พันธุกรรมการดื้อยาของ *Campylobacter coli* ที่แยกได้จากสุกรในประเทศไทย

นายชานนท์ เอกภาพโยธิน

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GENETICS OF ANTIBIOTIC RESISTANCE IN *CAMPYLOBACTER COLI* ISOLATED FROM SWINE IN THAILAND

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ศึกษารูปแบบและพันธุกรรมการดื้อยาของ *Campylobacter coli* จำนวน 83 เชื้อที่แยกได้จากสุกรที่ทราบค่าความเข้มข้นต่ำสุดของสารต้านจุลชีพ (MICs) สำหรับยาปฏิชีวนะ erythromycin, ciprofloxacin, nalidixic acid และ tetracycline แล้ว ตรวจหาการปรากฏของยีน *tetO* และ *intI1* ในเชื้อทุกตัว ตรวจการกลายพันธุ์ของยีน 23S rRNA ในเชื้อที่ดื้อต่อยา erythromycin จำนวน 44 ตัว และตรวจการกลายพันธุ์ในส่วน Quinolone Resistance Determining Region (QRDR) ของยีน *gyrA* และ *gyrB* ในเชื้อที่ดื้อต่อ ciprofloxacin และ/หรือ nalidixic acid จำนวน 60 ตัว ผลการวิจัยพบการปรากฏของยีน *tetO* ในเชื้อที่ดื้อต่อ tetracycline (97%, 65/67) และพบเชื้อที่มีการปรากฏยีน *intI1* จำนวน 4 เชื้อ (4.8%, 4/83) ซึ่งเชื้อทั้งหมดให้ amplicons ขนาด 1000 bp เมื่อเพิ่มจำนวน DNA ด้วย 5'-3' conserved sequence PCR และผลการตรวจหาลำดับเบสพบว่าเชื้อทุกตัวมียีน *aadA9* ซึ่งควบคุมการดื้อต่อยาในกลุ่ม aminoglycosides โดยเชื้อที่ดื้อต่อ erythromycin จำนวน 77% (34/44) มีการกลายพันธุ์ของ 23S rRNA โดยพบการกลายพันธุ์แบบ point mutations 5 รูปแบบ และรูปแบบที่พบมากที่สุดคือ A2230G (70%, 31/44) เชื้อทุกตัวที่ดื้อต่อ nalidixic acid และ/หรือ ciproflocxacin มีการกลายพันธุ์ในส่วน QRDR ของยีน *gyrA* และ 8.5% (5/60) พบการกลายพันธุ์ในยีน *gyrB* การกลายพันธุ์ที่พบมากที่สุดใน *gyrA* คือ C257T (93%, 56/60) ซึ่งเปลี่ยนกรดอะมิโน threonine ที่ตำแหน่ง 86 เป็น isoleucine ส่วนการกลายพันธุ์ที่พบมากที่สุดใน *gyrB* คือ A1144C (7%, 4/60) ซึ่งเปลี่ยนกรดอะมิโน lysine ที่ตำแหน่ง 381 เป็น glutamic acid

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต.....
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KEY WORD: *Campylobacter coli* / Campylobacteriosis / *gyrA* / *gyrB* / 23S rRNA / *tetO* / multidrug resistance / integrons

CHANON EKKAPOBYOTIN: GENETICS OF ANTIBIOTIC RESISTANCE IN *CAMPYLOBACTER COLI* ISOLATED FROM SWINE IN THAILAND. THESIS ADVISOR: ASST. PROF. RUNGTIP CHUANCHUEN, THESIS COADVISOR: ASSOC. PROF. ALONGKORN AMONSIN 63 pp.

This study was performed in a total of eighty-three *Campylobacter coli* strains isolated from swine, of which minimal inhibitory concentrations (MICs) for erythromycin, ciprofloxacin, nalidixic acid and tetracycline were known. All of the isolates were investigated for the presence of *tetO* and class 1 integrase. Forty-four erythromycin-resistant isolates were tested for mutations in 23S rRNA gene. Sixty isolates resistant to ciprofloxacin and/or nalidixic were examined for mutations in *gyrA* and *gyrB* genes. Most of the tetracycline-resistant isolates (97%, 65/67) possessed *tet(O)*. Four isolates (4.8%, 4/83) harbored class 1 integrons and all of them produced a 1,000-bp amplicon in a 5′-3′ conserved sequence PCR directed toward amplification of the gene cassettes. DNA sequencing demonstrated that all four isolates possessed the aminoglycoside resistance gene, *aadA9*. Most of erythromycin-resistant isolates (77%, 34/44) contained mutations in 23s RNA. Five of point mutations were found, of which the most common type was A2230G (70%, 31/44). For nalidixic acid and/or ciproflocxacin resistant strains, all carried mutations in Quinolone Resistance Determining Region (QRDR) of *gyrA* and 8.5% (5/60) contained mutations in QRDR of *gyrB*. The predominant mutation in *gyrA* was C257T (93%, 56/60) that converted threonine at position 86 to isoleucine. The point mutations in *gyrB* was A1144C (7%, 4/60) that converted lysine at position 381 to glutamic acid.

Department Veterinary Public Health Student's signature.....
Field of study Veterinary Public Health Advisor's signature.....
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LIST OF ABBREVIATIONS

bp	base pair
ºC	degree Celsius
C. coli	*Campylobacter coli*
C. jejuni	*Campylobacter jejuni*
DNA	deoxynucleic acid
DW	distilled water
e.g.	exempli gratia, for example
EDTA	ethylenediamine tetraacetic acid
et al.	Et alii, and others
g	gram (s)
h	hour (s)
i.e.	id est, that is
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
min	minute (s)
µl	microliter
µM	micromolar
ml	milliliter
mM	millimolar
PCR	Polymerase Chain Reaction
pH	the negative logarithm of hydrogen ion concentration
sec	second (s)
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
U	unit

CHAPTER I

INTRODUCTION

Swine is the major food producing animal in Thailand. In 2006, swine production has been estimated to be 13 million heads per year and Thai per capita pork consumption was 12.3 kg per year (Office of agricultural economics, 2006). However, swine is also the main source of food-borne pathogen for example, *Salmonella enterica*, *Listeria monocytogenes*, *Escherchia coli,* and *Campylobacter* spp. These bacterial pathogens can be transmitted to humans through the food chain.

Campylobacter is one of the major bacterial pathogens and a leading cause of foodborne disease in many countries including Thailand (Bodhidatta et al., 2002). Two *Campylobacter* species that commonly cause infections in humans including *Campylobacter jejuni* and *Campylobacter coli*. As *C. jejuni* is predominant in poultry, *C. coli* is most commonly associated with swine. *C. coli* is a commensal enteric bacterium in pigs (Kim et al., 2006). This bacterium generally does not cause a disease in pigs but it could cause gastrointestinal infection termed Campylobacteriosis in humans.

Campylobacteriosis is an infectious disease caused by bacteria of the genus *Campylobacter* (CDC, 2005). Most people who become ill with Campylobacteriosis get diarrhea, cramping, abdominal pain, and fever within 2 to 5 days after exposure to the organism. The diarrhea may be bloody and can be accompanied by nausea and vomiting. The illness typically lasts for 1 week. Some people who are infected with *Campylobacter* do not have any symptoms. In immunocompromised people, *Campylobacter* occasionally spreads to the bloodstream and causes a serious life-threatening infection (CDC, 2005). A previous study in Thailand showed that prevalence of *C coli* was 98%, 51%, and 60% in *C. coli* isolates from pigs, chicken, and farm workers, respectively (Padungtod et al., 2006). *C. coli* is associated with up to 15% of

human Campylobacteriosis cases (Moore et al., 2005) and infections with this pathogen in humans are commonly associated with consumption of pork (Keller and Perreten, 2006).

Campylobacteriosis is usually self-limited. That means that the patient could recover within 3-5 days without antibiotic treatment (Gibreel et al., 2005). However, in some groups of patients, i.e., those with severe illnesses, children under 5 years of age and immunocompromised patients, antibiotic treatment is required. Erythromycin, ciprofloxacin, nalidixic acid, and tetracycline are among the drugs of choice today (Butzler, 2004). However, rate of human infections caused by antimicrobial-resistance strains of *C. coli* has been increasing worldwide (Engberg et al., 2001). This makes clinical management of Campylobacteriosis more difficult, compromises antibiotic treatment of patients with bacteremia, prolongs illness and hospitalization, and increases cost of treatment.

Currently, antibiotic resistance in *C. coli* has become increasing in both developed and developing countries. In the United states, *C. coli* isolates from humans in 1997-2001 were resistant to nalidixic acid, ciprofloxacin and tetracycline in the rate of 36%, 30%, and 43%, respectively (Gupta et al., 2004; Luber et al., 2003). In European survey, *C. coli* strains from pigs were resistant to erythromycin (32.5%), nalidixic acid (14.8%), ciprofloxacin (14.1%), and tetracycline (19.4%) (Bywater et al., 2004; Saenz et al., 2000). In Thailand, antimicrobial resistance has become increasingly common in *C. coli* isolated from both humans and animals as well. The *C. coli* strains isolated from pigs and chicken showed high level resistance to erythromycin (56.9%), nalidixic acid (90.2%), ciprofloxacin (86.3%), and tetracycline (88.2%). The *C. coli* isolates from patients were also resistant to erythromycin (78.3%), nalidixic acid (65.2%), ciprofloxacin (69.6%), and tetracycline (34.8%), and approximately 92% showed resistance to multiple drugs (Padungtod et al., 2006).

Since antibiotic resistance in *C. coli* has been increasing in both humans and food animals, it is believed that the link between antibiotic resistant *C. coli* in humans

and animals exists. It has been hypothesized that the increase in the numbers of antibiotic resistant *C. coli* isolates in humans results from extensive use of antibiotics in food-animal production (Smith et al., 2002). In pig production, various antibiotics have been widely used for 3 main purposes which are treatment of infections, prophylaxis of diseases, and growth promotion. Such antibiotic use may increase selection pressure for antibiotic resistance, and distribution of genetics determinants in *C. coli*. (Phillips et al., 2004). As resistant *C. coli* isolates can be a direct threat to humans when their resistance phenotypes interfere with the efficacy of antibiotic treatment, they can be an indirect threat when their resistant determinants are transferred to other human pathogens. It is accepted that prove of the hypothesis will help to clarify the link of resistant *C. coli* along the food chain and benefit the creation of the plan to reduce antibiotic resistance. However, data on antibiotic susceptibility is not enough to verify the hypothesis. For this purpose, information of genetics of resistance is required. While data on genetics of antibiotic resistance of *C. coil* have been extensively reported in many countries (Keller and Perreten, 2006; Pratt and Korolik, 2005), such data of *C. coli* isolates in Thailand are unavailable. Currently, there is only one report of molecular mechanisms underlying antibiotic resistance in *C. jejuni* available in Thailand (Boonmar et al., 2007).

Therefore, the purpose of this study was to assess genetics of antibiotic resistance in *C. coli* isolated from swine in Thailand. The study focused on resistance to the clinically important antibiotics used for treatment of Campylobacteriosis that are macrolides, nalidixic acid, ciprofloxacin, and tetracyclines. Generally, mechanisms underlying antibiotic resistance could be chromosomal mutations or acquisition of resistance determinants depending on type of antibiotics. In this study, we determined i) mutations in *gyrA* and *gyrB* genes that are associated with resistance to fluoroquinolones. ii) mutations in 23S rRNA that is the target site of action of macrolides. iii) the presence of *tet(O)* gene that encodes Tet(O) responsible for resistance to tetracycline and iv) the presence of class 1 integrons that is a mobile genetic element and a major contributor to multidrug resistance in Gram-negative bacteria.

Results from this study provided genetic information of antibiotic resistance in *C. coli* isolates from swine in Thailand. It can be used to prove the link of antibiotic resistance between *C. coli* from pigs and humans when combined with data from the isolates along the food chain. This data can be used as a part of antibiotic resistance monitoring in Thailand and applied in risk analysis of antibiotic resistance. In addition, both data and techniques used in this study can be applied to further studies involving molecular mechanisms of antibiotic resistance in other *Campylobacter* species and other bacteria.

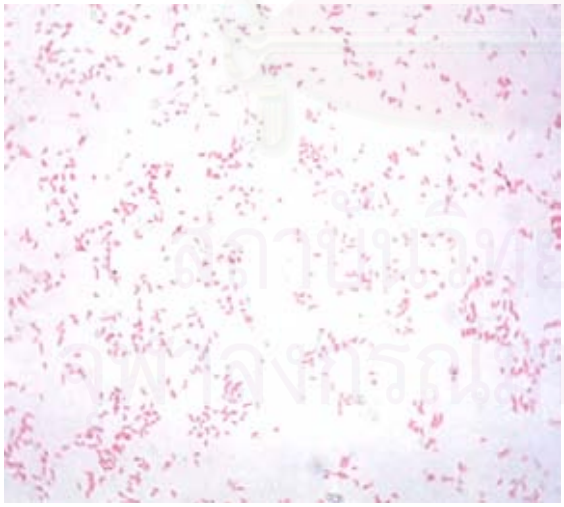
CHAPTER II

REVIEW LITERATURES

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

C. coli is a Gram-negative, slender, curved and motile rod shaped bacterium (Figure 1). It is 0.2 - 0.5 µm wide and 0.5 – 8.0 µm long. This bacterium possesses uni- or bi-polar flagellum, which yields “cork-screw” motility. *C. coli* is a microaerophilic organism that has a requirement for reduced levels of oxygen. This organism requires 3 - 5% oxygen and 2 - 10% carbon dioxide for optimal growth conditions and has an optimal growth temperature between 37 and 42 °C. *C. coli* is relatively fragile and sensitive to environmental stress (Corry et al., 1995). In general, it is carried in the intestinal tract of swine, poultry and cattle. Among these animals, swine are the most common reservoirs. *C. coli* does not usually cause infection in pigs but can cause food-borne disease in humans (Tam et al., 2003).

A.



B.

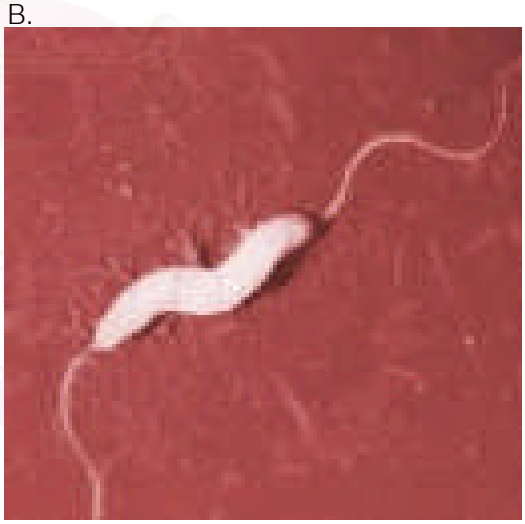


Figure 1. *Campylobacter* spp. A. Gram-stained smear (American Society for Microbiology, 1997) and B. Electron-microscope image (Institute of Microbiology, 2003)

Infection with *C. coli* is termed Campylobacteriosis. It is also often known as Campylobacter enteritis or gastroenteritis. The infective dose ranges between 500 and 10,000 cells depending on the strain, damages to cells from environmental stress and the susceptibility of the host (FSAI, 2002). *C. coli* infection causes diarrhea that may be watery or sticky and can contain blood and fecal leukocytes. Other symptoms that are often present include fever, abdominal pain, nausea, headache, and muscle pain. The illness usually occurs 2-5 days after ingestion of the contaminated food or water and generally lasts 7-10 days (FDA, 2002). Most infections are self-limited, therefore; antibiotic treatment is not always necessary. In addition, Campylobacteriosis is commonly associated with consumption of the contaminated pork, poultry meat, or untreated water (Keller and Perreten, 2006).

2. Occurrence of antibiotic resistant *C. coli*

Campylobacter is a leading cause of food-borne diseases, which are sporadic outbreak worldwide. Recently, a total of 183,961 cases of laboratory confirmed Campylobacteriosis were reported in the European Union. Over the world, the overall incidence of Campylobacteriosis was 48 per 100,000 populations that is slightly higher than that of Salmonellosis (ESFA, 2004). In the United States, *Campylobacter* is the major cause of food-borne diseases (Mead et al., 1999). In Thailand, *Campylobacter* was the most common pathogen found in children less than 12 years old with dysentery (28%) (Bodhidatta et al., 2002).

Since the 1991s, there has been evidence suggesting that the increased prevalence of *Campylobacter* with resistance to antibiotics, particularly fluoroquinolones, may be a result of the use of these agents in food animals (Endtz et al., 1991). In the United States, *C. coli* isolated from humans in 1997-2001 were resistant to erythromycin (8%), nalidixic acid (36%), ciprofloxacin (30%), and tetracycline (43%) (Gupta et al., 2004). In the European Union, including Denmark, Netherland, and Sweden, *C. coli* isolates from pigs were resistant to erythromycin (32.5%), nalidixic acid (14.8%), ciprofloxacin (14.1%), and tetracycline (19.4%) (Bywater et al., 2004). In Germany, *C.*

coli isolated from humans in 2001 were resistant to erythromycin (6.1%), ciprofloxacin (45.1%), and tetracycline (37.8%) (Luber et al., 2003).

In Thailand, several studies have demonstrated a number of *Campylobacter* strains that are resistant to various antimicrobial agents including ciprofloxacin and nalidixic acid (Padungtod et al., 2003). In a previous study, *C. coli* isolated from pigs and chicken showed high level resistance to erythromycin (56.9%), nalidixic acid (90.2%), ciprofloxacin (86.3%), and tetracycline (88.2%). *C. coli* isolates from patients were also resistant to erythromycin (78.3%), nalidixic acid (65.2%), ciprofloxacin (69.6%), and tetracycline (34.8%). Up to ninety-two percent of these isolates were multidrug resistant (Padungtod et al., 2006).

3. Genetics of antibiotic resistance in *C. coli*

In general, *C. coli* strains are intrinsically resistant to a number of antibiotics, i.e. bacitracin, novobiocin, rifampin, streptogramon B, trimethoprim, vancomycin, and cephalothin (Taylor and Courvalin, 1988). However, no information is currently available on the intrinsic mechanisms of such resistance. It has been proposed that some of intrinsic resistance may be involved in the inability of the drugs to penetrate the cells (Aarestrup and Engberg, 2001). Besides the intrinsic mechanisms of resistance, acquired resistance are also the important mechanisms in resistance *C. coli*. These include chromosomal mutations and acquisition of antibiotic resistance determinants i.e. transposon, plasmid and integrons (O'Halloran et al., 2004). The following sections describe mechanisms of resistance to erythromycin, fluoroquinolones, and tetracycline that are drugs of interest in this study.

3.1 Resistance to fluoroquinolones

The quinolones are a family of the broad-spectrum antibiotics. The parent of drugs in this group is nalidixic acid. The second generation of quinolones, fluoroquinolones, i.e., ciprofloxacin and levofloxacin are derived from the first compound

by a fluorine substitution at the C-6 position. This substitution increases their activities against Gram-negative bacteria. The bacterial targets of quinolones are the DNA gyrase (topoisomerases II) and topoisomerase IV. DNA gyrase consists of two subunits, GyrA and GyrB subunits encoded by the *gyrA* and *gyrB* genes, respectively. DNA topoisomerase IV also consist of two subunits, ParC and ParE subunits encoded by the *parC* and *parE* genes, respectively. Both gyrase and topoisomerase IV require ATP to complete the function (Payot et al., 2006). They temporarily break double-stranded DNA and cooperate to facilitate DNA replication and other key DNA transactions. DNA gyrase is essential for efficient DNA replication, transcription, and recombination, whereas topoisomerase IV plays a special role in chromosome segregation (Levine et al., 1998).

The mechanisms of action of quinolones and fluoroquinolones are inhibition of the function of DNA gyrase and DNA topoisomerase IV. In Gram-negative bacteria, DNA gyrase and DNA topoisomerase IV are the primary and secondary targets of fluoroquinolones, respectively. Resistance to these antibiotics arises from amino acid substitution in a target region of the corresponding topoisomerase termed the "Quinolone Resistance Determining Region (QRDR)" located within the DNA-binding domain on the surface of gyrase and topoisomerase protein (Payot et al., 2006). Such mutations may alter the interaction between the enzymes and the drugs resulting in the reduced sensitivity of each enzyme to quinolones. In *E. coli* and *Salmonella* spp., the common mutations have been reported to be located at positions 83 and 87 in the QRDR region of the GyrA subunit (Ge et al., 2005).

In *Campylobacter*, mutations in *gyrA* have been shown to confer decreased sensitivity of DNA gyrase to fluoroquinolones. Previous studies showed that fluoroquinolone resistance is mainly caused by a single step point mutation in the *gyrA* gene (C257T), which resulted in threonine at the position 86 being substituted with a isoleucine (Thr-86-Ile) (Ge et al., 2005; Wang et al., 1993). Other amino acid substitutions previously reported were Thr-86-Lys, Asp-90-Asn and Pro-104-Ser (Piddock et al., 2003). There was one report describing silent mutations in the *gyrB* gene associated with fluoroquinolones resistance in *C. jejuni* (Kinana et al., 2007). In *C. coli*,

only a silent mutations (A1471C) has been reported in *gyrB* gene. The effect on fluoroqiunolone resistance of the latter mutation remains to be determined (Piddock et al., 2003). For the *parC* gene, its role in fluoroquinolone resistance was shown only in one report (Gibreel et al., 1998). However, the gene has never been amplified and such results has never been repeated by other investigators (Bachoual et al., 2001; Piddock et al., 2003). To date, there is increasing evidence convincing that the secondary target (ParC) is absent in *Campylobacter* (Payot et al., 2006).

3.2 Resistance to macrolides

Macrolide compounds, for example; erythromycin and lincomycin inhibit bacterial growth by binding to bacterial ribosomes and interfering with protein synthesis. The mechanism of action of macrolide is the inhibition of protein synthesis via interfering with the translocation step. During protein synthesis, the newly synthesized peptide chain passes through the large ribosomal subunit (50S subunit) and runs from the peptidyl transferase center (domain V of 23S rRNA) to the back of the ribosome. This is the region where the macrolides including erythromycin, bind and make several common contacts around 23S rRNA (Poehlsgaard and Douthwaite, 2005). These results in blocking the entrance to tunnel in 50S subunit and inducing inhibit protein synthesis. The effect is inhibition of the translocation of the developing peptide chain from the A site to P site, which is required for the elongation of the peptide chain as the ribosome moves along the messenger RNA strand (Gibreel and Taylor, 2006).

Macrolide resistance can be based on several mechanisms including target modification by point mutation or methylation of 23S rRNA gene, hydrolysis of drugs and efflux pumps (Engberg et al., 2001). In *Campylobacter*, it has been attributed to nucleotide mutations at positions 2074 and 2075 in the peptidyl transferase region in domain V of the 23S rRNA target gene and these mutations probably reduce the interaction between the tunnel wall of the ribosome and the macrocyclic ring of macrolide (Gibreel et al., 2005; Gibreel and Taylor, 2006; Payot et al., 2006). Point mutation at position 2075 (A2075G) of the 23S rRNA was found to be associated with a

high levels of erythromycin-resistance phenotype in clinical strains of *C. jejuni* and *C. coli* (Niwa et al., 2001; Vacher et al., 2003). A previous study described transversion mutations at position 2074 (A2074C or A2074T) of the 23S rRNA. The A2074T conferred a lower level of resistance (Vacher et al., 2005).

In *C. coli*, point mutation at position 2230 (A2230G) in the peptidyl transferase region of the 23S rRNA gene was found to be associated with a high level of erythromycin resistance phenotype (Gibreel et al., 2005; Jensen and Aarestrup, 2001). Another mutation A2074C in the 23S rRNA gene was also associated to macrolide resistance (Vacher et al., 2003). A previous study has suggested that natural transformation has the potential to contribute to the dissemination of high-level resistance to erythromycin among *C. coli* strains colonizing food animals (Kim et al., 2006).

3.3 Resistance to tetracycline

Tetracycline is a broad-spectrum antibiotic, of which the function is to inhibit bacterial protein synthesis by blocking the attachment of the transfer RNA-amino acid to the ribosome. More precisely, it inhibits accommodation of aminoacyl-tRNA (t-RNA) into the ribosomal A site and therefore, prevents the addition of new amino acids to the growing polypeptide (Connell et al., 2003). The inhibitory activity of tetracycline is likely to result from its binding to a single high-affinity binding site that is located on the 30S ribosomal subunit (Chopra and Roberts, 2001). However, a recent study showed that the activity may be up to two tetracycline binding sites on the 30S subunit. (Spahn et al., 2001) Tetracycline can also inhibit protein synthesis in the eukaryotic host, but the intracellular concentration is less likely to reach the requirement because eukaryotic cells do not have a tetracycline uptake mechanism (Connell et al., 2003).

Resistance to tetracycline has been increasingly common in many pathogenic bacteria. Four general mechanisms of tetracycline resistance are currently known: (1) prevention of cytoplasmic accumulation by an energy-dependent efflux; (2) chemical

modification of tetracycline; (3) a mutation in the 16S rRNA affecting the specific binding site; and (4) ribosomal protection by a soluble protein. To date, *tet(O)* gene is the most commonly found in *Campylobacter,* but the other *tet* genes have not been identified in this pathogen at the time of studying.

Tet(O) protein encoded by the *tet(O)* gene is a member of ribosomal protection proteins that mediate tetracycline resistance (Alfredson and Korolik, 2007). Protein in this group has a molecular weight of approximately 70 kDa. Its amino acid sequence is similar to the ribosomal elongation factors, EF-G and EF-Tu, and was grouped into the translation factor superfamily of GTPases (Sanchez-Pescador et al., 1988). Tet(O) can displace tetracycline from the ribosome and increase the apparent dissociation constant of tetracycline binding to the ribosome. The ability of Tet(O) to dislodge tetracycline is strictly dependent on the presence of GTP (Connell et al., 2003). High-level tetracycline resistance is usually associated with the *tet*(O) gene carried on transmissible plasmids (Taylor and Courvalin, 1988). However, the *tet*(O) gene has also been found to be chromosomally located in *C. coli* (Lee et al., 1994). Gibreel et al. (2004) reported the chromosomally-mediated *tet(O)* in *C. jejuni* and *C. coli* strains that lacked plasmids, but not at such a high rate. It has been suggested that recombination between plasmid and chromosome or integration of a tetracycline resistance plasmid into the chromosome may occur (Pratt and Korolik, 2005).

3.4 Class 1 Integrons in *C. coli*

Integrons are the mobile genetic elements that play a major role in dissemination of multidrug resistance among Gram-negative bacteria. Nine classes of integrons have been identified so far, based on integrase gene homology (Lee et al., 2002) and more than sixty antibiotic resistance gene cassettes have been characterized within integrons (Fluit and Schmitz, 1999). Up to seven resistant gene cassettes have been found in a single integron (Naas et al., 2001).

Class 1 integrons are the predominant integrons in Gram-negative bacteria and have been reported in *Campylobacter* (O'Halloran et al., 2004). The typical structure of a class 1 integrons comprises two conserved segments (CS), 5'- and 3'- CS, flanking variable regions (Figure 2). The *intI1* gene encoding an integrase enzyme is located within the 5'-CS and responsible for the recombination of a gene cassette at a specific *att1* attachment site. Also within this 5'-CS region, there is a promoter that facilitates the efficient expression of integrated gene cassettes. The 3'-CS contains *qacEΔ1* encoding resistance to quaternary ammonium compounds and *sul1* encoding resistance to sulphonamide. *qacEΔ1* is a defective version of *qacE*, which is a multidrug efflux in the Small Multidrug Resistance (SMR) family. The gene cassettes located in variable regions are mobile and usually encode for antibiotic resistance (Hall and Collis, 1995).

Class 1 integrons may contain a number of recombined gene cassettes oriented in a classical "head to tail" arrangement and therefore conferring a multidrug-resistance (MDR). They can be found on the bacterial chromosome and conjugative plasmids that are more effective to transfer resistance gene cassettes. Gibreel and Skold (2000) reported the existence of chromosomally located integrons carrying a trimethoprim resistance gene cassettes (*dfr1* gene cassette) in *Campylobacter*. Lee et al. (2002) characterized *aadA4* gene encoding aminoglycoside adenyltransferase and confering resistance to aminoglycosides in *C. jejuni* isolates from the broiler chicken house environment. Recently, O'Halloran et al. (2004) reported the existence of class 1 integrons in *C. coli* isolated from human and poultry sources.

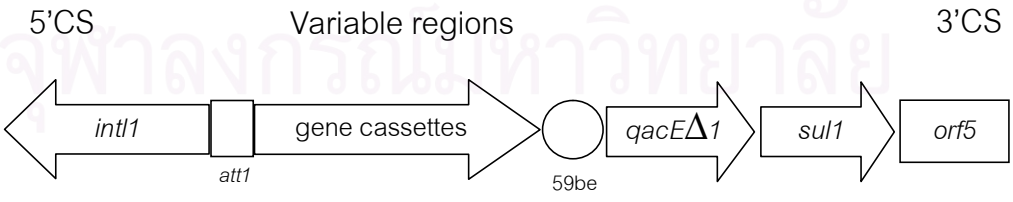


Figure 2. Structure of typical class 1 integrons. The 5'CS contain the *intI1* gene and 3'CS consists of *qacEΔ1*, *sul1* and ORF-5 of unknown function. 5' and 3' CS separated by a variable regions that usually contains one or more gene cassettes.

CHAPTER III

MATERIALS AND METHODS

In this study, the experiment was divided into 2 phases: phase I, confirmation of *C. coli* by multiplex PCR and DNA sequencing, and phase II, determination of genetics of antibiotic resistance in *C. coli*. The conceptual framework is shown in Figure 3.

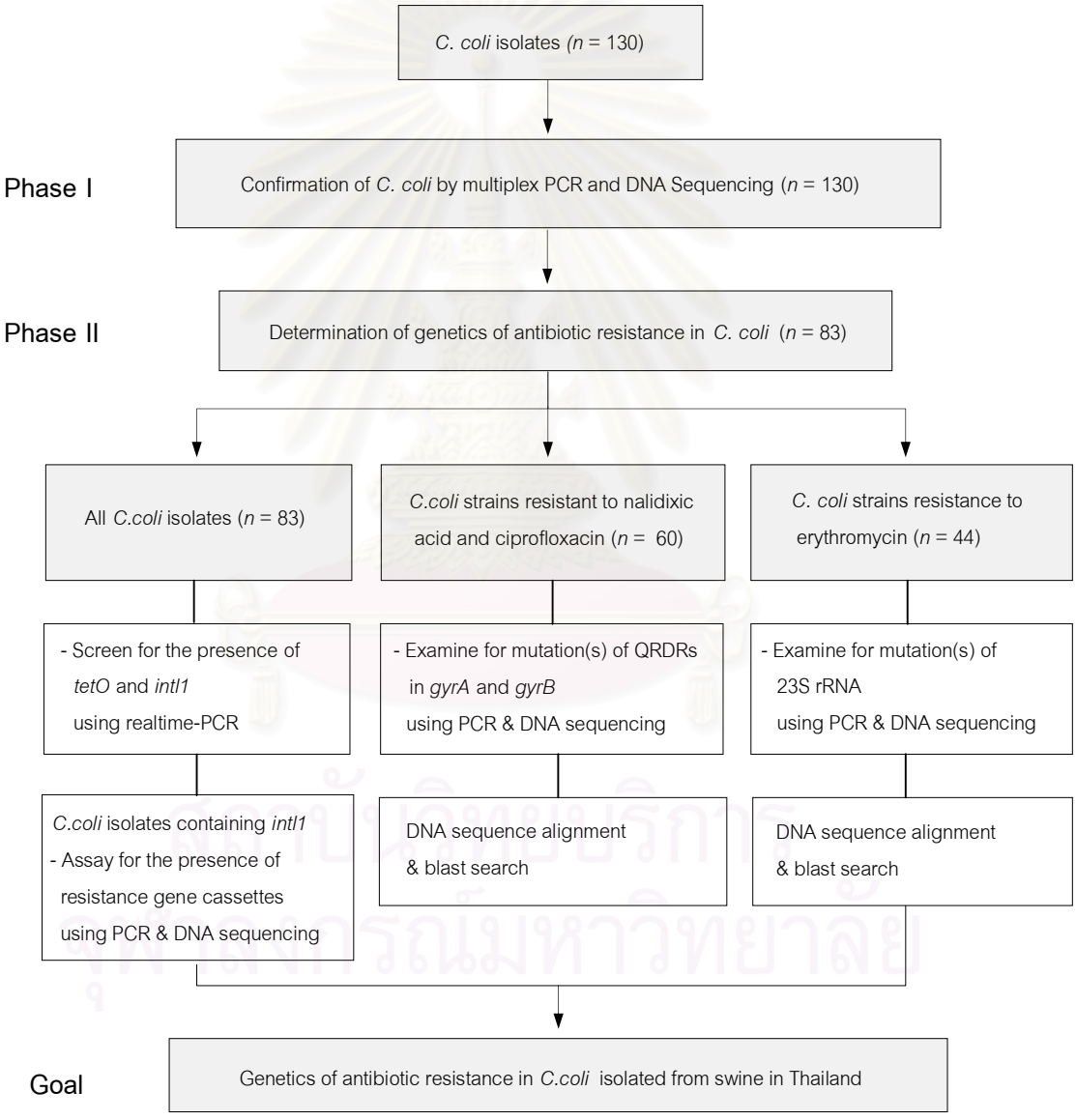


Figure 3. The conceptual framework in this study.

C. coli isolates and antimicrobial susceptibility testing

A total of 130 *C. coli* isolates were included in this study. They were isolated from pigs in northern Thailand during 2001-2003 and kept as stocks in the strain collection of Faculty of Veterinary Medicine, Chiangmai University, Chiangmai. All the isolates were recovered using the standard methods described in International Organization for Standardization (ISO 10272-1) and tested for their biochemical characteristics. They were subjected to the antimicrobial susceptibility test and minimum inhibitory concentrations (MICs) were determined for erythromycin, ciprofloxacin, nalidixic acid and tetracycline using two-fold agar dilution technique according to Clinical and Laboratory Standards Institute, CLSI, formerly NCCLS (NCCLS, 2000). The breakpoints of the drugs provided by the U.S. National Antimicrobial Resistance Monitoring System (NARM, 2003) were used to categorize *C. coli* into resistant and nonresistant and indicated as follows: nalidixic acid (≥32 µg/ml), ciprofloxacin (≥4 µg/ml), tetracycline (≥16 µg/ml) and erythromycin (≥8 µg/ml). All bacterial strains were stored as 20% glycerol stocks at -80°C, then shipped to Department of Veterinary of public health, Faculty of Veterinary Science, Chulalongkorn University for further investigations.

Phase I Confirmation of *C. coli*

All of the *Campylobacter* isolates were confirmed to be *C. coli* using multiplex PCR with genus-specific primers (16SF and 16SR) and species-specific primers (CCF and CCR).

All primers used in this study are listed in Table 1. Six primers including tetOF, tetOR, gyrBF, gyrBR, int1LF, and int1LR were designed in this study. Int1LF and int1LR were designed using Primer3 program available at http://hpc.ilri.cgiar.org/cgi-bin/primer3_www.cgi. The others were manually designed as suggested in Molecular cloning a laboratory manual (Sambrook and Russell, 2001). Locations on the correspondings genes of 6 primers designed in this study are shown in Appendix B.

For PCR amplification, template DNA of all isolates was prepared by the whole cell boiled lysate procedure (Wang et al., 2002). Briefly, bacteria were grown on Muller Hinton Agar (Difco, USA) supplemented with 10% sheep blood and incubated at 42 °C for 48 h in a microaerophilic atmosphere containing 5% O_2, 10% CO_2 and 85% N_2 using CampyGen™ gaspack (OXOID , Hampshire, England). A single colony was suspended in 50 µl of sterile distilled water and heated in a boiling water bath for 10 min. The suspension was centrifuged at 12,000 x g for 5 min. The supernatant was removed to a new Eppendorf tube and stored at -20 °C.

Table 1. Primers used in this study.

Primer	Sequence (5'-3')	Gene	Amplicon size (bp)	Reference
16SF	ACT CCT TTT CTT AGG GAA GAA TTC	16S rRNA	946	Neubauer and Hess, 2005
16SR	GTG GAG TAC AAG ACC CGG GAA	16S rRNA	946	Neubauer and Hess, 2005
CCF	GTA AAA CCA AAG CTT ATC GTG	*glyA*	126	Wang et al., 2003
CCR	TCC AGC AAT GTG TGC AAT G	*glyA*	126	Wang et al., 2003
tetOF	CAA AGG GGA ATC ACT ATC C	*tetO*	609	This study
tetOR	AAC CTG CCC GCA TAG TTC	*tetO*	609	This study
gyrAF	GAG TGT TAT TAT AGG TCG TGC	*gyrA*	274	Keller and Perreten. 2005
gyrAR	GGC ACT ATC ACC ATC ATC TAT AG	*gyrA*	274	Keller and Perreten. 2005
gyrBF	ACA AAG CCT TAA TGG CAG	*gyrB*	387	This study
gyrBR	CAT CAA CAT CCG CAT CTG	*gyrB*	387	This study
23SF	AAT TGA TGG GGT TAG CAT TAG CG	23S rRNA	316	Vacher, 2003
23SR	CAA CAA TGG CTC ATA TAC AAC TGG	23S rRNA	316	Vacher, 2003
Int1LF	CAG GAG ATC GGA AGA CCT	*intI1*	153	This study
Int1LR	TTG CAA ACC CTC ACT GAT	*intI1*	153	This study
5'CS	GGC ATC CAA GCA GCA AG	Variable regions	Variable	Levesque et al., 1995
3'CS	AAG CAG ACT TGA CCT GA	Variable regions	Variable	Levesque et al., 1995
qacEF	TAA GCC CTA CAC AAA TTG GGA GAT AT	*qacEΔ1–sul1*	1,198	Chuanchuen et al., 2007
sul1R	GGG TGC GGA CGT AGT CAG C	*qacEΔ1-sul1*	1,198	Chuanchuen et al., 2007

PCR assays were performed using PCR master mix of Eppendorf® MasterMix (Eppendorf, Hamberg, Germany) as suggested by the manufacturer. The PCR reactions were performed in 25 µl mixture consisting of 12.5 µl of Eppendorf® MasterMix 4.5 µl of sterile-distilled water, 1.5 µl of each primer at 10 µM and 5 µl of DNA. PCR amplifications were conducted on a PCR Sprint Thermocycler® (Thermo Electron Corporation®, Cambridge, UK). PCR thermocycing conditions were an initial denaturation at 94°C for 5 minutes, and 30 cycles of denaturation for 45 seconds at 94°C, primer annealing for 45 second at 55°C, and DNA extension for 45 seconds at 72°C and a final extention at 72°C for 5 minutes. PCR products were visualized on 1-1.5% agarose and gel purified using QIAQuick Gel Extraction kit (Qiagen, Hilden, Germany). Representative DNA samples were submitted for sequencing at Macrogen Inc. (Seoul, South Korea) to confirm specificity of primers.

Phase II Determination of genetics of antibiotic resistance in *C. coli*

The experiment in this phase included 1) determination of the presence of *tet(O)* gene, 2) determination of the presence of class 1 integrons, 3) examination of mutations of QRDRs in *gyrA* and *gyrB* genes, and 4) examination of mutations of 23S rRNA gene. For all steps, DNA template was prepared by the whole cell boiled lysate procedure as described above.

1. Determination of the presence of *tet(O)* gene

All the *C. coli* isolates were screened for the presence of *tet(O)* by PCR using specific primers tetOF and tetOR, which produced a 608 bp product. PCR reaction conditions for *tet(O)* were an initial denaturation at 95°C for 5 minutes, and 30 cycles of denaturation for 45 seconds at 94°C, primer annealing for 45 second at 55°C, and extension for 45 seconds at 72°C and a final extention at 72°C for 5 minutes. PCR products were gel purified using QIAQuick Gel Extraction kit (Qiagen, Hilden, Germany) and representative DNA samples were submitted for nucleotide sequencing. DNA from a tetracycline-resistant *C. coli* isolate that possesses *tet(O)* confirmed by DNA

sequencing was employed as a positive control and that from a tetracycline-susceptible strain was used as a negative control.

2. Integrons analysis

At this step, the presence of *intI1* was first identified. Then, the *intI1*-positive strains were investigated for the presence of gene cassettes that were later characterized. In the final, the presence of typical 3' conserved segment was examined. Locations of primers used for integrons analysis are shown in Figure 4. Experiments are described in detail below.

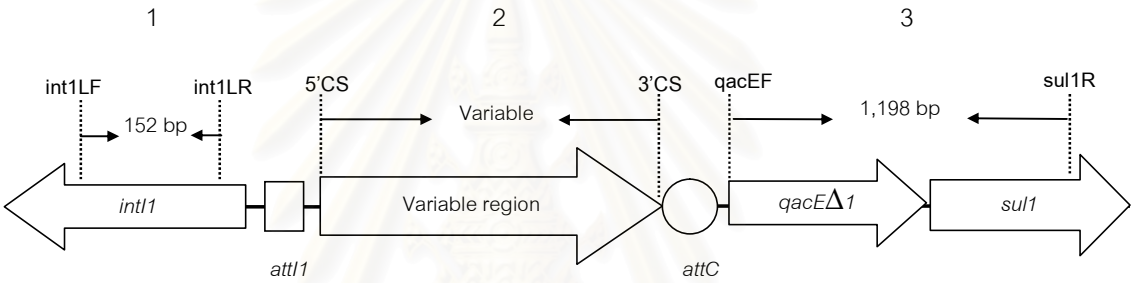


Figure 4. Schematic presentation of class 1 integrons showing locations of the primers and the resulting amplicons. The arrows indicate the direction of primers. The vertical-dashed lines indicate location of primers. The numbers 1, 2 and 3 show the order of PCR amplifications in this study.

All of the *C. coli* isolates were screened for the presence of class 1 integrase, *intI1*, by realtime PCR using specific primers int1LF and int1LR. PCR assays were carried out in a final volume of 20 µl using Biotools QuantiMix EASY SYG Kit (Biotools B&M Labs S.A., Madrid, Spain) according to the manufacturer's instructions. Each 20 µl reaction contained 10.0 µl of Biotools QuantiMix, 3.0 µl of water, 1.2 µl of each primer at 10 µM, 1.6 µl of $MgCl_2$ at 50 mM and 3 µl of DNA. PCR amplifications were performed on a Rotor-Gene™ 3000 Real Time Thermal Cycler (Corbett Research, Sydney, Australia). The PCR thermocycles were an initial denaturation at 94°C for 3 minutes, and 30 cycles of denaturation for 2 seconds at 94°C, primer annealing for 2 second at 52°C,

and extension for 8 seconds at 72°C. *Pseudomonas aeruginosa* P90 was used as positive control for *intI1* (Chuanchuen et al., 2007).

All the isolates containing class 1 integrase were assayed for the presence of resistance gene cassettes using 5'CS and 3'CS primers as previously described (Levesque et al., 1995). PCR reaction conditions were an initial denaturation at 95°C for 5 minutes, and 30 cycles of denaturation for 45 seconds at 95°C, primer annealing for 1 minute at 54°C, and extension for 3 minutes at 72°C and a final extention at 72°C for 5 minutes. The PCR amplicons were gel purified using QIAQuick Gel Extraction kit and submitted for DNA sequencing. Nucleotide sequence analysis was performed using the Blast algorithm available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

All strains containing class 1 integrase were examined for the presence of the 3'conserved regions using qacEF and sul1R primers as previously described (Chuanchuen et al., 2007). Thermal cycling conditions consisted of an initial denaturation at 95°C for 5 minutes, and 30 cycles of denaturation for 45 seconds at 95°C, primer annealing for 1 minute at 54°C, and extension for 3 minutes at 72°C and a final extention at 72°C for 5 minutes.

3. Examination of mutations of QRDRs in *gyrA* and *gyrB* genes

The *C. coli* isolates resistant to nalidixic acid and ciprofloxacin were examined for mutation(s) in the QRDRs of *gyrA* and *gyrB* using PCR and DNA sequencing. Amplifications of the QRDRs of the *gyrA* were performed using a primer set, gyrAF and gyrAR as previously described (Keller and Perreten, 2006). For the *gyrB* gene, the QRDR was PCR amplified using primers gyrBF and gyrBR. PCR thermocycling conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 45 second, 55°C for 45 second, and 72°C for 30 second, with a final step at 72°C for 5 minutes. After gel purification of the PCR products, all PCR products were submitted for nucleotide sequencing. Nucleotide sequence analysis was

performed using the NCBI Blast search. Nucleotide sequence of both strands were compared with the published DNA sequence of *C. coli*. (GenBank accession numbers AF092101 and AY330104 for *gyrA* and *gyrB* respectively) using the Chromas ver.1.45 and Seqman (DNA-STAR) program for analysis. Two *C. coli* isolates susceptible to both nalidixic acid and ciprofloxacin were also used as a negative control.

4. Examination of mutations in 23S rRNA

The *C. coli* strains resistant to erythromycin were examined for mutations in the 23S rRNA using PCR and DNA sequencing. Amplification of the 23S rRNA was performed using a primer set 23SF and 23SR as previously described (Vacher et al., 2003). PCR thermocycling conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 45 second, 55°C for 45 second, and 72°C for 45 second, with a final step at 72°C for 5 minutes. After gel purification, PCR products were submitted for sequencing. Nucleotide sequence were compared with the published DNA sequence of *C. coli* (GenBank accession numbers U09611). Two *C. coli* isolates susceptible to erythromycin were also used as a negative control.

Instruments and chemical substances

1. PCR assay
    1.1 Master Mix (Eppendorf®, Hamberg, Germany)
    1.2 DNA marker (Gibco®, Paisley, UK)
    1.3 Loading dye (Amersco®, Ohio, USA)
    1.4 Agarose gel (Molecular grade)
    1.5 Gel electrophoresis buffer (TAE)
    1.6 Ethidium Bromide 10 mg/ml (Sigma Aldrich Inc®, USA)
2. PCR cabinet
3. Thermocycler (Thermo electron corporation®, Cambridge, UK)
4. Rotor-Gene™ 3000 Real Time Thermal Cycler (Corbett Research, Sydney, Australia)
5. Gel electrophoresis system (OWL Scientific Inc®, USA)
6. Gel document system (Vilber Lourmat®, Marne La Valle, France)
7. PCR tubes and Microcentrifuge tube 1.5 ml
8. Centrifuge and Microcentrifuge
9. Micropipette and Micropipette tips
10. A -20°C refrigerator
11. A -80°C refrigerator
12. Experimental glasswares

CHAPTER IV

RESULTS

1. Confirmation of *C. coli* and antibiotic resistance patterns

Eighty-three (64%) from one hundred and thirty isolates were confirmed to be *C. coli* using multiplex PCR. The PCR amplification pattern for *C. coli* consisted of two PCR amplicons: 946-bp fragment for genus *Campylobacter* and 126-bp fragment for species *C. coli* (Figure 5). Nucleotide sequencing confirmed the specificity of primers used.

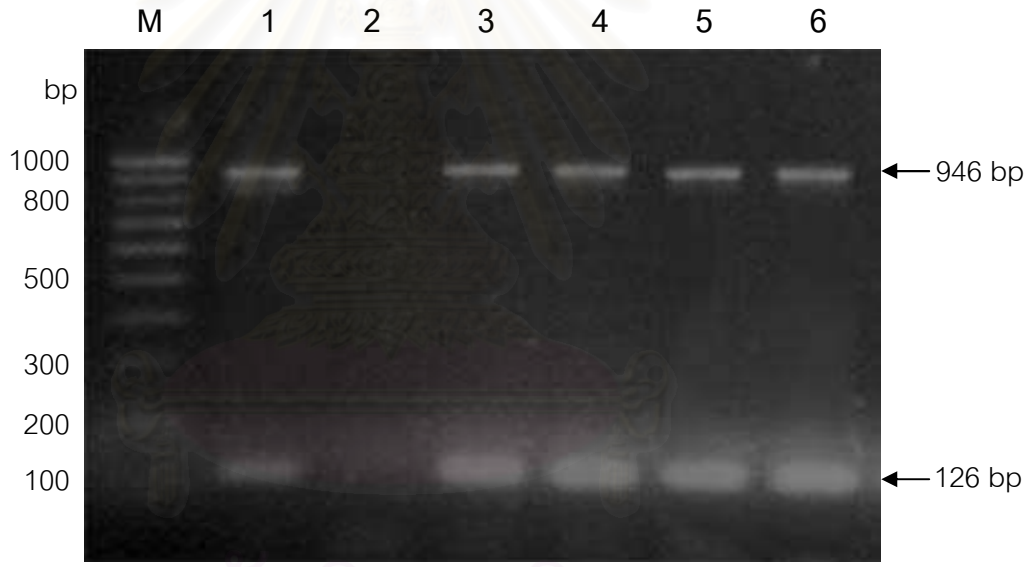


Figure 5. Confirmation of *C. coli* using multiplex PCR. DNA template was PCR amplified using primers specific for genus (16SF and 16SR) and species (CCF and CCR) that generated the amplicons size of 946 and 126 bp, respectively. Lane M, 100-bp marker; Lane 1, *C. coli* positive control; Lane 2, negative control; Lane 3-6, The confirmed *C. coli* isolates.

All isolates in this study showed resistance to at least one antibiotic tested. Resistance to nalidixic acid (84%) and ciprofloxacin (86%) were most commonly found, followed by resistance to tetracycline (81%) and erythromycin (66%). Forty-nine percent of the isolates showed resistance to all antibiotics tested. The antibiotic-resistance patterns were also analyzed. All of the isolates could be grouped into 9 antibiotic resistance patterns (Table 2). The most frequent multiple resistance pattern was CIP-ERY-NAL-TET (49.4%).

Table 2. Antibiotic resistance pattern of *C. coli* isolates (*n*=83)

Antibiotic resistance pattern	No. of isolates (%)
Sensitive to all antibiotics tested	3 (3.6)
ERY	7 (8.4)
CIP-ERY	2 (2.4)
CIP-NAL	2 (2.4)
ERY-TET	1 (1.2)
CIP-ERY-NAL	2 (2.4)
CIP-ERY-TET	1 (1.2)
CIP-NAL-TET	23 (27.7)
ERY-NAL-TET	1 (1.2)
CIP-ERY-NAL-TET	41 (49.4)
Total	83 (100)

CIP, ciprofloxacin; ERY, erythromycin; NAL, nalidixic acid; TET, tetracycline.

2. Determination of genetics of antibiotic resistance in *C. coli*

2.1. The presence of the *tet (O)* gene

Ninety-seven percent (65/67) of the tetracycline-resistant strains possessed *tet(O)* and none of the tetracycline-sensitive strains carried this gene. The PCR amplification product from a tetracycline-resistant, *C. coli* strains, CAC004 was sequenced and confirmed to be the *tet(O)* nucleotide sequence reported in GenBank (data not show). DNA from this isolate was employed as a positive control for screening of the presence of *tet(O)* (Figure 6).

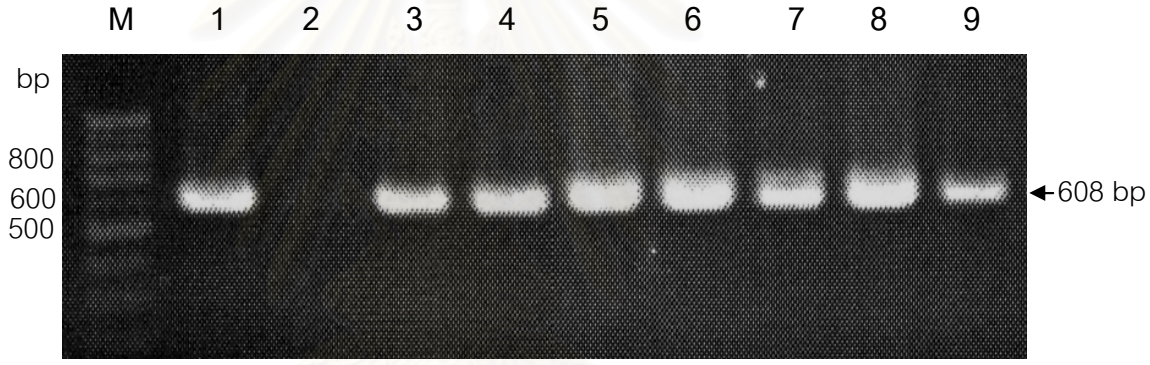


Figure 6. Screening for the presence of the *tet(O)* gene. DNA template was PCR amplified using tetOF and tetOR primers that generated the amplicon size of 608 bp. Lane M, 100-bp marker; Lane 1, positive control of *tet(O)*; Lane 2, negative control; Lane 3-9 the *tet(O)* containing *C. coli* strains.

2.2. The presence of class 1 integrons

The *intI1* gene was identified in 4 of 83 isolates (4.8%) by realtime PCR. All the isolates containing *intI1* were further assayed for the presence of inserted gene cassettes using 5'-3' conserved sequence PCR. The results showed that all of them produced a single band of 1,000-bp amplicon (Figure 7).

Figure 7. PCR Amplification of variable regions in the *intI1*-positive strains. DNA template was PCR amplified using 5'CS and 3'CS primers. Lane M, 1kb DNA ladder marker; Lane 1-4, the 1000-bp amplicons of variable regions from the *intI1* positive strains.

The nucleotide analysis revealed that the amplicon sequences were identical to the nucleotide sequence of *aadA9* encoding an aminoglycoside adenyltransferase that confers streptomycin/spectinomycin resistance. All the *intI1*-positive strains contained the *qacEΔ1-sul1* genetic organization. Therefore, they all had the typical 3' conserved region of class 1 integrons.

2.3. Mutations in QRDRs of the *gyrA* and *gyrB* genes

Sixty of *C. coli* isolates resistant to nalidixic acid and ciprofloxacin were examined for mutations within the QRDRs of *gyrA* and *gyrB*. Mutations in both genes are shown in Table 3. Seven point mutations were identified in *gyrA*, 4 of which were silent mutations. Replacement of C at position of 257 with T in *gyrA* leading to a Thr-86-Ile substitution in GyrA was the most common (93%) mutations followed by a Gly-119-Ser mutation (83%), which was due to point mutations C-357-T (75%) or G-355-T (8%). Sixty-four percent of the isolates carrying a Thr-86-Ile substitution had an additional mutation Gly-119-Ser, which was a result of a point mutation at position 355.

Five *C. coli* isolates (8%) resistant to nalidixic acid and ciprofloxacin habored a mutation in the *gyrB* gene. These point mutations were A-1144-C and A-1145-G leading to a Lys-381-Glu and Lys-381-Arg, respectively. All the isolates with mutations in *gyrB* additionally harbored mutation(s) in *gyrA*, Thr-86-Ile substitution (1/5) and Thr-86-Ile substitution and Gly-119-Ser substitution (4/5). One isolate had up to 4 point mutations including double point mutations in *gyrA* and *gyrB* genes that were C-257T and G-355-A in *gyrA* and A-1144-C and A-1145G in *gyrB*. There were no mutations in QRDR of the quinolone susceptible *C. coli* control strains in comparison to the QRDR of the reference strain.

Table 3. Mutations observed in the *gyrA* and *gyrB* gene sequences from the quinolone-resistant *C. coli* isolates (*n* =60)

Gene	Mutation		No. (%)
	Nucleotide substitution	Amino acid substitution	
gyrA	C-252-T	Silent mutation	3 (5)
	C-257-T	Thr-86-Ile	56 (93)
	T-297-C	Silent mutation	31 (52)
	C-345-A	Silent mutation	59 (98)
	G-355-A	Gly-119-Ser	45 (75)
	C-357-T	Gly-119-Ser	5 (8)
	T-360-C	Silent mutation	1 (2)
gyrB	A-1144-C	Lys-381-Glu	4 (7)
	A-1145-G	Lys-381-Arg	1 (2)

2.4. Mutations in 23S rRNA

To date, the complete genome sequence of *C. coli* is not available. Therefore, detection of a potential macrolide-associated mutation in domain V of the 23S rRNA gene was analyzed by amplifying a 316 bp fragment of the target gene, corresponding to positions 2024 to 2340 in the published sequence of 23S rRNA of *C. coli* (GenBank accession no. U09611). This region covers domain V of the 23S rRNA. In this study, this specific region of 44 erythromycin-resistant isolates was amplified and sequenced to detect mutations associated with the resistance. Several sequence variations were found in the amplified area.

Thirty-four (77%) erythromycin-resistant strains had at least one mutation in 23S rRNA sequences and no mutations were observed in ten erythromycin-resistant strains. There were no mutations in 23S rRNA of the erythromycin sensitive control. An A-2230-G substitution appeared to be the most common mutation (70.4%) in erythromycin resistant *C. coli* isolates (Table 4). The other mutations including T-2268-C (6.8%), C-2252-T (15.9%), G-2277-A (11.3%), and A-2278-G (11.3%) were also identified. Only three strains had a T-2268-C single point mutation and erythromycin MIC value of these strains varied from 8 to 256 μg/ml. Seven isolates had more than one point mutation. Five from these isolates carried up to 4 point mutations (i.e. A-2230-G, C-2252-T, G-2277-A and A-2278-G) in the region and only two isolates carried two point mutations (i.e. A-2230-G and C-2252-T). However, the erythromycin MIC value (256 μg/ml) of these isolates was not different from MIC value (8-256 μg/ml) of the isolates with a single point mutation of the same base substitution.

Table 4. Mutations observed in the 23S rRNA gene sequences from the erythromycin resistant *C. coli* isolates (*n* =44)

Gene	Mutation	No. (%)
	Nucleotide substitution	
23S rRNA	A-2230-G	31 (70.4)
	T-2268-C	3 (6.8)
	C-2252-T	7 (15.9)
	G-2277-A	5 (11.3)
	A-2278-G	5 (11.3)

CHAPTER V

DISCUSSION

Multi-resistance to nalidixic acid, ciprofloxacin, erythromycin, and tetracycline in *Campylobacters* must be considered to be highly undesirable since *Campylobacters* are common bacterial cause of infectious intestinal disease in humans, and these antibiotics are generally considered to be the drugs of choice for treatment of Campylobacteriosis. Infections with these resistant strains are associated with increased risk of invasive illness or death. As antibiotics have been extensively used in production of pigs, this major food animal is the main carrier of *C. coli*. A widely-increased incidence of resistant *C. coli* in both humans and animals warrants the immediate action to control and prevent abuse of antibiotics in swine production. For the strategy, mechanisms underlying resistance need to be understood. Therefore, genetics of antibiotic resistance in *C. coli* were investigated in this study.

In this study, PCR amplification was successful in detecting *tet(O)*. Since 97% of tetracycline resistant strains harbored *tet(O)* and none of tetracycline susceptible strains carried the gene, it indicated that the presence of this gene was well corresponding to tetracycline resistance in *C. coli* tested. The similar findings were previously reported in many countries (Pratt and Korolik, 2005). The *tet(O)* gene was identified in all tetracycline-resistant *C. coli* isolated from pig in North Carolina (Thakur and Gebreyes, 2005), from chicken in Taiwan (Lee et al., 1994), from humans in Poland (Wardak et al., 2007) and from chicken, pig, and humans in Australia (Pratt and Korolik, 2005). These support that *tet(O)* gene plays an important role in tetracycline resistance in *C. coli* isolates (Aarestrup and Engberg, 2001).

As most of the *tet(O)* genes identified in *Campylobacter* are carried on transmissible plasmids (Taylor and Courvalin, 1988), it has also been found to be chromosomally located (Gibreel et al., 2004). A previous study demonstrated that sixty-seven percent of the tetracycline resistant *C. jejuni* clinical isolates contained plasmids

and all contained the *tet(O)* gene. However, due to the strain loss, localization and transferability of *tet(O)* was not determined in this study. Two tetracycline resistant isolates in our collection did not carry *tet(O)*. The high level MIC of tetracycline in these strains may be due to expression of other resistance mechanism(s). One of the possible mechanisms could be the CmeABC efflux system, which is a multidrug efflux pump contributing to multidrug resistance in *Campylobacter*. This active efflux pump extrudes the antibiotic substrates out of the cells and therefore, the intracellular concentration of antibiotics will not reach the requirement for inhibiting cell growth. The antibiotic substrates of the CmeABC include ciprofloxacin, nalidixic acid, erythromycin, tetracycline, cefotaxime, rifampin, chloramphinical and gentamicin (Tauch et al., 2002). Further investigations are required to elucidate the responsible mechanisms for resistance to tetracycline in these strains.

It is well known that class 1 integrons play a major role in multi-drug resistance in several Gram-negative bacterial species. However, our findings showed that class 1 integrons were found in only 4 isolates of *C. coli*. This low incidence of the integrons is corresponded to the results of a previous study in the Netherlands reporting that no class 1 integrons were detected in *Campylobacter* isolates (van Essen-Zandbergen et al., 2007). These data indicate that class 1 integrons may be getting to play a role in dissemination of antibiotic resistance among intestinal microflora of swine in Thailand. This is the first report of these mobile genetic elements in *C. coli* in Thailand.

All the *intI1* positive isolates in the present study consisted of the *aadA9* gene cassettes array. The *aadA9* gene encoding for aminoglycoside adenyltransferase confering resistance to streptomycin and spectinomycin. However, an aminoglycoside resistance phenotype was not investigated due to the sample loss. In a previous study, class 1 integrons with a recombined gene cassette containing the aminoglycoside-resistance gene, *aadA2* were found in 14% of *C. coli* isolated from humans and poultry (O'Halloran et al., 2004). In Georgia, 21% of *C. jejuni* isolated from the broiler chicken house environment possessed the integrase 1 gene and only 5 isolates containing the aminoglycoside resistance gene, *aacA4* (Lee et al., 2002). From our knowledge, the

aadA9 gene associated with class 1 integrons have never been reported in *C. coli* and to date, only the aminoglycoside resistance gene cassettes *aadA2* and *aadA4* has been identified in *Campylobacter*. As the use of aminoglycoside therapy may be considered as a treatment option for *Campylobacter*-related infections, our data supported that the possibility now exists for treatment failure to occur due to these mobile genetic elements. The *aadA9* gene was previously located on R-plasmid from *Corynebacterium glutamicum* (Tauch et al., 2002). This gene cassette was also detected in class 1 integrons in *Arthrobacter protophormiae* isolated from pigsties in Denmark (Agerso and Sandvang, 2005). The presence of the identical gene cassette in the different bacterial species suggest that the gene cassettes or integrons have been exchanged intra and inter species and, therefore, play a role in dissemination of antimicrobial resistance among bacteria (Hsu et al., 2006).

In *Campylobacter*, the fluoroquinolone resistance is mediated by mutations within the QRDR of the *gyrA* gene (Engberg et al., 2001). The major mutation associated with high-level resistance to fluoroquinolone is a single point mutation C257T in the *gyrA* gene, which results in threonine 86 being substitutioned with a isoleucine (Thr-86-Ile) (Beckmann et al., 2004; Engberg et al., 2001; Zhang et al., 2003). This is in agreement with our findings showing that a point mutation C257T was the most common (93%) mutation in the *gyrA* gene in quinolone resistant strains, followed by a Gly-119-Ser mutation (83%).

Other point mutations within QRDR of *gyrA* that have been less frequently found include those resulting in Thr-86-Lys, Thr-86-Ala, Asp-90-Asn and Pro-104-Ser (Keller and Perreten, 2006). Double mutations, Thr-86-Ile/Pro-104-Ser and Thr-86-Ile/Asp-90-ASN associated with high resistance level to fluoroquinolone have also been reported (Payot et al., 2006). However, multiple GyrA substitutions have been rarely described (Beckmann et al., 2004; Piddock et al., 2003; Wang et al., 1993). In the present study, Gly-119-Ser substitution was determined up to 83%, but their role in quinolone resistance have not been reported before. Double point mutation with Thr-86-Ile/Gly-119-Ser were found in many isolates (68%) but ciprofloxacin and nalidixic acid MIC

values of these strains were not different from those with only single point mutation of Thr-86-Ile. This suggests that additional mutation Gly-119-Ser may not have an effect or have only the marginal effect to the increased level of quinolone MIC. In addition, strains with up to 4 silent mutations, C252T, T297C, C345A and T360C in QRDR of *gyrA* were identified in our study. The results were in accordance with a previous study showing that silent mutations in QRDR of *gyrA* were not involved in quinolone resistance in *Campylobacter* (Kinana et al., 2007). It was also suggested that these silent mutations were not linked with fluoroquinolone resistance (Alonso et al., 2004).

Despite having identical amino acid substitution in the QRDR of *gyrA*, the MIC for fluoroquinolone of *C. coli* varied greatly (from 4 to 32 µg/ml for ciprofloxacin and from 32 to 128 µg/ml for nalidixic acid). This suggested that other factors contributing to the resistance phenotype may exist. It has been suggested that, similar to *Enterobacteriaceae*, additional mutations in *gyrB* or *parC* genes may increase the ultimate MIC conferred by the critical amino acid change within the GyrA QRDR (Friedman et al., 2001; Wang et al., 1993). A Previous study, high-level fluoroquinolone resistance in *E. coli* has been reported to be cause by substitution mutation Asp-426-Asn and Lys-447-Glu in GyrB (Ruiz, 2003). In *Campylobacter*, only silent mutations (A1471C) have been found in *gyrB* gene of *C. coli* and *C. jejuni* so far (Piddock et al., 2003). Our results showed that 5 resistant strains harbored an additional mutation in the *gyrB* gene, i.e., A-1144-C and A-1145-G leading to a Lys-381-Glu and Lys-381-Arg respectively. These five isolates also had a point mutation C257T in *gyrA*, however; the ciprofoxacin MIC value (4-16 µg/ml) and nalidixic acid MIC value (16-128 µg/ml) of these isolates were not different from that of the strains with single point mutation. Moreover, silent mutation in *gyrB* were not found in this study. These findings suggest that the mutation in *gyrB* may not affect the increased fluoroquinolone MIC value. Thus, the effect on fluoroqiunolone resistance of these mutations remain to be determined.

Topoisomerase IV, i.e., ParE and ParC subunits have been shown to be a secondary target for fluoroquinolone action in *E. coli* and *Salmonella* (Levine et al., 1998). High-level quinolone resistance in these two pathogens is commonly associated

with at least one mutation in *gyrA* and another mutation in the A subunit gene of topoisomerase IV (Payot et al., 2006). Up to date, The *parC* homologue has been reported in only one literature (Gibreel et al., 1998). Several laboratories have reported experimental attempts to amplify *parC* using primers and conditions described by Gribeel et al. (1998) but all were unsuccessful (Cooper et al., 2002; Payot et al., 2002; Piddock et al., 2003). It is, therefore, convincing that the secondary target (topoisomerase IV) may be absent in *Campylobacter*. The absence of a secondary target for fluoroquinolones in *Campylobacter* leads to a situation where a unique modification in the GyrA subunit (Thr-86-Ile) is sufficient to confer fluoroquinolone-resistant phenotype in *Campylobacter* at least in *C. coli* and *C. jejuni* (Bachoual et al., 2001; Luo et al., 2003).

High-level macrolide resistance in *Campylobacter* has mainly been attributed to mutations in domain V of the 23S rRNA target gene at position 2074 and 2075 (Gibreel and Taylor, 2006). In this study, a transitional mutation A2230G in the 23S rRNA gene was the most common mutation (70%), identified in erythromycin resistant *C. coli* isolates. This is in agreement with previous studies in *C. coli* isolates from pigs in Denmark (Jensen and Aarestrup, 2001), from sheep, poultry, cattle, and humans in Canada (Gibreel et al., 2005), from turkey in California (Chan et al., 2007), and from humans in Ireland (Corcoran et al., 2006). *C. coli* is well-known for the ability to acquire exogenous DNA by natural transformation, which occurs when a host organism acquires naked DNA from the extracellular environment (Wilson et al., 2003). A previous study suggested that natural transformation has the potential to contribute to the dissemination of high-level resistance to erythromycin among *C. coli* strains that harbored the point mutation A2075G in the 23S rRNA gene (Kim et al., 2006). The authors demonstrated that transformation of chromosome DNA fragments from a *C. coli* strain contains A2230G in to an erythromycin susceptible strain could generate an erythromycin resistant *C. coli* derivative. Because the mutation A2230G in the 23S rRNA gene was the most common found in this study, it may promote the chance of acquisition of erythromycin resistance. In this scenario, if the erythromycin-resistant *C. coli*, carrying a mutation A2230G in 23S rRNA die and release free DNA fragments to environment,

erythromycin-susceptible stains can acquire those free DNA from environment. Homologous recombination may occur resulting in strains resistant to erythromycin. Therefore, the role of transformation in dissemination of resistance to erythromycin worths further investigation.

Additional mutations including C-2252-T, G-2277-A and A-2278-G were found in 7 erythromycin-resistant strains in this study. These mutations were not found in negative control strain and they have never been shown to cause macrolide resistance in *Campylobacter* isolates. However, the erythromycin MIC value (256 µg/ml) of these strains was not different from MIC value (8-256 µg/ml) of those isolates with single point mutation A2230G. Instead, these mutations may be due to sequence variation of each *C. coli* strains (Jensen and Aarestrup, 2001). Since the 23S rRNA sequences from only two erythromycin susceptible strains were analyzed, it is not enough to verify the latter suggestion. Therefore, sequence analysis of 23S rRNA in a large number of erythromycin susceptible strains is suggested. In addition, a single point mutaion T-2268-C were also found in this study and expressed resistance to erythromycin (MICs 8-256 µg/ml). These point mutation was the first reported in a erythromycin resistant *Campylobacter*. These findings suggest that a single point mutation T-2268-C in 23S rRNA may affect to increase the erythromycin MIC value of *C. coli*.

The erythromycin-resistant isolates contains mutations showed the wide range MIC of erythromycin varying from 8 to 256 µg/ml and there were ten resistant isolates with no point mutations in 23S rRNA. From these results, it demonstrated that other resistance mechanism, for instance; multidrug efflux pumps may contribute to the resistance phenotype in these strains and act in synergy with the mutations to confer a high level of resistance to erythromycin (Cagliero et al., 2005). Thus, over expression of this efflux system may confer resistace to erythromycin in these strains. Synergy between efflux pump CmeABC and the point mutation of 23S rRNA may also increase the ultimate MIC value of erythromycin in *C. coli*.

Genetics of antibiotic resistance in *C. coli* in this study described that point mutations in the *gyrA* and 23S rRNA were the major mechanism for resistance to fluoroquinolone and macrolide, respectively. The *tet(O)* genes and class 1 integrons were also presented and may have the potential to horizontal transfer among these pathogens and can play an important role in distribution of antibiotic resistance. Taken together, the results indicate that dissemination of antibiotic resistance in *C. coli* is attributed to clonal and horizontal spread. As transmission of the resistant pathogens through the food chain poses a real threat to public health, projects to promote the appropriate use of antimicrobials in veterinary medicine and animal agriculture are necessary. The findings warrant further studies to determine and monitor antimicrobial resistance along the food chain.

Conclusion and suggestions

In this study, our findings demonstrated the major mechanisms conferring resistance to clinically-important antibiotics erythromycin, quinolones, and tetracycline in *C. coli* isolated from pig in Thailand. The mechanisms include a C257T mutation in the QRDR of *gyrA* for fluoroquinolone resistance, a transitional mutation A2230G in the 23S rRNA for erythromycin resistance and the presence of *tet(O)* gene for tetracycline resistance. In addition, the results also revealed class 1 intergrons with the *aadA9* gene. As most of resistance mechanisms identified in this study were not different from those previously reported, the *aadA9* gene array is a novel resistant gene cassette identified in *C. coli*.

Data obtained in this study could be beneficially used as follows:

1. To be applied as part of risk analysis of antibiotic resistance. As risk analysis of antibiotic resistance is mandatory for new drugs that will be used in animals, the genetics data of antibiotic resistance for *C. coli* is still limited. Data from other countries could not be always used due to differences in antibiotic use in different geographic areas. Therefore, antibiotic resistance in bacteria needs to be systematically studied.

2. To be a part of resistance monitoring program. Currently, most antibiotic resistance surveillance in Thailand depends on results of susceptibility test. Molecular epidemiological data is very limited. Therefore, studies of resistance mechanism would improved the survillence of antibiotic resistance in the country.

3. To prove the link of antibiotic resistance. To date, no absolute conclusion of the hypothesis has been made and more informative data are in demand. The results could be used as a part of the hypothesis proved. However, studies of the pathogen along the food chain are still needed.

From the results of this study, the suggestion for further studies could be as follows:

1. Antibiotic resistant *C. coli* strains that did not have mutation of the target sites (*gyrA*, *gyrB* and 23S rRNA) and antibiotic resistance determinants *tet(O)* and class 1 integrons) were also found in this study. This indicated that high level MIC of these strains may be due to other resistance mechanisms including other target mutations, multidrug efflux systems (CmeABC), and other mobile genetic elements (class 2 and class 3 integrons) which may play an important role in resistance to antibiotics. These mechanisms remains to be examined. Understading mechanisms responsible for antimicrobial resistance will facilitate the design of strategies to control antibiotic-resistant *Campylobacter*.

2. Data on genetics of antibiotic resistance of *C. coli* along the food chain will help to creation of the plan to reduce antibiotic resistance. Therefore, the studies of genetics of antibiotic resistances in *C. coli* isolated from other sources, e.g., from humans, pork, environment, and in more numbers of *C. coli* are recommended.

3. Study of genetic relatedness of *C. coli* isolates along the food chain should be performed in order to prove the link of antibiotic resistance in *C. coli* along the food chain. This will also provide the support.

4. Study of resistance gene tranfer in *C. coli*, especially, *tet(O)* gene and class 1 integrons should be performed. This will help to elucidate the route of disssemination of antibiotic resistance among bacteria.

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APPENDICES

APPENDIX A

Reagents and preparations

Reagents for PCR reaction

1. The Eppendorf MasterMix (2.5x) (Eppendorf®, Hamberg, Germany) contains

- Taq DNA Polymerase	62.5	U/ml
- KCl	125	mM
- Tris-HCl pH 8.3	75	mM
- $Mg(OAc)_2$	3.75	mM
- Igepal® -CA630	0.25	%
- each dNTP	500	µM

Reagents for agarose gel electrophoresis

1. 10 mg/ml Ethidium bromide

- Ethidium bromide	1	g
- Distilled deionized water	1,000	ml

Add 1 g of ethidium bromide to 100 ml of distilled deionized water. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap container in aluminum foil or transfer to a dark bottle and store at room temperature.

2. 50X TAE (Tris-Acetate buffer) 1000 ml contains

- Tris base	242.0	g
- Glacial acetic acid	57.1	ml
- 0.5 M EDTA pH 8.0	100.0	ml
- Distilled deionized water	1,000	ml

Add 242 g of Tris base, 57.1 ml of Glacial acetic acid and 100 ml of 0.5 M EDTA pH 8.0 to 500 ml of distilled deionized water and then adjust the final volume to 1,000 ml. Sterilize the solution by autoclaving.

3. 0.5 M EDTA, pH 8.0 1000 ml contains

- Disodium ethylene diamine tetraacetate. $2H_2O$	186.1	g
- Distilled deionized water	800.0	ml

Add 186.1 g of disodium ethylene diamine tetraacetate. $2H_2O$ to 800 ml of distilled deionized water. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH. Dispense into aliquots and sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to 8.0 by the addition of NaOH.

4. 1 M Tris HCl, pH 8.0 1000 ml contains

- Tris (ultrapure)	121.1	g
- Distilled deionized water	800.0	ml

Dissolve 121.1 g of Tris in 800 ml of distilled deionized water. Adjust the pH to 8.0 by adding conc. HCL 42.0 ml and then adjust the final volume to 1,000 ml. Sterilize the solution by autoclaving and store the solution at room temperature.

APPENDIX B

Locations of primers on the corresponding genes designed in this study

1. TetOF and TetOR primers

```
1     ATGAAAATAA TTAACTTAGG CATTCTGGCT CACGTTGACG CAGGAAAGAC AACA TTAACG
61    GAAAGTTTAT TGTATACCAG TGGTGCAATT GCAGAACTAG GGAGCGTAGA TGAA GGCACA

                                          tetOF
                                     ---------->
121   ACAAGGACAG ATACAATGAA TTTGGAGCGT CAAAGGGGAA TCACTATCCA GACAGCAGTG
181   ACATCTTTTC AGTGGGAGGA TGTAAAAGTC AACATTATAG ATACGCCAGG CCAT ATGGAT
241   TTTTTGGCGG AAGTATACCG TTCTTTATCC GTATTAGACG GAGCAGTATT ATTA GTTTCT
301   GCAAAGGATG GCATACAGGC ACAGACCCGT ATACTGTTTC ATGCACTACA GATA ATGAAG
361   ATTCCGACAA TTTTTTTCAT CAATAAAATT GACCAAGAGG GGATTGATTT GCCA ATGGTA
421   TATCGGGAAA TGAAAGCAAA GCTTTCTTCG GAAATTATAG TGAAGCAAAA GGTT GGGCAG
481   CATCCCCATA TAAATGTAAC GGACAATGAC GATATGGAAC AGTGGGATGC GGTA ATTATG
541   GGAAACGATG AACTATTAGA GAAATATATG TCAGGGAAAC CGTTTAAAAT GTCA GAACTA
601   GAACAGGAAG AAAACAGGAG ATTCCAAAAC GGAACGTTAT TTCCCGTTTA TCAC GGAAGC
661   GCTAAAAACA ATCTGGGGAT TCGGCAGCTT ATAGAAGTAA TTGCCAGTAA ATTT TATTCA

                                <----------
                                    tetOR
721   TCAACGCCTG AAGGTCAATC TGAACTATGC GGGCAGGTTT TTAAGATTGA ATATTCGGAA
781   GAAAGACAAC GTCTTGCATA TGTACGCCTT TATGGCGGAA TCCTGCATTT GCGG GATTCG
841   GTTAGAATAT CGGAAAAGGA AAAAATAAAA ATTACAGAAA TGTGTACTTC AATA AATGGT
901   GAATTATGTA AAATTGATAA GGCTTATTCC GGGGAAATTG TTATTTTGCA AAAT GAGTTT
1021  TTGAAGCTAA ATAGTGTTCT TGGAGATACA AAGCTATTGC CACAGAGAGA GAGA ATTGAA
```

Figure A-1. Locations and nucleotide sequence of tetOF and tetOR primers on *tet(O)* gene. The arrows indicated positon and direction of primers.

2. Int1LF and int1LR primers

```
1     CTACCTCTCA CTAGTGAGGG GCGGCAGCGC ATCAAGCGGT GAGCGCACTC CGGCACCGCC
61    AACTTTCAGC ACATGCGTGT AAATCATCGT CGTAGAGACG TCGGAATGGC CGAGCAGATC
121   CTGCACGGTT CGAATGTCGT AACCGCTGCG GAGCAAGGCC GTCGCGAACG AGTGGCGGAG
181   GGTGTGCGGT GTGGCGGGCT TCGTGATGCC TGCTTGTTCT ACGGCACGTT TGAAGGCGCG
241   CTGAAAGGTC TGGTCATACA TGTGATGGCG ACGCACGACA CCGCTCCGTG GATCGGTCGA
301   ATGCGTGTGC TGCGCAAAAA CCCAGAACCA CGGCCAGGAA TGCCCGGCGC GCGGATACTT
361   CCGCTCAAGG GCGTCGGGAA GCGCAACGCC GCTGCGGCCC TCGGCCTGGT CCTTCAGCCA
421   CCATGCCCGT GCACGCGACA GCTGCTCGCG CAGGCTGGGT GCCAAGCTCT CGGGTAACAT
481   CAAGGCCCGA TCCTTGGAGC CCTTGCCCTC CCGCAAGATG ATCGTGCCGT GATCGAAATC

                          Int1LR
                      ------------>
541   CAGATCCTTG ACCCGCAGTT GCAAACCCTC ACTGATCCGC ATGCCCGTTC CATACAGAAG
601   CTGGGCGAAC AAACGATGCT CGCCTTCCAG AAAACCGAGG ATGCGAACCA CTTCATCCGG

                                   Int1LF
                                  <------------
661   GGTCAGCACC ACCGGCAAGC GCCGCGCCGG CCGAGGTCTT CCGATCTCCT GAAGCCAGGG
721   CAGATCCGTG CACAGCACCT TGCCGTAGAA GAACAGCAAG GCCGCCAATG CCTGACGATG
781   CGTGGAGACC GAAACCTTGC GCTCGTTCGC CAGCCAGGAC AGAAATGCCT CGACTTCGCT
841   GCTGCCCAAG GTTGCCGGGT GACGCACACC GTGGAAACGG ATGAAGGCAC GAACCCAGTG
901   GACATAAGCC TGTTCGGTTC GTAAGCTGTA ATGCAAGTAG CGTATGCGCT CACGCAACTG
961   GTCCAGAACC TTGACCGAAC GCAGCGGTGG TAACGGCGCA GTGGCGGTTT TCAT
```

Figure A-2. Locations and nucleotide sequence of int1LF and int1LR primers on *intI1* gene. The arrows indicated positon and direction of primers.

3. gyrBF and gyrBR primers

```
1     ATGCAAGAAA ATTACGGTGC GAGTAATATT AAAGTCCTAA AAGGCCTAGA AGCT GTTAGA
61    AAACGCCCAG GTATGTATAT AGGAGATACA AACATAGGCG GACTTCATCA TATG ATTTTA
121   GAAGTTGTGG ATAATTCTAT CGATGAAGCT ATGGCAGGAC ATTGTGATAC TATA GATGTA
181   GAAATCACTA CTGAAGGAAG CTGTATAGTT AGTGATAATG GTCGTGGTAT TCCT GTTGAT
241   ATGCACCCAA CTGAAAATAT GCCAACTTTA ACTGTTGTTT TAACTGTCCT ACAT GCAGGG
      . . . . . .
1021  GTGCGTCCTA TAGTTTCAAA AGCAAGTTTT GAATATTTGA CTAAATATTT TGAA GAAAAT

                                      gyrBF
                                  ---------->
1081  CCTATCGAAG CTAAAGCTAT AATGAACAAA GCCTTAATGG CAGCTAGAGG AAGAGAAGCA
1141  GCGAAAAAAG CTAGAGAATT AACGCGTAAA AAAGAAAGTT TAAGCGTAGG AACT TTACCA
1201  GGGAAATTAG CTGATTGTCA AAGTAAAGAT CCAAGTGAAA GTGAAATTTA TCTT GTAGAA
1261  GGGGATTCTG CAGGAGGTTC TGCAAAACAA GGTAGAGAAA GATCTTTCCA AGCT ATACTG
1321  CCTTTGCGTA GTAAAATTTT AAATGTTGAA AAAGCAAGAC TAGATAAAAT TTTA AAATCT
1381  GAGCAAATTC AAAATATGAT TACCGCTTTT GGCTGTGGTA TAGGTGAAGA TTTT GATCTT

                                      gyrBR
                                  <----------
1441  TCAAAACTTA GATATCATAA AATCATCATA ATGACAGATG CGGATGTTGA TGGATCACAT
1501  ATACAAACCT TGCTTTTAAC TTTCTTCTTC CGTTTTATGA ATGAACTTGT AGCA AATGGA
1561  CATATTTATC TAGCACAACC ACCTTTATAT CTTTATAAAA AAGCTAAAAA GCAA ATTTAT
2161  TTAAAAGATG AAAAAGCTTT GAGCGAATAC CTGATAGAAA CAGGAATAGA AGGT TTAAAC
2221  TATGAAGGTA TAGGAATGAA TGATTTAAAA GATTATTTAA AAATCGTTGC AGCT TATCGT
2341  GCGATTTTAA AAGATCTTGA AAAGCGTTTT AATGTGATTT CTGTGATACG CTAT ATGATA
2401  GAAAATTCAA ATTTAGTTAA AGGAAATAAT GAAGAATTAT TTAGTGTAAT CAAA CAATTT
```

Figure A-3. Locations and nucleotide sequence of gyrBF and gyrBR primers on *gyrB* gene. The arrows indicated positon and direction of primers.

APPENDIX C

Mutations in *C. coli* isolates

Table A. Mutations within the quinolone resistance-determining region (QRDR) of *gyrA* gene from 60 fluoroquinolone-resistant *C. coli* isolates.

ID[a]	Nucleic acid codons and corresponding amino acids of *C. coli* QRDR of *gyrA*											
	Codon/	Amino acid	Codon/	Amino acid	Codon/	Amino acid	Codon/	Amino acid	Codon/	Amino acid	Codon/	Amino acid
AF092101[b]	GGC	Gly-84	ACT	Thr-86	TTT	Phe-99	ATC	Ile-115	GGC	Gly-119	GCT	Ala-120
1. CAC022[c]	- - -	-	- - -	-	- - -	-	- - -	-	- - -	-	- - -	-
2. CAC029[c]	- - -	-	- - -	-	- - -	-	- - -	-	- - -	-	- - -	-
3. CAC001	- - T	-	- T -	Ile-86	- - C	-	- - A	-	A - T	Ser-119	- - -	-
4. CAC002	- - -	-	- T -	Ile-86	- - -	-	- - A	-	- - -	-	- - -	-
5. CAC006	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
6. CAC007	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
7. CAC008	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - C	-

[a] The isolate names for each isolate are indicated.

[b] AF092101 is the accession number for the sequence of *gyrA* of wild type *C. coli* in GenBank.

[c] *C. coli* strains susceptible to both nalidixic acid and ciprofloxacin used as a negative control.

Table A. Cont.

ID	Nucleic acid codons and corresponding amino acids of *C. coli* QRDR of *gyrA*											
	Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid	
AF092101[b]	GGC	Gly-84	ACT	Thr-86	TTT	Phe-99	ATC	Ile-115	GGC	Gly-119	GCT	Ala-120
8. CAC009	- - T	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
9, CAC013	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
10. CAC018	- - -	-	- T -	Ile-86	- - C	-	- - A	-	- - -	-	- - -	-
11. CAC019	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
12. CAC023	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
13. CAC024	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
14, CAC025	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
15. CAC026	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - T	Ser-119	- - -	-
16. CAC028	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
17. CAC030	- - -	-	- - -	-	- - C	-	- - A	-	A - -	Ser-119	- - -	-
18. CAC031	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
19. CAC032	- - -	-	- T -	Ile-86	- - -	-	- - A	-	- - -	-	- - -	-
20, CAC033	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
21, CAC034	- - -	-	- T -	Ile-86	- - C	-	- - A	-	- - -	-	- - -	-

Table A. Cont.

ID	Nucleic acid codons and corresponding amino acids of *C. coli* QRDR of *gyrA*											
	Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid	
AF092101[b]	GGC	Gly-84	ACT	Thr-86	TTT	Phe-99	ATC	Ile-115	GGC	Gly-119	GCT	Ala-120
22. CAC037	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
23. CAC038	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
24. CAC039	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
25. CAC040	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
26. CAC041	- - -	-	- T -	Ile-86	- - C	-	- - A	-	- - -	-	- - -	-
27. CAC042	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
28. CAC044	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
29. CAC045	- - -	-	- T -	Ile-86	- - -	-	- - A	-	- - -	-	- - -	-
30. CAC046	- - -	-	- T -	Ile-86	- - C	-	- - A	-	- - -	-	- - -	-
31. CAC047	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
32. CAC048	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
33. CAC049	- - -	-	- T -	Ile-86	- - C	-	- - A	-	- - -	-	- - -	-
34. CAC050	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
35. CAC051	- - -	-	- T -	Ile-86	- - C	-	- - A	-	- - -	-	- - -	-

Table A. Cont.

ID	Nucleic acid codons and corresponding amino acids of *C. coli* QRDR of *gyrA*											
	Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid	
AF092101[b]	GGC	Gly-84	ACT	Thr-86	TTT	Phe-99	ATC	Ile-115	GGC	Gly-119	GCT	Ala-120
36. CAC052	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
37. CAC053	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
38. CAC054	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - T	Ser-119	- - -	-
39. CAC055	- - -	-	- T -	Ile-86	- - -	-	- - A	-	- - -	-	- - -	-
40. CAC057	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
41. CAC058	- - -	-	- - -	-	- - C	-	- - A	-	A - -	Ser-119	- - -	-
42. CAC059	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
43. CAC060	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
44. CAC061	- - -	-	- T -	Ile-86	- - C	-	- - A	-	- - -	-	- - -	-
45. CAC062	- - -	-	- T -	Ile-86	- - -	-	- - A	-	- - -	-	- - -	-
46. CAC063	- - -	-	- T -	Ile-86	- - -	-	- - A	-	- - -	-	- - -	-
47. CAC064	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
48. CAC065	- - -	-	- - -	-	- - C	-	- - A	-	A - -	Ser-119	- - -	-
49. CAC066	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - T	Ser-119	- - -	-

Table A. Cont.

ID	Nucleic acid codons and corresponding amino acids of *C. coli* QRDR of *gyrA*											
	Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid		Codon / Amino acid	
AF092101[b]	GGC	Gly-84	ACT	Thr-86	TTT	Phe-99	ATC	Ile-115	GGC	Gly-119	GCT	Ala-120
50. CAC067	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - T	Ser-119	- - -	-
51. CAC068	- - T	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
52. CAC069	- - -	-	- T -	Ile-86	- - -	-	- - A	-	- - -	-	- - -	-
53. CAC073	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
54. CAC074	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
55. CAC075	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
56. CAC078	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
57. CAC079	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-
58. CAC080	- - -	-	- - -	-	- - C	-	- - A	-	- - -	-	- - -	-
59. CAC081	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
60. CAC082	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - T	Ser-119	- - -	-
61. CAC083	- - -	-	- T -	Ile-86	- - C	-	- - A	-	A - -	Ser-119	- - -	-
62. CAC084	- - -	-	- T -	Ile-86	- - -	-	- - A	-	A - -	Ser-119	- - -	-

Figure B. Sequences of 386-bp PCR fragment of the *gyrB* gene from 5 resistant isolates habored a mutation.

ID[a]		1120	1130	1140	1150	1160	1170	1180
AY330104[b]	1111	GCTTTAATGG CAGCTAGAGG AAGAGAAGCA GCAAAAAAAG CAAGAGAATT AACGCGTAAA AAAGAAAGCT						
CAC022[c]	1111	GCTTTAATGG CAGCTAGAGG AAGAGAAGCA GCAAAAAAAG CAAGAGAATT AACGCGTAAA AAAGAAAGCT						
CAC029[c]	1111	GCTTTAATGG CAGCTAGAGG AAGAGAAGCA GCAAAAAAAG CAAGAGAATT AACGCGTAAA AAAGAAAGCT						
CAC002	1111	GCTTTAATGG CAGCTAGAGG AAGAGAAGCA GCA**C**AAAAAG CAAGAGAATT AACGCGTAAA AAAGAAAGCT						
CAC006	1111	GCTTTAATGG CAGCTAGAGG AAGAGAAGCA GCA**CG**AAAAG CAAGAGAATT AACGCGTAAA AAAGAAAGCT						
CAC007	1111	GCTTTAATGG CAGCTAGAGG AAGAGAAGCA GCA**C**AAAAAG CAAGAGAATT AACGCGTAAA AAAGAAAGCT						
CAC009	1111	GCTTTAATGG CAGCTAGAGG AAGAGAAGCA GCA**C**AAAAAG CAAGAGAATT AACGCGTAAA AAAGAAAGCT						
CAC013	1111	GCTTTAATGG CAGCTAGAGG AAGAGAAGCA GCA**C**AAAAAG CAAGAGAATT AACGCGTAAA AAAGAAAGCT						

[a] The isolate names for each isolate are indicated.

[b] AY330104 is the accession number for the sequence of *gyrB* of wild type *C. coli* in GenBank**.**

[c] *C. coli* strains susceptible to both nalidixic acid and ciprofloxacin used as a negative control.

Bold letters indicate either the position of the defined point mutation .

Figure C. Sequences of 316-bp PCR fragment of an internal area of the 23S rRNA from 34 erythromycin resistant isolates.

ID[a]		2230	2240	2250	2260	2270	2280	2290	2300
U09611[b]	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC022[c]	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC029[c]	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC002	2221	CAAGACGGA**G** AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC003	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC006	2221	CAAGACGGA**G** AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC007	2221	CAAGACGGA**G** AGACCCCGTG GACCTTTACT A**T**AGCTTGAC ACTGCTATTT GGATAA**AG**AT GTGCAGGATA GGTGGGAGGC							
CAC008	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC013	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC015	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTA**C**TT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC019	2221	CAAGACGGA**G** AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC023	2221	CAAGACGGA**G** AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC028	2221	CAAGACGGA**G** AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC030	2221	CAAGACGGA**G** AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							

[a] The isolate names for each isolate are indicated.

[b] U09611 is the accession number for the sequence of 23S rRNA of wild type *C. coli* in GenBank**.**

[c] *C. coli* strains susceptible to erythromycin used as a negative control.

Bold letters indicate either the position of the defined point mutation for macrolide resistance.

Figure C. Cont.

ID		2230	2240	2250	2260	2270	2280	2290	2300
U09611	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC031	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC037	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC038	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ATAGCTTGAC ACTGCTATTT GGATAAAGAT GTGCAGGATA GGTGGGAGGC							
CAC040	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC044	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ATAGCTTGAC ACTGCTATTT GGATAAAGAT GTGCAGGATA GGTGGGAGGC							
CAC047	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC048	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC049	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC051	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC054	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC055	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC057	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC058	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC059	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ATAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							

Figure C. Cont.

Name		2230	2240	2250	2260	2270	2280	2290	2300
U09611	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC060	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC061	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC062	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC064	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ATAGCTTGAC ACTGCTATTT GGATAAAGAT GTGCAGGATA GGTGGGAGGC							
CAC065	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC066	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC069	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC072	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTACTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC073	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC075	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC077	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTACTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC078	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC079	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ATAGCTTGAC ACTGCTATTT GGATAAAGAT GTGCAGGATA GGTGGGAGGC							
CAC080	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC081	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							

Figure C. Cont.

Name		2230	2240	2250	2260	2270	2280	2290	2300
U09611	2221	CAAGACGGAA AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC082	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ATAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC083	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC084	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							
CAC085	2221	CAAGACGGAG AGACCCCGTG GACCTTTACT ACAGCTTGAC ACTGCTATTT GGATAAGAAT GTGCAGGATA GGTGGGAGGC							

BIOGRAPHY

Mr. Chanon Ekkapobyotin was born on February 22, 1981 in Bangkok, Thailand. He graduated from the Faculty of Veterinary Science, Chulalongkorn University, Thailand in 2005. After that, he enrolled the degree of Master of Science in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University in 2006.