Mensa, J. and Vila, J. 2002. High prevalence of nalidixic acid resistant, ciprofloxacin susceptible phenotype among clinical isolates of *Escherichia coli* and other Enterobacteriaceae. Diagn. Microbiol. Infect. Dis. 42(4): 257-261.

Sabarinath, A., Tiwari, K.P., Deallie, C., Belot, G., Vanpee, G., Matthew, V., Sharma, R. and Hariharan, H. 2011. Antimicrobial resistance and phylogenetic groups of commensal *Escherichia coli* isolates from healthy pigs in Grenada. WebmedCentral Vet. Med. 2(5): WMC001942.

Sallen, B., Rajoharison, A., Desvarenne, S. and Mabilat, C. 1995. Molecular epidemiology of integron-associated antibiotic resistance genes in clinical isolates of enterobacteriaceae. Microb. Drug Resist. 1(3): 195-202.

Schierack, P., Steinruck, H., Kleta, S. and Vahjen, W. 2006. Virulence factor gene profiles of *Escherichia coli* isolates from clinically healthy pigs. Appl.Environ. Microbiol. 72(10): 6680-6686.

Seputiene, V., Povilonis, J., Ruzauskas, M., Pavilonis, A. and Suziedeliene, E. 2010. Prevalence of trimethoprim resistance genes in *Escherichia coli* isolates of human and animal origin in Lithuania. J. Med. Microbiol. 59(3): 315-322.

Shaheen, B.W., Oyarzabal, O.A. and Boothe, D.M. 2010. The role of class 1 and 2 integrons in mediating antimicrobial resistance among canine and feline clinical *E. coli* isolates from the US. Vet. Microbiol. 144(3-4): 363-370.

Sherley, M., Gordon, D.M. and Collignon, P.J. 2004. Evolution of multi-resistance plasmids in Australian clinical isolates of *Escherichia coli*. Microbiol. 150(5): 1539-1546.

Singh, R., Schroeder, C.M., Meng, J., White, D.G., McDermott, P.F., Wagner, D.D., Yang, H., Simjee, S., Debroy, C., Walker, R.D. and Zhao, S. 2005. Identification of antimicrobial resistance and class 1 integrons in Shiga toxin-producing *Escherichia coli* recovered from humans and food animals. J. Antimicrob. Chemother. 56(1): 216-219.

Stannarius, C., Burgi, E., Regula, G., Zychowska, M.A., Zweifel, C. and Stephan, R. 2009. Antimicrobial resistance in *Escherichia coli* strains isolated from Swiss weaned pigs and sows. Schweiz Arch Tierheilkd. 151t(3): 119-125.

Stokes, H.W. and Hall, R.M. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. 3(12): 1669-1683.

Stokes, H.W., O'Gorman, D.B., Recchia, G.D., Parsekhian, M. and Hall, R.M. 1997. Structure and function of 59-base element recombination sites associated with mobile gene cassettes. Mol. Microbiol. 26(4): 731-745.

Sunde, M. and Norstrom, M. 2005. The genetic background for streptomycin resistance in *Escherichia coli* influences the distribution of MICs. J. Antimicrob. Chemother. 56(1): 87-90.

Swick, M.C., Morgan-Linnell, S.K., Carlson, K.M. and Zechiedrich, L. 2011. Expression of multidrug efflux pump genes *acrAB-tolC*, *mdfA*, and *norE* in *Escherichia coli* clinical isolates as a function of fluoroquinolone and multidrug resistance. Antimicrob. Agents Chemother. 55(2): 921-924.

Tollefson, L., Altekruse, S.F. and Potter, M.E. 1997. Therapeutic antibiotics in animal feeds and antibiotic resistance. Rev. Sci. Tech. 16(2): 709-715.

van Den Bogaard, A.E., London, N. and Stobberingh, E.E. 2000. Antimicrobial resistance in pig faecal samples from the Netherlands (five abattoirs) and Sweden. J. Antimicrob. Chemother. 45(5): 663-671.

van Essen-Zandbergen, A., Smith, H., Veldman, K. and Mevius, D. 2007. Occurrence and characteristics of class 1, 2 and 3 integrons in *Escherichia coli*, *Salmonella* and *Campylobacter* spp. in the Netherlands. J. Antimicrob. Chemother. 59(4): 746-750.

Vashist, J., Vishvanath, Kapoor, R., Kapil, A., Yennamalli, R., Subbarao, N. and Rajeswari, M.R. 2009. Interaction of nalidixic acid and ciprofloxacin with wild type and mutated quinolone-resistance-determining region of DNA gyrase A. Indian J. Biochem. Biophys. 46(2): 147-153.

Vasilakopoulou, A., Psichogiou, M., Tzouvelekis, L., Tassios, P.T., Kosmidis, C., Petrikkos, G., Roma, E.S., Charvalos, E., Passiotou, M., Avlami, A. and Daikos, G.L. 2009. Prevalence and characterization of class 1 integrons in *Escherichia coli* of poultry and human origin. Foodborne Pathog. Dis. 6(10): 1211-1218.

Vu-Khac, H., Holoda, E., Pilipcinec, E., Blanco, M., Blanco, J.E., Dahbi, G., Mora, A., Lopez, C., Gonzalez, E.A. and Blanco, J. 2007. Serotypes, virulence genes, intimin types and PFGE profiles of *Escherichia coli* isolated from piglets with diarrhoea in Slovakia. Vet. J. 174(1): 176-187.

Wang, X.M., Jiang, H.X., Liao, X.P., Liu, J.H., Zhang, W.J., Zhang, H., Jiang, Z.G., Lu, D.H., Xiang, R. and Liu, Y.H. 2010. Antimicrobial resistance, virulence genes, and phylogenetic background in *Escherichia coli* isolates from diseased pigs. FEMS Microbiol. Lett. 306(1): 15-21.

Wannaprasat, W., Padungtod, P. and Chuanchuen, R. 2011. Class 1 integrons and virulence genes in *Salmonella enterica* isolates from pork and humans. Int. J. Antimicrob. Agents. 37(5): 457-461.

Wittig, W., Prager, R., Stamm, M., Streckel, W. and Tschape, H. 1994. Expression and plasmid transfer of genes coding for the fimbrial antigen F107 in porcine *Escherichia coli* strains. Zentralbl Bakteriol. 281(2): 130-139.

Woodall, C.A. 2003. DNA transfer by bacterial conjugation. Methods Mol. Biol. 235:61-65.

Wu, S., Dalsgaard, A., Hammerum, A.M., Porsbo, L.J. and Jensen, L.B. 2010. Prevalence and characterization of plasmids carrying sulfonamide resistance genes among *Escherichia coli* from pigs, pig carcasses and human. Acta. Vet. Scand. 5247.

Yang, H., Chen, S., White, D.G., Zhao, S., McDermott, P., Walker, R. and Meng, J. 2004. Characterization of multiple-antimicrobial-resistant *Escherichia coli* isolates from diseased chickens and swine in China. J. Clin. Microbiol. 42(8): 3483-3489.

Zhang, W., Zhao, M., Ruesch, L., Omot, A. and Francis, D. 2007. Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. Vet. Microbiol. 123(1-3): 145-152.

APPENDICES

APPENDIX A

Mutations found in the *gyrA* and *parC* genes sequences from the ciprofloxacin resistant *E. coli* isolates (n=14)

Gene	Mutation		No. (%)
	Nucleotide substitution	Amino acid substitution	
gyrA	C-248-T	Ser-83-Leu	9 (64.3)
	C-255-T	Silent mutation	11 (78.6)
	T-258-C	Silent mutation	1 (7.1)
	G-259-A	Asp-87-Asn	9 (64.3)
	T-267-C	Silent mutation	13 (92.9)
	C-273-T	Silent mutation	11 (78.6)
	A-281-C	Gln-94-Pro	1 (7.1)
	T-300-C	Silent mutation	11 (78.6)
	T-333-C	Silent mutation	7 (50.0)
parC	G-173-T	Ser-58-Ile	11(78.6)
	A-207-G	Silent mutation	7 (50.0)
	G-342-A	Silent mutation	1 (7.1)
	T-384-A	Silent mutation	1 (7.1)
	G-393-A	Silent mutation	2 (14.3)

APPENDIX B

Association between antimicrobial resistance phenotypes of *intI1* positive and *intI1* negative *E.coli* isolates

Antimicrobial agents	*IntI1* (+) ve No (%) of isolates		*IntI1* (-) ve No (%) of isolates		P-value	Chi-square
	Resistant	Susceptible	Resistant	Susceptible		
Ampicillin	231 (91)	20 (8)	84 (90)	9(10)	0.615	0.257
Chloramphenicol	222 (88)	29 (12)	51 (55)	42 (45)	0.000	46.791
Ciprofloxacin	140 (56)	111 (44)	40 (43)	53 (57)	0.035	4.433
Gentamicin	145 (58)	106 (42)	73 (78)	20 (22)	0.000	12.558
Streptomycin	200 (80)	51 (20)	84 (90)	9 (10)	0.021	5.336
Sulfamethoxazole	178 (71)	73 (29)	54 (58)	39 (42)	0.024	5.104
Tetracycline	247 (98)	4 (2)	84 (90)	9 (10)	0.000	12.195
Trimehoprim	236 (94)	15 (6)	37 (40)	56 (60)	0.000	1.219E2

APPENDIX C

Distribution of resistance phenotypes among 4 phylo groups

Phylognetic group (No.)	No. (%) of isolates							
	AMP	CHP	CIP	GEN	STR	SUL	TET	TRI
A(28)	25 (89.3)	28 (100)	14 (50)	8 (25.6)	28 (100)	24 (85.7)	28 (100)	28 (100)
B1(282)	257 (91.1)	212 (57.2)	151 (53.6)	184 (65.3)	222 (78.7)	189 (67)	269 (95.4)	211 (74.3)
B2(24)	23 (95.8)	23 (95.8)	5 (20.8)	16 (66.7)	24 (100)	19 (79.2)	24 (100)	24 (100)
D(10)	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	0 (0)	10 (100)	10 (100)
Total (344)	315 (91.6)	273 (79.4)	180 (52.3)	218 (63.4)	284 (82.7)	232 (67.4)	331 (96.2)	273 (79.4)
Chi-Square	1.744	16.871	18.881	20.929	15.978	26.497	2.970	19.670
P-value	0.627	**0.001**	**0.000**	**0.000**	**0.001**	**0.000**	0.369	**0.000**

APPENDIX D

Distribution of 10 virulence genes among 4 phylogenetic groups

Phylo. groups(No.)	Positive No. (%) of isolates in virulence genes									
	estA	*faeG*	*estB*	*elt*	*fedA*	*fasA*	*sepA*	*paa*	*astA*	*eaeA*
A(28)	0	0	10	19	0	28	0	1	10	3
	(0)	(0)	(35.7)	(67.9)	(0)	(100)	(0)	(3.6)	(35.7)	(10.7)
B1(282)	2	16	76	146	54	281	50	77	110	33
	(0.7)	(5.7)	(27)	(51.8)	(19.2)	(99.5)	(17.7)	(27.3)	(39)	(11.7)
B2(24)	0	0	0	24	0	24	0	0	0	9
	(0)	(0)	(0)	(100)	(0)	(100)	(0)	(0)	(0)	(37.5)
D(10)	0	0	0	10	4	5	0	0	0	0
	(0)	(0)	(0)	(100)	(40)	(50)	(0)	(0)	0)	(0)
Total(344)	2	16	86	199	58	338	50	78	120	45
	(0.6)	(4.7)	(25)	(57.9)	(16.9)	(98.3)	(14.5)	(22.7)	(34.9)	(13.1)
Chi-square	0.442	3.689	13.62	30.19	15.41	1.400 E2	12.86	19.24	20.33	14.701
P-value	0.931	0.297	**0.003**	**0.000**	**0.001**	**0.000**	**0.005**	**0.000**	**0.000**	**0.002**

Appendix E

MIC values of antimicrobial agents for 144 *E.coli* isolates which contain plasmids

Group	Isolate No.	MIC (µg/ml)							
		AMP	CHP	CIP	GEN	STR	SUL	TET	TRI
1	33.1	16	8	0.125	0.25	1	2	2	4
	33.2	8	8	0.125	0.25	1	2	2	1
	33.4	16	8	0.5	2	1	32	2	2
	33.8	8	8	0.125	0.25	1	2	2	1
	33.9	8	8	0.125	0.25	1	2	2	1
	33.5	8	8	16	2	1	32	2	1
	33.6	8	8	16	16	1	16	2	1
	33.7	8	8	16	32	1	8	2	1
	33.3	>512	8	16	2	128	256	2	2
2	50.1	512	64	0.125	0.25	1	128	512	1
	50.2	512	128	0.125	0.25	1	32	512	1
	50.3	512	64	0.125	2	1	128	512	1
	50.4	512	64	0.125	4	1	32	512	1
	50.5	>512	64	1	1	1	2	512	1
	50.7	>512	64	0.125	0.25	1	4	512	1
	50.9	>512	64	0.125	2	1	4	512	1
	50.10	512	64	0.125	0.25	1	4	512	1
3	12.7	>512	16	0.125	0.5	32	32	64	1
4	34.2	16	64	0.25	0.5	16	8	256	>512
	12.6	>512	16	0.25	256	2	512	64	1
5	52.1	1	128	0.125	0.5	1	4	256	>512
	52.2	1	64	0.125	0.5	1	4	256	>512
	52.3	1	64	0.125	0.5	1	4	256	>512
	52.4	1	64	0.125	0.5	1	4	256	>512
	52.5	1	64	0.125	0.5	1	4	128	>512
	52.6	1	64	0.125	0.5	1	4	256	>512
	52.7	1	64	0.125	0.5	1	4	256	>512
	52.8	1	128	0.125	0.5	1	4	256	>512
	52.9	1	128	0.125	0.5	1	4	256	>512
	52.10	1	128	0.125	0.5	1	4	256	>512
6	50.6	>512	64	16	1	1	16	512	1
	50.8	>512	64	16	2	1	4	512	1

*The MIC value above the breakpoints are underlined

Group	Isolate	MIC (µg/ml)							
	No.	AMP	CHP	CIP	GEN	STR	SUL	TET	TRI
7	53.6	>512	64	0.125	2	1	8	256	>512
	53.7	>512	64	0.125	2	1	8	256	>512
	53.8	>512	64	0.125	2	1	8	256	>512
	53.9	>512	64	0.125	2	1	8	256	>512
	53.10	>512	64	0.125	2	1	8	256	>512
8	30.2	>512	8	0.125	2	256	1024	128	1
	30.3	>512	8	0.25	0.5	128	1024	128	1
	30.4	>512	8	0.25	2	128	1024	128	1
	30.6	>512	8	0.125	2	256	1024	128	1
	30.7	>512	8	0.125	4	256	1024	128	1
9	34.3	16	>512	16	2	16	16	256	>512
	58.1	>512	>512	0.25	>256	>256	512	256	>512
10	114.3	4	64	0.25	1	64	256	256	>512
	114.4	4	64	0.25	0.5	32	256	256	>512
	114.5	4	64	0.25	0.5	32	256	256	>512
11	116.1	16	64	0.5	2	32	256	256	>512
	116.4	16	128	0.25	1	256	256	512	>512
	116.5	16	128	0.25	1	64	256	512	>512
12	10.1	>512	128	32	256	64	1024	64	1
	10.4	>512	128	32	256	64	1024	64	1
	10.5	>512	128	32	256	64	1024	64	1
13	92.1	>512	128	8	128	256	256	512	>512
14	118.2	>512	128	32	256	128	256	512	>512
	118.3	>512	128	32	>256	128	256	512	>512
	118.4	>512	128	16	256	64	256	512	>512
	118.5	>512	128	32	>256	128	256	256	>512
15	13.3	>512	256	128	32	128	256	256	>512
16	18.1	>512	256	256	32	128	256	64	>512
17	22.1	>512	128	128	16	128	256	64	>512
18	54.1	>512	512	128	8	4	512	128	>512
	54.2	>512	512	128	8	4	512	128	>512
19	49.1	>512	>512	64	2	256	512	512	>512
20	58.9	>512	>512	16	4	>256	512	256	>512
	29.3	>512	128	0.25	32	128	1024	128	>512
	29.8	>512	128	0.125	16	128	1024	64	>512
	29.9	>512	128	0.125	8	128	1024	64	>512

*The MIC value above the breakpoints are underlined

Group	Isolate	MIC (µg/ml)							
	No.	AMP	CHP	CIP	GEN	STR	SUL	TET	TRI
	116.3	16	128	0.25	8	>256	256	512	>512
21	6.1	>512	512	32	0.5	>256	1024	64	>512
	6.2	>512	512	32	0.5	>256	1024	64	>512
	6.3	>512	512	32	0.5	>256	1024	64	>512
	6.4	>512	512	32	0.5	>256	1024	64	>512
	6.5	>512	512	32	0.5	>256	1024	64	>512
	6.6	>512	512	32	0.5	>256	1024	64	>512
	6.7	>512	512	32	0.5	>256	1024	64	>512
	6.8	>512	512	32	0.5	>256	1024	64	>512
	6.9	>512	512	32	0.5	>256	1024	64	>512
	6.10	>512	512	32	0.5	>256	1024	64	>512
22	71.1	>512	128	32	1	>512	1024	256	>512
23	72.2	>512	128	32	1	>512	1024	128	>512
24	31.6	>512	64	0.5	16	128	1024	64	>512
25	32.1	>512	64	0.5	256	64	1024	256	>512
	32.5	>512	64	0.5	256	32	512	256	>512
	32.7	>512	64	0.5	256	32	512	256	>512
	32.9	>512	64	0.5	256	32	512	256	>512
26	59.7	128	>512	1	8	128	512	512	>512
27	60.1	>512	>512	0.5	>256	128	512	512	>512
	60.2	>512	>512	0.5	>256	>256	512	128	>512
	60.3	>512	>512	0.5	>256	>256	512	128	>512
	60.4	>512	>512	0.5	>256	>256	512	128	>512
	60.5	>512	>512	0.5	>256	>256	512	128	>512
	60.6	>512	>512	0.5	>256	>256	512	128	>512
	60.7	>512	>512	0.5	>256	>256	512	128	>512
	60.8	>512	>512	0.5	>256	>256	512	128	>512
	60.9	>512	>512	0.5	>256	>256	512	256	>512
	60.10	>512	>512	0.5	>256	>256	512	128	>512
28	31.9	>512	64	0.5	64	32	1024	64	>512
29	106.4	>512	128	8	64	128	512	512	>512
30	13.1	>512	32	0.5	256	128	1024	128	>512
	13.2	>512	32	0.5	256	128	1024	128	>512
31	76.2	>512	128	2	64	256	>1024	256	>512
	76.4	>512	128	2	32	256	1024	256	>512
	76.6	>512	128	0.5	64	256	1024	256	>512

Group	Isolate	MIC (µg/ml)							
	No.	AMP	CHP	CIP	GEN	STR	SUL	TET	TRI
32	51.1	>512	0	16	32	32	512	512	>512
33	55.1	>512	8	128	256	>256	512	256	>512
34	56.1	>512	8	16	256	32	512	512	>512
35	84.3	>512	4	16	>256	>256	512	256	>512
36	75.10	>512	16	4	64	256	>1024	256	>512
37	54.3	>512	512	128	8	4	512	128	>512
38	55.4	>512	256	64	256	32	256	256	>512
39	56.7	>512	>512	32	256	128	512	512	>512
40	57.1	>512	>512	32	>256	256	512	512	>512
	57.3	>512	>512	32	>256	64	512	512	>512
	57.4	>512	>512	32	>256	256	512	512	>512
	57.5	>512	>512	32	>256	128	512	512	>512
	57.6	>512	>512	32	>256	>256	512	512	>512
	57.7	>512	>512	16	>256	>256	512	512	>512
	57.8	>512	>512	32	>256	>256	512	512	>512
	57.9	>512	>512	32	>256	>256	512	512	>512
	57.10	>512	>512	32	>256	>256	512	512	>512
41	61.2	>512	>512	16	256	128	512	512	>512
42	84.1	>512	128	16	>256	>256	512	256	>512
43	106.1	>512	128	0.25	>256	128	512	512	>512
44	10.9	>512	256	32	256	128	1024	64	>512
45	71.7	>512	256	64	32	>512	1024	256	>512
46	72.10	>512	128	32	16	256	1024	128	>512
47	73.1	>512	128	32	16	256	1024	256	>512
48	75.1	>512	32	4	256	512	1024	256	>512
49	76.1	>512	128	8	64	512	>1024	256	>512
50	51.3	512	4	0.5	32	32	256	512	>512
	51.4	512	8	0.5	32	32	256	512	>512
	51.9	512	16	0.5	32	32	256	512	>512
51	51.7	512	8	0.5	32	16	512	512	>512
52	51.2	512	4	1	32	32	512	512	>512
	51.5	512	4	0.5	32	32	512	512	>512
	51.8	512	16	0.5	32	32	512	512	>512
	51.10	512	8	0.5	64	32	512	512	>512
53	51.6	512	64	0.5	32	32	512	512	>512
54	29.2	>512	128	0.125	32	64	16	8	>512

*The MIC value above the breakpoints are underlined

Group	Isolate	MIC (µg/ml)							
	No.	AMP	CHP	CIP	GEN	STR	SUL	TET	TRI
55	29.4	>512	128	0.25	32	256	16	256	>512
	29.5	>512	128	0.125	32	256	16	128	>512
	29.6	>512	128	0.125	16	32	16	128	>512
56	118.1	>512	8	32	256	128	256	512	>512

*The MIC value above the breakpoints are underlined

Applendix F

Fifty-six plasmid profiles of *E. coli* isolates (*n*=144)
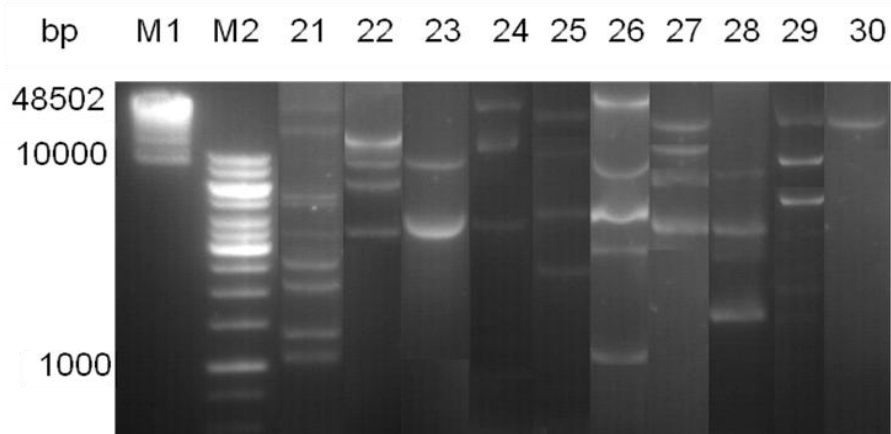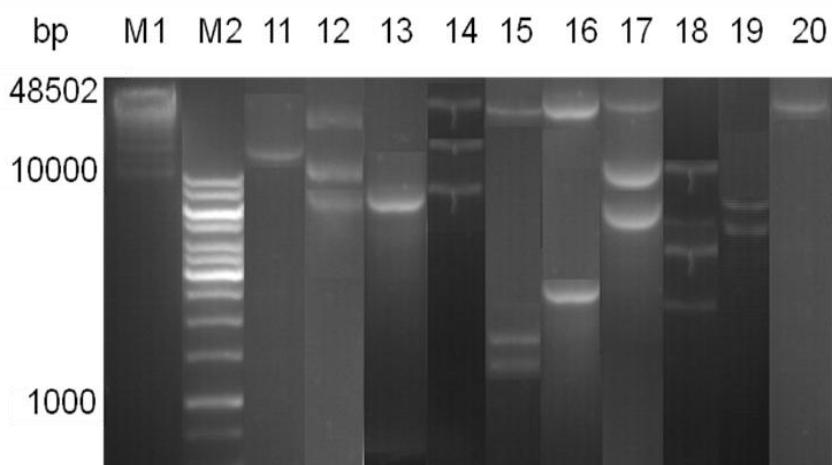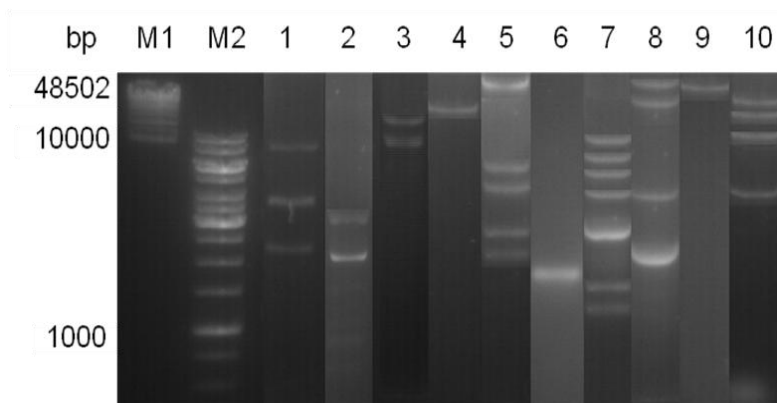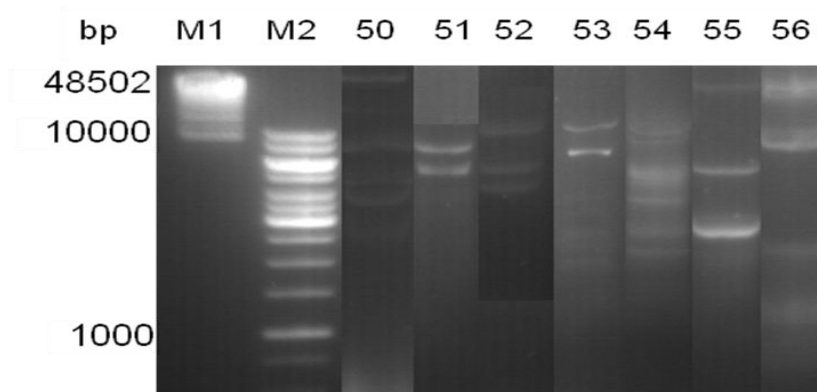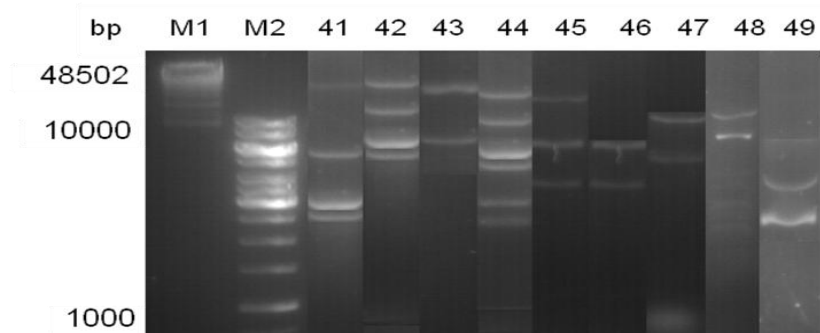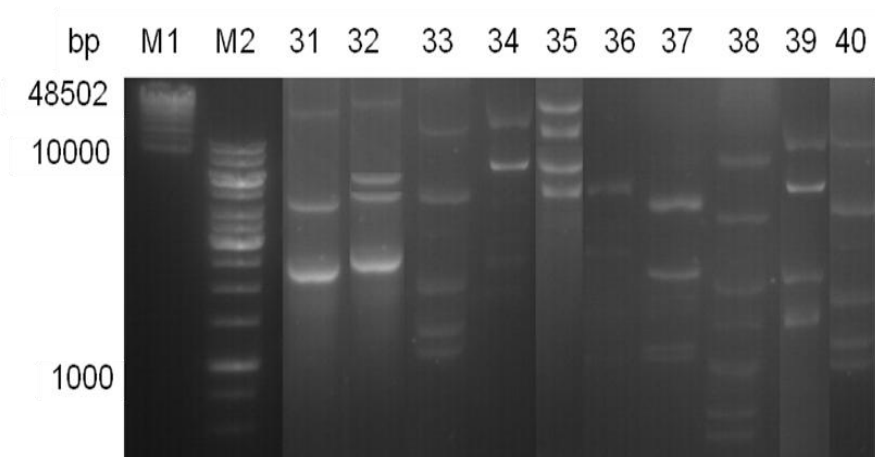
Appendix G

Bacterial Growth Media, Instruments and chemical substances

1. Bacterial growth media

    1.1 Brilliant-Green agar (Difco)

- Proteose peptone No.3	10.0g
- Yeast extract	3.0g
- Lactose	10.0g
- Saccharose	10.0g
- Sodium chloride	5.0g
- Agar	20.0g

    1.2 Luria-Bertani agar (Difco)

- Tryptone	10.0g
- Yeast extract	5.0g
- Sodium chloride	5.0g
- Agar	15.0g

    1.3 Luria-Bertani broth (Difco)

    1.4 Mueller-Hinton agar (Difco)

- Beef extract powder	2.0g
- Acid digest of caesin	17.5g
- Starch	1.5g
- Agar	17.0g

    1.5 Xylose Lysine Deoxycholate agar (Difco)

- Yeast extract	3.0g
- L-lysine	5.0g
- Xylose	3.75g
- Lactose	7.5g
- Saccharose	7.5g
- Sodium desoxycholate	2.5g
- Ferric ammonium citrate	0.8g

- Sodium Thiosulfate	6.8g
- Sodium chloride	15.0g
- Phenol red	0.08g

2. PCR assay

    2.1 MasterMix (Fermentas®)

- Taq DNA polymerase in reaction buffer	0.05units/µl
- $MgCl_2$	4mM
- dNTPs (dATP, dCTP, dGTP, dTTP)	0.04mM of each

    2.2 DNA marker (Fermentas®)

    2.3 Loading Dye (Fermentas®)

    2.4 Agarose gel (Sigma-Aldrish®)

- Agarose (ultra pure)	1.2g
- Adjusted 1x TBE volume to 100 ml	

    2.5 Tris–acetate/EDTA

    2.6 Ethidium Bromide 10mg/ml (Sigma-Aldrish®)

- Ethidium bromide	1.0g
- Distilled water	1,000.0ml

    2.7 50x TAE (Tris-Acetate buffer) 1,000 ml contains

- Tris base	242.0g
- Glacial acetic acid	57.1g
- 0.5M EDTA pH 8.0	100.0ml
- Distilled water	1,000.0ml

    2.8 PCR Tpersonal combi model® (Biometra®)

3. Other chemicals

    3.1 Lysozyme (Biobasic Inc®)

    3.2 Phosphate buffer saline (Diagxotics®)

    3.3 Sodium dodecyl sulfate (Amresco®)

    3.4 Ribonuclease A (Fermentas®)

    3.5 Restriction endonuclease enzymes (Fermentas®)

BIOGRAPHY

Miss Khin Khin Lay, a Myanmar student, was born in Amarapura, Mandalay Region, Myanmar, on 10th May 1975. She obtained Bachelor of Veterinary Science degree and Master of Veterinary Science degree from the University of Veterinary Science, Yezin, Myanmar in 2000 and 2004 respectively. She worked as an Assistant Lecturer in the Department of Animal Husbandry at the University of Veterinary Science from 2001 to 2004. She studied at Faculty of Veterinary Medicine, Chiang Mai University (CMU) in Thailand and Freie Universitaet Berlin (FUB) in Germany for her Master of Veterinary Public Health from 2005-2007. She then worked as Lecturer in the Department of Animal Husbandry at the University of Veterinary Science, Yezin. In 2009, she got the scholarship "The Graduate Scholarship Program for Faculty Members from Neighboring Countries" for giving an opportunity to Ph.D study at Veterinary Public Health Program, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Thailand.