MOLECULAR EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* AND ENTEROCOCCI FROM ANIMALS, VEGETABLES AND WATER

WIPAWADEE SIANGLUM

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(MEDICAL TECHNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2007**

Thesis
Entitled

MOLECULAR EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* AND ENTEROCOCCI FROM ANIMALS, VEGETABLES AND WATER

……
Miss Wipawadee Sianglum
Candidate

……
Assist. Prof. Wijit Wonglumsom,
Ph.D. (Food Science)
Major-Advisor

……
Lect. Kanokwan Kittiniyom,
Ph.D. (Pathology)
Co-Advisor

…….....
Assist. Prof. Potjanee Srimanote,
Ph.D. (Molecular Microbiology)
Co-Advisor

……
Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies

……
Lect. Ratana Lawung,
Ph.D.
Chair
Master of Science Programme in
Medical Technology
Faculty of Medical Technology

Thesis
Entitled

MOLECULAR EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* AND ENTEROCOCCI FROM ANIMALS, VEGETABLES AND WATER

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Medical Technology)
on
27 February, 2007

……...
Miss Wipawadee Sianglum
Candidate

……...
Lect. Seksun Samosornsuk,
Ph.D. (Biomedical Science)
Chair

…….
Assist.Prof. Chanwit Tribuddharat,
M.D., Ph.D.
(Microbiology and Immunology)
Member

……...
Assist. Prof. Wijit Wonglumsom,
Ph.D. (Food Science)
Member

…….
Assist. Prof. Potjanee Srimanote,
Ph.D. (Molecular Microbiology)
Member

……...
Lect. Kanokwan Kittiniyam,
Ph.D. (Pathology)
Member

……
Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

……...
Assoc. Prof. Chatchai Sornchai,
M.D. M.Sc. (Microbiology)
Dean
Faculty of Medical Technology
Mahidol University

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my advisor, Assist. Prof. Dr. Wijit Wonglumsom, for her kindness, valuable suggestion, expert guidance and extensive support.

I would like to express my appreciation to Assist. Prof. Potjanee Srimanote for her carefulness, valuable encouragements and suggestion. I also specially thanks to Dr. Kanokwan Kittiniyom for her kindness, warm suggestion and supportive encouragement throughout my study.

I would like to express my appreciation to all the lecturers and teachers for their valuable knowledge and suggestion.

I would like to thank all of my colleagues at the Faculty of Medical Technology, Mahidol University, for their friendship, cheerfulness, friendly advice, kind assistance and giving the nice memory during my study.

I am grateful for the Graduate Scholarship of the Faculty of Medical Technology, Mahidol University partly supported during the time of my study.

I am indebted to Dr. George A. Jacoby, Lahey Clinic, Massachusetts, and Assist. Prof. Amornrut Leelaporn, Department of Microbiology, Faculty of Medicine Siriraj Hospital, for valuable support for some materials in this study.

Finally, I am deeply grateful to my lovely family, particularly my parents and my younger sister for their support, understanding, encouragement and love. Without their attention and dedication, I would not have gone through some difficulties throughout my study.

Wipawadee Sianglum

MOLECULAR EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* AND ENTEROCOCCI FROM ANIMALS, VEGETABLES AND WATER

WIPAWADEE SIANGLUM 4736810 MTMT/M

M.Sc. (MEDICAL TECHNOLOGY)

THESIS ADVISORS: WIJIT WONGLUMSOM, Ph.D.,
KANOKWAN KITTINIYOM, Ph.D., POTJANEE SRIMANOTE, Ph.D.

ABSTRACT

The spread and emergence of antimicrobial-resistant bacteria has emerged as a public health major problem worldwide. The usage of antimicrobials for prevention and treatment in humans and animals possibly contributes to an increasing occurrence of resistant bacteria. An epidemiology of resistant genes in fecal-indicator bacteria including *Escherichia coli* and enterococci originating from humans, pets, vegetables, water and chickens was studied using dot blot hybridization. Resistant genes of *tetM*, *tetA*, *catI*, *cmlA*, *sulI* and *qnrA* in *E. coli* isolates and *tetM*, *tetL*, *ermB*, *aadA* and *vatD* in enterococci were determined. Two multiplex-polymerase chain reaction (PCR) for simultaneous detection of five resistant genes for *E. coli* and of three resistant genes for *Enterococcus* spp. were developed. Moreover, a duplex PCR for species identification of *E. faecium* and *E. faecalis* was successfully developed in this study. Among *E. coli* isolates, *tetA* was frequently observed followed by *cmlA*, *sulI*, *tetM* and *catI*, respectively. High prevalence of resistant genes including *tetA* (71.3%), *catI* (10.4%) and *sulI* (34.8%) was observed in isolates from chickens, while the highest frequency of *tetM* (31.4%) and *cmlA* (33.3%) was seen in isolates from rivers. Among enterococci isolates, *tetM* was highly observed followed by *tetL* and *ermB*, respectively. Isolates from chickens carried a large amount of resistant genes, *tetM*, *tetL* and *ermB*, for more than 80% and up to 75% contained three genes together. Various plasmid profiles were observed in *E. coli* especially from isolates of chickens. Mutational analysis on quinolone resistance determining region (QRDR) of *gyrA* showed 10 single-strand conformation polymorphism (SSCP) patterns with 9 patterns found in vegetables and water and 7 patterns in pets. Amino acid changes in position 83 and/or 87 were revealed in 4 patterns with alteration of Ser-83-Leu and Asp-87-Asn. The pattern with the double mutation was detected only in human and pet isolates and they were not found in ciprofloxacin susceptible isolates. The molecular characterization by identification of resistant genes indicated high degree of resistant gene distribution among *E. coli* and enterococci.

KEY WORDS: ANTIBIOTIC RESISTANCE/ RESISTANCE GENES/
MUTATIONAL ANALYSIS/ *ESCHERICHIA COLI*
ENTEROCOCCUS SPP.
118 P.

ระบาดวิทยาทางอณูชีววิทยาของการดื้อยาต้านจุลชีพใน *ESCHERICHIA COLI* และ ENTEROCOCCI ที่แยกได้จากสัตว์ ผัก และน้ำ (MOLECULAR EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* AND ENTEROCOCCI FROM ANIMALS, VEGETABLES AND WATER)

วิภาวดี เสียงล้ำ 4736810 MTMT/M

วท.ม. (เทคนิคการแพทย์)

คณะกรรมการควบคุมวิทยานิพนธ์: วิจิตร วงค์ล่ำซำ, Ph.D., กนกวรรณ กิตตินิยม, Ph.D., พจนีย์ ศรีมาโนชญ์, Ph.D.

บทคัดย่อ

การเกิดขึ้นและการแพร่กระจายของแบคทีเรียที่ดื้อยาต้านจุลชีพเป็นปัญหาด้านการสาธารณสุขที่สำคัญทั่วโลก การเพิ่มขึ้นของแบคทีเรียดื้อยาเป็นผลมาจากการใช้ยาต้านจุลชีพทั้งในแง่การป้องกันและการรักษาในคนและสัตว์ การวิจัยนี้ได้ศึกษาระบาดวิทยาของยีนดื้อยาในแบคทีเรียที่เป็นดัชนีชี้วัดการปนเปื้อนของอุจจาระ ได้แก่ *Escherichia coli* และ enterococci ที่แยกได้จากคน สัตว์เลี้ยง ผักสด น้ำ และไก่ ด้วยวิธี dot blot hybridization โดยการตรวจยีนดื้อยา *tetA tetM catI cmlA sulI* และ *qnrA* ใน *E. coli* และตรวจยีนดื้อยา *tetM tetL ermB aadA* และ *vatD* ใน enterococci งานวิจัยนี้ได้พัฒนาวิธี multiplex-polymerase chain reaction (PCR) สำหรับตรวจหายีนดื้อยาทั้ง 5 ชนิดพร้อมกันใน *E. coli* และตรวจหายีนดื้อยา 3 ชนิดใน enterococci นอกจากนี้ได้พัฒนาวิธีการ duplex PCR เพื่อการวินิจฉัยสปีชีส์ระหว่าง *E. faecalis* และ *E. faecium* ใน *E. coli* พบว่ามียีนดื้อยา *tetA* ในอัตราสูงสุด รองลงมาตามลำดับคือ *cmlA sulI tetM* และ *catI* โดย *E. coli* ที่แยกจากไก่พบ *tetA* สูงสุดคือร้อยละ 71.3 *catI* ร้อยละ 10.4 และ *sulI* ร้อยละ 34.8 ในขณะที่ *E. coli* ที่แยกจากแม่น้ำพบ *tetM* สูงสุดคือ ร้อยละ 31.4 และ *cmlA* ร้อยละ 33.3 ส่วนใน enterococci พบว่ามียีนดื้อยา *tetM* ในอัตราสูงสุด รองลงมาคือ *tetL* และ *ermB* ตามลำดับ โดยที่แยกได้จากไก่พบ *tetM tetL* และ *ermB* มากกว่าร้อยละ 80 และพบทั้งสามยีนดื้อยานี้ร่วมกันสูงถึงร้อยละ 75 *E. coli* ที่แยกได้จากไก่พบความหลากหลายของ plasmid profile มากที่สุดเมื่อเทียบกับที่แยกได้จากแหล่งอื่นๆ ในการศึกษาการเปลี่ยนแปลงของนิวคลีโอไทด์บริเวณ quinolone resistance determining region (QRDR) ของยีน *gyrA* ด้วยวิธี single strand conformation polymorphism (SSCP) พบทั้งหมด 10 รูปแบบ โดยพบในตัวอย่างผักสดและน้ำ 9 รูปแบบ และในตัวอย่างสัตว์เลี้ยง 7 รูปแบบ มี 4 รูปแบบที่มีการเปลี่ยนแปลงของกรดอะมิโน ในตำแหน่งที่ 83 จาก Ser ไปเป็น Leu และ/หรือ ในตำแหน่งที่ 87 จาก Asp ไปเป็น Asn ซึ่งการเปลี่ยนแปลงของกรดอะมิโนทั้งสองตำแหน่งพร้อมกันพบเฉพาะใน *E. coli* ที่แยกได้จากคนและสัตว์เลี้ยง โดยแบคทีเรียเหล่านี้ไม่ไวต่อยา ciprofloxacin การศึกษาลักษณะของอณูชีววิทยาตรวจหายีนดื้อยาแสดงให้เห็นถึงการแพร่กระจายอย่างมากของยีนดื้อยาต้านจุลชีพใน *E. coli* และ enterococci.

118 หน้า

CONTENTS

Page

CONTENTS (CONTINUED)

Page

CONTENTS (CONTINUED)

Page

LIST OF TABLES

Page

LIST OF TABLES (CONTINUED)

Page

LIST OF FIGURES

Page

LIST OF FIGURES (CONTINUED)

Page

LIST OF ABBREVIATIONS

AMP	Ampicillin
Asn	Asparagine
Asp	Aspartic acid
BCIP	5-bromo-4 chloro-3 idolyl phosphate
bp	Base pair (s)
°C	Degree's Celsius
CIP	Cipofloxacin
CRO	Ceftriaxone
dATP	Deoxyadenosine 5'- triphosphate
dCTP	Deoxycytosine 5'- triphosphate
dGTP	Deoxyguanosine 5'- triphosphate
dNTP	Deoxynucleoside triphosphate
dTTP	Deoxythymidine 5'- triphosphate
dUTP	Deoxyuridine 5'- triphosphate
DNA	Deoxyribonucleic acid
DIG	Digoxigenin
E	Erythromycin
EDTA	Ethylene diamine tetraacetic acid
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
h	Hour(s)
I	Intermediate
LB	Luria Bertani
Leu	Leucine
mg	Milligram
MIC	Minimal inhibitory concentration

LIST OF ABBREVIATIONS (CONTINUED)

min	Minute(s)
ml	Milliliter
mM	Millimolar
µg	Microgram(s)
µl	Microliter(s)
NA	Nalidixic acid
NBT	Nitroblue-tetrazolium salt
pmol	Picomole(s)
PCR	Polymerase chain reaction
QRDR	Quinolone resistant determining region
R	Resistant
RT	Room temperature
S	Susceptible
SDS	Sodium dodecyl sulphate
Ser	Serine
SSCP	Single-strand conformation polymorphism
SXT	Sulfamethoxazole-trimethoprim
TE	Tetracycline
TE	Tris-EDTA buffer
TSB	Tryptic soy broth
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume

CHAPTER I
INTRODUCTION

An emergence of antimicrobial resistance has been recognized as a major problem of public health worldwide. An important consequence of proper use, misuse and overuse of antibiotics is an emergence and a dissemination of antimicrobial resistant bacteria in humans, animals and environments (1-3). Many surveillance programs have been set up for monitoring of drug resistance after antimicrobial agents were used in human and veterinary medicine, which were used as growth promoters in animal husbandry, fish farming and other fields (4). These programs were focused mainly on human pathogens, agents of zoonosis and normal intestinal flora from animals (5-8). After an introduction of antimicrobials, most studies showed an increasing of the resistance in pathogenic bacteria and commensal bacteria. The commensal bacteria could be a reservoir of resistance genes for pathogenic bacteria. Resistance commensal bacteria of food animals might be contaminated in meat products and reach to humans not only by direct contact of the microorganisms but also through a consumption of food products of animal origin (9). One potential source of acquired resistance genes is a therapeutic use of antimicrobial agents in veterinary medicine or an application as growth promoters in a conventional animal fattening (10). In the countries that used avoparcin, a glycopeptide antibiotic like vancomycin, as an antimicrobial growth promoter (AMGP), vancomycin resistance enterococci (VRE) were found in food animals fed with avoparcin and in the faecal flora of healthy humans and pet animals (2). In Thailand, the use of chicken manure as a fertilizer in integrated chicken-fish farms caused an alteration of the species composition of enterococci in farms and increasing of the prevalence of drug resistance within each species (4).

Monitoring of the prevalence of antimicrobial resistance in indicator bacteria, such as *E. coli* and enterococci in different sources including animals, healthy

humans, patients, environment compartments, waste, surface water sediments and sludge, indicated that clonal spread of resistant strains was occurred (8).

Moreover, resistance genes of the resistant bacteria could be transferred from animals to humans and disseminated into an environment. A relationship of the usage of an antibiotic and the dissemination of resistant bacteria from animals to humans has been described by Hummel *et al.* (11). An occurrence of resistance in the commensal microorganisms are considered to be a good indicator for selective pressure excited by antibiotic use in that population and for the resistance problems found in pathogens (2, 12).

The complexity balance of micro flora in different habitats within the ecosystem can potentially cause the high transferability of resistance genes among bacteria occupying the habitats. The spread of plasmid encoding an antimicrobial resistance gene in *E. coli* from chickens to human handlers (1, 13) or of antimicrobial-resistance microorganisms from poultry to human has been reported (1). The occurrences of resistance genes may be through horizontal or lateral gene transfer among bacteria of different genera and family or through vertical gene transfer occurring when an organism receives genetic material from its ancestor (generations to the next generations of the bacterial clonality) (14). Regarding antibiotic resistance genes in *E. coli,* integrons are very important in terms of the mechanisms of resistance and in the dissemination of resistance genes (7). Integrons are known to be associated with multiple-drug resistance in enteric organisms and specifically class 1 integrons have been shown to be important in the dissemination of *intI*, *sulI* and one or more antimicrobial resistance gene cassettes among gram-negative bacteria (15). The varying sizes of integrons were found in the *E. coli* isolates and they contained resistance genes for aminoglycosides, trimethoprim, and *β*-lactams (15-17). More recently, Wang *et al.* (18) reported the occurrence of a gene in a class 1 integron, termed *qnr*, which provides low-level quinolone resistance. The most frequent mechanism in quinolone resistance of *E. coli* includes alteration in genes that encode subunits of the quinolone targets of DNA gyrase (in *gyrA* and *gyrB* genes) and topoisomerase IV (in *parC* and *parE* genes). Mutation in *gyrB* and *parE* gene are less common than that in *gyrA* or *parC.*

Therefore, the objectives of this study are to investigate the correlation of plasmid profiles of *E. coli* and antimicrobial susceptibility patterns, to detect the resistance genes of tetracycline (*tetA* and *tetM*), chloramphenicol (*catI and cmlA*), sulfonamide (*sulI*) and quinolone (*qnrA*), and to assess the *gyrA* mutations related to quinolones resistance in *E. coli* isolates. For enterococci, the resistance genes for aminoglycoside (*aadA*), tetracycline (*tetM* and *tetL*), macrolide (*ermB)* and streptogramin *(vatD*) were detected. The simultaneous identification of *E. faecium* and *E. faecalis* and also the detection of resistance genes were modified and developed to be a multiplex PCR. All of the bacterial isolates were collected from humans, pets (dogs and cats), fresh vegetables, ice, water and chickens.

The objectives of this study:

1. To investigate the resistance genes in *E. coli* and enterococci by using dot blot hybridization and to study the correlation between resistance genes and antimicrobial susceptibility profiles.

2. To develop multiplex PCR for detection of antimicrobial resistance genes of *E. coli* and enterococci and for identification of *E. faecalis* and *E. faecium*.

3. To study the correlation of plasmid banding pattern of *E. coli* isolates and antimicrobial susceptibility profiles.

4. To determine point mutation(s) in *gyrA* of *E. coli* by using PCR Single-Strand Conformational Polymorphism (PCR-SSCP) analysis and to study the correlation with antimicrobial susceptibility test of quinolone agents.

CHAPTER II
LITERATURE REVIEW

2.1 *Escherichia coli* and *Enterococcus* spp.

2.1.1 *Escherichia coli*

Escherichia coli is a species in the genus *Escherichia*, which contains mostly motile Gram-negative bacilli (Figure 2.1-2.2). They are the most common facultative bacteria that live in lower intestines of warm-blooded animals and humans, and they are necessary for the proper digestion of food in the intestine. *E. coli* can be recovered easily from clinical specimens on general or selective media at 37°C under aerobic conditions. From stool samples, *E. coli* are usually recovered on MacConkey and eosin methylene-blue agar, which are selective for isolation of bacteria in the *Enterobacteriaceae* and are differentiative for enteric organisms on the basis of their morphology (19). For epidemiologic or clinical purposes, *E. coli* strains are often selected from the agar plates after presumptive visual identification of their typical colonies. However, this method should be used only with caution, because only about 90% of *E. coli* strains are lactose positive. Some diarrheagenic *E. coli* including EIEC strains, are typically lactose negative. The indole test is positive in 99% of *E. coli* and is the best test for differentiation from other members of the *Enterobacteriaceae* (20). Because *E. coli* is ubiquitous in human and animal feces, the presence of this species in water and food is considered to be an indicator of fecal contamination. *E. coli* is a part of the bowel flora of healthy human. However, in the debilitated or immunosuppressed host, or in injure gastrointestinal barriers, normal non-pathogenic strains of *E. coli* can cause infections. Urinary tract infections, bacteremia, meningitis and diarrheal diseases are the most frequent clinical syndromes. Moreover, the healthy human may be susceptible to an infection by one of several highly adapted *E. coli* clones that together have evolved the ability to cause a broad spectrum of human diseases (19).

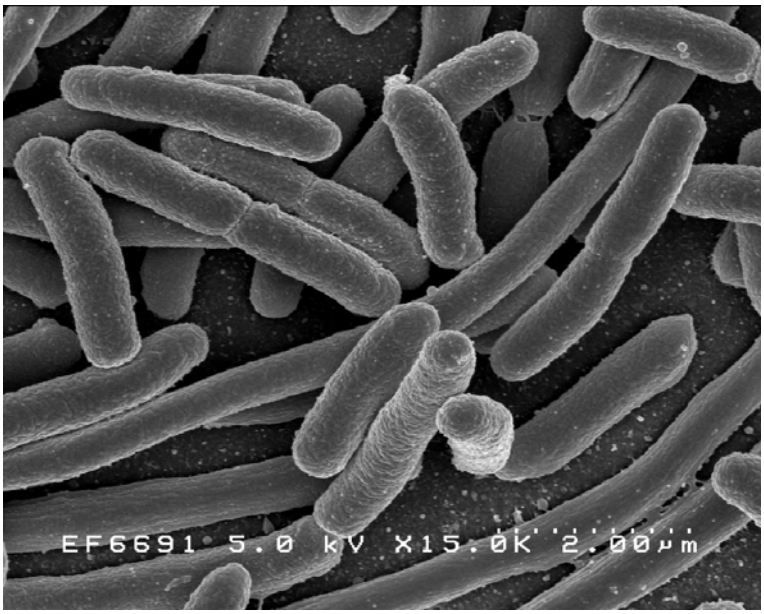


Figure 2.1 The scanning electron microscopy of *Escherichia coli* (21)**.**

Figure 2.2 Gram-negative bacilli of *Escherichia coli* (22).

2.1.2 Resistance to antimicrobials

Various frequencies of antibiotic resistance were detected in *E. coli* isolated from different sources, such as humans, broiler, swine, pets, foods. These differences could reflect the specific use of antibiotics in these groups. Saenz *et al.* (23) have described the high frequency of nalidixic acid-, ciprofloxacin- and gentamicin-resistance, which were 88%, 38 % and 40%, respectively, in *E. coli* isolates from broilers, and which were 53, 13 and 17%, respectively from foods. High levels of resistance to trimethoprim–sulphamethoxazole and tetracycline have been found in *E. coli* isolates from broilers, pigs and foods and most of *E. coli* isolates from avian and swine origins were resistant to tetracyclines, aminoglycosides, and sulphonamides (Figure 2.3). Many *E. coli* isolates from swine were resistant to chloramphenicol and it was also observed in 53% of *E. coli* isolates from swine farms in Oklahoma, USA (7).

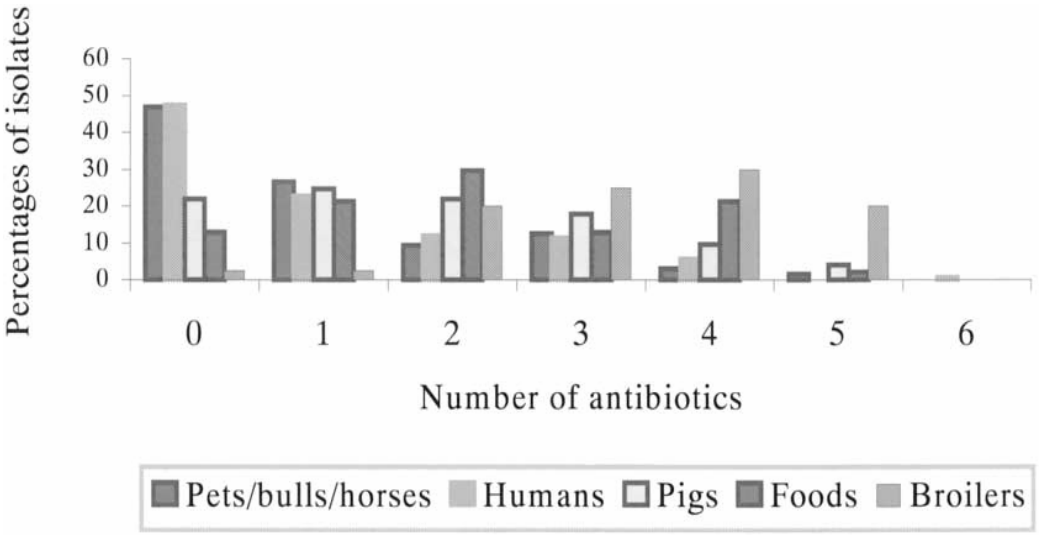


Figure 2.3 Resistance of *E. coli* isolates of different origins to a variety of antibiotics (the antibiotics considered were: nalidixic acid, ampicillin, tetractycline, gentamicin, trimethoprim–sulphamethoxazole, chloramphenicol). X-axis represents the number of antibiotics to which isolates are resistant (23).

2.2 *Enterococcus* spp.

2.2.1 *Enterococcus* spp.

Genus *Enterococcus* was mainly related to the "streptococci of fecal origin. Enterococci are Gram positive cocci that occur singly, in pairs, and in short chains. The cells are sometimes cocco-bacillary when Gram stains are prepared from a growth on agar plate. Enterococci are facultatively anaerobic and optimum growth occurs at 35ºC. Most strains grow between 10 ºC and 45 ºC. All strains grow in broth containing 6.5% NaCl and hydrolyze esculin in a presence of 40% bile salts. Motility is observed in some species. Catalase test appears negative while a pseudocatalase is sometimes produced and a weak effervescence is observed in catalase test when a strain of *E. faecalis* is grown on a blood containing medium. Nearly all strains are homofermentative without gas production and lactic acid is the end of glucose fermentation. Most strains produce a cell-wall associated glycerol teicoic acid antigen that is identified as the streptococcal group D antigen (19).

Enterococci are ubiquitous bacteria that are predominantly habitats of gastrointestinal tract in humans and animals and are commonly found in environments contaminated with human and animal faecal materials, such as urban sewage, plants, recipient water and soil receiving fertilizers of animal origin as well as in food products from animals (24). Enterococci are less common in other organic sites, such as in genitourinary tract and the oral cavity. *E. faecalis* and *E. faecium* have been consistently identified as the dominant *Enterococus* spp. in human faeces and they are the causative agents of the vast majority of the enterococcal infections in humans (25). *E. avium*, *E. cecorum*, *E. dispar*, *E. durans*, *E. galllinarum*, *E. gilum, E. hirae*, *E. mundtii*, *E. pallens* and *E. casseliflavus*, are also isolated from human sources (19). Enterococci are normally considered as bacteria with a low pathogenicity and enterococal infections occur often in elderly patients who have serious underlying medical problems and in the immunocompromised patients who have had prolong hospitalization and have received broad spectrum antimicrobial therapy and have used invasive devices .

The enterococci have become the second most common bacterium isolated from nosocomial urinary and wound infections, and the third most common cause of nosocomial bacteremia (26). *E. faecalis* frequently causes infections within the peritoneal cavity, especially following penetrating trauma such as gunshot wounds, knife wounds, and surgical wounds, urinary tract infections, kidney infections, prostate infections, and infections of damaged or compromised skin, such as diabetic or decubitus ulcers, burns, and surgical wounds. Other opportunistic fecal streptococci include *E. faecium* and *E. durans*.

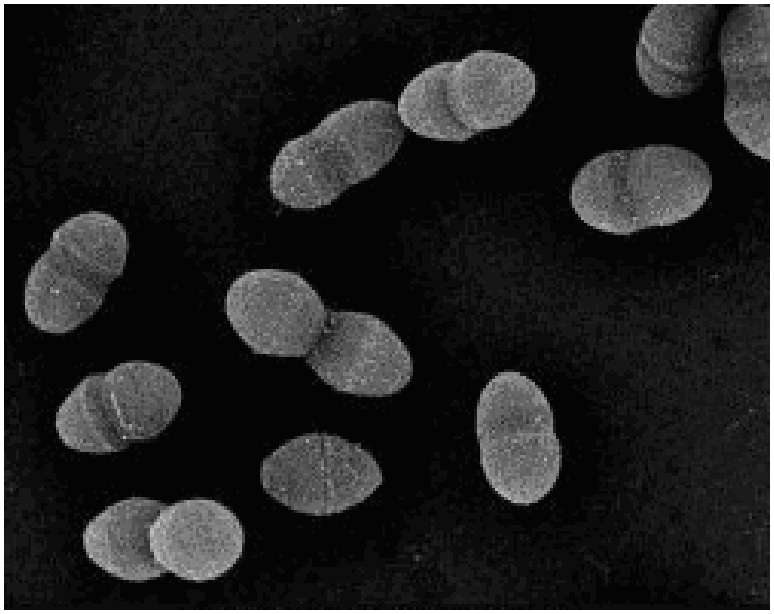


Figure 2.4 The scanning electron microscopy of *Enterococcus* spp.(27).

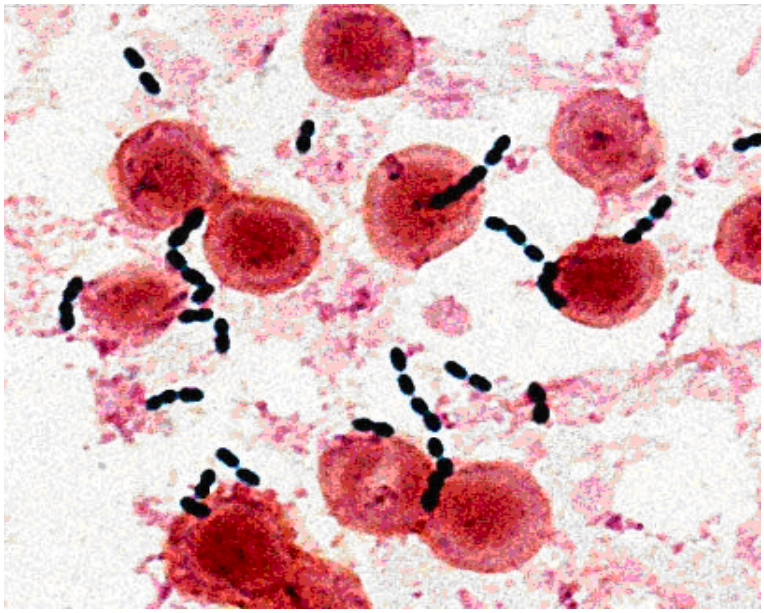


Figure 2.5 Gram-positive cocci of *Enterococcus* spp. in blood culture (28)**.**

2.2.2 Resistance to antimicrobials

The increasing incidence of antimicrobial resistant enterococci is due to the unsuitable use of antibiotic both in agriculture, as growth promoters, and in human health care system. Antibiotic resistant enterococci are particularly important problem of treatment in human infection. Studies have shown that certain strains of enterococci are resistant to expensive and potent antibiotics such as vancomycin (29), (30), (31). This is particularly worrisome for the medical community since these antibiotics are given as a last resort to fight severe bacterial infections. Enterococci are naturally resistant to cephalosporines, aminoglycosides (low-level type), polymyxins, lincomycin and clindamycin (mostly). Additionally, enterococci often possess resistance or intermediate susceptibility to quinolones. Furthermore, the bacteria are able to acquire resistances to macrolides, tetracyclines, chloramphenicol, trimethoprim/sulfamethoxazole, rifampicin, aminoglycosides (high-level type) and ampicillin (32). At the end of the 1980s, the first glycopeptide-resistant enterococci (GRE) were detected and infections caused by these strains could hardly be treated.

2.3 Antimicrobial compounds

2.3.1 Mechanisms of Antibiotic action

Antimicrobials are chemical compounds produced by actinomycetes, fungi or bacteria that interfere with some essential bacterial structure. Antibiotics process without effects on eukaryotic host so the infectious agents that can be called having selective toxicity (33). The major classes of antimicrobials may be categorized according to their mechanisms of action's principle including inhibition of bacterial cell wall synthesis, inhibition of bacterial protein and folic acid synthesis, inhibition of nucleic acid synthesis, and disruption of the bacterial cell membrane structure.

2.3.1.1 Inhibition of bacterial cell wall synthesis

The peptidoglycan of cell wall protects the bacteria from bursting in response to the high osmotic strength of cytoplasmic contents relating to the external medium, therefore destruction of the cell wall can lead to bacterial lysis (34). Gram-negative and gram-positive bacteria have a peptidoglycan as a part of their cell-wall structure. The peptidoglycan layer of gram-positive bacteria is generally thicker than that of gram-negative bacteria. Transglycosidase and transpeptidase are enzymes processing for peptidoglycan crosslinking of glycan and peptide strands, respectively, β-lactams target site. The action of β-lactam antibiotics called penicillin-binding proteins (PBPs) (35) apparently attack and acylates the active site of the transpeptidase leading to inactivation of the enzymes. Vancomycin, glycopeptides antibiotics, also targets peptidoglycan by inhibiting cell-wall synthesis. It binds to the D-Ala-D-Ala portion of pentapeptides and thereby keeps the substrate away from transglycosidase and transpeptidase (34). Binding appears to inhibit both transglycosilation which are and transpeptidation the two final steps in the peptidoglycan synthesis.

2.3.1.2 Inhibition of bacterial protein synthesis

Protein synthesis is catalyzed by ribosomes that are composed of two necleoproteins, 30S and 50S subunits, containing about two-thirds RNA and one-thirds protein (36). The bacterial ribosome is a good target for antimicrobials because it differs appreciably from ribosomes of mammalian cells. This can explains an effectiveness and a selective toxicity of many clinical importance drugs. Many steps involved in protein biosynthesis, including initiation, elongation and termination, can be blocked. Some antibiotics interact with the conserved sequences of 16S rRNA of the 30S subunit such as aminoglycosides and tetracyclines and some antibiotics the 23S rRNA of the 50S subunit such as macrolides, chloramphenicol, lincosamides and quinupristin-dalfopristin) have been found (26, 37).

2.3.1.4 Inhibition of folic acid metabolisms

Sulfonamide is a structure analog of *p*-aminobenzaic cid (PABA), which is essential for bacterial folic acid synthesis. Sulfonamides compete with the PABA for the enzyme dihydropteroate synthetase, and the effect of the sulfonamide may be overcome by adding excess PABA. Sulfonamide inhibits growth of bacteria, but not killing them. Trimethoprim is a folate antagonist. Its similarity is close enough to confuse the relevant bacterial enzyme. Bacterial dihydrofolate reductase is many times more sensitive to trimethoprim than the enzyme in human does (34). The combination of sulfonamide and trimethoprim drugs change them to act as bactericidalactivity while each antimicrobial agent is bacteriostatic when used individually (19).

2.3.1.5 Inhibition of DNA synthesis

This class of antibiotics inhibits bacterial DNA replication that is an essential process for all organisms. Quinolones, work by binding to and inhibiting the activity of topoisomerase II, DNA gyrase and topoisomerase IV, these enzymes involve in an interconversion of a topologically differ form of the DNA. The DNA topoisomerases are categorized into type I and type II. Type I topoisomerase transiently breaks one strand at a time, called a nick, while type II topoisomerase breaks both strands at the same time (38). The new quinolone member continues to be developed and approved,

such as norfloxacin, ciplofloxacin, ofloxacin, and gatifloxacin, also called fluoroquinolones, due to the present of a fluorine substituent (39).

2.3.1.6 Inhibition of RNA synthesis

All organisms need transcription process to decode genetic information from DNA to RNA. Rifampicin or rifampin is a semisynthetic version of rifamycin B isolated from a strain of *Amycolatopsis meditertanea*, formerly known as *Streptomyces mediteranea or Nocardia mediteranea* (34). It works by inhibiting bacterial DNA dependent RNA polymerase and is used against gram-positive bacteria and mycobacterium. Rifampicin inhibits RNA polymerase by binding to the β-subunit of the enzyme at an allosteric site, not at the active site, as shown by resistant mutations in clinical isolates of *Mycobacterium tuberculosis* and *M. leprae* (40). Resistance to rifampicin can be occurred by point mutations in *rpoB* gene (37).

2.3.1.7 Disruption of cell membrane structure

Cell membranes including the outer membrane of gram-negative bacteria, are essential to all organisms. The polymyxins, cationic antimicrobial peptides, are a group of cyclic peptides with a fatty acid chain attached to the peptide an they attack the cytoplasmic membrane of gram-positive and gram-negative bacteria, and outer membrane of gram-negative bacteria (41). The interaction of the cationic peptide and the membrane disrupts membrane organization and causes increased permeability of cell components, finally leading to leads to cell-killing (41).

2.3.2 Development of antimicrobial resistance

Two types of antimicrobial resistance development include intrinsic and acquired resistance. Intrinsic resistance is the natural resistance of bacteria process to some antibiotics due to their cell structures. Organisms with intrinsic resistance are usually exhibited low virulence but they still persist in the environment because they are resistant to so many agents. Bacteria can persist in hospitals that have a high selective pressure by using a lot of antibiotics so multi-drug resistant bacteria are naturally selected as acquire resistance. This resistance can be due to mutation in genetic material or the acquisition of resistance genes from other resistant bacteria. Lateral or horizontal gene transfer (HGT) is a process of genetic material, such as plasmid, integrons and transposons (42) exchange between individual bacteria. The horizontal gene transfer can occur through three possible including conjugation, transformation and transduction. Conjugation are occurred when there is direct cell-cell contact that connection formed by sex-pilus between two bacteria that does not need to be closely related. Transformation is a transfer of small pieces of DNA from the external environment to the bacteria. Transduction is a transfer of genes by a bacteriophage. The transformation and transduction tend to be occured mainly between members of the same species and also between members of very closely related bacteria (26) (Figure 2.6).

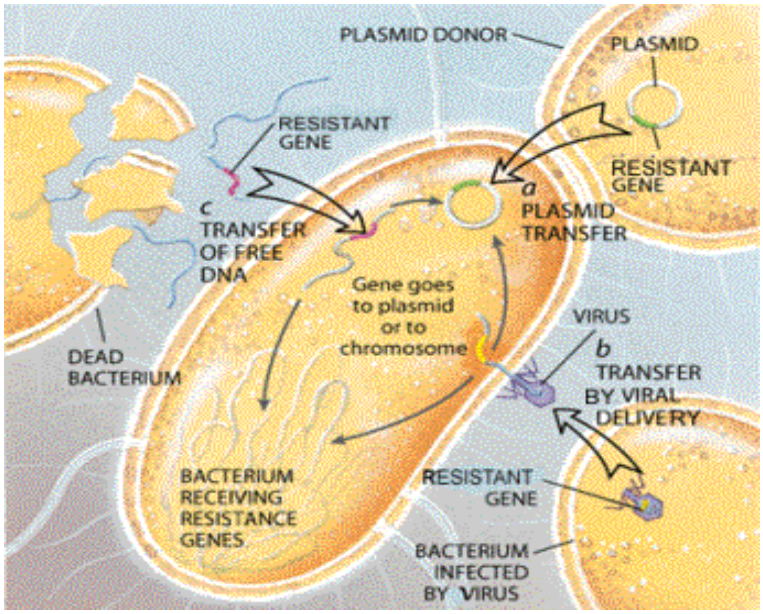


Figure 2.6 Horizontal gene transfer (101).

2.3.3 Mobile genetic elements

2.3.3.1 Plasmids

Transmission of antibiotics resistance, often to several drugs simultaneously, from one bacterium to another is attributed to R factors or plasmids. Plasmids are self-replicating double-strand DNA circles because they contain at least one DNA sequence that serves as an origin of replication, or *ori* (a starting point for DNA replication) which enables the plasmid DNA to be raplicated dependently from chromosome. Plasmids are vary in size between <2 and >100 kb that can code for resistance to antimicrobial agents, disinfectants, heavy metal cations, anions, nucleic acid-binding substances or bacteriocins, and also for metabolic or virulence propoties (43). Large plasmids can carry a *tra* gene complex that enables them to move from one host cell to another by conjugation, through a direct connection formed by a sex pilus. Not all plasmids are capable of self-transfer (26). Plasmids usually act as vecters for transposons and integrons/gene cassettes (43). They may be one copy, for large plasmids, to hundreds of copies of the same plasmid in a single cell, or even thousands of copies, for some artificial plasmids selected for high copy number (such as the pUC series of plasmids) (21).

2.3.3.2 Transposons

Transposons or jumping genes are facilitates a movement of genes in both directions of chromosomes and plasmids, and between bacteria independently of homologous recombination even of different genera. They also vary in their structures and sizes Transposons are flanked by DNA segments known as insertion sequences (IS), which encode the enzyme transposase that catalyzes transposition and provide the ends recognized by transposase when it cuts and pasts the DNA during an insertion event (26). Larger transposons often carry one or more additional genes, the most are antibiotic resistance genes.

2.3.3.3 Integrons

Integron is a genetic element that is similar to transposons but integron is possesses a site, *attI*, at which for addition of DNA in the form of gene cassettes. The 5' segment encodes a site-specific recombinase or called integrase that can facilitate the site-specific recombination events (42) and also the promoter for the expression of the cassette-borne genes (43). Gene cassettes do not have the replication systems or transposition systems, but they move by site-specific recombination. There are several classes of integrons which class I and class II being the most frequently detected (44). Integrons can carry several different genes cassettes therefore they play an important role in a dissemination of multiple antimicrobial resistance genes.

2.3.4 Biochemical mechanisms of resistance

Several mechanisms have been developed by bacteria in order to deal with antibiotics and all require either the modification of existing genetic materials or the acquisition of new genetic materials. Resistance to antimicrobials may enable a bacterium to produce some enzymes that destroy the antibacterial drugs, to modify the drug's target site, to express efflux systems preventing the agents from the reaching its intracellular targets, or to produce an alternative metabolic pathway bypassesing the action of the drug.

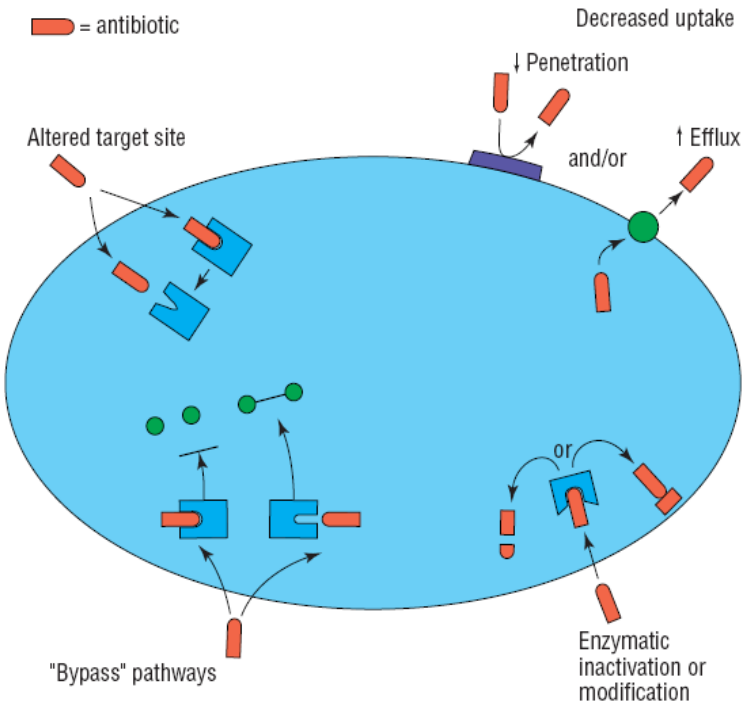


Figure 2.7 Four major biochemical mechanisms of antimicrobial resistance (45).

2.3.5 Antimicrobial agents

2.3.5.1 AMINOGLYCOSIDES

Aminoglycosides were first introduced in 1944. They are broad-spectrum and effective antibiotics with many desirable properties for the treatment of life-threatening infections. The main agents of aminoglycosides are gentamicin, streptomycin, tobramycin, netilmicin, neomycin, amikacin and framycetin . They are trisaccharides with amino groups. Structurally, each of these aminoglycosides contains two or more amino sugars linked by glycosidic bonds to an aminocyclitol ring nucleus (19).

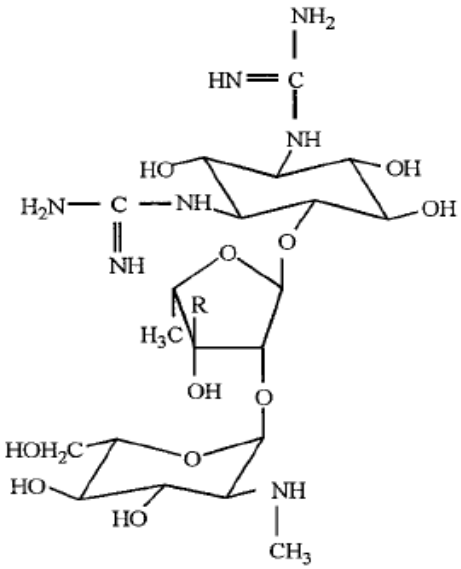


Figure 2.8 Structural formulae of streptomycin and derivatives (46).

Mechanisms of Aminoglycosides Action and Resistance

Aminoglycosides are commonly used in the treatment of the infections by both gram-negative and gram-positive organisms. Their penetration through the cell membrane of the bacterium depends partly on oxygen-dependent active transport by a polyamine carrier system and they have minimal action against anerobic organisms. They bind to the ribosomes and thus interfere with protein synthesis. The bacterial ribosome is the good target of the antibiotics because it differs obviously from ribosomes of mammalian cells. Aminoglycosides act by binding the 30S subunit of the bacterial ribosome. This does not prevent the 30S subunit from binding mRNA and placing a tRNA in the P site. But 50S subunit does not join the 30S to form the active ribosome and no protein synthesis occurs. Aminoglycosides are bactericidal because protein synthesis is essential for continued viability of a bacterium (37).

Resistance to aminoglycosides is widespread. Enzymatic modification is the most common type of aminoglycoside resistance and results in high-level resistance. It is often due to enzymatic inactivation by *N*-acetyltransferases (AAC) which use acetyi-coenzyme A as donor and effect aminofunctions, and *O*-nucleotidyltransferases (adenylyltransferases) (ANT) and *O*-phosphotransferases (APH). Aminoglycoside-resistant strains often emerge as a result of acquiring plasmid-borne genes encoding aminoglycoside-modifying enzymes. Moreover, many of these genes are associated with transposons which aid in the rapid dissemination of drug resistance across species boundaries (47). Several studies have described the association of genes encoding aminoglycoside-modifying enzymes with integrons sequences, including *ant(2")-Ia (aadB)*, *aac(3)-Ia (aacC1)*, *ant(3")-Ia (aadA, aad(3")(9))*, and *aac(6')-Ia (aacA1)*. Other mechanisms include altered ribosome biding sites and loss of permeability. Mutations at the site of aminoglycoside attachment may interfere with ribosomal binding. Resistance to streptomycin can occur by this mechanism since this agent binds to a single site on the 30S subunit of the ribosome (48). Some strains of *Pseudomonas aeruginosa* and other gram-negative bacilli exhibit aminoglycoside resistance due to a transport defect or membrane impermeabilization. This mechanism is likely chromosomally mediated and results in cross-reactivity to all aminoglycosides (49). The level of resistance is moderate. Infective endocarditis due to enterococci with high levels of resistance to aminoglycosides is increasing

commonly. All enterococci have low-level resistance to aminoglycosides because of their anaerobic metabolism. In the treatment of bacterial endocarditis, a beta-lactam drug is also used synergistically to facilitate aminoglycoside penetration into the cell. When high-level resistance occurs, it is typically due to the production of inactivating enzymes by the bacteria. Because of the increasing frequency of this resistance, all enterococci should be tested for antibiotic susceptibility (49).

2.3.5.2 TETRACYCLINES

The tetracyclines discovered in 1940s are antibiotics that inhibit protein synthesis by preventing the attachment of aminoglycocyl-tRNA to the ribosomal acceptor (A) site. Tetracyclines are broad-spectrum agents exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites. The favorable antimicrobial properties of these agents and the absence of major adverse side effects has led to their extensive use in the therapy of human and animal infections (50).

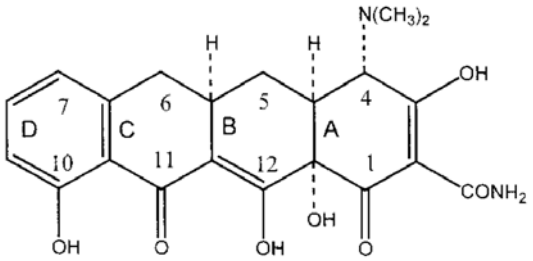


Figure 2.9 Structure of 6-deoxy-6-demethyltetracycline, the minimum tetracycline pharmacophore (50).

Mechanisms of Tetracyclines Action and Resistance

Tetracyclines are compounds consisting of four fused cyclic six membered rings. Tetracyclines probably penetrate bacterial cells by passive diffusion. Tetracycline acts by binding to the 30S ribosomal subunit, resulting in the inhibition of protein synthesis (50). The effect of binding is to misrepresent the A site and prevent the alignment of aminoacylated tRNA with the codon on mRNA. A growing number of bacterial species have acquired resistance to the bacteriostatic activity of tetracycline. In 1989, a describing the nomenclature for tetracycline resistance determinants which employed letters of the English alphabet was published by Levy et al. (51).

To date, at least 24 tetracycline resistance (*tet*) determinants and three oxytetracycline resistance (*otr*) determinants, first found in oxytetracyline- producing *Streptomyces*, have been described and characterized, with new *tet* determinants being identified continually (37). Most of the resistance genes encode for one of the two

important mechanisms of tetracycline resistance, either efflux or ribosomal protection. Protection of the ribosome from the action of tetracycline as a mechanism of tetracycline resistance was discovered in streptococci. Tetracycline resistance can result from the production of a protein that interacts with the ribosome such that protein synthesis is unaffected by the presence of the antibiotic. The determinants *tetM, tetO, tetB(P), tetQ, tetS, tetT, tetW*, and *otrA* confer resistance to tetracycline, doxycycline, and minocycline (37). The *tetX* gene codes for an enzyme which inactivates tetracyclines. Efflux is mediated by energy-dependent efflux pumps. The other important mechanism involves an elongation factor G like protein that confers ribosome protection. Oxidative destruction of tetracycline has been found in a few species. Nevertheless, the enzymatic inactivation of the antibiotic is not thought to be important in nature (52). Efflux determinants from gram-negative bacteria (*tetA* to *tetE, tetG,* and *tetH*) have a common genetic organization that is different from that in gram-positive bacteria. They all contain a structural gene and a repressor gene that are expressed in opposite directions from overlapping operator regions. The gram-positive *tetK* and *tetL* genes encoding tetracycline efflux proteins are regulated by mRNA attenuation in a similar way to that described for gram-positive *erm* genes encoding rRNA methylase and *cat* genes encoding chloramphenical acetyltransferases (37).

2.3.5.3 CHLORAMPHENICOL

Chloramphenicol is an antibiotic derived from the bacterium *Streptomyces venezuelae* and is now produced synthetically. Chloramphenicol inhibits protein synthesis by binding to the 50S ribosomal subunit, inhibits the peptidyltransferase step in protein synthesis and preventing the transpeptidation process of peptide chain elongation (19).

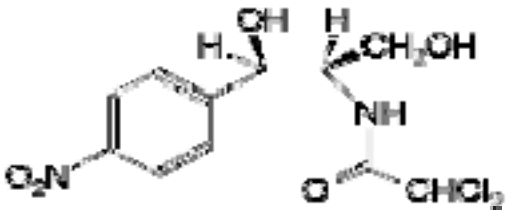


Figure 2.10 Chemical structure of chloramphenicol.

Mechanism of Chloramphenicol Action and Resistance

Resistance to chloramphenicol is generally due to inactivation of the antibiotic by a chloramphenicol acetyltransferase. The *cat* genes of gram-negative and gram-positive bacteria show little homology, and a variety of different enzymes have been described. The gene is most commonly found on plasmids. Sometimes decreased outer membrane permeability or active efflux is observed in gram-negative bacteria (37). Two early studies looked at cross-hybridization of *cat* determinants from staphylococcal plasmids and a group B *Streptococcus* plasmid with streptococal, enterococcal, and pneumonoccal isolates. Although cross-resistance was observed, no clear conclusions about the presence of specific *cat* determinants could be made. The study have been described the use of degenerate primers in a PCR assay to enable the amplification of an internal fragment of the *cat* genes in gram-positive cocci (53). The assay was able to detect three classes of *cat* gene present in staphylococci, *catP*, and a streptococcal *cat* gene. *catQ* was not detected. The six *cat* genes accounted for the chloramphenicol resistance in 12 streptococci tested, but only 3 *cat* genes accounted for the resistance in 10 enterococcal isolates. Another resistance to chloramphenicol may be mediated non-enzymatically through the *cmlA* or *flo* genes, which both

encode putative drug efflux pumps. The *flo* gene is similar in primary structure to *cmlA* and confers resistance to both florfenicol and chloramphenicol. It has previously been shown to be disseminated on large plasmids among genetically diverse strains of *E. coli*. But neither chloramphenicol acetyltransferase nor *cmlA* confers resistance to florfenicol (54). Detection of chloramphenicol resistance determinants has received little attention, because chloramphenicol is little used for the treatment of severe infections. Only a limited number of small-scale studies have used molecular techniques to investigate the distribution of *cat* genes.

2.3.5.4 MACROLIDES

Macrolides have been used since the early 1950s, with erythromycin being the prototypical antibiotic of this class for over 30 years. Their chemical structures consist of a macrocyclic lactone ring attached to two sugar moieties, desosamide and clasinose. Erythromycin is a naturally occurring 14-membered macrolides derived from *Streptomyces erythraeus*.

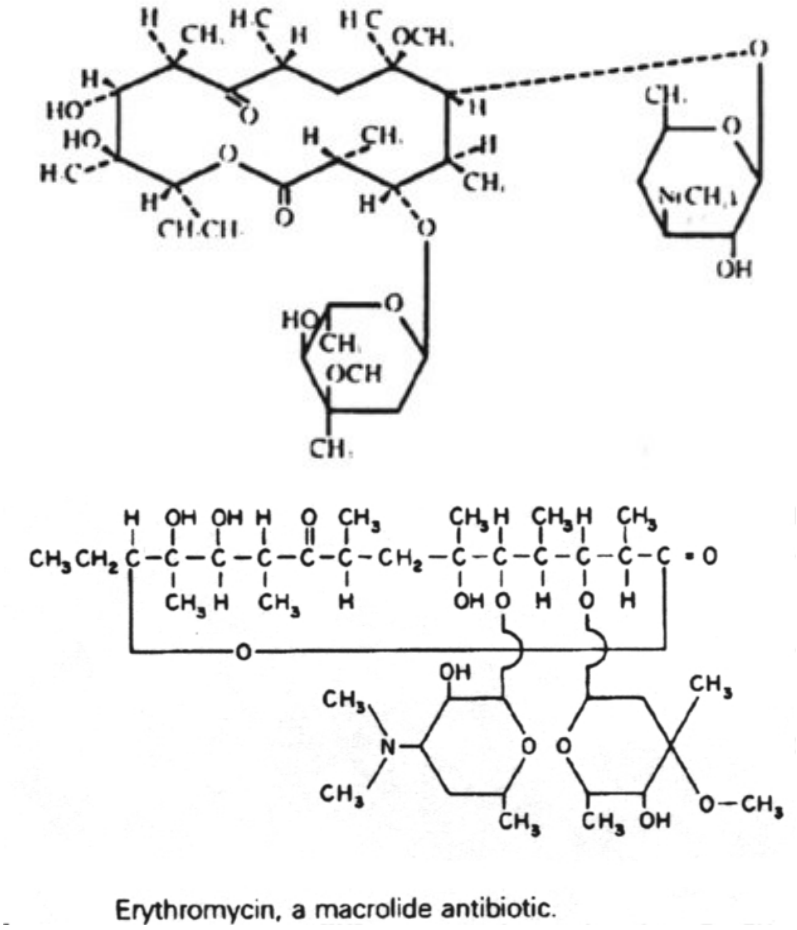


Figure 2.11 Structure of erythomycin, a macrolide antibiotic.

Mechanisms of Macrolide Action and Resistance

Macrolides are generally bacteriostatic agents that inhibit bacterial RNA-dependent protein synthesis. They might be the bactericidal at high drug concentration and against a low inoculum of bacteria. These agents bind reversibly to the 23S rRNA of the 50S ribosomal subunits of the susceptible organisms, thereby, blocking the translocation reduction of polypeptide chain elongation. These present of rRNA methylases is the primary mechanism of macrolide resistance and confer macrolide-lincosamide-streptogramin B (MLS_B) coresistance. Efflux is also now common mechanism of resistance of streptococci. Other uncommon mechanisms of resistance to macrolides include the production of macrolide- inactivating enzymes (esterase, phosphorylase, glycosidase) and mutation in 23S rRNA and the ribosomal protein (19).

An ATP-dependent efflux system that pumps macrolides out of the cell has been found in *Staphylococcus* species. This efflux system works only on some of the macrolides. The deduced amino acid sequence of the gene share homology with known ATP-dependent transport proteins. The study by Schmitz et al.(55), 75 European erythromycin-resistance *Enterococcus faecium* isolates were screen by PCR of resistance gene. *ermB* was the most prevalent resistance found, follow by *ermA* (93 and 4%, respectively). The combination of *ermA* and *ermB* was detected in two of 75 isolates (3%). The recent study have analyzed 113 erythromycin-resistance enterococci isolated from human and animal origins and found the *ermB* to be prevalence in 88% of the enterococci test (56).

2.3.5.5 STREPTOGRAMINS

Antibiotics of the streptogramin class are produced predominantly by members of the genus *Streptomyces* as a complex of structurally unrelated group A and group B compounds. Group A compounds are M (macrolactones). Group B components are (cyclic hexadepsipeptides).

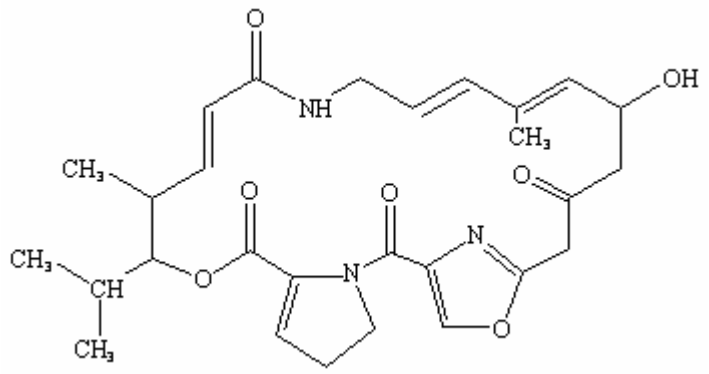


Figure 2.12 Chemical structure of streptogramin A.

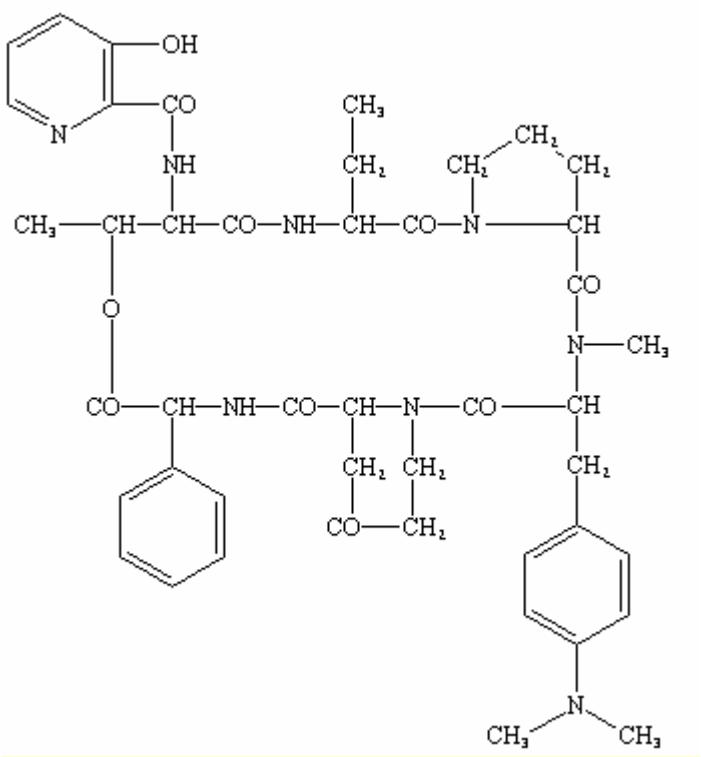


Figure 2.13 Chemical structure of streptogramin B.

Mechanisms of Streptogramin Action and Resistance

Group A and group B compounds are produced jointly in nature and bind synergistically to different target sites on the 50S ribosomal subunit to inhibit bacterial protein elongation. Although the A and B compounds are individually bacteriostatic to sensitive strains, the mechanism of streptogramin inhibition is synergistic and is frequently bactericidal (57). The streptogramins block the translation of mRNA into protein. Both group A and group B molecules bind to the peptidyl-transferase domain of the bacterial ribosome. The group B molecule stimulates the dissociation of peptidyl-tRNA from the ribosome and may interfere with the passage of the completed polypeptide away from the peptidyl-transferase centre. The group A molecule inhibits the elongation of the polypeptide chain by preventing both the binding of aminoacyl-tRNA to the ribosomal A site and the formation of the peptide bond. When the two types of molecule are used in combination, the binding of the group A molecule alters the conformation of the ribosome such that the affinity of the ribosome for the B molecule is increased (58).

Resistance to streptogramins is mediated by: enzymatic modification of the antibiotic; active transport or efflux mediated by an ATP-binding protein; and alteration of the target site. The most commonly known resistance to streptogramins is the MLS_B resistance conferred by the *erm* genes (59). These genes encode an enzyme that dimethylates an adenine residue in the 23S ribosomal RNA, results in decreased binding of macrolides, lincosamides and streptogramins of group B (60). In 1999, the Food and Drug Administration approved the use of the streptogramin and quinupristin/dalfopristin (Synercid) to treat infections due to vancomycin-resistant *E. faecium*. The use of analogues of human antimicrobials in food-animal production has given rise to concerns involving the development and transmission of resistance among bacterial pathogens (60). In the case of streptogramin antimicrobials, virginiamycin, an analogue of quinupristin/dalfopristin, has been used in animal production for >20 years (61).

In enterococci resistance to streptogramin A compounds is mediated via related acetyltransferases *vatD* and *vatE*. Resistance against streptogramins B is either encoded by the widespread *ermB* gene cluster conferring resistance to macrolide-lincosamide-streptogramin B antibiotics or via expression of the *vgbA* gene, which

encodes a staphylococcal-type lactonase. *E. faecalis* is intrinsically resistant to streptogramins (62).

2.3.5.6 SULFONAMIDES

Sulfonamides were the first effective systemic antimicrobial agents used in United Stats; they were introduced during the 1930s (33). They are derived from sulfanilamide, which has chemical similarities to *p*-aminobenzoic acid, a factor essential for bacterial folic acid synthesis. Various substitution at the sulfonyl radical attached to the benzene ring nucleus enhance the antibacterial activity and also determine the pharmacologic properties of the drug.

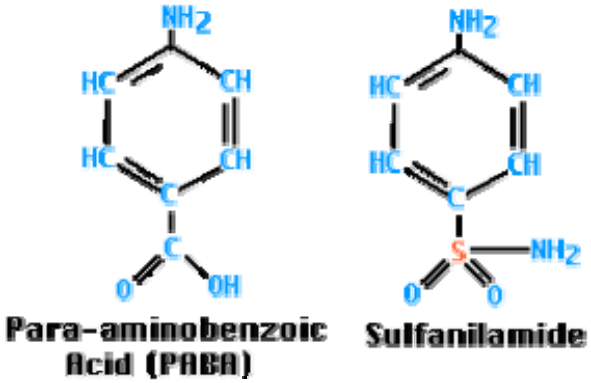


Figure 2.14 Sulfonamides, sulfanamides are structurally similar to *para*-aminobenzoic acid (PABA).

Sulfonamides competitively inhibit the bacterial modification of *p*-aminobenzoic acid into dihydrofalate reductase. This sequential inhibition of folate metabolism ultimately prevent of synthesis of bacterial DNA. Folate is required for the synthesis of the precursors of DNA and RNA both in bacteria and mammals, but mammals gain their folic acid from food whereas bacteria need to synthesize it. Human purine synthesis is not affected significantly by sulfonamide (19). The current generation of sulfonamides is sulfamethoxazole, which is used together with trimethoprim The bactericidal effect of combination of sulfonamides and trimethoprim remains clinically relevant in the treatment of many bacterial infection, such as urinary and respiratory tract infections.

Mechanisms of Sulfonamide Action and Resistance

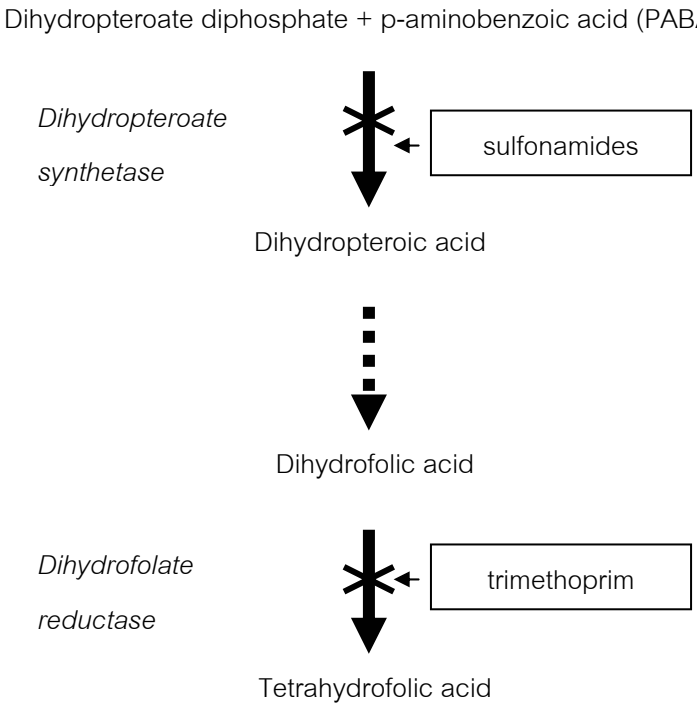


Figure 2.15 Mechanisms of sulfonamide action**.**

Resistance to sulfonamides arises from mutations in the enzymes inhibited by these antibiotics. The mutation form of the enzyme no longer binds the antibiotic with a higher affinity than its natural substrate. Mutation conferring to resistance to sulfonamides or to trimethoprim occurs rather frequently, but simultaneous double mutations that confer resistance to both types of antibiotic occur only rarely. For this reason, a combination of trimethoprim and one of the sulfonamides is currently used for antibacterial therapy. DANMAP *et al.* (2003) have been reported the potential transfer of sulfonamide resistance *E. coli* from animals, directly or via handling of raw meat, to humans is therefore undesirable Sulfonamide resistance is often encoded by *sulI* and *sulII* in *Enterobacteriaceae*. The *sulI* genes are part of the 3' conserved segment of Class 1 integrons, which are the most frequently detected integrons in *Enterobacteriaceae* (42).

2.3.5.7 QUINOLONES

In the early 1960s, the discovery of the naphthyridine agent, nalidixic acid, was known for the series of quinolone. Nalidixic acid was first introduced as a therapeutant for urinary tract infections caused by gram-negative organisms. The second generation of quinolone named fluoroquinolone (Figure 2.14), such as ciplofloxacin and ofloxacin, resulted from fluorination, primarily at the position C6 (39). This new group of antibiotics have poor activity against streptococci and anaerobes, which constitute a majority of microflora of the mouth, colon and vaginal tract. Therefore, they are less likely than others agents to disrupt the normal flora (26). Quinolone are commonly used in both clinical and veterinary medicine, either for medical reasons or as growth promoters. Their extensive use has results in rapidly develop resistance to these agents. The quinolone resistance levels are higher for the narrow-sprectrum quinolone nalidixic acid than for the board-spectrum fluoroquinolones reaching up to 15-20% for nalidixic acid and 10% for fluoroquinolone in several surveys (63).

Mechanisms of Quinolone Action and Resistance

The quinolone targets are different in gram-negative and gram-positive. In gram-negative organism, DNA gyrase seem to be the primary target for all quinolones. In gram-positive organism, topoisomerase IV or DNA gyrase is the primary target depending on the on the quinolone considered; i.e., the quinolone structure determines the mode of antibacterial action. Thus, the primary target seems to depend on the bacterial species as well as on the quinolone structure.

Quinolones act by binding to complexes that form between DNA and gyrase or topoisomerase IV. Shortly after binding, the quinolones induce a conformational change in the enzyme. The enzyme breaks the DNA and the quinolone prevents re-ligation of the broken DNA strands. The enzyme is trapped on the DNA resulting in the formation of a quinolone– enzyme–DNA complex. Quinolone–gyrase–DNA complex formation rapidly inhibits DNA replication and is consistent with gyrase acting ahead of replication forks. However, inhibition of replication by quinolone–topoisomerase IV–DNA complexes occurs slowly, consistent with the enzyme being located behind the replication forks. Complex formation reversibly inhibits DNA and

cell growth and is thought to be responsible for the bacteriostatic action of the quinolones. Lethal action is not reversible and is thought to be a separate event from complex formation. Higher quinolone concentrations are required to kill cells rather than to inhibit growth or form complexes and some quinolones inhibit growth better than others but are less effective at killing (39)

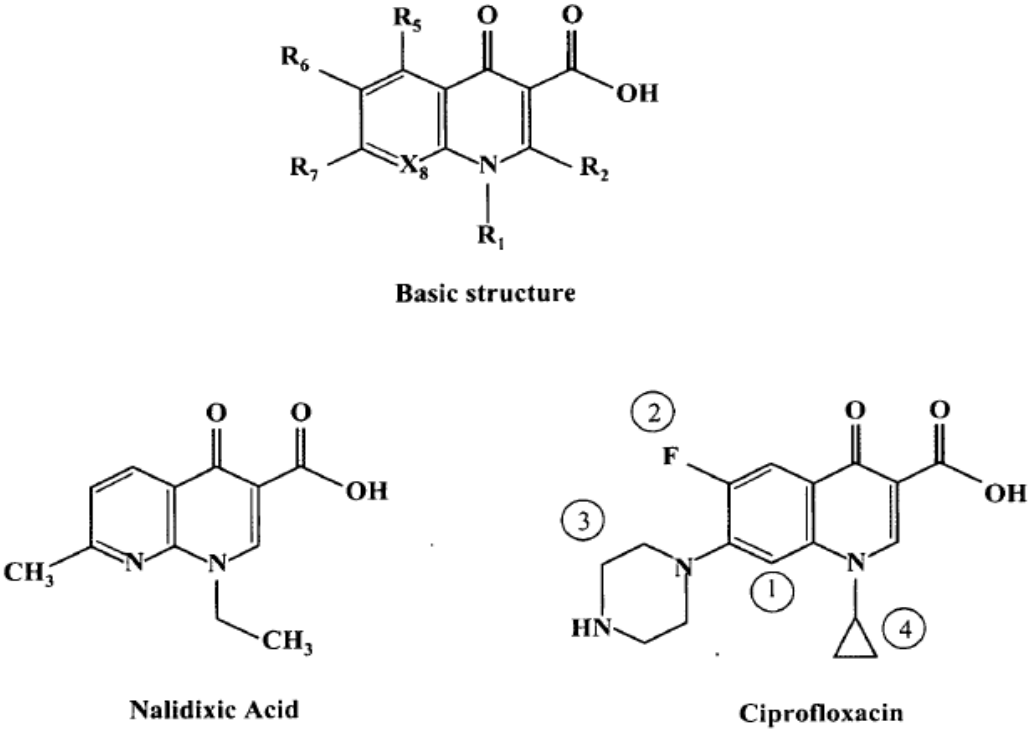


Figure 2.16 The pharmacophore change from nalidixic acid to ciprofloxacin.
1. Modification of naphthyridone into quinolone reduces plasma protein binding.
2. Addition of the fluorine atom was shown to increase quinolone activity against DNA gyrase and facilitate penetration into the cell. 3. Introduction of a piperazine results in a longer half-life. 4. Replacement of the N-1 ethyl group by cyclopropyl group can enhance potency against gram-positive and gram-negative bacteria (39).

Targets for the quinolone agents

DNA gyrase

DNA gyrase is a tetramer of two A and two B subunits, encoded by the *gyrA* and *gyrB* genes, respectively. *gyrA* and *gyrB* are located at 48 and 83 min, respectively, on the *E. coli* genome, but in some bacteria they are adjacent to each other and *oriA*; this configuration is known as the quinolone resistance determining region (QRDR). DNA gyrase is responsible for introducing negative supercoils into DNA and for relieving topological stress arising from the translocation of transcription and replication complexes along DNA. It acts by wrapping DNA into a positive supercoil and then passing one region of duplex DNA through another via DNA breakage and rejoining. This is an ATP-dependent process. In the presence of ATP, the process is driven forward, increasing supercoiling. In the absence of ATP, the process is driven back again, relaxing the DNA. Keeping the DNA chromosome wound into loops facilitates the movement of replication forks (64).

Topoisomerase IV

Topoisomerase IV is a homologue of DNA gyrase, comprising four subunits, two of C and two of E, encoded by the *parC* and *parE* genes, respectively. The topoisomerase IV locus was described in 1990. The reaction mechanism of topoisomerase IV is similar to that of gyrase but topoisomerase IV binds to DNA crossovers rather than wrapping DNA. Topoisomerase IV is primarily involved in decatenation, the unlinking of replicated daughter chromosomes (64).

Quinolone resistance in *E. coli*

Alteration of target enzymes is the most dominant factors in expression of quinolone resistance. In *E. coli*, the most extensively studied organism, amino acid substitutions involved in the development of quinolone resistance have been described for *GyrA/GyrB* and *ParC/ParE*. The small region from codons 67 to 106 of *GyrA* in *E. coli* was specified the quinolone resistance-determining region (QRDR) (Table 2.1).

Although quinolones are thought to interact primarily with the A subunit of DNA gyrase, mutations have also been discovered in the B subunit which also confer quinolone resistance in some species such as *E. coli*. However, the frequency of *gyrB* mutations, compared to *gyrA*, has been shown to be relatively low in most species (65).

Table 2.1 Mutations described in *GyrA* subunits of quinolone-resistance strains of *E. coli* (65).

Codon[a]	Wild type	Mutant
GyrA		
51	**Ala**	**Val**
67	**Ala**	**Ser**
81	**Gly**	**Cys, Asp**
82	**Asp**	**Gly**
83	**Ser**	**Leu, Trp, Ala, Val**
84	**Ala**	**Pro, Val**
87	**Asp**	**Asn, Gly, Val, Tyr, His**
106	**Gln**	**Arg, His**

a Mutations in other codons, such as codon 93, have been described, but their role in development of resistance to quinolones remains unclear

Mechanisms of quinolone resistance in *E. coli*

To date, two major chromosomally mediated mechanisms of quinolone resistance are established: alterations in the targets of quinolone and decreased accumulation inside the bacteria due to impermeability of the membrane and/or over expression of efflux pump systems. Moreover, the mobile elements have the potential fro horizontal transfer the *qnr* gene which confers the resistance to quinolone (39).

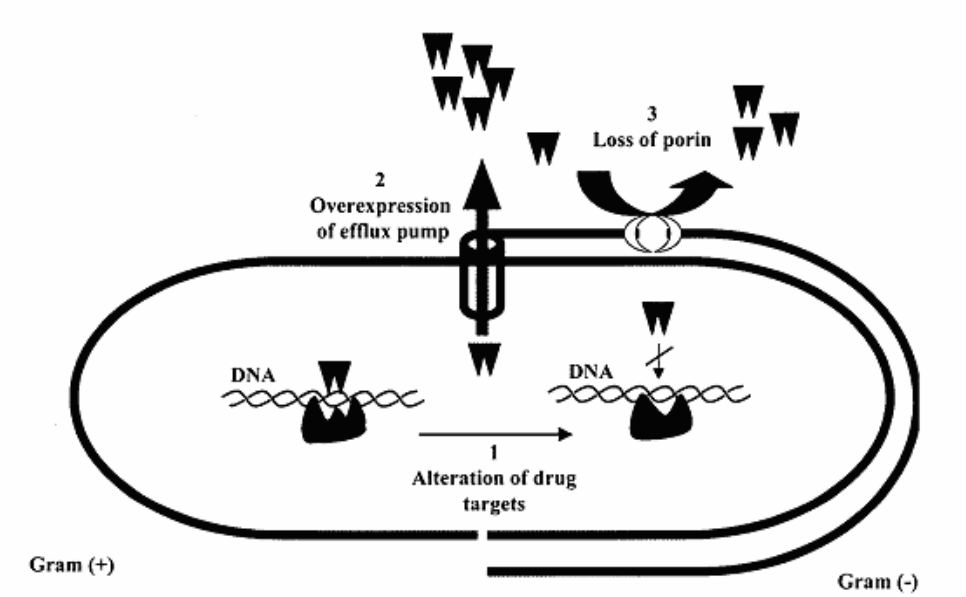
Drug target alternations

Quinolone antibiotics exert their antibacterial effects by inhibiting the type II topoisomerases, DNA gyrase and topoisomerase IV, the major targets of quinolone because mutation of these gene result in antibiotic resistance. Resistance of quinolone appears to be caused mainly by alteration in *gyrA* gene of the DNA gyrase and in the *parC* gene of the topoisomerase IV. Multiple mutations occur within a single chromosome gene, resulting in mutants associated with variable MICs.

Different substitutions of one or several amino acids in the *gyrA* gene result in a wild range of ciprofloxacins MIC (from 0.32 to≥256 μg/mL). Alterations described in the *GyrA* of *E. coli* are predominantly in the so-called quinolone resistance determining region (QRDR), between positions 67 and 106 (Table 2.1) near the Tyr122, which binds to transiently cleaved DNA. Recently, position 51, a region outside the QRDR, has been proposed as a novel point mutation resulting in decreased susceptibility to the quinolones.

Mutations in codons number 67, 81, 82, 83, 84, 87 and 106 of *gyrA* have been observed to be responsible for the development of quinolone resistance in *E. coli.* However some of these mutations within the QRDR (e.g. in *E. coli* mutations at positions 67, 82 and 106), have only been described in laboratory obtained quinolone-resistant mutants (39). A similar QRDR has also been reported in *parC*. According to studies of mutant *E. coli* selected *in vitro*, mutations in gyrA result first in substitution of Ser83 follow by Asp87. Results of surveys of mutant *E. coli* found in vivo appear consistent with the stepwise occurrence of mutation observed *in vitro*; nearly all single-site mutants have substitutions at Ser83 and most isolates with substitutions at Asp87 also have substitution at Ser83 (39).

It should be pointed out that alterations in the QRDR of DNA gyrase and topoisomerase IV are normally associated with higher MICs than for to wild-type strains. Nevertheless, such alterations do not always increase the MICs, especially of the newer fluoroquinolones, such as gemifloxacin, gatifloxacin, and moxifloxacin, above currently used NCCLS breakpoints.



Major molecular mechanisms of fluoroquinolone resistance: (1) alterations of target enzymes (DNA gyrase and/or topoisomerase IV) to prevent the drug binding; (2) overexpression of efflux pumps to decrease accumulation of drugs; and (3) loss of porins to reduce permeability of drugs in gram-negative bacteria. = fluoroquinolones; = target enzymes, DNA gyrase, and/or topoisomerase IV; = mutated target enzymes.

Figure 2.17 Molecular mechanisms of fluoroquinolone resistance (39).

Decreased uptake

Decreased quinolone uptake may be associated with two factors: an increase in the bacterial impermeability to these antibacterial agents or the over expression of efflux pumps. Although mutations in drug targets are the primary mechanism for fluoroquinolone resistance, increasing the activity of efflux pump also contributes to resistance, especially for high level resistance. Efflux pumps, involved in quinolone resistance also confer resistance to other non-structure related antibiotics. The multiple antibiotic resistance (mar) locus has been reported to be responsible for quinolone resistance of *E .coli*.

Quinolones may cross the outer membrane in two different ways: through specific porins or by diffusion through the phospholipid bilayer. The degree of diffusion of a quinolone is greatly associated with, and dependent on, its level of hydrophobicity. All quinolones may cross the outer membrane through the porins, but only those with a greater level of hydrophobicity may diffuse through the

phospholipidbilayer. Thus alterations in the composition of porins and/or in the lipopolysaccharides may alter susceptibility profiles. In lipopolysaccharide-defective mutants, increased susceptibility to hydrophobic quinolones has been described, without alterations in the level of resistance to the hydrophilic quinolones. Alterations in membrane permeability are usually associated with decreased expression of porins. This has been described both in *E. coli* and other Gram-negative bacteria (65).

Transferability of quinolone resistance

There have been reports describing the presence of quinolone resistance genes on plasmids. However, in the strains described by Munshi *et al.* (66). Recently, a plasmid in *Klebsiella pneumoniae* has been described, capable of conferring quinolone resistance when transferred to a recipient strain. Tran and Jacoby (67) have demonstrated that the plasmid contains a novel gene from the USA, which they named *qnrA*, that encodes a protein of 218 amino acids belonging to the pentapeptide repeat family. The gene product protects the DNA gyrase from quinolone inhibition, although its effect on topoisomerase IV is unclear. This gene is flanked by ORF513, an ORF previously identified in some integrons, suggesting that the *qnr* gene may be located within an integron. The presence of *qnr* increases the resistance to nalidixic acid and fluoroquinolones by four to eight folds. The low prevalence of this gene was described in a long series of Gram-negative microorganisms, mainly *K. pneumoniae* and *E. coli*, from nineteen different geographical origins around the world. The *qnr* gene was only found in six strains in 1994 and four *K. pneumoniae* and the *E. coli* in 1995, which all of them collected from the same geographical origin at University of Alabama in the USA. Although no studies of clonality among the *K. pneumoniae* strains was carried out. The exact mechanism of DNA gyrase protection conferred by *qnr* has been established (65). *qnrA2* is another *qnrA* determinant that differ from *qnrA1* by a few amino acid substitutions. *qnrA2* has been identified from *K. oxytoca* isolated from China (GenBank accession number AY675584) (63). Recently, *qnrB* and variants have been identified from *Citobacter koseri, Escherichia coli, Enterobacter cloacae* and *K. pneumoniae* from the USA and India with several isolates carrying both *qnrA*-like and *qnrB*-like genes (68). A *qnrS* determinant was

identified from a *Shigella flexneri* isolates in Japan (69). QnrB and QnrS also have the pentapeptide repeat family of proteins share 40% and 59% amino acid identity with QnrA, respectively (63).

2.3.6 Detection of mutations of quinolone resistance

Various methods have been reported to detect point mutations in target genes including sequence-specific oligonucleotide probe hybridization, sequencing of the target genes, RFLP, radioisotopic or non-radioisotopic SSCP analysis, mismatch amplification mutation assay PCR and allele-specific PCR in combination with RFLP. The previous study, Tokue *et al.* (70) studied 36 of *S. aureus* isolates by non-radioisotopic SSCP (nRI-SSCP) for the presence of point mutations in the *gyrA* gene. Direct DNA-sequencing analysis of the PCR amplified DNA fragments confirmed the results obtained by nRI-SSCP. The authors concluded that the use of the nRI-SSCP method allowed relatively rapid analysis of DNA from a large number of strains. Yoon-Hee Park *et al.* (71) analyzed the mutation in the *gyrA* and *mar* gene of *E. coli* from the patients with leukemia who received the quinolone as the regimen of selective gut decontamination (71). The PCR-SSCP analysis revealed various mobility patterns with various conformational changes in QRDR and there were possibly the result of at least one point mutation in that area . Several investigators have applied PCR-SSCP analysis to detect mutations in *gyrA* and also other antimicrobial resistance genes in *E. coli*. Alonso *et al.* designed a non-radioactive PCR–SSCP with a single PCR step to differentiate point mutant derivatived from wild-type bla_{TEM} genes in *E. coli*. The author concluded that PCR-SSCP with a single PCR step was the suitable tool for rapid detection of genes encoding TEM- and TEM-derived β-lactamases (67). Thus, PCR-SSCP analysis is a simple and useful method for the detection of point mutations associated with antimicrobial resistance.

2.3.7 Detection of antimicrobial resistance genes

The genetic of antimicrobial resistance has been explained completely or in part for many organism-antimicrobial agent combinations. As a result, molecular methods for assessing antimicrobial resistance have been developed, and many of these are being used or become the part of standard testing in clinical microbiology laboratories. Polymerase chain reaction (PCR) assay has been the most commonly used nucleic acid amplification technique in the detection of antimicrobial resistance genes and their genetic support. Convential PCRs, defined as a separate amplification and post PCR detection assays, have been described for most resistance determinants. Several reports have described the use of these techniques for detection of resistance determinants and surveillance of antimicrobial resistant bacteria. For example, the newer aminoglycoside genes reported in gram positive organisms are those encoding phosphotransferase, e.g., *aph*(2")-*Ib* and *aph*(2")-*Ic,* which mediate high-level gentamicin resistance in enterococci, and well-known streptomycin resistance gene of gram negative bacteria (*aadA*) (7). Vancomycin resistance is mediated by several different determinants, including *vanA, vanB, vanC, vanD, vanE* and *vanG* can be differentiated and tracked the epidemiologic spread. DNA probe to the *cat* genes commonly found in gram negative organisms (*catI, catII* and *catIII*) and the *catP* and *catQ* genes from *Clostridium difficile* and *C. perfringens* have been described. There are two major sulfonamide resistance genes, *sulI* and *sulII* (72). Both have been cloned, sequenced and proved by Radstrom *et al.* (73). For tetracycline resistance, PCR assays have been developed for the *tetA, tetB, tetC, tetD, tetE, tetF, tetH, tetK, tetL, tetM, tetN, tetO, tetQ, tetS, tetU* and *tetV* determinants (19), (74).

Other significant technical developments include multiplex PCR assays using more than one primer set for simultaneous detection of several antimicrobial resistance genes. Multiplex PCR was used to be the first molecular assays to detect glycopeptide resistance in enterococci (75). Primers were designed for the simultaneous detection of *vanA*, *vanB*, and *vanC-1* and another primer set for detection of *vanC-2* and *vanC-3* in the multiplex PCR. Also in that year a second set of primers specific for *vanA*, *vanB*, and *vanC-1* was published by Miele *et al*. (76). Two years later a third set of primers for useing in a multiplex PCR was published by Patel et al.(77). Genomic DNA was isolated and two multiplex PCRs (primer set for

ermA, *ermB*, and *ermC*, together with *msrA/msrB*, as well as a primer set for *ereA* and *ereB* in a second separate PCR) were performed as described by Sutcliffe *et al*. (78). After confirmation of the presence of an *erm* gene, single PCRs were performed to verify the class of the *erm* gene, either *ermA*, *ermB*, or *ermC*. The most prevalent resistance gene in *S. aureus* was *ermA* (67%), followed by *ermC* (23%) and *msrA/msrB* (6%).

CHAPTER III
MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment and Instrument

3.1.1.1 Autoclave, Model Hiclave HV, Becthai, Thailand.

3.1.1.2 Incubator for 35° C, Model Jermaks, USA.

3.1.1.3 Shaking incubator, J.P. SELECTA, SPAIN.

3.1.1.4 Electrophoresis sets (horizontal) & power supply, ThermoEC, USA.

3.1.1.5 Electrophoresis sets (vertical) & power supply, BIO-RAD, USA.

3.1.1.6 Safty carbinet, Model BH 120, GELMAN SCIENCES.

3.1.1.7 Sonicator, Model 450 SONIFIER, BRANSON ULTRASONICS, USA.

3.1.1.8 Freezer, Model Forua-86C ULT Freezer ThermoEC, USA.

3.1.1.9 Hot air oven, MEMMERT, WEST GERMANY.

3.1.1.10 Waterbath, Model 6032011, J.P. SELECTA, SPAIN.

3.1.1.11 PCR, PTC200, DNA Engine BIO-RAD

3.1.1.12 Centrifuge, Model Allegra X-12R, Beckman coulter.

3.1.1.12 Hybridizer, Model HB-1000, UVP laboratory, Upland, CA, USA.

3.1.1.14 Automatic pipettes

2-20 µl, BIO-RAD, USA.

20-200 µl, BIO-RAD, USA.

100-1000 µl, BIO-RAD, USA

3.1.2 Chemical and biological reagents

3.1.2.1 Reagents for genomic DNA extraction

- Normal saline
- Phenol
- Isopropanol
- 70% ethanol

3.1.2.2 Reagents for PCR and agarose gel electrophoresis

- 10X PCR buffer, Fermentus, USA.
- Molecular weigth marker, Fermentus, USA.
- $MgCl_2$, Fermentus, USA.
- dNTP mix, Fermentus, USA.
- *Taq* polymerase, Fermentus, USA.
- 6x loading dye, Fermentus, USA.
- Agarose
- 1x TAE buffer

3.1.2.3 Reagents for dot blot hybridization

- DIGHigh Prime DNA labeling kit (Roche Diagnostics GmbH, Germany).
- Klenow fragment, Fermantus, USA.
- Sodium Hydroxide (NaOH)
- Maleic acid (Sigma)
- Tween20 (Sigma)
- Sodium dodecyl sulfate (SDS) (Sigma)

3.1.2.4 Reagents for PCR-SSCP

- 10x TBE
- 40% acrylamide: bisacrylamide stock solution (37.5:1)
- 10% (w/v) Ammonium persulfate
- SSCP loading dye
- Fixing solution (10% acetic acid)
- 0.1% Silver nitrate solution
- Developing solution

3.2 Methods

3.2.1 Source of bacterial isolates

Totally 313 isolates of *E. coli* and 258 isolates of enterococci from previous studies collection (79, 80), isolate during March to June 2005, were studied. The sources of bacterial isolates were faecal samples of pets, including dogs and cats, humans (pet's owners), fresh vegetables, edible ice, chicken cecal contents collected in Bangkok and its vicinity. *E. coli* isolates originated from rivers of Chaopraya river, Morn canal, Chuk Pra canal, Bangkoknoi canal and BangkokYai canal were collected during February to March 2006. The susceptibility testing of the isolates from humans, pets, vegetables, ice and chickens have been tested by disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) recommendation. In *E. coli,* antimicrobial disks were ampicillin (10 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg), tetracycline (30 µg) and trimethoprim-sulfamethoxazole 1.25/23.75 (25 µg) were used. Antimicrobial susceptibility testing of enterococci also have been examined with ampicillin (10 µg), vancomycin (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), erythromycin (15 µg) and gentamycin (10 µg or 120 µg).

3.2.2 Genomic DNA extraction

Genomic DNA of bacterial isolates were extracted by a modified of phenol-chloroform methods. *E. coli* and enterococci from stock cultures were grown overnight in LB broth and TSB, respectively. For *E. coli*, three millilitres of the overnight culture, in microcentrifuge tubes, were spun down at 10,000 rpm for 1 min. The supernatant were decanted and the pellets were resuspended in 0.5 ml NSS by vortexing. The cold phenol (0.5 ml) were added in the tube and inversely mixed. The next step, the tube was spun at RT at 10,000 rpm for 5-10 min. The supernatant were transferred into the new tube and the cold isopropanol (0.6 ml) were added for precipitation of DNA. The pellet were washed and dissolved in 60 µl of sterile water and kept at 4ºC until used.

A process of DNA extraction of enterococci was similar to the method of *E. coli* extraction except in the step of bacterial cell lysis. Before adding a cold isopropanol, the pellets of enterococci were suspended in 557 µl of 10 mM Tris-1 mM EDTA, and 10 µl of a 50 mg/ml solution of egg white lysozyme and were incubated at 37ºC for 30 min. The bacteria were lysed by adding 30 µl of 10% of SDS and 3 µl of 20 mg/ml of proteinase K solution. The solutions were mixed well before incubating at 30 min and then continued with a step of the DNA extraction by using of *E. coli.*

3.2.3 Dot blot hybridization and PCR

3.2.3.1 Preparation of DNA probes

The 10 ng to 3 µg purified DNA fragments was put in a PCR tube. Then, these fragments were denatured at 95ºC for 10 min and immediately cooled on ice. Two microlitter of hexanucleotide mixture, 2 µl of 10x dNTP labeling mixture and 1 µl of Klenow fragment (1 U/µl) were added to the DNA mixture and made up to 20 µl with deionized water. The reactions were incubated at 37ºC overnight and stopped by heating at 65ºC for 10 min or adding 2 µl of 0.2 mM EDTA. The labeled DNA was kept in -20 ºC until used. The DNA labeling efficiency testing was performed according to the manufacturer's instruction.

3.2.3.2 Preparation of the whole cell DNA and dot blot filter for hybridization

Each bacterial isolates was inoculated in 96-wells microtitre plates containing 200 µl of LB broth and incubated at 37ºC until a heavy growth (16 h or overnight for *E. coli* and 24 to 48 h for *Enterococcus* spp.) After incubation, the cells were pelleted by centrifugation (Beckman coulter) at 2000 x g for 10 min. Supernatant was discharged and then the pellet was resuspended and lysed by adding 10 µl of the 1x TE buffer and 5 µl of 10% SDS was added and mixed. In lysis step of entercocci cells, the pellets in each well were suspended in 37.13 µl of 10 mM Tris-1 mM EDTA, and 0.67 µl of a 50 mg/ml solution of egg white lysozyme and were incubated at 37ºC for 30 min. The bacteria were lysed by the addition of 2 µl of 10% of SDS and 0.2 µl of 20 mg/ml proteinase K solution. The solutions were mixed well before incubating 30 min and were then continued with the next step, as *E. coli*. After cell

lysing, 50 µl of 0.5 M NaOH and 1.5 M NaCl were added and the microtitire plates were centrifuged again for 10 min in the bench centrifuge. Three to five microlitters of supernatant or 1.5 µl of chromosomal DNA of each strain was applied to the nylon membrane (Hybond N^+ membrane). The transferred DNA was dried at RT (25ºC) and fixed to nylon membrane by putting on the filter paper and were then soaked with 0.4 N NaOH for 20 min. The membranes were rinsed by using 2xSSC and pre-hybridized or kept in 4ºC until used.

3.2.3.3 Dot blot hybridization

The membranes were put into hybridization bottles pre-hybridized in 20 ml pre-hybridization solution at 42ºC for at least 1 h in hybridizer. Hybridization solution was prepared before hybridization step by heating 20 ml of hybridization solution containing 19 µl of DNA probe at 95ºC for 10 min (68ºC for 10 min for reused probe). The pre-hybridization solution was decanted and replaced with pre-heat probe hybridization solution at 42ºC overnight. After hybridization, the probe solution was decanted and stored at -20ºC for reused. The membrane filters were washed twice in 20 ml prewarmed 2xSSC containing 0.1% SDS at RT under constant shaking followed by washing twice with prewarmed 0.5xSSC containing 0.1% SDS at 65ºC under constant shaking. The filters were washed briefly in 10 ml of 1x Maleic acid buffer and were then incubated in 1x blocking solution for 30 min at RT. After incubation, the membranes were washed twice in washing buffer at RT followed by incubated with anti-digoxigenin-Fab fragment-Alkaline Phosphatase conjugate (diluted 1:5000 in 1x Maleic acid buffer, pH 7.5) for at least 30 min at RT. The membranes were washed 3 times with washing buffer for 15 min to remove unbound antibody-conjugated at RT. The filters were equilibrated by detection buffer at RT and were then incubated in the dark at RT using the colour detection solution and then, the colour reaction was stopped by tap water followed by soaking in the 1x TE buffer.

3.2.4 Multiplex PCR assay

3.2.4.1 PCR oligonucleotide primers

According to the dot blot hybridization results of resistance genes detection, multi-primer sets were developed and modified to use for simultaneous identification of species specific for *E. faecalis* (D-Ala:D-Ala ligase) and *E. faecium*, to detect three resistance genes including *tetM*, *tetL* and *ermB* for enterococci isolates and to detect five resistance genes for *E. coli* including *tetA, tetM, catI*, *cmlA* and *sulI*.

3.2.4.2 PCR amplification

Genomic DNA of *E. coli* and entercocci were used as the templates for PCR detection. Three PCR master mixes consisting of different primer sets were prepared. Group I contained primers for *E. faecalis* and *E. faecium*; group II was *tetA, tetM, catI*, *cmlA* and *sulI* primers; and group III was *tetM*, *tetL* and *ermB* primers. The PCR mixture consisted of 1X of *Taq* DNA polymerase reaction buffer, 1.5mM of $MgCl_2$, 0.2 mM of dNTPs and 1U of *Taq* DNA polymerase. The primer (Table 4.1) mixing ratios were as follows, group I used 0.4:0.4 of *E. faecalis*:*E. faecium*, group II used 0.1:0.4:0.2:0.4:0.2 of *tetA*:*tetM*:*catI*:*cmlA*:*sulI* and group III used 0.2:0.2:0.1 for *tetM*:*tetL*:*ermB* in μM concentration per a reaction. PCR was performed in the final volume of 25 μl per a reaction. Amplifications were initiated with denaturation at 95ºC for 5 min, followed by 30 cycles of 95ºC for 1 min, 50ºC, 51 ºC and 57 ºC for primer group I, II and III, respectively, for 1 min, and 72ºC for 1 min, and ended with a final extension at 72ºC for 7 min. The PCR products were checked by electrophoresis on 2.5% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. A 100-bp DNA ladder was used as a molecular weight marker.

Table 3.1 Oligonucleotide primers and gene-specific probes used for species level identification of *E. faecalis* and *E. faecium*.

Primer group I	Primer sequence (5'-3')	PCR product size (bp) (gene-specific probe)	Reference
E. faecalis			
E. faecalis-F	ATCAAGTACAGTTAGTCTT	941	(10)
E. faecalis-R	ACGATTCAAAGCTAACTG		
E. faecium			
E. faecium-F	TTG AGG CAGACC AGA TTGACG	658	(75)
E. faecium-R	TAT GAC AGC GAC TCCGAT TCC		

Table 3.2 Oligonucleotide primers and resistance gene-specific probes used for amplification of antimicrobial resistance genes of *E. coli*.

Primer group II	Primer sequence (5'-3')	PCR product size (bp) (gene-specific probe)	Reference
Tetracycline			
tetA-F	GCTACATCCTGCTTGCCTTC	280	This study
tetA-R	GGCAGGCAGAGCAAGTAGAG		
tetM-F	ACAGAAAGCTTATTATATAAC	171	
tetM-R	TGGCGTGTCTATGATGTTCAC		(74)
Chloramphenicol			
catI-F	CCACCGTTGATATATCCCAA	587	This study
catI-R	CATTCTGCCGACATGGAA		
cmlA-F	CTCTTGTTTGGACCGCTA	847	This study
cmlA-R	AGAAGTAGACTGCCGTGA		
Sulfonamide			
sulI-F	CCGATGAGATCAGACGTA	505	This study
sulI-R	CCCAGATCCTTTACAGGA		
Quinolones			
qnrA-F	TCAGCAAGAGGATTTCTCA	627	(102)
qnrA-R	GGCAGCACTATTACTCCCA		

Table 3.3 Oligonucleotide primers resistance gene-specific probes used for amplification of antimicrobial resistance genes of *Enterococcus* spp..

Primer group III	Primer sequence (5'-3')	PCR product size (bp) (gene-specific probe)	Reference
Tetracycline			
tetL-F	TTATTCAAGGGGCTGGTGCA	505	This study
tetL-R	ACCATAGAGACAAACCCTGC		
tetM-F	ACAGAAAGCTTATTATATAAC	171	This study
tetM-R	TGGCGTGTCTATGATGTTCAC		
Erythromycin			
ermB-F	GGTAAAGGGCATTTAACGACGA	316	This study
ermB-R	GCAACCCTAGTGTTCGGTGA		
Streptomycin			
aadA-F	ATCGCCGAAGTATCGACTCA	664	This study
aadA-R	CTTCAAGTATGACGGGCTGA		
Streptogramin			
vatD-F	CTCAAAAGGGTGGATGGTCA	421	This study
vatD-R	ACGAGGAGTAAAGCCTGGAA		

3.2.4.3 DNA sequencing

The random amplified products for each target DNA fragment were purified using the Qiagen gel extrection kit (F. Hoffmannla Roche Ltd., Valencia, USA). Their sequences were confirmed by DNA sequencing with the oligonucleotide primers used fro PCR. All of the nucleotide sequences were compared with sequences in the GenBank database by use of the BLAST program.

3.2.5 Plasmid profile analysis

All genomic DNA samples of *E. coli* were isolated from humans (pet's owner), pets (cats and dogs), fresh vegetables and ice, were resolved by electrophoresis on 0.8% agarose gel in 1x Tris acetate EDTA (17) buffer at a constant voltage of 100 V for 1 h and 1 kb DNA ladder were used as a size marker. After electrophoresis, the agarose gel were stained in an ethidium bromide for 30 min and a plasmid band was visualized under an ultraviolet light. The detection and sizing of plasmids were performed and the correlation between plasmid banding profiles, antibimicrobial susceptibility patterns and resistance gene patterns of *E. coli* isolates was analyzed.

3.2.6 PCR-SSCP analysis

3.2.6.1 PCR amplification

A 248 bp fragment of *gyrA* gene from *E. coli* strains were amplified by PCR. Genomic DNA from each was subjected to PCR for *gyrA* gene by using the following oligonucleotide primers: 5' GTACTTTACGCCATGAACG 3' and 5' ATTTTCGCCAGACGGA 3' (Table 4.2). The PCR mixture (25 µl per a reaction) contained 2 µl of genomic DNA, 1X of *Taq* DNA polymerase reaction buffer, 1.5mM of $MgCl_2$, 0.2 mM of dNTPs, 10 pmol of each primers and 1U of *Taq* DNA polymerase. Amplifications were initiated with denaturation at 95ºC for 5 min, followed by 30 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec, and ended with a final extension at 72ºC for 7 min. The purity of the PCR products was checked by electrophoresis on 2% agarose gel and visualized under an ultraviolet light after staining with ethidium bromide. A 100-bp DNA ladder was used as a molecular weight marker.

Table 3.4 Oligonucleotide primers used for amplification of of *E. coli gyrA* in this study.

Primer name	Primer sequence (5'-3')	PCR product size (bp)	Reference
E. coli gyrA			
gyrA-F	GTACTTTACGCCATGAACG	247	(81)
gyrA-R	ATTTTCGCCAGACGGA		

3.2.6.2 Preparation of SSCP polyacrylamide gel

The 10% non-denaturing polyacrylamide gel was prepared from the mixture of 2.5 ml of stock 40% acrylamide: bisacrylamide (37.5:1), 0.5 ml of 10x TBE, 1 ml of 50% glycerol, 5.89 ml of sterile distilled water, and 100µl of 10% Ammonium persulphate and 10 µl of TEMED. The mixture was poured into the space between two glass-plates and immediately inserted a plastic comb at the top of the plates for making sample wells, trying to avoid forming of air bubbles or spilling that might make error for DNA running. The gel was formed to polymerize for at least 30 min, then removed the comb and assembled for performing the electrophoresis.

3.2.6.3 Preparation of denaturing PCR-SSCP samples and SSCP-gel electrophoresis

A mixture of 2 µl of PCR products of *gyrA* gene, 3µl of 0.5x TBE, and 5µl of 2x SSCP loading buffer was heated at 95ºC for 10 min for denaturing the double-strand DNA and then immediately cooled on ice for 5 min in order to separate the single-strand DNA from each other. The denatured DNA mixture was applied into each well of the SSCP-polyacrylamide gel and run the vertical electrophoresis under RT (25ºC) with the constant current of 20 mA in 0.5xTBE buffer for 3.5 hr. A 100 bp DNA ladder was used as a marker that processed the same way as samples preparation except in the heating step.

3.2.6.4 Silver staining of SSCP gel

After electrophoresis step, SSCP gel was visualized by silver staining. SSCP gel was first placed in the fixing solution (10% acetic acid) and agitated for 20 min, then rinsed 3 times with distilled water for 2 min each. The gel was transferred to staining step with 0.1% silver nitrate for 30 min and rinsed with distilled water before transferring to developing solution (3% w/v of Na_2CO_3 and 0.15% v/v of formaldehyde). Until all bands could be visualized, the reaction was stopped by adding with a fixing solution (10% acetic acid) for 1 minute and followed by washing the gel with distilled water for 2 min before drying gel in cellophane at RT. The different SSCP band patterns of single-strand DNA was observed and classified.

3.2.6.5 DNA sequencing

The PCR products of *gyrA* fragment of each different SSCP band patterns was purified by using gel extraction kit and were then sent to performed DNA sequencing. The oligonucleotides used for PCR were also used as primers for sequencing reaction. Analysis of all nucleotide sequences of mutations in *gyrA E. coli* were performed by using alignment and comparison with the reference nucleotide sequence of the *E. coli gyrA* gene (GenBank accession no. X57147). The vector NTI verion 8 is the software program was used for multi-DNA sequences alignment.

3.2.7 Statistical analysis

Comparisons of the associations between the data were performed by using chi-square test (χ^2 test) (SPSS software, version 14) with confidence interval of 95% (*p*-value <0.05).

This statistic was used to analyze the correlation between;

1. Antimicrobial resistance genes and sample sources
2. Antimicrobial resistance genes and antimicrobial susceptibility test

CHAPTER IV
RESULTS

4.1 Molecular detection of antimicrobial resistance genes from *E. coli* by dot blot hybridization

In the present study, the identification of resistance genes by using dot blot hybridization (Figure 4.1) of 313 *E. col*i isolates from humans (n=23), pets (cats and dogs) (n=41), vegetables and ice (n=83), chickens (n=115) and rivers (n=51) were investigated. For all *E. coli* strains, *tetA* (n=171) was mostly observed followed by *cmlA* (n=73), *sulI* (n=64), *tetM* (n=32) and *catI* (n=23), respectively, whereas *qnrA* was not detected. Among *E. coli* isolates from animal sources, most of resistance genes were observed in chickens more frequency than in pets, including *tetA*, *catI*, *cmlA* and *sulI* while only *tetM* was showed higher percentages.

Of 313 *E. coli* isolates, isolates from chicken origin showed the highest detection rates of *tetA* (71.3%), *catI* (10.4%) and *sulI* (34.8%) resistance genes while *E. coli* from rivers were observed the highest frequency of *tetM* (31.4%) and *cmlA* (33.3%) gene (Table 4.1).

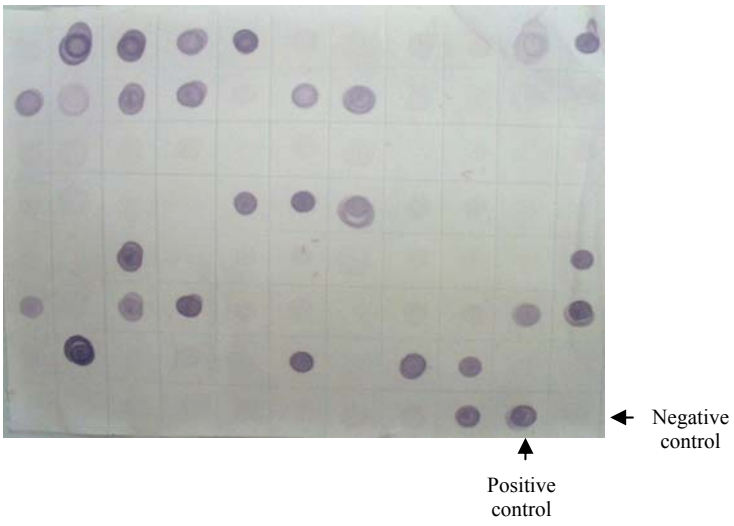


Figure 4.1 Demonstration of DNA hybridization by using random primer labelling.

Table 4.1 Frequency of antibiotic resistance genes among *E. coli* isolates from each samples sources.

Sample sources	Resistance genes of *E. coli* isolates (%)					
	tetA	*tetM*	*catI*	*cmlA*	*sulI*	*qnrA*
Humans (n=23)	12 (52.2)	1 (4.3)	0	3 (13.1)	4 (17.4)	0
Pets (cats and dogs) (n=41)	13 (35.1)	4 (9.8)	1 (2.4)	5 (13.5)	2 (4.9)	0
Vegetables and ice (n=83)	28 (32.2)	5 (6.0)	7 (8.4)	22 (25.3)	8 (9.6)	0
Chickens (n=115)	82 (71.3)	6 (5.2)	12 (10.4)	26 (22.6)	40 (34.8)	0
Rivers (n=51)	36 (70.6)	16 (31.4)	3 (5.9)	17 (33.3)	10 (19.6)	0
Total (n= 313)	171	32	23	73	64	0
Chi-square	41.555	30.772	4.829	6.428	26.601	
df	4	4	4	4	4	
p-value	0.000	0.000	0.305	0.169	0.000	

4.1.1 The correlation between antimicrobial resistance genes of *E. coli* isolates and sample sources

The correlation of each antimicrobial resistance gene, isolation sources of *E. coli* and antibiotic susceptibility testing were analyzed by Chi-square test (χ^2 test) with confidence interval of 95% (p-value $\leq$ 0.05). *tetA, tetM* and *sulI* genes had the significant correlation (p-value$\leq$0.05) with five sample sources of *E. coli* (p-value =0.000) (Table 4.1), whereas *catI* and *cmlA* showed no significant correlation with the sample sources (p-value =0.305 and p-value = 0.169, respectively).

Analyzation of the correlation between susceptibility testing of tetracycline agent and genotypic assay with *tetA* and *tetM* genes and of chloramphinicol drug with *catI* and *cmlA* genes was studied. Susceptibility test of tetracycline agents in all *E. coli* strains showed the significant correlation with *tetA* gene (p-value = 0.000), whereas showed no significant correlation with *tetM* (p-value = 0.051) (Table 4.2). This no significant correlation migth be due to the small number of samples effecting to the calculation by the software program. In chloramphinecol, the association between phenotypic testing and genetic detection showed the significant correlations for both *catI* and *cmlA* genes (p-value = 0.000 and p-value = 0.000) as shown in Table 4.3.

Table 4.2 The correlation of antimicrobial susceptibility test of tetracycline agent and resistance genes in 262 *E. coli* isolates from humans, pets, vegetables, ice and chickens.

Resistance gene	**Antimicrobial susceptibility test**				**Chi-square test**
	TE-S	**TE-I**	**TE-R**	**Total**	
tetA	2	0	133	135	Chi-square = 70.568 df = 1 *p*-value = 0.000
tetM	0	0	16	16	Chi-square = 3.799 df = 1 *p*-value = 0.051
Total	2	0	149	151	

TE-S = Tetracycline susceptible, TE-I = Tetracycline intermediate and TE-R = Tetracycline resistant.

Table 4.3 The correlation of antimicrobial susceptibility test of chloramphenicol and resistance genes in 262 *E. coli* isolates from humans, pets, vegetables, ice and chickens.

Resistance gene	**Antimicrobial susceptibility test**				**Chi-square test**
	C-S	**C-I**	**C-R**	**Total**	
catI	1	2	53	56	Chi-square = 82.377 df = 2 *p*-value = 0.000
cmlA	2	0	18	20	Chi-square = 20.142 df =2 *p*-value = 0.000
Total	3	2	71	76	

C-S = Chloramphenicol susceptible, C-I = Chloramphenicol intermediate and C-R = Chloramphenicol resistant.

4.2 Molecular detection of antimicrobial resistance genes from enterococci by dot blot hybridization

In this study, the identification of resistance genes by using dot blot hybridization of 258 enterococci isolates from humans (n=23), pets (cats and dogs) (n=36), vegetables and ice (n=99), chickens (n=100) were investigated. *E. coli* isolates from humans, pets, vegetables and ice sample sources, *tetM* (n=141) was mostly found in these group while *ermB* was observed (n=115) the similar percentage of resistance gene with *tetL* (n=106). However, *E. coli* isolates from chickens were found very high percentage of *ermB* (92.0%) when compared to other sources (humans, pets, vegetables and ice) and also showed the mostly observed of resistance genes for *tetL* (83.0%) and *tetM* (84.0%) (Table 4.4). The number of resistance genes in individual enterococcal isolates shown in Table 4.5. Occurance of one resistance gene showed similar percentages among enterococci islated from humans, pets, vegetables and ice and chickens (17.4%, 16.7%, 12.1% and 12.0%, respectively.). In addition, the enterococci isolates with all of three genes, including *tetM*, *ermB* and *tetL* showed the highest rates (75%) in enterococci isolates from chicken origin (Table 4.4).

Table 4.4 Frequency of antibiotic resistance genes among enterococci isolates from each sample source.

Sample sources	Resistance genes of enterococci isolates (n=258), (%)				
	tetL	*tetM*	*ermB*	*aadA*	*vatD*
Humans (n=23)	6 (26.1)	12 (52.2)	5 (21.7)	0	0
Pets (cats and dogs) (n=36)	13 (36.1)	20 (55.6)	11 (30.6)	0	0
Vegetables and ice (n=99)	13 (13.1)	25 (25.3)	8 (8.1)	0	0
Chickens (n=100)	83 (83.0)	84 (84.0)	92 (92.0)	0	0
Total (258)	115	141	106	0	0
Chi-square	103.61	69.348	151.86		
df	3	3	3		
p-value	0.000	0.000	0.000		

Table 4.5 The number of antimicrobial resistance gene(s) in individual enterococci isolates from each sample source.

Sample sources	Resistance gene(s) of entercocci isolates (%)			
	1 gene	2 genes	3 genes	Not detected
Humans (n=23)	4 (17.4)	8 (34.8)	1 (4.3)	10 (43.5)
Pets (n=36)	6 (16.7)	10 (27.8)	6 (16.7)	14 (38.9)
Vegetable and water (n=99)	12 (12.1)	9 (9.1)	6 (6.1)	72 (72.7)
Chicken (n=100)	12 (12.0)	10 (10.0)	75 (75.0)	3 (3.0)
Total (n=258)	34 (13.2)	37 (14.3)	88 (34.1)	99 (38.4)

4.2.1 The correlation between antimicrobial resistance genes of enterococci isolates and sample sources

The correlation of each antimicrobial resistance gene, sources of entercocci isolates and drug susceptibility test were analyzed by Chi-square test (χ^2-test) with confidence interval of 95% (p-value ≤ 0.05). All of 3 resistance genes, *tetM, tetL* and *ermB*, showed the significant correlation (p-value ≤ 0.05) between four sample sources of enterococci including humans, pets, vegetables, ice and chckens (p-value =0.000) (Table 4.4). The correlation between antimicrobial susceptibility test and resistance genes of erythromycin agents was studied. The *ermB* gene had the significant correlation with the susceptibility test of erythromycin (p-value = 0.000) (Table 4.5).

Table 4.6 The correlation of antimicrobial susceptibility test of erythromycin agent and resistance gene in enterococci isolates.

Resistance gene	**Antimicrobial susceptibility test**				**Chi-square test**
	E-S	**E-I**	**E-R**	**Total**	
ermB	0	4	112	116	Chi-square = 131.72 df = 2 *p*-value = 0.000
Total	0	4	112	116	

E-S = Erythromycin susceptible, E-I = Erythromycin Intermediate and

E-R = Erythromycin resistant.

4.2.2 Species specific identification of *Enterococcus* spp. by using dot blot hybridization

The total of 258 enterococci isolates that have been identified as *Enterococcus* spp. by biochemical testing from the previous study, were positive with *E. faecalis* specific probe for 69% (n=178) and with *E. faecium* specific probe for 16.3% (n=42).

Isolation rates of *E. faecalis* were higher than that of *E. faecium* in all sample sources including humans (11.1%), pets (16.1%), vegetables and water (31.1%) and chickens (41.7%). *Enterococcus* spp. other than *E. faecalis* and *E. faecium* were found as 15.9% (n=41) in this study. However, five samples tested colonies showed double positive results for two species specific probes (Table 4.7).

Table 4.7 Species-specific identification of *Enterococcus* spp.

Sample sources	A Number of isolates (%)		
	E. faecalis	*E. faecium*	Non *E. faecalis* and non *E. faecium*
Humans (n=23)	20 (11.1)	2 (4.8)	3 (7.3)
Pets (n=36)	29 (16.1)	3 (7.1)	5 (12.2)
Vegetable and ice (n=99)	56 (31.1)	20 (47.6)	24 (58.5)
Chicken (n=100)	75 (41.7)	17 (40.5)	9 (21.1)
Total (n=258)	180 (100) (69.8)	42 (100) (16.3)	41 (100) (15.9)

Remark: Five isolates showed positive results for both *E. faecalis* and *E. faecium*.

4.3 Multiplex PCR for resistance genes detection and species identification

4.3.1 Optimization of PCR reaction

4.3.1.1 Effect of annealing temperature

The PCR products of different annealing temperatures using primer group III (*tetM*, *tetL* and *ermB* primers) were showed in Figure 4.2. Six annealing temperatures for PCR reactions ranged between 45°C to 60°C were studied. Lane 4 and 5 showed the absence of *tetM* product because of using the three lower annealing temperatures (51 ºC, 48 ºC and 45 ºC). The appropriate annealing temperature of the PCR reaction for all of three loci (*tetM, tetL* and *ermB* genes) was at 57°C as shown in lane 2. This annealing temperature showed specific products that had an efficiency to amplify all of 3 expected genes. For other multiplex PCR primer sets, group I (*E. faecalis* and *E. faecium*)and group II (*tetA, tetM, suII, catI* and *cmlA*) (Figure 4.3), effect of annealing temperatures were studied as well and their optimal temperatures were 50ºC and 51ºC, respectively.

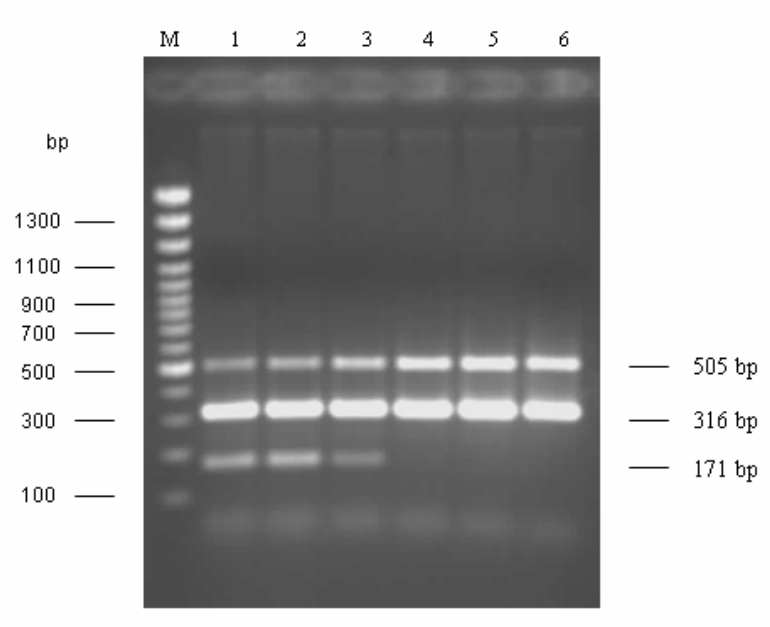


Figure 4.2 Multiplex PCR optimization of *tetM* (171 bp), *tetL* (505 bp) and *ermB* genes (316 bp) using six annealing temperatures. Lane 1: at 60°C, lane 2: at 57°C, lane 3: at 54°C, lane 4: at 51°C, lane 5: at 48°C and lane 6: at 45°C.

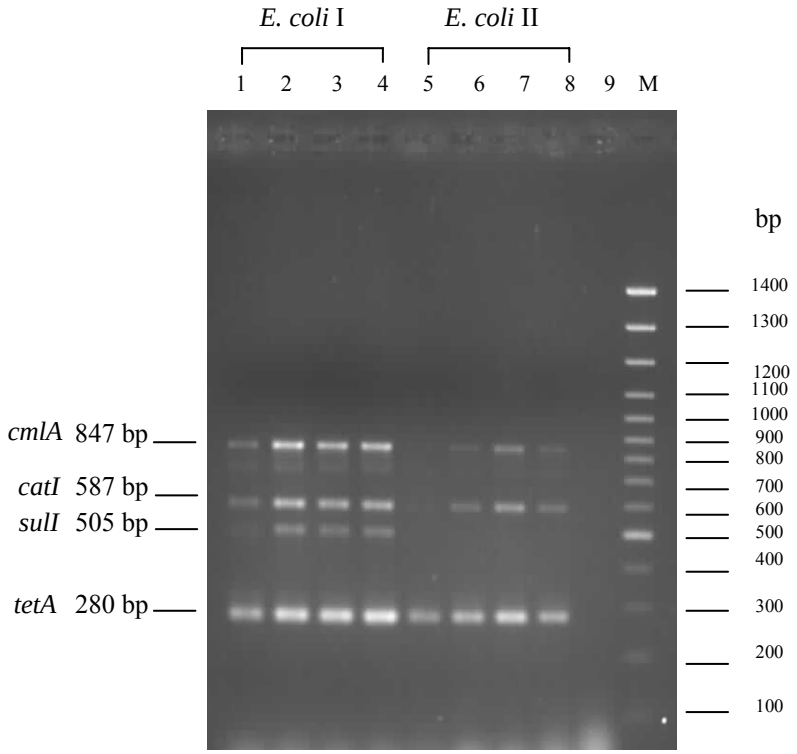


Figure 4.3 Demonstration of the effect of different annealing temperatures of primer group I in appearance of expected PCR products of two *E. coli* isolates. Lane 1 and 5: at 60°C, lane 2 and 6: at 57°C, lane 3 and 7: at 51°C and lane 4 and 9: at 46°C; lane M: 100 kb DNA ladder.

4.3.1.2 Effect of the primer concentrations

In order to amplify all of the target resistance genes to produced similar amounts of the specific PCR products, the individual primer pairs were optimized. The optimization of a concentration of each specific primer, *ermB* and *tetL*, were tested by deviding into 3 ratios (1:2, 1:3 and 1:4) in different concentrations. Group A, the ratio of *ermB:tetL* was 1:2 of with concentrations of 0.1:0.2, 0.2:0.4 and 0.3:0.6 μM; group B, the ratio of *ermB:tetL* was 1:3 with concentrations of 0.1:0.3, 0.2:0.6 and 0.3:0.9 μM; and group C, the ratio of *ermB:tetL* was 1:4 with concentrations of (0.1:0.4, 0.2:0.8 and 0.3:1.2 μM). The reactions were performed with the same PCR condition, the same component and the same DNA template concentration. The results of the optimization of primer concentration shown in Figure 4.4. The most proper primer concentrations for different of three enterococcal resistance genes were 0.2:0.1 ratios of *:tetL:ermB* in μM concentration per reaction (Figure 4.4, lane 1). The optimization of primer concentrations for multiplex PCR of *E. coli* resistance genes and identification to the species level of *E. faecalis*. and *E. faecium* were performed in the same way of enterococci (data not shown). For the optimal concentration of were 0.2:0.2:0.1 rations of *tetM:tetL:ermB* (primer set I).

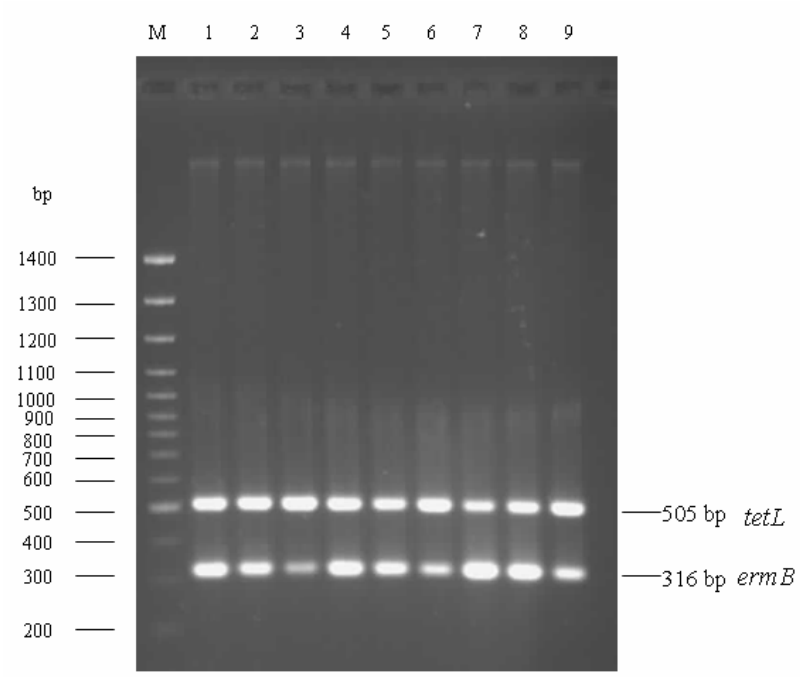


Figure 4.4 The effect of different concentrations of individual primers for enterococcal resistance genes. Lane M, the 100 bp DNA ladder; lane 1, 4 and 7 showed the ratio of *ermB:tetL* was 1:2 in different concentrations (0.1:0.2, 0.2:0.4 and 0.3:0.6); lane 2, 5 and 8 showed the ratio of *ermB:tetL* was 1:3 in different concentrations (0.1:0.3, 0.2:0.6 and 0.3:0.9); and lane 3, 6 and 9 showed the ratio of *ermB:tetL* was 1:4 in different concentrations (0.1:0.4, 0.2:0.8 and 0.3:1.2).

4.3.2 Amplification of antimicrobial resistance genes in *E. coli* and enterococci isolates

Twenty isolates of *E. coli* and entercocci were randomly selected for resistance genes detection using the developed multiplex PCR. For *E. coli* isolates, the samples were simultaneously amplified by specific oligonucleotide primer pairs for 5 antimicrobial resistance genes including *tetM, tetA, sulI, catI* and *cmlA.* The positive control were previously amplified by the specific primers. The target PCR products of each positive control strains were confirmed by DNA sequencing and then compared with the sequences in GenBank database used the BLAST program. All of the 5 specific PCR products (171 bp, 280 bp, 505 bp, 587 bp and 847 bp) were observed with comparison to the positive controls (Figure 4.5). In enterococi isolates, 3 primer pairs for *tetM, tetL* and *ermB* were used and the target fragments were 171 bp, 316 bp and 505 bp, respectively. All expected targets were amplified, however, a few non-specific bands also occurred (Figure 4.6).

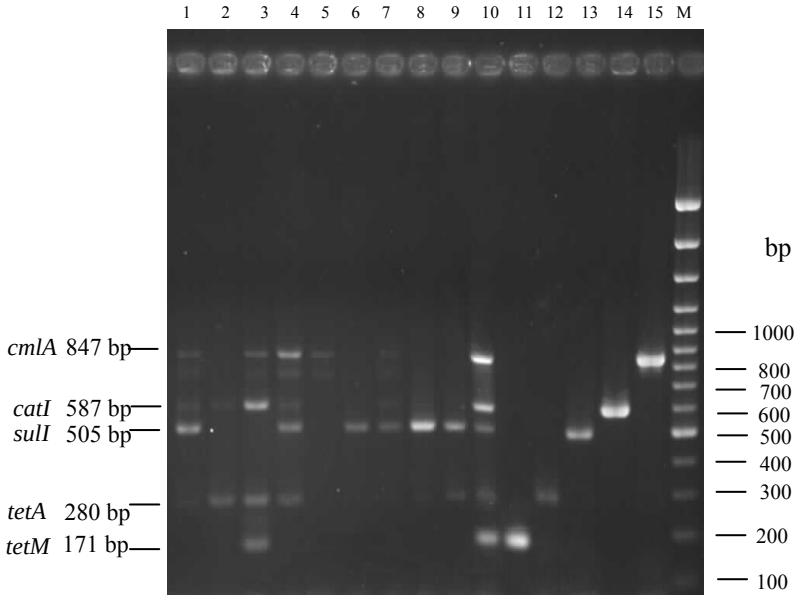


Figure 4.5 Multiplex PCR analysis of *E. coli* DNA from chicken origin, using *tetM*, *tetA, catI, cmlA* and *sulI* primers. Lane 1 and 4: *E. coli* isolates with *tetA*, *sulI* and *cmlA*; lane 2: *E. coli* isolate with *tetA* and *catI*; lane 3: *E. coli* isolate with *tetM*, *tetA*, *catI* and *cmlA*; lane 5: *E. coli* isolate with *cmlA*; lane 6 and 8: *E. coli* isolates with *sulI*; lane 7: *E. coli* isolate with *sulI*, *catI* and *cmlA*; lane 9: *E. coli* isolate with *sulI* and *tetA*; lane 10: PCR product from a mixture of *tetM*, *tetA*, *sulI*, *catI* and *cmlA*; and lane 11, 12, 13, 14, and 15: PCR products of *tetM*, *tetA*, *sulI*, *catI* and *cmlA*, respectively.

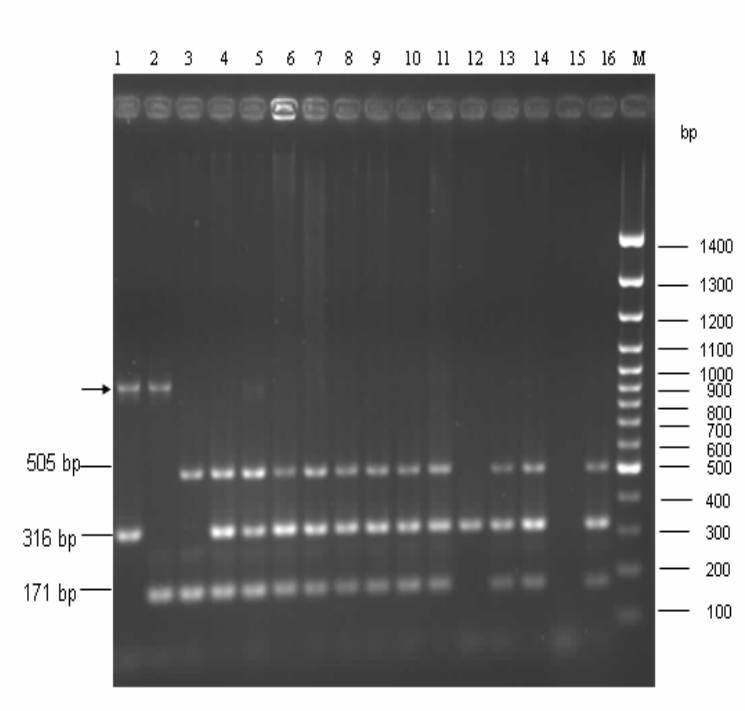


Figure 4.6 PCR analysis of DNA from enterococci isolates containing resistance genes of *tetM* (171 bp), *ermB* (316 bp) and *tetL* (505 bp). Lane : 1, entercoccus with *ermB* gene; lane 2, enterococcus with *tetM* gene; lane 3 entercocci with *tetM* and *tetL*; lane 4-11 and 13-14 entercocci with *tetM*, *ermB* and *tetL*; lane M, 100 bp DNA ladder used as the molecular weight marker; lane 12 entercocci with *ermB* gene; lane 15, no template DNA (negative control); lane 16, positive control strain.

* The arrow shown non-specific bands at lane 1 and 2

4.3.3 Amplification using *E. faecalis* and *E. faecium* specific primers

Species identification of *E. faecalis* and *E. fecium* were determined by duplex PCR as shown in Figure 4.7. Twenty strains were randomly selected for amplification with the *E. faecalis* and *E. faecium* primers. *E. faecium* and *E. faecalis* were produced the PCR products of 941 bp and 658 bp, respectively. The negative cotrol, no DNA template reaction, at lane 10 showed no bands for two species, *E. faecalis* and *E. faecium*. Results using mixed isolates to prepare PCR amplification, without subculturing before selection of one colony produced mixed positive bands for both two species (Figure 4.7). The PCR amplification of twenty two isolates showed accordance results with hybridization method.

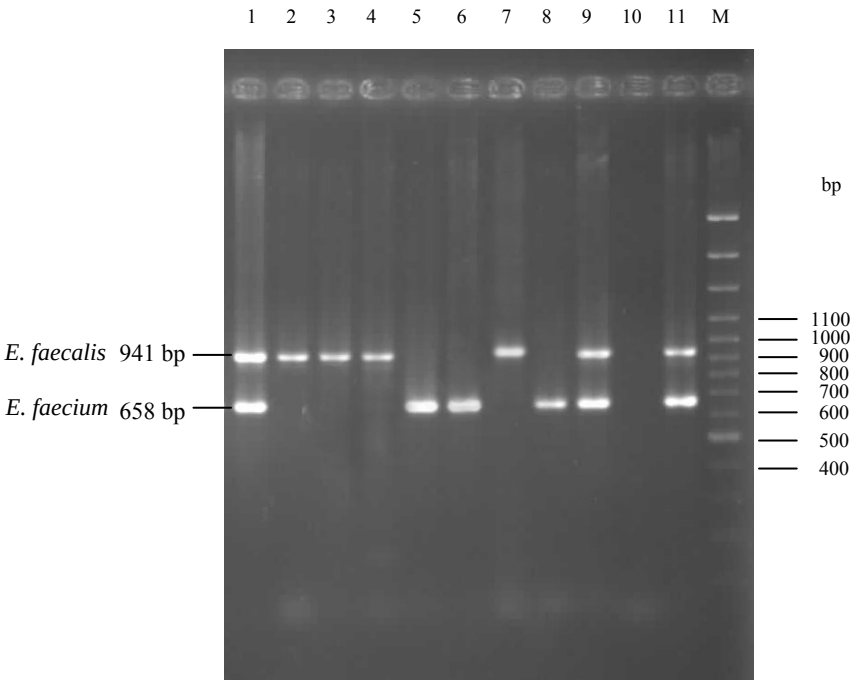


Figure 4.7 Identification to the species level of *Entercoccus* spp. by PCR analysis. Lane : 1 and 11, positive control for *E. faecalis* and *E. faecium*; 2-4, *E. faecali*; 5-6 *E. faecium*; 7, *E. faecalis*; 8, *E. faecium*; 9, the isolates showed positive for both *E. faecalis* and *E. faecium*; 10, no template control (negative control); M, 100 bp DNA ladder used as the molecular weight marker.

4.4 Antimicrobial susceptibility patterns and plasmid profile analysis of *Escherichia coli* Isolates

4.4.1 plasmid profile analysis

The sizes of plasmid bands between 1–100 kb were investigated in 255 *E. coli* isolates originated from humans and pets (60 isolates), vegetables and water (83 isolates) and chicken cecal contents (112 isolates). All isolates were subjected to plasmid analysis by agarose gel electrophoresis with ethidium bromide strianing and visualized by UV light (Figure 4.8). Various plasmid patterns were found in *E. coli* isolates from three sample sources, especially in *E. coli* isolated from chicken. A low number of strains containing plasmid bands were observed in *E. coli* isolated from human and pet sources and from vegetables and water 9 isolates, 14.8% and 15 isolates, 18%. The *E. coli* isolates from there sources showed one plasmid band with sizes between 1.8 – 9.4 kb or two plasmid bands with sizes between 1.6 – 4.8 kb, and only 2 isolates 2.4% from vegetable and water group occurring more than two plasmids, ranging from 1.9 to 3.2 kb. Sixty seven (59.8%) isolates of *E. coli* from chickens were detected one or more plasmid bands with sizes ranging from 1 to 100 kb (Table 4.8). Therefore, the plasmid patterns of the strains in chicken group were classified into 8 plasmid patterns (Pattern I to VIII) as shown in Table 4.8.

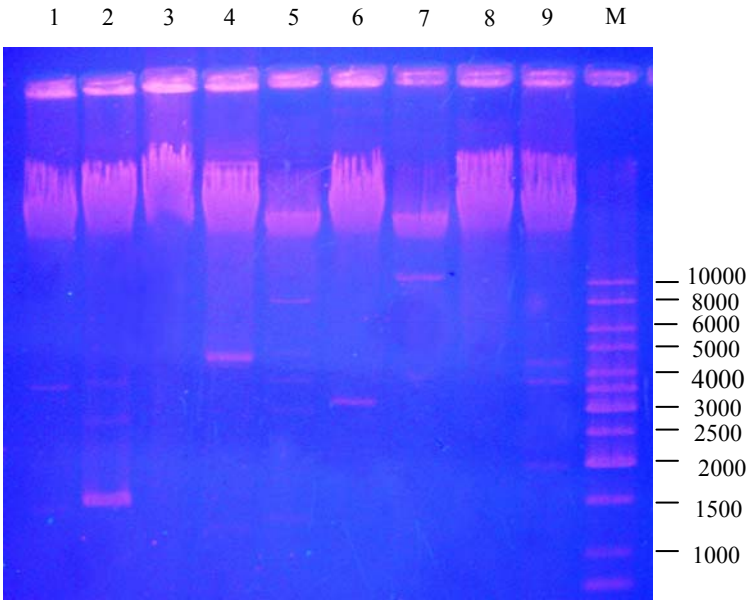


Figure 4.8 Agarose gel electrophoresis of plasmid DNA (1-100 kb) from *E. coli* isolates. Lanes 1-9, isolates from chicken cecal contents; M, Molecular weight marker (1 kb ladder).

4.4.2 Correlation of antibiotic susceptibility patterns and plasmid profiles

Plasmid profiles in correlation with antibiotic susceptibility and resistance genes detected by dot blot hybridization were analyzed. A total of 15 different susceptibility patterns were observed in all plasmid profiles of *E. coli* isolated from chicken. All isolates were resistant to nalidixic acid, 95.5% resistance to tetracycline, 83.6% resistance to ampicillin, 82.1% resistance to trimethoprim-sulfamethoxazole, 77.6% resistance to ciprofloxacin, 50.7% resistance to chloramphenicol and only 3 isolates resistance to ceftriaxone (Table 4.8). Only one pair of isolates were showed the identical plasmid pattern (Pattern VIII) and were observed the same plasmid profile with only resistance to nalidixic acid. For plasmid patterns III, V, VII and VIII very few isolates contained plasmid(s) but showed an absence of any resistance gene.

Table 4.8 Plasmid profiles and antibiotic resistance patterns of *E. coli* isolats from feces of chicken

Plasmid pattern (s)	Number of Plasmid (s)	Plasmid size (kb)	Number of isolates (n=67)	Drug resistance*	Resistance genes (n)
Pattern I	1	1.0-2.0	1	AMP, TE, NA	*tetA* (n=1)
			1	AMP, TE, NA, CIP	*tetA* (n=1)
			6	AMP, TE ,NA, CIP,SXT	*tetA* (n=4)
					tetA, sulI (n=2)
			1	AMP, TE, NA, C, SXT	*tetA , sulI, cmlA, catI* (n=1)
			3	AMP, TE, NA, CIP,C, SXT	*tetA, sulI, catI* (n=1)
					tetA, cmlA, cat (n=2)
Pattern II	1	2.1-3.0	2	TE, NA	*tetA* (n=1)
					tetA, sulI (n=1)
			1	AMP, TE, NA, SXT	*tetA, sulI* (n=1)
			2	AMP, TE, NA, CIP, C	*tetA* (n=1)
					sulI (n=1)
			1	AMP, TE, NA, CIP, SXT	*sulI* (n=1)
Pattern III	1	3.1-5.0	2	AMP, TE, NA, SXT	no resistance gene (n=1)
					tetA (n=1)
			1	AMP, TE, NA,CIP, SXT	*tetA, sulI* (n=1)
			1	TE, NA, CIP, SXT, C	*tetA, cmlA* (n=1)
			4	AMP, TE, NA,CIP, SXT, C	*tetA, cmlA* (n=1)
					tetA, sulI (n=1)
					tetA, sulI, catI (n=1)
					cmlA, sulI, catI (n=1)
Pattern IV	1	>5.0	1	AMP, TE, NA, SXT, C	*tetA, tetM* (n=1)
			1	AMP, TE, NA, SXT, CIP	*tetA, sulI* (n=1)
			1	AMP, TE, NA, SXT, CIP, C	*tetA, cmlA* (n=1)

* Tests for Amphicillin 10 μg (AMP_{10}), Chloramphenical 30 μg (C_{30}), Ciprofloxacin 5 μg (CIP_5), Ceftriaxone 30 μg(CRO_{10}), nalidixic acid 30 μg (NA_{30}), Trimethoprim- Sulfamethorazole 1.25/23.75 (SXT_{25}) and Tetracycline 30 μg (TE_{30})

Table 4.8 Plasmid profiles and antibiotic resistance patterns of *E. coli* isolats from feces of chicken (Continued)

Plasmid pattern (s)	Number of Plasmid (s)	Plasmid size (kb)	Number of isolates (n=67)	Drug resistance*	Resistance genes (n)
Pattern V	2	1.0-3.0	1 1 1 3	AMP, TE, NA, SXT C, TE, NA, SXT, CIP AMP, TE, NA, SXT, CIP AMP, TE, NA, SXT, CIP, C	*tetA, sulI* (n=1) *tetA* (n=1) *tetA, sulI* (n=1) *tetA, sulI* (n=1) *tetA, sulI, cmlA* (n=1)
Pattern VI	2	1 band (1.0-3.0) and 1 band (>3.0)	1 1 1 1 5	TE, NA TE, NA, SXT AMP, TE, NA, SXT AMP, TE, NA, CIP, SXT AMP, TE, NA, CIP, SXT, C	*tetA* (n=1) *tetA, tetM, sulI* (n=1) *tetA* (n=1) *tetA (n=1)* *tetA* (n=1) *tetA, cmlA* (n=1) *tetA, sulI* (n=2) *tetA, tetM* (n=1)
Pattern VII	2	>3.0	2 1 1	NA AMP, TE, NA, CIP AMP, TE, NA, CIP, C	no resistance gene (n=2) *tetA* (n=1) *sulI* (n=1)
Pattern VIII	>2	1.0-100	1 2 1 1 3 1 7 3	TE, NA, CIP, SXT TE, NA, CIP, SXT, C AMP, TE, NA,CIP AMP, NA, CIP, SXT, C AMP, TE, NA, SXT, CIP AMP, TE, NA, SXT, C AMP, TE, NA, CIP, SXT, C AMP, TE, NA, CIP, SXT, CRO	no resistance gene (n=1) *tetA, cmlA* (n=1) *tetA, sulI* (n=1) no resistance gene (n=1) *cmlA* (n=1) *tetA* (n=2) *tetA, sulI* (n=1) *tetA, sulI* (n=1) *tetA* (n=3) *tetA, cmlA* (n=2) *tetA, sulI* (n=1) *tetA, sulI, cmlA* (n=1) no resistance gene (n=1) *tetA, sulI* (n=2)

* Tests for Amphicillin 10 µg (AMP_{10}), Chloramphenical 30 µg (C_{30}), Ciprofloxacin 5 µg (CIP_5), Ceftriaxone 30 µg(CRO_{10}), nalidixic acid 30 µg (NA_{30}), Trimethoprim- Sulfamethorazole 1.25/23.75 (SXT_{25}) and Tetracycline 30 µg (TE_{30})

4.5 PCR-SSCP analysis of *gyrA* mutations in *E. coli* isolates

4.5.1 Antimicrobial susceptibility patterns of *E. coli* isolates

Total of 133 *E. coli* isolates were obtained during March to June, 2005 from 34 faecal samples of pets (dogs and cats), 19 pet's owner, 59 of vegetable samples and 21 of ice samples in Bangkok and its vicinity. The isolates were performed for quinolone susceptibility testing by the standard disk diffusion method and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) by the previous study. All *E. coli* isolates were examined for resistance to nalidixic acid (20 μg) and ciprofloxacin (5 μg) by using *E. coli* ATCC 25922 as the control strain.

Susceptibility test of resistant *E. coli* isolates from humans, pets, vegetables and ice to nalidixic acid were found 6 (31.6%), 11 (32.4%), 21 (35.6%) and 12 (57.1%) isolates, repectively and 1 (5.3%), 8 (23.5%), 13 (22.0%) and 4 (19.0%) isolates, respectively were resistant to ciprofloxacin (Table 4.9). The susceptibility test for nalidixic acid showed similar percentages of resistance among humans, pets and vegetables sample groups but the high percentages were observed in ice sample group. For ciprofloxacin susceptibility test, the similar percentage were found among pets, vegetables and ice while the lower resistance percentage was found particularly in humans (Figure 4.9).

Table 4.9 Antimicrobial susceptibility pattern of 133 *E. coli* isolates by disk diffusion method.

Susceptibility test	Number of isolates (%)							
	Nalidixic acid				Ciprofloxacin			
	Human (n=20)	Pet (n=34)	Vegetable (n=59)	Ice (n=21)	Human (n=19)	Pet (n=34)	Vegetable (n=59)	Ice (n=21)
Susceptible	12 (63.2)	22 (64.7)	36 (61.0)	7 (33.3)	16 (84.2)	26 (76.5)	43 (72.9)	15 (71.4)
Intermediate	1 (5.3)	1 (2.9)	2 (3.4)	2 (9.5)	2 (10.5)	0 (0.0)	3 (5.1)	2 (9.5)
Resistant	6 (31.6)	11 (32.4)	21 (35.6)	12 (57.1)	1 (5.3)	8 (23.5)	13 (22.0)	4 (19.0)

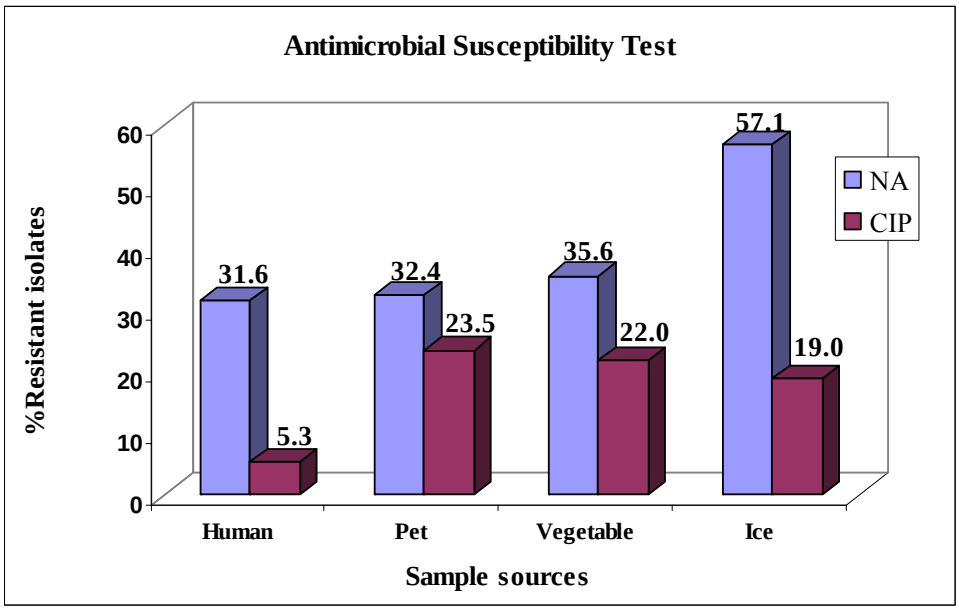


Figure 4.9 Percentage of quinolne resistant *E. coli* originating from human, pet, vegetable and ice determinated by disk diffusion test.
NA = nalidixic acid, CIP = ciprofloxacin.

4.5.2 PCR amplification of *E. coli gyrA* gene

All of 133 *E. coli* isolates were amplified by specific *gyrA* gene primers (Figure 4.10) with 248 bp of PCR product size. The PCR fragments of *E. coli gyrA* were continued to analyze by SSCP method.

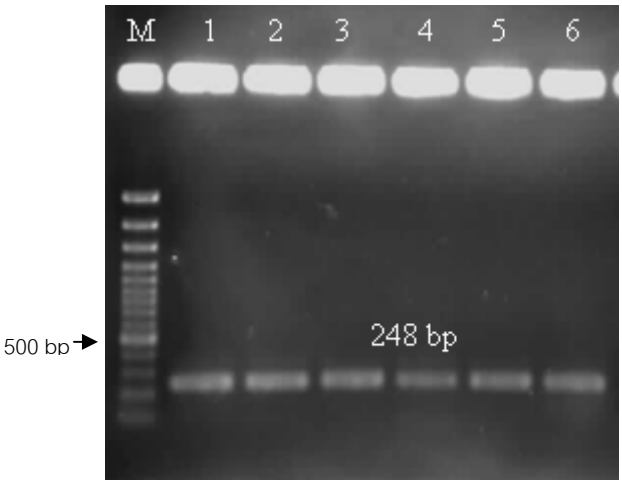


Figure 4.10 PCR product of 248 bp of *gyrA* gene were run on 2.0% agarose gel under UV light after staining by ethidium bromide. Lane M, 100 bp DNA ladder marker; lane 1-6, 248 bp of *gyrA* PCR products.

4.5.3 PCR-SSCP patterns of *E. coli* isolates

E. coli gyrA were analyzed by PCR-SSCP analysis showing the different bands based on the different types of nucleic acid sequences. The results showed 10 different SSCP band patterns of *E. coli gyrA*. They were name as patten E1, E2, E3, E4, E5, E6, E7, E8, E9 and E10 (Figure 4.11). *E. coli* isolates from fresh vegetables and ice were presented more variety patterns (9 patterns) than the strains isolated from pets (7 patterns) and humans (5 patterns). Pattern E1, E3, E4, E5, E6 and E9 were presented the silent mutation in various amino acid positions. Only 4 out of 10 SSCP-patterns (E2, E7, E8 and E10) have been identified mutations representing changes in amino acids at positions 83 and 87 (Figure 4.13). Ser-83 to Leu and Asp-87 to Asn

mutations have been observed in only nalidixic acid resistant strains, and were not found in nalidixic acid susceptible strains (Table 4.10). Asp-87-Asn was presented as a double mutation with Ser-83-Leu which was detected only in *E. coli* isolated from pets and humans and was not found in nalidixic acid and ciprofloxacin susceptible strains (Table 4.10). *E. coli* isolates that showed double mutation and resistance to ciprofloxacin were found only in pets sample among all nalidixic acid resistant *E. coli* isolates, approximately 24.5% (12/49) showed the mutations (Figure 4.12).

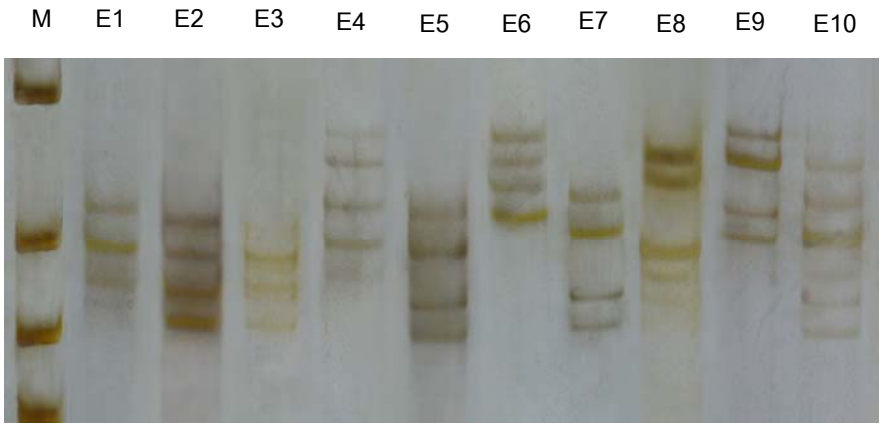


Figure 4.11 PCR-SSCP-patterns in the QRDR of *gyrA* gene of *E. coli* isolates (E1-E10). Lane M, 100 bp DNA ladder; lane 1,3-6 and 9, Pattern E1, E3-6 and E9 showed no change of amino acid of *gyrA* gene ; lane 2, E2 mutants with Ser→Leu changed at codon 83 and Asp →Asn changed at codon 87; lane 7, E7 mutants with Ser→Leu changed at codon 83 ; lane 8, E8 mutants with Ser→Leu changed at codon 83 and lane 10, E10 mutants with Ser→Leu changed at codon 83 .

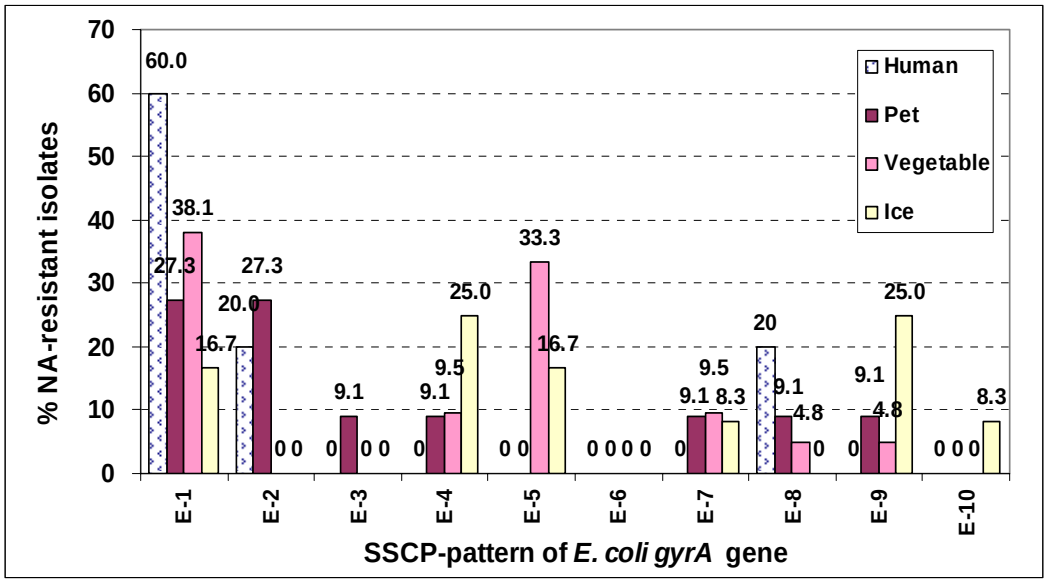


Figure 4.12 Percentages of nalidixic acid (NA) resistant *E. coli* isolates classified by SSCP-patterns found in different sample sources.

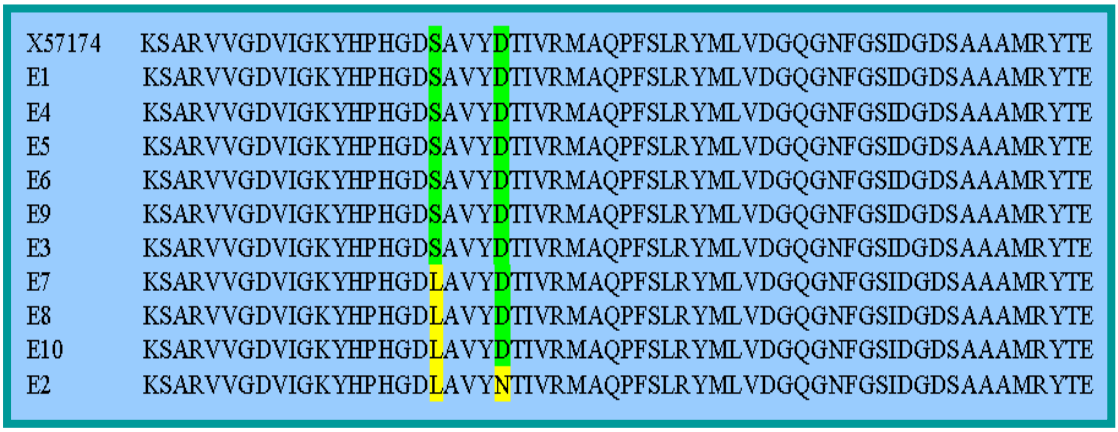


Figure 4.13 Partial amino acid sequences of *GyrA* from different SSCP-patterns (E1-E10). Amino acid changes in GyrA protein were detected in *E. coli* strains belonging to each pattern. Alignment of amino acid from 10 different SSCP-patterns of *E. coli gyrA* showed the different amino acids in the codon 83 and 87. The *gyrA* amino acid sequence was compared with that previously reported (GenBank accession no. X57174).

Table 4.10 Percentages of *E. coli* isolates classified by SSCP-patterns and antibiogram phenotype of 133 isolates from humans, pets, vegetables and ice.

SSCP Patterns	No. of isolates (%)			No. of isolates (%)			Amino acid change
	NA-S	NA-I	NA-R	CIP-S	CIP-I	CIP-R	
E-1	67 (85.9)	4 (66.7)	16 (32.7)	78 (77.2)	3 (42.9)	6 (24.0)	No change
E-2	–	–	4 (8.2)	–	1 (14.3)	3 (12.0)	83 (Ser-Leu), 87 (Asp-Asn)
E-3	–	–	1 (2.0)	–	–	1 (4.0)	No change
E-4	4 (5.1)	1 (16.7)	6 (12.2)	6 (5.9)	2 (28.6)	3 (12.0)	No change
E-5	2 (2.6)	–	9 (18.4)	3 (3.0)	–	8 (32.0)	No change
E-6	–	1 (16.7)	–	–	1 (14.3)	–	No change
E-7	–	–	4 (8.2)	2 (2.0)	–	2 (8.0)	83 (Ser-Leu)
E-8	–	–	3 (6.1)	2 (2.0)	–	1 (4.0)	83 (Ser-Leu)
E-9	5 (6.4)	–	5 (10.2)	9 (8.9)	–	1 (4.0)	No change
E-10	–	–	1 (2.0)	1 (1.0)	–	–	83 (Ser-Leu)
Total	78 (100)	6 (100)	49 (100)	101 (100)	7 (100)	25 (100)	

The single-strand conformational polymorphism patterns of *gyrA* gene in *E. coli:* E1, E2, E3, E4, E5, E6, E7, E8, E9 and E10.

NAL-S = nalidixic acid sensitive, NAL-I = nalidixic acid intermediate resistant, NAL-R = nalidixic acid resistant

CIP-S = ciprofloxacin sensitive, CIP-I = ciprofloxacin intermediate resistant, CIP-R = ciprofloxacin resistant

CHAPTER V
DISCUSSION

Molecular detection of antimicrobial resistance genes in *E. coli* and enterococci

Detection of antimicrobial resistance genes using dot blot hybridization

In the present study, 6 resistance genes including *tetM, tetA, sulI, catI*, *cmlA* and *qnrA* in 313 *E. coli* strains and 5 resistance genes, such as *tetM, tetL, ermB, aadA* and *vatD* in 258 enterococci isolated from humans, pets, vegetables, ice and chicken sample sources and *E. coli* isolated from river source were detected by dot blot hybridization with Dioxygenin (DIG) labeled DNA probes. Each positive control strain was amplified by specific oligonucleotide primers and the PCR products were confirmed by sequencing. The isolates that showed negative results of PCR amplification were used as negative control strains. Genomic DNA of *E. coli* isolates and bacterial lysate of enterococci were used for resistance genes detection by DNA hybridization assay which have used for screening and epidemiology purpose in a diversity of genera by several researchers (6, 17, 62, 73, 82). This study demonstrated that microflora from healthy humans, pets (cats and dogs) and chickens were bearing antimicrobial resistance genes. Of greater concern are reported some of these *E. coli* and enterococci isolates are resistant to antibiotics that are used for treatment human infections. The resistance determinants may reside on mobile DNA elements, such as plasmids that can be transferred to human pathogens or to the resident human gut flora (2, 12, 54, 74, 83).

Tetracycline resistance is often due to the acquistion of the genes (50). The two important mechanisms of tetracycline resistance are energy-dependent efflux and ribosomal protection (51, 74). The *tetA* gene, one of the most widespread among gram-negative bacteria of human and veterinary origin, coded for an efflux protein that confers resistance to tetracycline (43, 84). Among all of 6 resistance genes in *E. coli* isolates, *tetA* was found as the highest frequency of resistant tetracycline determinants in this study. The *tetL* efflux gene of gram-positive bacteria confers resistance to tetracycline (19). Ribosomal protection proteins are the other major

mechanism of tetracycline resistance. The *tetM* is one of the best characterized of these proteins (19, 74) The *tetM* and *tetL* was highly observed (n=141 and n=115, respectively) in enterococci isolates especially the strains from chickens (n=84 and n=83, respectively). These genes are generally found on transmissible plasmids, that occasion become integrated into a chromosome and may undergo recombination with other resistance plasmids. Tetracyclines have long been excluded from animal feeding and agriculture in Thailand but *tet* genes were still observed in vegetables, chickens and river in this study. One reason to explain is that because of the existance of corresponding resistance genes due to the horizontal gene transfer. The presence of tetracycline resistance in *E. coli* and enterococci isolates from a variety of sources agreed with the finding of other studies on the antimicrobial resistance of strains from a variety of different sources throughout the world (82, 85, 86). The presence of tetracycline resistance in *E. coli* from a varity of sourecs agree with finding of other studies on the antimicrobials resistance of *E. coli* from different sources throughout the world (82).

Resistance to chloramphenicol is commonly due to an inactivation of the antibiotic by chloramphenicol aceyltransferase. The *catI* gene that is the most commomly observed on plasmids has been described (37). Another mechanism observed in gram negative bacteria is active efflux encoded by *cmlA*. In this study showed more frequency of *cmlA* gene than *catI* in each sample source. Resistance to macrolide in enterococci is mainly due to two resistance mechanisms including modification of the ribosomal target of the antibiotic owing to methylation or mutation and efflux of macrolides. Methylation is encoded by the *ermB* gene (87). Schmmitz *et al.* (55) reported the screening of 75 European erythromycin-resistance *E. faecium* isolates, *ermB* (93%) was the most resistance gene found followed by *ermA* (4%). The *ermB* is often linked to the *tetM* gene (88). Two resistance genes include *aadA* and *vatD* conferring resistance to streptogramin A, were not detected in any enterococci isolates by dot blot hybridization in this study. However, the using of PCR method using specific-oligonucliotide primers for *aadA* and *vatD* to amplify 20 random enterococci isolates that randomly selected from chicken samples showed 2 positive isolates for *aadA* amplification, whereas amplification of *vatD* gene showed negative results. Simjee *et al.*(83) have reported no appearance of *vatD* but high

prevalence of *vatE* among US quinupristin-dalfopristin-resistant *E. faecium* isolates recovered from retail meats in the Greater Washington DC area, whereas *vatD* has been found in European countries (89). Sulfonamide resistance is often encoded by *sulI* and *sulII* gene in *Enterobacteriaceae* (103). Recently a third gene, *sulIII,* was found to encoded sulfonamide resistant bacteria. *SulI* gene are part of the 3' conserved segments of class I integrons, which are the most frequency detected integrons in *Enterobacteriaceae* (42). The *sulI* gene was detected in 17.4% (n=4), 4.9% (n=2), 8.6% (n=8), 34.8% (n=40) and 19.6%(n=10) of isolates from humans, pets, vegetables and ice, chicken and river, respectively. The presence of *sul* gene of *E. coli* isolates from each environment sample source especially in the gut flora of healthy humans is should be concerned.

A combination of two resistance mechanisms may lead to high resistance levels that are equal to the sum of the levels conferred by each mechanism individually (additive resistance) (43). In this study, the combination of *tetA* and *tetM* were observed in 29 *E. coli* isolates (9.3%), whereas *tetM* and *tetL* combination were showed in 106 enterococci isolates (41.1%). For combination of *catI* and *cmlA* in *E. coli* were found 11 isolates (3.5%).

Multiple resistance genes were investigated in this study, especially in *E. coli* and enterococci isolates from chickens. The results may increase a concern of a possible development of resistance to antimicrobial agents in microflora organisms and may be a presumption evidence for a usage antibiotics in animal feed or therapeutic application that results in the higher distribution of antimicrobial resistance genes in poutry than other sample sources. The common antimicrobial drugs used in food and companion animals are from five major classes (43): β-lactams, tetracyclines, aminoglycosides, macrolides and sulfonamides. Additionally, fluoroquinolones have been avialable in some European countries for more than 20 years and used in large animal in the United States in 1998 (43). It should be also concerned about the spread of resistance genes in environment including vegetables that may be contaminated from water, soil, and rivers. In the present study, the multiple resistance genes were found in high percentages, especially in both *E. coli* and enterococci isolated from chicken origin.

The combination of molecular characterization of antimicrobial resistance genes or mutations and the genetic elements involved in the dissemination of these genes gave an effective way to identify the spreading of antibiotic resistance gene. DNA detection showed whether an antimicrobial resistance gene present in a sample. The presence and absence of certain genes corresponded to particular phenotypes, however, it did not necessarily imply that the particular strain was resistant or susceptible as were reported in this study. Several antimicrobials earlier used as growth promoters have been banned in several countries. In Thailand the usage of antimicrobials is not well controlled. Obviously, the control use of antimicrobial agents is a prerequisite to limit the emergence of drug resistance bacteria. Additional research is certainly needed to better understanding the mechanisms behind bacterial resistance to antimicrobial and underlying mechanisms, such as the transfer of genetic material between bacteria in an environment. The results indicated that the resistance genes may not always express resistance to antimicrobial therefore phenotype based method of resistance organisms can under estimate the levels of resistance organisms.

Detection of antimicrobial resistance genes by multiplex PCR

Multiplex PCR is a useful technique for genetic screening, microsatellite analysis and other applications that is necessary to amplify several PCR fragments in a single reaction (77, 78). The optimization are required because primer–dimer and the non specific products may interfere with the amplification of specific products. This study reported on the modification and development of multiplex PCR method to detect antimicrobial resistance genes of 20 *E. coli* isolates (*tetM, tetA, sulI, catI* and *cmlA* gene) and 20 enterococci isolates (*tetM, tetL* and *ermB* gene) with the simultaneous specific primers. Each primer sets, all primers were designed to have similar anealing temperatures. The relative concentration of primers was found to be an important factor in an amplification of products from each organisms in a single reaction. Other critical factors in the multiplex PCR including the concentrations of PCR buffer, such as magnesium chloride, and deoxyribonucleotide triphosphates, and cycling temperatures (90). A few non-specific PCR products also were found in the PCR reaction in this study. However, all of the expected PCR products could be amplified for all of the target sites in the same reaction. As the purpose was to assess the method

for use as a simple and rapid diagnosis, the multiplex PCR might be a useful tool for detection method for study of resistance genes. The ability to combine different primer sets in a multiplex PCR not only allows the detection of resistance genes but also provides the ready means for distinguishing the nature of resistance determinants.

Molecular identification in species specific level of *Enterococcus*

Species identification of *Enterococcus* spp. by the traditional phenotypic method can be processed by requiring numerous tests including growing in various media, biochemical testing, motility and pigmentation. These methods often required longer time of incubation and many steps before determination. Molecular techniques for species identification have been developed, such as PCR and sequencing. Several genes, such as these encoding for heat shock protein 60, elongation factor EF-TU, D-Ala: D-Ala ligase and manganese-dependent superoxide dismutase, that have the species-specific regions, have been used for species identification (10, 91). The strains from non-human sources, such as environment and food, may not be confirmed by the criteria used for standard phenotypic characterization. In this study, PCR based method using multi-oligonucleotide primer sets has been developed for species identification between *E. faecalis* and *E. faecium* that have been examined by biochemical testing to be *Enterococcus* spp. Of 258 enterococci isolates from humans, pets, vegetables, ice and chicken sources, 69.8% *E. faecalis* and 16.3% *E. faecium* were observed in this study. Similar to other reports, *E. faecalis* is the most commonly observed followed by *E. faecium* in clinical isolates and environment (75, 92). Non *E. faecalis* and non *E. faecium* was observed for 15.9% and these isolates might be other species of *Enterococcus*. Five strains were found 2 positive bands for both *E. faecalis* and *E. faecium* amplification that might caused from the direct using of enterococci strains from stock culture media (kept at RT) to culture for genomic DNA extraction and preparating the bacterial cell lysate for dot blot hybridization without selection of only one colony by subculturing on agar first.

Plasmid profile analysis

Antimicrobial resistance genes are often located on transmissible plasmids in *E. coli* and other normal flora. Antibiotic pressure of selective pressure can promote the transfer of these plasmids to other *E. coli* strains and to other enteric pathogens (93). The common method for analyzing of plasmid profiles is agarose gel electrophoresis (94-96). In this thesis, the 1-100 kb plasmids of *E. coli* strains collected from humans, animals, vegetables, and water sources were analyzed by using genomic DNA for agarose gel electrophoresis. Because the small size plasmids (1-100 kb) studied in this thesis could be detected in genomic DNA running in agarose gel electrophoresis, therefore the plasmid DNA were not used for this purpose. A high variety of plasmid patterns (plasmid pattern I-VIII) was freuencly observed from *E. coli* isolated from cecal content of chicken than from humans, pets, vegetables and ice origin. Plasmid profile analysis of the isolates from chickens revealed that most of the strains showed more than 2 plasmids (plasmid pattern VIII) that had multi drug resistance to 4-6 antimicrobials. For plasmid pattern I-IV, comparison among 4 ranges of plasmid size of one plasmid band found in *E. coli* isolates, the larger plasmid f (plasmid pattern IV) (>5.0 kb) were resistant to many antibiotics (5-6 agents), whereas the isolates of other three patterns (plasmid pattern VIII) were resistant to 2-6 agents. Therefore, number of antimicrobial resistance in *E. coli* isolates might be related with the size of plasmids. Of 67 isolates from chicken sources, sixty-five strains were observed multiple antibiotic resistances. There has been increasing concern of the possible of development of resistance to antimicrobial agents in *E. coli* as the result of the use of such agents in chicken feed and ability of horizontal resistance genes transfer among bacteria in environment. Plasmids may appear in various shapes and forms: circular, partially cleaved or linear and multimeric as concatamers or catenates that are based on the molecular geometry and the degree of coiling of plasmids with an intact circular double strand (97). All of these forms may be observed in the genomic DNA preparation. However, the predominant bacterial plasmid forms are covalently closed circular (ccc) form, the open circular (op) form, also called “nicked form”, and the linear form (typically obtained by unique restriction digestion). The various forms of plasmids may interfere on the analysis of plasmid profiles in this study.

One to five resistance genes including, *tetA*, *tetM; sulI, cmlA* and *catI* were observed in most *E. coli* isolates, while 7 isolates showed no detection of any antimicrobial resistance genes. Among these isolates, three of them grouped in plasmid pattern VIII with more than 2 plasmid bands observed. The high incidence of plasmids may reflect the prophylactic and therapeutic uses of antimicrobial agents and the plasmid analysis of *E. coli* isolates is useful for epidemiological study.

Analysis of *gyrA* mutations related to quinolone resistance in *E. coli* isolates

Escherichia coli is one of the most relevant species for quinolone resistance. The primary target of quinolones in *E. coli* has been identified to be the DNA gyrase, specifically the GyrA subunit (98) termed the Quinolone Resistance-Determining Region (QRDR). This enzyme is composed of two A and two B subunits encoded by *gyrA* and *gyrB* genes, respectively. In *E. coli*, DNA gyrase includes amino acid between positions 51 -106 with "hot spots" for amino acid mutation at position 83 and 87. In this study, non-radioactive single-strand conformation polymorphism (SSCP) method and sequencing were employed to investigate the presence of mutations in the QRDR of *gyrA* gene 133 *E. coli* isolates originating from pets, humans, vegetables and ice. The susceptibility test for nalidixic acid showed the highest percentage of resistance in ice, whereas humans, pets and vegetables showed the similar percentages, approximately 32-36%. For ciprofloxacin susceptibility test, *E. coli* isolates from humans origin showed lower percentage of resistance than pets, vegetables and ice. The results showed 10 different SSCP-patterns (E1-E10). Only 4 out of 10 SSCP-patterns showed the mutation at amino acid positions 83 and/or 87.

Among all nalidixic acid resistant *E. coli* isolates, approximately 23.5% showed the mutations, therefore the other quinolone resistant mechanisms may also be involved. In this study, the *qnrA*, plasmid mediated quinolone resistance, in *E. coli* isolates from humans, pets, vegetables, ice and water also was observed, however no detection for any *E. coli* isolates from these environmental sources. Although quinolone are thought to interact primarily with the A subunit of DNA gyrase, mutations have also been discovered in the B subunit, which also confers quinolone resistance in *E. coli*. However, the frequency of *gyrB* mutations, has been found to be

lower than *gyrA* mutation in most species (7, 39, 99). Changing in the cell envelope of gram negative bacteria, particularly in outer membrane has been associated with decreasing uptake and increasing resistance to quinolones (37). However, decreased uptake has not been demonstrated to be a mechanism of resistance for gram positive bacteria. Increased efflux as a mechanism of quinolone resistance, mainly lower level resistance, has been observed in quinolone resistance bacteria (98, 99). Several studies pointed out that the alteration in the QRDR of DNA gyrase and topoisomerase IV are normally associated with higher MICs than in wild type strains (38-40, 64, 100). The different environment and inducers may affect to the variety of nucleotide changes in *gyrA* gene of *E. coli* isolates originating from different sources. Moreover, the *gyrA* mutations might be used as efficient indicators for nalidixic acid resistance.

CHAPTER VI
CONCLUSION

1. The study of antimicrobial resistance genes is interesting for diagnostic purpose, espectially in this study multiplex PCR has been developed the rapid detection of 5 resistance genes including *tetM, tetA, sulI, catI* and *cmlA* in *E. coli* isolates and 3 resistance genes for *tetM, tetL* and *ermB* in enterococci. This is useful for several genes markers detection.

2. The comparison of phenotypic and genotypic testing for antimicrobial resistance genes in *E. coli* and enterococci isolates showed interesting data about the significant correlation between resistance genes found and antimicrobial susceptibility test by disk diffusion method. In *E. coli* isolates, *tetA* and *tetM* showed significant correlation with susceptibility test of tetracycline, *catI* and *cmlA* showed significant correlation with susceptibility test of chloramphenical. The *sulI* showed significant correlation with susceptibility test of trimethoprim–sulphamethoxazole. For enterococci isolates, the significant correlation of *ermB* and susceptibility test of erythromycin was observed (p-value <0.05).

3. The significant different could be observed between each sample source and *tetM, tetA* and *sulI* in *E. coli* strains and *tetM, tetL* and *ermB* in enterococci isolates, while the remaining resistance genes (*catI* and *cmlA)* showed no significant different (p-value <0.05) among sample sources.

4. In this study molecular epidemiology tool was used to study the antibiotic resistance problem and the results demonstrated that this approach has the potential for identifying reservoirs and determining the identities of many classes of antibiotic resistance genes in a variety of sources.

5. A few studies reported incidence of resistance genes in microorganisms from environment sources, especially in Thailand. Therefore, the detection of antimicrobial resistance genes in indicator bacteria including *E. coli* and enterococci from different environment sources (healthy humans, pets, vegetables, ice, chicken and river) is beneficial for epidemiological and basic research study.

6. The wide dissemination of antimicrobial resistance genes in bacterial isolates from various sources may because of the usage of antimicrobials and the horizontal gene transfer of resistance genes, particularly resistance gene cassettes transfer which may cause the transportation of multiple antibiotics resistance among normal flora and pathogenic bacteria in human. It is important that guideline for prudent use of antimicrobial agents are adopted and surveillance program are established.

7. Plasmid profile analysis might reflected the prophylactic and therapeutic uses of antimicrobial agents and the plasmid analysis of *E. coli* isolates is useful for epidemiological study.

8. The susceptibility test for nalidixic acid showed similar percentages of resistance among human, pet and vegetable sample groups but the high percentage was observed in ice sample group. For ciprofloxacin susceptibility test, the similar percentages were found among pet, vegetable and ice, while the lower resistance percentage was found particularly in humans.

9. PCR-SSCP analysis is a simple, rapid and useful method for the detection of point mutations associated with quinolone resistant *E. coli* , such as *gyrA*, and the investigation of epidemiological surveillance of organisms involved in outbreaks or for the epidemiological resistance studies.

10. The *gyrA* mutation within QRDR may be utilized as useful markers for quinolone resistance *E. coli* detection that benefit to the effective diagnosis, treatment, monitoring and prevention of antimicrobial resistance.

REFERENCES

1. Miles TD, McLaughlin W, Brown PD. Antimicrobial resistance of *Escherichia coli* isolates from broiler chickens and humans. BMC Vet Res 2006;2:7.
2. van den Bogaard AE, Stobberingh EE. Epidemiology of resistance to antibiotics. Links between animals and humans. Int J Antimicrob Agents 2000;14(4):327-35.
3. van den Bogaard AE, London N, Driessen C, Stobberingh EE. Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. J Antimicrob Chemother 2001;47(6):763-71.
4. Petersen A, Dalsgaard A. Species composition and antimicrobial resistance genes of *Enterococcus* spp, isolated from integrated and traditional fish farms in Thailand. Environ Microbiol 2003;5(5):395-402.
5. Boerlin P, Travis R, Gyles CL, Reid-Smith R, Janecko N, Lim H, *et al*. Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. Appl Environ Microbiol 2005; 71(11):6753-61.
6. Lanz R, Kuhnert P, Boerlin P. Antimicrobial resistance and resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland. Vet Microbiol 2003;91(1):73-84.
7. Yang H, Chen S, White DG, Zhao S, McDermott P, Walker R, *et al*. Characterization of multiple-antimicrobial-resistant *Escherichia coli* isolates from diseased chickens and swine in China. J Clin Microbiol 2004;42(8):3483-9.
8. Saenz Y, Brinas L, Dominguez E, Ruiz J, Zarazaga M, Vila J, *et al*. Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. Antimicrob Agents Chemother 2004;48(10):3996-4001.

9. Lemcke R, Bulte M. Occurrence of the vancomycin-resistant genes *vanA, vanB, vanCl, vanC2* and *vanC3* in *Enterococcus* strains isolated from poultry and pork. Int J Food Microbiol 2000;60(2-3):185-94.
10. Aarestrup FM, Agerso Y, Gerner-Smidt P, Madsen M, Jensen LB. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagn Microbiol Infect Dis 2000;37(2):127-37.
11. Hummel R, Tschape H, Witte W. Spread of plasmid-mediated nourseothricin resistance due to antibiotic use in animal husbandry. J Basic Microbiol 1986;26(8):461-6.
12. Blake DP, Hillman K, Fenlon DR, Low JC. Transfer of antibiotic resistance between commensal and pathogenic members of the Enterobacteriaceae under ileal conditions. J Appl Microbiol 2003;95(3):428-36.
13. Tejedor Junco MT, Gonzalez Martin M, Pita Toledo ML, Lupiola Gomez P, Martin Barrasa JL. Identification and antibiotic resistance of faecal enterococci isolated from water samples. Int J Hyg Environ Health 2001;203(4):363-8.
14. Adhikari RP, Kalpana KC, Shears P, Sharma AP. Antibiotic resistance conferred by conjugative plasmid in *Escherichia coli* isolated from community ponds of Kathmandu Valley. J Health Popul Nutr 2000;18(1):57-9.
15. Hsu SC, Chiu TH, Pang JC, Hsuan-Yuan CH, Chang GN, Tsen HY. 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 2006;27(5):383-91.
16. Zhao S, White DG, Ge B, Ayers S, Friedman S, English L, *et al*. Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. Appl Environ Microbiol 2001;67(4):1558-64.

17. Kang HY, Jeong YS, Oh JY, Tae SH, Choi CH, Moon DC, *et al*. Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. J Antimicrob Chemother 2005;55(5):639-44.

18. Wang M, Tran JH, Jacoby GA, Zhang Y, Wang F, Hooper DC. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. Antimicrob Agents Chemother 2003;47(7):2242-8.

19. Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH. Manual of clinical microbiology 8th ed. Washington, D.C. : ASM Press, 2003.

20. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 1998;11(1):142-201.

21. Rocky Mountain Laboratories, NIAID, NIH. Scanning electron micrograph of *Escherichia coli*, grown in culture and adhered to a cover slip [Internet]. United States Department of Health and Human Services (US); [cited 2007 Jan 10]. Available from: http://en.wikipedia.org/wiki/Image:*EscherichiaColi*_NIAID.jpg

22. Fankhauser DB, editor. Gram Stain Images [Internet]. [place unknown]; 2005 Jan 5 [modified 2005 Jan 6; cite 2006 Dec 15]. Available from: http://biology.clc.uc.edu/fankhauser/Labs/Microbiology/Gram_Stain/Gram_stain_images/index_gram_stain_images.html

23. Saenz Y, Zarazaga M, Brinas L, Lantero M, Ruiz-Larrea F, Torres C. Antibiotic resistance in *Escherichia coli* isolates obtained from animals, foods and humans in Spain. Int J Antimicrob Agents 2001;18(4):353-8.

24. Franz CM, Holzapfel WH, Stiles ME. Enterococci at the crossroads of food safety? Int J Food Microbiol 1999;47(1-2):1-24.

25. Mundy LM, Sahm DF, Gilmore M. Relationships between enterococcal virulence and antimicrobial resistance. Clin Microbiol Rev 2000;13(4):513-22.

26. Salyers AA, Whitt. DD. Bacterial Pathogenesis: A Molecular Approach. 2 ed. Washington, D.C: ASM Press, 2001.

27. Shankar N, editor. *Enterococcus* spp. [Internet]. California (CA); Joint Genome Institute; c1997-2007 [updated 2005 Sep 8; cite 2006 Dec 15]. Available from: http://www.jgi.doe.gov/News/news_elinks.htm

28. Delisle G, Tomalty L, edithors. [Internet]. Washington: American Society for microbiology; 2002 Jan 1 [cited 2006 Oct 10]. Figure 1 *Enterococcus faecalis* in a blood culture. Available from: http://www.microbelibrary.org/ASMOnly/details.asp?id=383&Lang=
29. Shepard BD, Gilmore MS. Antibiotic-resistant enterococci: the mechanisms and dynamics of drug introduction and resistance. Microbes Infect 2002;4(2):215-24.
30. DeLisle S, Perl TM. Vancomycin-resistant enterococci: a road map on how to prevent the emergence and transmission of antimicrobial resistance. Chest 2003;123(5 Suppl):504S-18S.
31. Bonten MJ, Willems R, Weinstein RA. Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infect Dis 2001;1(5):314-25.
32. Klare I, Witte W. Glycopeptide resistant enterococci. Occurrence, distribution, resistance transmission, significance. Wien Klin Wochenschr 1997;109(9):293-300.
33. Walsh C. Where will new antibiotics come from? Nat Rev Microbiol 2003;1(1):65-70.
34. Yoneyama H, Katsumata R. Antibiotic resistance in bacteria and its future for novel antibiotic development. Biosci Biotechnol Biochem 2006;70(5):1060-75.
35. Spratt BG, Cromie KD. Penicillin-binding proteins of gram-negative bacteria. Rev Infect Dis 1988;10(4):699-711.
36. Ban N, Nissen P, Hansen J, Moore PB, Steitz TA. The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. Scienc. 2000;289(5481):905-20.
37. Fluit AC, Visser MR, Schmitz FJ. Molecular detection of antimicrobial resistance. Clin Microbiol Rev 2001;14(4):836-71.
38. Maxwell A. DNA gyrase as a drug target. Trends Microbiol 1997;5(3):102-9.
39. Chen FJ, Lo HJ. Molecular mechanisms of fluoroquinolone resistance. J Microbiol Immunol Infect 2003;36(1):1-9.

40. Spratt BG. Resistance to antibiotics mediated by target alterations. Science 1994;264(5157):388-93.

41. Hancock RE, Chapple DS. Peptide antibiotics. Antimicrob Agents Chemother 1999;43(6):1317-23.

42. Carattoli A. Importance of integrons in the diffusion of resistance. Vet Res 2001;32(3-4):243-59.

43. Aarestrup FM. Antimicrobial Resistance in Bacteria of Animal Origin. Washington, D.C. : ASM Press, 2006.

44. Roe MT, Vega E, Pillai SD. Antimicrobial resistance markers of class 1 and class 2 integron-bearing *Escherichia coli* from irrigation water and sediments. Emerg Infect Dis 2003;9(7):822-6.

45. Hawkey PM. The origins and molecular basis of antibiotic resistance. Bmj 1998;317(7159):657-60.

46. Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM. Aminoglycosides: activity and resistance. Antimicrob Agents Chemother 1999;43(4):727-37.

47. Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol Rev 1993;57(1):138-63.

48. Chow JW. Aminoglycoside resistance in enterococci. Clin Infect Dis 2000;31(2):586-9.

49. Dworkin RJ. Aminoglycosides for the treatment of gram-negative infections: therapeutic use, resistance and future outlook. Drug Resist Updat 1999;2(3):173-179.

50. Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 2001;65(2):232-60.

51. Levy SB, McMurry LM, Burdett V, Courvalin P, Hillen W, Roberts MC, *et al*. Nomenclature for tetracycline resistance determinants. Antimicrob Agents Chemother 1989;33(8):1373-4.

52. Roberts MC. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol Rev 1996;19(1):1-24.

53. Pepper K, de Cespedes G, Horaud T. Heterogeneity of chromosomal genes encoding chloramphenicol resistance in streptococci. Plasmid 1988;19(1):71-4.
54. Bischoff KM, White DG, Hume ME, Poole TL, Nisbet DJ. The chloramphenicol resistance gene *cmlA* is disseminated on transferable plasmids that confer multiple-drug resistance in swine *Escherichia coli*. FEMS Microbiol Lett 2005;243(1):285-91.
55. Schmitz FJ, Sadurski R, Kray A, Boos M, Geisel R, Kohrer K, *et al*. Prevalence of macrolide-resistance genes in *Staphylococcus aureus* and *Enterococcus faecium* isolates from 24 European university hospitals. J Antimicrob Chemother 2000;45(6):891-4.
56. Jensen C, Jordan M, Shapiro R, Scantlebury V, Hakala T, Fung J, *et al*. Interaction between tacrolimus and erythromycin. Lancet 1994;344(8925):825.
57. Sugantino M, Roderick SL. Crystal structure of Vat(D): an acetyltransferase that inactivates streptogramin group A antibiotics. Biochemistry 2002;41(7):2209-16.
58. Bonfiglio G, Furneri PM. Novel streptogramin antibiotics. Expert Opin Investig Drugs 2001;10(2):185-98.
59. Leclercq R, Courvalin P. Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. Antimicrob Agents Chemother 1991;35(7):1267-72.
60. Thal LA, Zervos MJ. Occurrence and epidemiology of resistance to virginiamycin and streptogramins. J Antimicrob Chemother 1999;43(2):171-6.
61. Roberts MC. Distribution of macrolide, lincosamide, streptogramin, ketolide and oxazolidinone (MLSKO) resistance genes in Gram-negative bacteria. Curr Drug Targets Infect Disord 2004;4(3):207-15.
62. Werner G, Klare I, Witte W. Molecular analysis of streptogramin resistance in enterococci. Int J Med Microbiol 2002;292(2):81-94.
63. Nordmann P, Poirel L. Emergence of plasmid-mediated resistance to quinolones in Enterobacteriaceae. J Antimicrob Chemother 2005;56(3):463-9.

64. Hawkey PM. Mechanisms of quinolone action and microbial response. J Antimicrob Chemother 2003;51 Suppl 1:29-35.

65. Ruiz J. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother 2003;51(5):1109-17.

66. Munshi MH, Sack DA, Haider K, Ahmed ZU, Rahaman MM, Morshed MG. Plasmid-mediated resistance to nalidixic acid in *Shigella dysenteriae* type 1. Lancet 1987;2(8556):419-21.

67. Alonso R, Fernandez-Aranguiz A, Colom K, Cisterna R. Non-radioactive PCR-SSCP with a single PCR step for detection of inhibitor resistant beta-lactamases in *Escherichia coli*. J Microbiol Methods 2002;50(1):85-90.

68. Jacoby GA, Walsh KE, Mills DM, Walker VJ, Oh H, Robicsek A, *et al*. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob Agents Chemother 2006;50(4):1178-82.

69. Hata M, Suzuki M, Matsumoto M, Takahashi M, Sato K, Ibe S, *et al*. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrob Agents Chemother 2005;49(2):801-3.

70. Tokue Y, Sugano K, Saito D, Noda T, Ohkura H, Shimosato Y, *et al*. Detection of novel mutations in the *gyrA* gene of *Staphylococcus aureus* by nonradioisotopic single-strand conformation polymorphism analysis and direct DNA sequencing. Antimicrob Agents Chemother 1994;38(3):428-31.

71. Yoo JH, Huh DH, Choi JH, Shin WS, Kang MW, Kim CC, *et al*. Molecular epidemiological analysis of quinolone-resistant *Escherichia coli* causing bacteremia in neutropenic patients with leukemia in Korea. Clin Infect Dis 1997;25(6):1385-91.

72. Kerrn MB, Klemmensen T, Frimodt-Moller N, Espersen F. Susceptibility of Danish *Escherichia coli* strains isolated from urinary tract infections and bacteraemia, and distribution of *sul* genes conferring sulphonamide resistance. J Antimicrob Chemother 2002;50(4):513-6.

73. Radstrom P, Swedberg G, Skold O. Genetic analyses of sulfonamide resistance and its dissemination in gram-negative bacteria illustrate new aspects of R plasmid evolution. Antimicrob Agents Chemother 1991;35(9):1840-8.

74. Aminov RI, Garrigues-Jeanjean N, Mackie RI. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl Environ Microbiol 2001;67(1):22-32.

75. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol 1995;33(5):1434.

76. Miele A, Bandera M, Goldstein BP. Use of primers selective for vancomycin resistance genes to determine *van* genotype in enterococci and to study gene organization in *VanA* isolates. Antimicrob Agents Chemother 1995;39(8):1772-8.

77. Patel R, Uhl JR, Kohner P, Hopkins MK, Cockerill FR. Multiplex PCR detection of *vanA, vanB, vanC*-1, and *vanC*-2/3 genes in enterococci. J Clin Microbiol 1997;35(3):703-7.

78. Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother 1996;40(11):2562-6.

79. Chiasatit C, Kananuruk K. Antimicrobial Resistance of *Salmonella, Campylobacter* and commensal bacteria in chicken cecal contents from retail markets in Bangkok and vicinity in 2005. B. Sc. Term paper (Clinical Microbiology), Faculty of Medical Technology, Mahidol University 2005.

80. Kottajak O, Boonyakhet S. Antimicrobial resistance of *Escherichia coli* and enterococci isolated from vegetable and ice in Bangkok and vicinity. B.Sc. Term paper (Clinical Microbiology), Faculty of Medical Technology, Mahidol University 2005.

81. Pleumsud J. Mutational analysis of Quinolone Resistance in *Campylobacter* and *Salmonella* isolated from diarrheal patient in Thialand. M. Sc. Thesis (Clinical Microbiology), Faculty of Medical Technology, Mahidol

University 2005.

82. Maynard C, Bekal S, Sanschagrin F, Levesque RC, Brousseau R, Masson L, *et al*. Heterogeneity among virulence and antimicrobial resistance gene profiles of extraintestinal *Escherichia coli* isolates of animal and human origin. J Clin Microbiol 2004;42(12):5444-52.
83. Simjee S, White DG, Wagner DD, Meng J, Qaiyumi S, Zhao S, *et al*. Identification of *vat(E)* in *Enterococcus faecalis* isolates from retail poultry and its transferability to *Enterococcus faecium*. Antimicrob Agents Chemother 2002;46(12):3823-8.
84. Karami N, Nowrouzian F, Adlerberth I, Wold AE. Tetracycline resistance in *Escherichia coli* and persistence in the infantile colonic microbiota. Antimicrob Agents Chemother 2006;50(1):156-61.
85. Schroeder CM, Meng J, Zhao S, DebRoy C, Torcolini J, Zhao C, *et al*. Antimicrobial resistance of *Escherichia coli* O26, O103, O111, O128, and O145 from animals and humans. Emerg Infect Dis 2002;8(12):1409-14.
86. Sayah RS, Kaneene JB, Johnson Y, Miller R. Patterns of antimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic- and wild-animal fecal samples, human septage, and surface water. Appl Environ Microbiol 2005;71(3):1394-404.
87. Nys S, Tjhie JH, Bartelds AI, Heijnen ML, Peeters MF, Stobberingh EE. Erythromycin resistance in the commensal throat flora of patients visiting the general practitioner: a reservoir for resistance genes for potential pathogenic bacteria. Int J Antimicrob Agents 2005;26(2):133-7.
88. Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J, Seppala H. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob Agents Chemother 1999;43(12):2823-30.
89. Soltani M, Beighton D, Philpott-Howard J, Woodford N. Mechanisms of resistance to quinupristin-dalfopristin among isolates of *Enterococcus faecium* from animals, raw meat, and hospital patients in Western Europe. Antimicrob Agents Chemother 2000;44(2):433-6.

90. Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques 1997;23(3):504-11.
91. Poyart C, Quesnes G, Trieu-Cuot P. Sequencing the gene encoding manganese-dependent superoxide dismutase for rapid species identification of enterococci. J Clin Microbiol 2000;38(1):415-8.
92. Jackson CR, Fedorka-Cray PJ, Barrett JB. Use of a genus- and species-specific multiplex PCR for identification of enterococci. J Clin Microbiol 2004;42(8):3558-65.
93. Olsvik O, Sorum H, Birkness K, Wachsmuth K, Fjolstad M, Lassen J, *et al*. Plasmid characterization of *Salmonella typhimurium* transmitted from animals to humans. J Clin Microbiol 1985;22(3):336-8.
94. Meyers JA, Sanchez D, Elwell LP, Falkow S. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J Bacteriol 1976;127(3):1529-37.
95. Vatopoulos AC, Mainas E, Balis E, Threlfall EJ, Kanelopoulou M, Kalapothalki V, *et al*. Molecular epidemiology of ampicillin-resistant clinical isolates of *Salmonella enteritidis*. J Clin Microbiol 1994;32(5):1322-5.
96. Ang-Kucuker M, Tolun V, Helmuth R, Rabsch W, Buyukbaba-Boral O, Torumkuney-Akbulut D, *et al*. Phage types, antibiotic susceptibilities and plasmid profiles of *Salmonella typhimurium* and *Salmonella enteritidis* strains isolated in Istanbul, Turkey. Clin Microbiol Infect 2000;6(11):593-9.
97. Schmidt T, Friehs K, Schleef M, Voss C, Flaschel E. Quantitative analysis of plasmid forms by agarose and capillary gel electrophoresis. Anal Biochem 1999;274(2):235-40.
98. Jacoby GA. Mechanisms of resistance to quinolones. Clin Infect Dis 2005;41 Suppl 2:S120-6.
99. Hooper DC. Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis 2001;7(2):337-41.

100. Friedman SM, Lu T, Drlica K. Mutation in the DNA gyrase A Gene of *Escherichia coli* that expands the quinolone resistance-determining region. Antimicrob Agents Chemother 2001;45(8):2378-80.

101. Levy SB. The challenge of antibiotic resistance. Sci Am 1998;.53-46:(3)278

102. Robicsek A, Sahm DF, Strahilevitz J, Jacoby GA, Hooper DC. Broader distribution of plasmid-mediated quinolone resistance in the United States. Antimicrob Agents Chemother 2005;49(7):3001-3.

103. Hammerum AM, Sandvang D, Andersen SR, Seyfarth AM, Porsbo LJ, Frimodt-Moller N, *et al*. 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 2006;106(2):235-7.

APPENDIX

Reagent Preparation

1. 50x TAE (pH 7.8)

Trise-base	242 g
Na_2EDTA	37.2 g
Glacial acetic acid	57.1 ml

Adjust volume to 1 L with deionized water. The solution was mixed and kept at room temperature.

2. 10x TBE

0.89 M Trise-base	216 g
0.89 M Boric acid	110 g
50 mM EDTA	18.6 g

Adjust volume to 2 L with deionized water. The solution was mixed and kept at room temperature.

3. 40% acrylamide: bisacrylamide stock solution (37.5:1)

Acrylamide	38.93 g
Bris-acrylamide	1.07 g

All components were dissolved at 65°C for 10 minutes, filtered through a 0.45 µl filter membrane and kept at 4°C.

4. 10% (w/v) Ammonium persulphate

Ammonium persulphate	0.1 g
Distilled water to	1 ml

The mixer was dissolved and kept at -20°C

5. 2x SSCP loading dye

Formamide	950 µl (95%)
Bromphenol blue	10 µl (0.05%)
Xylene cyanol	10 µl (0.05%)
1M EDTA µl	20 µl (20 mM EDTA)
1M NaOH µl	10 µl (10 mM NaOH)

Adjust volume with distilled water to 1 ml.

The mixer was dissolved and kept at -20°C.

6. Fixing solution (10% glacial acetic acid)

Glacial acetic acid	100 ml
Distilled water	900 ml

The solution was mixed and kept at room temperature.

7. Developing solution

Sodium carbonate ($Na_2CO_{3)}$	3 g
Distilled water	100 ml

The solution was mixed and kept at -20°C for at least 2 h and adds 20 µl of 10mg/ml sodium thiosulfate and 1.5 ml of 37% formaldehyde before use.

8. 0.1% Silver nitrate solutions

Silver nitrate	1 g

Adjust volume with distilled water to 1 L

Add 1.5 ml of 37% formaldehyde before use.

Note: should be freshly prepared

9. 0.4 M NaOH

NaOH	1.6 g
Distilled water	100 ml

The solution was mixed and kept at room temperature.

10. 20X SSC (0.3 M Na-Citrate / 3 M NaCl pH 7.4)

0.3 M Tris-sodium citrate	88.2 g
3 M NaCl	175.3 g
Distilled water	900 ml

The solution was mixed, adjusts pH to 7 with NaOH, autoclave at 121°C 15 minutes and kept at 4°C.

11. 10x dNTP labeling mix

10mM dATP	1 µl
10mM dCTP	1 µl
10mM dGTP	1 µl
10mM dTTP	0.65 µl
1 mM DIG-11-dUTP	2.9 µl
Distilled water	3.45 ml

The solution was mixed and kept at -20°C.

12. 10x Maleic acid buffer

Maleic acid	116.1 g
NaCl	87.6 g
NaOH	80 g

The solution was prepared by processing on ice bath and was then added distilled water 700 ml

Adjust pH to 7.5 with 5 M NaOH and make up to 1 L with distilled water
The solution was mixed and kept at room temperature.

13. Washing buffer (1x Maleic acid buffer and 0.03% Tween20)

10x Maleic acid buffer	50 ml
Tween20	1.5 ml
Distilled water	450 ml

The solution was mixed and kept at 4°C.

14. 1x Blocking solution (diluted 10x blocking solution in 1x Maleic acid buffer)

10x Maleic acid buffer	3 ml
10x Blocking solution	3 ml
Distilled water	24 ml

The solution was kept at -20°C.

15. Pre-hybridization solution

Formamide (50% (v/v))	10 ml
20x SSC (1% (w/v))	6 ml
10% SDS (6x SSC)	2 ml
50x Denhardt's solution (5x)	2 ml
5 mg/ml Herring sperm DNA (100 µg/ml)	0.4 ml

The solution was mixed and kept at -20°C until use.

16. Hybridization solution

Formamide (50% (v/v))	10 ml
20x SSC (1% (w/v))	6 ml
10% SDS (6x SSC)	2 ml
50x Denhardt's solution (5x)	0.4 ml
5 mg/ml Herring sperm DNA (100 µg/ml)	0.4 ml
5 mg/ml Heparin (100 µg/ml)	0.4 ml
Sterile deionized water	0.8 ml

The solution was mixed and kept at -20°C until use.

17. 10x Detection buffer

Tris base	6.055 g
NaCl	2.92 g
$MgCl_2 \cdot 6H_2O$	5.08 g
Distilled water	400 ml

Adjust pH to 9.5 with 5 M NaOH

Made up to 500 ml with distilled water

18. Colour detection solution (Total volume 10 ml)

75 mg/ml nitroblue tetrazolium (NBT)	45 µl
50 mg/ml 5-bromo-4-chloro-3 indolyl phosphate, toluidinium salt (BCIP)	35 µl
1x detection buffer	10 ml

BIOGRAPHY

NAME	Miss Wipawadee Sianglum
DATE OF BIRTH	24 March 1979
PLACE OF BIRTH	Ubonratchathani, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 1998-2001 Bachelor of Science (Medical Technology) Mahidol University, 2004–2006 Master of Science (Medical Technology)
FELLOWSHIPS	Thesis is partly supported by the Graduate Scholarship of the Faculty of Medical Technology, Mahidol University
ADDRESS	320/6 Burapanai Road, Meung, Ubonratchathani, 34000 Email: wipawadeeka@hotmail.com wipawadeeka@yahoo.com