MOLECULAR CHARACTERIZATION AND EPIDEMIOLOGICAL STUDY OF MULTIDRUG RESISTANT PATHOGENIC *ESCHERICHIA COLI* HUMAN ISOLATES FROM 2001-2010

SUPAPORN RUKSASIRI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (MICROBIOLOGY) FACULTY OF GRADUATE STUDIES MAHIDOL UNIVERSITY 2015

Thesis
entitled

MOLECULAR CHARACTERIZATION AND EPIDEMIOLOGICAL STUDY OF MULTIDRUG RESISTANT PATHOGENIC *ESCHERICHIA COLI* HUMAN ISOLATES FROM 2001-2010

……..
Miss Supaporn Ruksasiri
Candidate

……..
Assoc. Prof. Chanwit Tribuddharat, M.D.,
Ph.D. (Microbiology and Immunology)
Major advisor

…….
Assoc. Prof. Chertsak Dhiraputra, M.D.,
M.Sc. (Microbiology)
Co-advisor

……..
Asst. Prof. Iyarit Thaipisuttikul, M.D.,
Ph.D. (Genetics)
Co-advisor

…….
Asst. Prof. Kanokwan Kittiniyom,
Ph.D. (Pathology)
Co-advisor

……..
Prof. Patcharee Lertrit, M.D.,
Ph.D. (Biochemistry)
Dean
Faculty of Graduate Studies
Mahidol University

…….
Assoc. Prof. Pattarachai Kiratisin, M.D.,
Ph.D. (Microbiology and Immunology)
Program Director
Master of Science Program in Microbiology
Faculty of Medicine Siriraj Hospital
Mahidol University

Thesis
entitled

MOLECULAR CHARACTERIZATION AND EPIDEMIOLOGICAL STUDY OF MULTIDRUG RESISTANT PATHOGENIC *ESCHERICHIA COLI* HUMAN ISOLATES FROM 2001-2010

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Microbiology)
on
June 30, 2015

……..
Miss. Supaporn Ruksasiri
Candidate

……..
Assoc. Prof. Pintip Pongpech,
Ph.D. (Medical Microbiology)
Chair

……..
Assoc. Prof. Chertsak Dhiraputra, M.D.,
M.Sc. (Microbiology)
Member

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

……..
Asst. Prof. Iyarit Thaipisuttikul, M.D.,
Ph.D. (Genetics)
Member

……..
Asst. Prof. Kanokwan Kittiniyom,
Ph.D. (Pathology)
Member

……..
Prof. Patcharee Lertrit, M.D.,
Ph.D. (Biochemistry)
Dean
Faculty of Graduate Studies
Mahidol University

……..
Prof. Prasit Watanapa, M.D.,
Ph.D. (Surgery), FRCS, FACS
Dean
Faculty of Medicine Siriraj Hospital
Mahidol University

ACKNOWLEDGMENTS

First and foremost, I would like to express my heartfelt gratitude and appreciation to my advisor Assoc. Prof. Chanwit Tribuddharat for his valuable guidance, support, and encouragement throughout this study. This thesis could not be successfully completed without his kindness. I am also very grateful to him for giving me the opportunity to be his student which gave me a valuable experience.

I am sincerely grateful to my thesis committee: Assoc. Prof. Chertsak Dhiraputra, Assoc. Prof. Pintip Pongpech, Asst. Prof. Iyarit Thaipisutikul, and Asst. Prof. Kanokwan Kittiniyom for their valuable suggestions of this thesis.

I would like to express my sincere thankfulness to my bosses, COL Carl J Mason, former Chief of Enteric Diseases Department, AFRIMS and Dr. Ladaporn Bodhidatta, Asst. Chief, Department of Enteric Diseases, AFRIMS, for giving me the opportunity to further my academic degree and also for their advice, generosity, and encouragement which guided me to reach the completion of this study.

I would like to express my special thanks to Mrs. Kamonporn Poramathikul for her goodwill and good suggestion which helped me a lot throughout my graduate study.

I would like to express my special thanks to Dr. Oralak Serichantalergs for providing facilities and materials for dot-blot hybridization technique and also for suggestion in this study.

I would like to thank to Dr. Pimnapar Neesanant for her consultation throughout this study and Mrs. Piyarat Pootong for her dot-blot hybridization technique training.

I would like to thank Siriraj Graduate Thesis scholarship from Faculty of Medicine Siriraj Hospital, Mahidol University for research grant in this study.

I would like to thank Armed Forces Research Institute of Medical Sciences (AFRIMS) for research support in this study.

I would like to thank Mrs. Kesanee Kanchanalai for a private donation to support this study.

I would like to give my warmest thank to my colleagues at AFRIMS for their understanding and support.

I also thank all of the graduate students at the Department of Microbiology, Faculty of Medicine Siriraj Hospital for their friendship, support, and sharing experiences with me.

Finally, my special gratitude is express to my family for their love, belief, and support which has allowed me to overcome the difficulties.

Supaporn Ruksasiri

MOLECULAR CHARACTERIZATION AND EPIDEMIOLOGICAL STUDY OF MULTIDRUG RESISTANT PATHOGENIC *ESCHERICHIA COLI* HUMAN ISOLATES FROM 2001-2010

SUPAPORN RUKSASIRI 5437663 SIMI/M

M.Sc. (MICROBIOLOGY)

THESIS ADVISORY COMMITTEE: CHANWIT TRIBUDDHARAT, M.D., Ph.D., CHERTSAK DHIRAPUTRA M.D., M.Sc., IYARIT THAIPISUTIKUL, M.D., Ph.D., KANOKWAN KITTINIYOM, Ph.D.

ABSTRACT

Multidrug resistant (MDR) diarrheagenic *Escherichia coli* (DEC) is a dangerous causative agent of diarrhea in children and travelers. Nevertheless, little is known about the molecular characteristics of these bacteria in a community setting. This study assessed the prevalence and molecular characterization of class 1 integrons and epidemiology of 200 MDR diarrheagenic *E. coli*: enteroaggregative *E. coli* (EAEC); enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroinvasive *E. coli* (EIEC); and shiga-like toxin producing *E. coli* (STEC) isolates from indigenous children, native adults and travelers in Thailand, Cambodia, Maldives, Nepal, and Vietnam in 2001-2010. Using PCR specific primers to a class 1 integrase (*intI1*) gene, 5′-3′ conserved sequence (5′CS-3′CS), and dot-blot hybridization, class 1 integrons were found in 70.5% (62/88) and 76.4% (84/110) in adults and children, respectively. Resistance gene cassettes identified in this study were *aadA1*, *aadA2*, and *aadA5* (for streptomycin resistance); *dfrA12* and *dfrA17* (for trimethoprim resistance); and *linF* (for lincosamide resistance). Epidemiological study was performed to understand genetic relatedness and investigate the possible clonal spread among these bacteria by using plasmid profiling, Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR), and high throughput multi-locus sequence typing (HiMLST) incorporated with space-time disease surveillance. Using Achtman MLST typing scheme, this study have designed the specific PCR primers to 7 housekeeping genes, excepting *adk* to obtain optimal amplicon sizes for 454 sequencing system. Among three typing techniques, plasmid profiling gave the lowest discriminatory power (DI, 0.956) whereas DI of ERIC PCR is very similar to HiMLST (0.973 vs 0.979). The results showed high diversity in each group of *E. coli* pathotypes, whereas ST182 was predominant as the ETEC, suggesting some ETEC strains were clonal expansion. The clonal spread included the possible outbreaks, the persistence clones, and the international spread clones. Moreover, many STs were matched with strains from food-producing animals in *E. coli* MLST database. These findings highlight the role of clonal expansion among these bacteria in human and animals. In conclusion, this study revealed the long-term genetic relatedness among community-acquired MDR diarrheagenic *E. coli* isolates harboring class 1 integrons, and suggested the circulation of these resistance elements in the community. These results provide crucial public health information for surveillance and control of antibiotic resistance pathogens in this part of Asia. A cost-effective high-throughput MLST (HiMLST) absolutely generates the robust MLST on a large-scale sequencing, which can be applied for new strategies in MLST studies using next-generation sequencing (NGS) or new affordable applications.

KEY WORDS: *ESCHERICHIA COLI*/ INTEGRON/ HIGH THROUGHPUT MULTI-LOCUS SEQUENCE TYPING/ CLONAL SPREAD/ NEXT-GENERATION SEQUENCING

140 pages

การตรวจระดับอณูชีวโมเลกุลและการศึกษาระบาดวิทยาของเชื้อ *Escherichia coli* ชนิดก่อโรคที่ดื้อต่อยาต้านจุลชีพหลายขนานจากคนในช่วงปี 2001-2010

MOLECULAR CHARACTERIZATION AND EPIDEMIOLOGICAL STUDY OF MULTIDRUG RESISTANT PATHOGENIC *ESCHERICHIA COLI* HUMAN ISOLATES FROM 2001-2010

สุภาพร รักษาสิริ 5437663 SIMI/M

วท.ม. (จุลชีววิทยา)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: ชาญวิทย์ ตรีพุทธรัตน์, M.D., Ph.D., เชิดศักดิ์ ธีระบุตร, M.D., M.Sc., ไอยฤทธิ์ ไทยพิสุทธิกุล, M.D., Ph.D., กนกวรรณ กิตตินิยม, Ph.D.

บทคัดย่อ

เชื้อ *Escherichia coli* (*E. coli*) ชนิดก่อโรคท้องเสียที่ดื้อยาหลายขนานเป็นสาเหตุสำคัญของโรคอุจจาระร่วงในเด็กและนักท่องเที่ยว อย่างไรก็ตามข้อมูลด้านลักษณะทางอณูชีวโมเลกุลของเชื้อเหล่านี้ในชุมชนยังมีน้อย การศึกษาครั้งนี้ได้ประเมินความชุกและลักษณะทางอณูชีวโมเลกุลของ class 1 integrons และระบาดวิทยาของเชื้อ *E. coli* ชนิดก่อโรคท้องเสียที่ดื้อยาหลายขนาน จำนวน 200 ตัวอย่างที่แยกได้จากเด็กชนพื้นเมืองและผู้ใหญ่รวมทั้งชาวบ้านและนักท่องเที่ยวในประเทศไทย กัมพูชา มัลดีฟส์ เนปาล และเวียดนามช่วงปี ค.ศ. 2001-2010 ซึ่งได้แก่ enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) และ shiga-like toxin producing *E. coli* (STEC) โดยวิธีเพิ่มสารพันธุกรรมแบบลูกโซ่ (PCR) ซึ่งใช้ไพรเมอร์ที่จำเพาะต่อยีน *intI1*, 5'CS-3'CS และวิธี dot-blot hybridization โดยยีนเป้าหมายคือ *intI1* ตรวจพบ class 1 integrons 70.5% (62/88) และ 76.4% (84/110) ในผู้ใหญ่และเด็กตามลำดับ ยีนดื้อยาที่พบในการศึกษาครั้งนี้ได้แก่ *aadA1*, *aadA2* และ *aadA5* (ดื้อต่อยาสเตร็ปโตมัยซิน) *dfrA12* และ *dfrA17* (ดื้อต่อยาไตรเมโทพริม) และ *linF* (ดื้อต่อยาในกลุ่มลินโคซาไมด์) การศึกษาทางระบาดวิทยาเพื่อเข้าใจความสัมพันธ์ทางพันธุกรรมและสืบสวนการแพร่กระจายตามชนิดของสายพันธุ์ (clonal spread) ในแบคทีเรียเหล่านี้โดยใช้ plasmid profiling, Enterobacterial Repetitive Intergenic Consensus PCR (ERIC PCR) และ high throughput multi-locus sequence typing (HiMLST) ร่วมกับการเฝ้าระวังโรคโดยอาศัยพื้นที่สัมพันธ์กับเวลา (space-time disease surveillance) ไพรเมอร์จำเพาะต่อ 7 ยีนที่มีความจำเป็นต่อการดำรงชีพของเชื้อ (housekeeping genes) ได้ถูกออกแบบสำหรับเพิ่มสารพันธุกรรมแบบลูกโซ่ (PCR) ยกเว้นยีน *adk* โดยใช้แบบแผนการแยกชนิด MLST ตาม Achtman (Achtman MLST typing) เพื่อให้ได้ขนาดของ DNA (amplicons) ที่เหมาะสำหรับระบบ 454 sequencing ในบรรดา 3 เทคนิคการแบ่งชนิด plasmid profiling มีอำนาจการจำแนกสายพันธุ์ (discriminatory power) ต่ำสุด (DI, 0.956) ในขณะที่อำนาจการจำแนกของวิธี ERIC PCR คล้ายกับวิธี HiMLST มาก (0.973 vs 0.979) ผลการศึกษาแสดงให้เห็นถึงความหลากหลายในแต่ละกลุ่มของเชื้อ *E. coli* ชนิดก่อโรค ยกเว้น ST182 ทุกตัวอย่างเป็น ETEC จึงกล่าวได้ว่า ETEC บางสายพันธุ์มีการแพร่กระจายตามชนิดของสายพันธุ์นั้น (clonal spread) ในการศึกษานี้พบการแพร่กระจายตามชนิดของสายพันธุ์ได้แก่ สายพันธุ์ที่น่าจะระบาด (possible outbreaks) สายพันธุ์ที่คงอยู่ (persistence clones) และสายพันธุ์ข้ามประเทศ (international spread clones) นอกจากนี้หลาย STs ยังจับคู่กับสายพันธุ์จากสัตว์เพื่อผลิตเป็นอาหารที่มีในฐานข้อมูล *E. coli* MLST การค้นพบนี้ได้เน้นบทบาทของการแพร่กระจายตามสายพันธุ์ในแบคทีเรียเหล่านี้ทั้งในมนุษย์และสัตว์ โดยสรุปการศึกษาครั้งนี้แสดงให้เห็นถึงความสัมพันธ์ทางพันธุกรรมในระยะยาวของเชื้อ *E. coli* ชนิดก่อโรคท้องเสียที่ดื้อยาหลายขนานแบบมี class 1 integrons และชี้ให้เห็นถึงการหมุนเวียนของ class 1 integrons ในชุมชน ผลการศึกษาเหล่านี้เป็นข้อมูลสำคัญทางสาธารณสุขสำหรับการเฝ้าระวังและการควบคุมเชื้อโรคดื้อยาปฏิชีวนะในเอเชียภูมิภาค ด้วยราคาที่มีประสิทธิภาพของเทคนิค HiMLST ทำให้การแบ่งชนิดแบบ MLST สำหรับโครงการหาลำดับทางพันธุกรรมขนาดใหญ่แข็งแกร่งขึ้นอย่างแท้จริง ซึ่งสามารถปรับใช้สำหรับกลยุทธ์ใหม่ในการศึกษา MLST โดยใช้ next-generation sequencing (NGS) หรืองานประยุกต์ใหม่ในราคาที่สามารถจ่ายได้

140 หน้า

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF TABLES (cont.)

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS

Abbreviation	Term
AAFs	Adherence fimbriae
AACs	Aminoglycoside acetyltransferases
ABC	ATP-binding cassette
A/E	Attaching-and effacing
AGPs	Antimicrobial growth promoters
AMEs	Aminoglycoside-modifying enzyme
AM	Ampicillin
ANTs	Aminoglycoside nucleotidyltransferases
APHs	Aminoglycoside phosphotransferases
AST	Antibiotic susceptibility testing
ATCC	American Type Culture Collection
BFP	Bundle-forming pili
C	Chloramphenicol
cAMP	Cyclic adenosine monophosphate
CATs	Chloramphenicol acetyltransferases
CAZ/CLA	Ceftazidime-clavulanic acid
CDC	Center for Control Disease and Prevention
CFs	Fibrillar colonizing factor
CIP	Ciprofloxacin
CRO	Ceftriaxone
CT	Cholera enterotoxin
CTX	Cefotaxime
CTX/CLA	Cefotaxime-clavulanic acid

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
CLSI	Clinical and Laboratory Standards Institute guidelines
DAEC	Diffusely adherent *E. coli*
DAF	Decay-accelerating factor
DEC	Diarrheagenic *E. coli*
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DI	Discriminatory power index
DIG	Digoxigenin
EAEC	Enteroaggregative *E. coli*
EAF	Enteropathogenic *E. coli* adherence factor
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBLs	Extended-spectrum β-lactamases
ETEC	Enterotoxigenic *E. coli*
emPCR	Emulsion polymerase chain reaction
ERIC PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
G	Sulfisoxazole
GN	Gentamycin
HC	Hemorrhagic colitis
Hfr	High frequency of recombination
HiMLST	High throughput multilocus sequence typing

LIST OF ABBREVIATIONS(cont.)

Abbreviation	Term
HUS	Hemolytic Uremic Syndrome
ICEs	Integrative and conjugative elements
K	Kanamycin
LEE	Locus of enterocyte effacement
LINs	Lincosamide nucleotidyltransferases
LTs	Heat-labile toxins
MATE	Multidrug and toxic effects
MDR	Multidrug resistance
MFS	Major facilitator superfamily
MGTs	Glycosyltransferases
MID	Multiplex identifier
MPHs	Macrolide phosphotransferases
MR	Methyl Red
N	Neomycin
NA	Nalidixic acid
NCBI	National Center for Biotechnology Information
NDM	New Delhi metallo β-lactamase
NGS	Next-generation sequencing
PAIs	Pathogenicity islands
PABA	Para-aminobenzoic acid
Pet	Plasmid-encoded toxin
PMQR	Plasmid-mediated quinolone resistance
PRPs	Pentapeptide repeat proteins
QRDR	Quinolone-resistance determining region

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
RND	Resistance nodulation cell division subfamily
S	Streptomycin
SMR	small multidrug regulator subfamily
SXT	Trimethoprim-sulfamethoxazole
ST	Sequence type
STEC	Shiga toxin-producing *E. coli*
STs	Stable toxins
STX	shiga toxin
TE	Tetracycline
Tn	Transposon
VATS	Virginiamycin acetyltransferases
VP	Voges-Proskaeur
VT	verocytotoxin
VTEC	verotoxin-producing *E. coli*

CHAPTER I
INTRODUCTION

Escherichia coli is the major facultative anaerobe of the normal gastrointestinal tract in human and animals, but it is also the most frequent human bacterial pathogen that causes urinary tract infection, sepsis, meningitis, and enteric/diarrheal disease (1). Diarrheagenic *E. coli* (DEC) is among the most common bacterial agents of diarrheal diseases that remains a major public health problem in developing countries, especially for children under five years old and in the international travelers who visit those countries (2). Normally, DEC is one of the etiology of foodborne diseases that transmitted by person-to-person contact and consumption of contaminated food and water. There are six pathotypes of DEC, enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) or verocytotoxin-producing *E. coli*, enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC).

Nowadays, the prevalence of antimicrobial resistant bacteria has dramatically increased globally and become the major public health issues. Particularly, multidrug resistant (MDR) *E. coli* is associated with increased morbidity and mortality, worldwide (3, 4). MDR *E. coli* from human and food-producing animals was increased from 7.2% in 1950s to 63.6% in 2000s and co-resistant phenotype was reported among tetracycline, streptomycin, sulfonamide, ampicillin, and chloramphenicol (5). Although there are many causes of emergence of multidrug resistant strains, the most important cause is the overuse of antibiotics worldwide across all ecosystems over the past decades, including in humans, animals, aquaculture, and agriculture (6).

The intensive use of antimicrobials in both of human and veterinary medicine, as well as agriculture and aquaculture, is associated with an emerging resistance against therapeutic drugs, followed by the selection of virulence and

resistance genes carrying bacterial strains, and dissemination of resistant strain in humans, animals, and the environment (7-10). Bacteria acquire resistance from genetic mutations and horizontal gene transfer by mobile genetic elements (MGEs), e.g., plasmids, transposons, integrons, and bacteriophages. However, antibiotic resistance determinants are carried mostly by mobile genetic elements (11).

In recent years, integrons and gene cassettes play a role in the spread of antibiotic resistance. They are genetic elements able to recognize and capture mobile gene cassettes carrying antibiotic resistance genes via site-specific recombination. Although integrons cannot transpose with their antibiotic genes by themselves, their spread is associated with mobile genetic elements, such as transposons and conjugative plasmids. Among five different classes of mobile integrons based on the sequence homology of integrases, the class 1 integron is the most widespread and mostly found in clinical isolates, especially in Gram-negative bacteria (12). Class 1 integrons usually comprise two conserved segments that flank the variable gene cassette region. The 5'-conserved segment contains the *intI1* gene (*intI1*), the *attI* site, and the common promoter Pc, whereas the 3'-conserved segment includes an antiseptic resistance gene (*qacEΔ1*), a sulfonamide resistance gene (*sul1*), and an ORF5 (*orf5*) of unknown function (13). The prevalence of class 1 integrons is high in both of clinical isolates and commensal flora from human and animals, especially in Gram-negative bacteria (14). Nevertheless, still little is known about genetic relatedness among class 1 integrons carrying DEC strains.

Besides horizontal gene transfer, the dissemination of antimicrobial resistance genes can be mediated by clonal expansion. Thereby, understanding of clonal relatedness between bacterial strains is essential for surveillance and control of their spread. Epidemiological study can be useful for tracking of the sources and routes of pathogens, and can be used to investigate an outbreak situation, understand pathogenesis of an infection. Importantly, it contributes to the effectiveness of monitoring and surveillance systems and provides significant data to public health control strategies (15). Nowadays, molecular typing methods have become necessary in epidemiological investigation and can be used to determine genetic relationship among bacterial strains. Therefore, this study was conducted to investigate the prevalence and characterization of class 1 integrons in 200 MDR diarrheagenic *E. coli*

isolates from human among 5 Asian countries during the past 10 years (from year 2001-2010) by using PCR specific primers to a class 1 integrase (*intI1*) gene, 5′-3′ conserved sequence (5′CS-3′CS), and dot-blot hybridization. Three different molecular typing methods, including plasmid profile, enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC PCR), and high throughput multi-locus sequence typing (Hi-MLST) with *454* technology, were performed for investigation of the possible clonal spread of community-acquired MDR diarrheagenic *E. coli* from that period.

CHAPTER II
OBJECTIVES

The objectives of this study are as follow

1. To determine the presence of class 1 integrons in multidrug resistant pathogenic *E. coli* isolates from human among Asian countries from year 2001-2010.

2. To characterize class 1 integrons and antimicrobial resistance gene cassettes.

3. To investigate the possible clonal spread of community-acquired multidrug resistant pathogenic *E. coli* with long term period.

CHAPTER III
LITERATURE REVIEW

3.1 *Escherichia coli*

3.1.1 History

E. coli was first isolated from feces of a child by the German-Austrian pediatrician and bacteriologist Theodor Escherich in 1885, called "*Bacterium coli commune*" because it is a Gram-negative rod bacterium and found in lower intestine of human and mammals. In 1919, *Escherichia coli* was named to honor Theodor Escherich for his discovery and abbreviated as *E. coli*. It was considered to be a commensal organism of large intestine until 1935, there has been reported of *E. coli* causing outbreak of diarrhea among infants (16).

3.1.2 Taxonomy

E. coli belongs to:

Kingdom Eubacteria
Phylum Proteobacteria
Class Gammaproteobacteria
Order Enterobacteriales
Family Enterobacteriaceae
Genus *Escherichia*
Species *Escherichia coli*

3.1.3 Cell structure and physiology

E. coli is facultative anaerobic gram negative bacilli with 1-3 μm long and 0.25-1.0 μm in diameter. It is versatile and well adapted to various conditions. It grows at 15-48ºC with optimal temperature is 37-42ºC, and pH range from 5.5-8.0. Most of them are commonly motile by using peritrichous flagella and ferment glucose,

lactose, mannitol, sorbitol, arabinose, maltose, xylose, trehalose, and mannose including utilize nitrates to nitrites.

3.1.4 Habitat

The common habitat of *E. coli* is in the gastrointestinal tract of humans and warm-blooded animals. It usually colonizes an infant's gastrointestinal within 40 hours after birth. However, it cans survive outside the body for a short time in fecal contaminated environments such as soil, sediment, and water, which used as indicator for the presence of recent fecal contamination.

3.1.5 Identification

E. coli can be cultured easily from clinical specimens on selective and non- selective media at 37ºC under aerobic condition. It can be identified by using phenotypic properties and biochemical tests. *E. coli* is an oxidase negative and catalase positive bacteria. Most of them ferment lactose, positive for indole, Methyl Red (MR), and motile, whereas citrate and Voges-Proskaeur (VP) tests are negative. However, some pathogenic *E. coli* especially Enteroinvasive *E. coli* (EIEC) strains are typically lactose negative and non-motile (1). Identification of pathogenic *E. coli* strains requires detection of its unique virulence properties such as phenotypic assay, e.g., HEp-2 adherence assay and molecular detection method, e.g., nucleic acid probe, and specific PCR.

3.1.6 Pathogenicity and evolution

E. coli is usually mutualism with its host organism; however there are adapted *E. coli* that acquired specific virulence determinants to increase ability to cause the diseases (17). Pathogenic *E. coli* is associated with virulence-related plasmids, bacteriophages, and chromosomal pathogenicity islands (PAIs) (Figure 3.1). They are responsible for three major diseases in human; urinary tract infection, sepsis/ meningitis, and gastroenteritis. The six pathotypes of *E. coli* that cause diarrheal diseases are enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) or verocytotoxin-producing *E. coli* (VTEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely

adherent *E. coli* (DAEC) (Figure 3.2). They are transmitted by person-to-person contact and consumption of contaminated food and water such as raw meat, milk, fruit, and vegetable.

3.1.6.1 Enteropathogenic *E. coli* (EPEC)

EPEC is a leading cause of infantile diarrhea in developing countries. The symptoms caused by EPEC infection including profuse watery diarrhea, vomiting, and low-grade fever. It induces the attaching-and effacing (A/E) histopathology, which associated with three stages of pathogenesis including (i) localized adherence involving bundle-forming pili (BFP), located on EPEC adherence factor (EAF) plasmid, (ii) signal transduction by type III secretion system, which is encoded by several genes on the locus of enterocyte effacement (LEE) pathogenicity island and (iii) intimate bacterial attachment with pedestal formation by intimin protein, encoded by *eae* gene on LEE (Figure 3.2a).

3.1.6.2 Enterohaemorrhagic *E. coli* (EHEC)

EHEC cans cause severe disease such as Hemorrhagic Colitis (HC), which can progress to Hemolytic Uremic Syndrome (HUS) leading to kidney failure, hemolytic anemia, and thrombocytopenia. Although EHEC is a subset of shiga toxin-producing *E. coli* (STEC) or verotoxin-producing *E. coli* (VTEC), which produces shiga toxin (Stx) or verocytotoxin (VT), it contains LEE pathogenicity island that is homologous with those of EPEC. Like an EPEC, most EHEC strains also induce attaching-and effacing (A/E) lesions, however the severity of the disease is associated with shiga toxin (Stx), encoded by bacteriophage (Figure 3.2b). Among many different serotypes of STEC, EHEC O157:H7 is the most important pathogen in North America, Japan, and parts of Europe, whereas other serotypes are significant in other developed countries (18).

3.1.6.3 Enterotoxigenic *E. coli* (ETEC)

ETEC is a crucial bacterial cause of diarrhea in travelers and children in developing world. The diarrhea is watery without blood and fever. Pathogenesis of ETEC is due to the production of enterotoxins LTs, STs, or

combination of these. Heat-labile toxins (LTs), which are similar in structure and function to the cholera enterotoxin (CT) of *V. cholerae*, consist of A subunit and pentamers molecules of B (AB_5 toxin). The B subunit binds to the ganglioside receptor GM1 and GD1b (Figure 3.2c), and A subunit targets α-subunit of G protein, which regulates adenylate cyclase. This leads to increased level of cyclic adenosine monophosphate (cAMP) resulting in diarrhea (17). Stable toxins (STs) are small peptide toxins and resistant to boiling for 30 minutes. There are two unrelated classes; STa and STb, which are associated with human and animal diseases, respectively. STa comprise two variants, ST1a or STp is found in *E. coli* isolates from both human and animals, and ST1b or STh that is found in human isolates only. STa binds to guanylyl cyclase receptor leading to elevation of cyclic guanosine monophosphate and effecting to increased chloride secretion and decreased NaCl absorption. ETEC also adheres to small bowel enterocyte mediated by one or more proteinaceous fimbrial or fibrillar colonizing factor (CFs), which are species-specific (1).

3.1.6.4 Enteroaggregative *E. coli* (EAEC)

EAEC is considered to be the second most common cause of travelers' diarrhea and implicated in both acute and chronic diarrheal diseases among children and adults in developed and developing countries (19, 20). The clinical manifestation of EAEC infection is watery diarrhea with or without blood and mucus. It is characterized by the formation of stacked-brick, which is aggregative pattern of adherence mediated by aggregative adherence fimbriae (AAFs) that are encoded on pAA plasmid (Figure 3.2d). Several cytotoxins and enterotoxins are secreted by EAEC to cause mucosal damage such as plasmid-encoded toxin (Pet), *Shigella* enterotoxin1 (ShET1), and EAEC heat-stable enterotoxin (EAST1), which is homologous to ETEC STa (17).

3.1.6.5 Enteroinvasive *E. coli* (EIEC)

EIEC closely resembles *Shigella* in their biochemistric, genetic, and pathogenesis mechanisms. Like *Shigella* spp., EIEC is invasive pathogen that possesses a plasmid related to pInv plasmid. It is able to invade and destroy colonic tissue mediated the mechanisms of epithelial cell penetration, lysis of the

phagosome, intracellular multiplication, movement, and migration into adjacent cell (1) (Figure 3.2e). The symptom of EIEC infection is watery diarrhea, but some cases present dysentery syndrome including blood, mucus, tenesmus, and fever (1).

3.1.6.6 Diffusely adherent *E. coli* (DAEC)

DAEC forms a diffuse adherence (DA) pattern by interaction between F1845, the genes encoding the fimbria and the host cell called decay-accelerating factor (DAF), which is located on intestinal and urinary epithelial cell. Leading to signal transduction, it induces the growth of enterocytes as finger-like cellular projection (Figure 1.2 e), which wraps around bacterium resulting in the elongation and damage of brush border microvilli (18). The symptom is watery diarrhea without blood or fecal leucocytes.

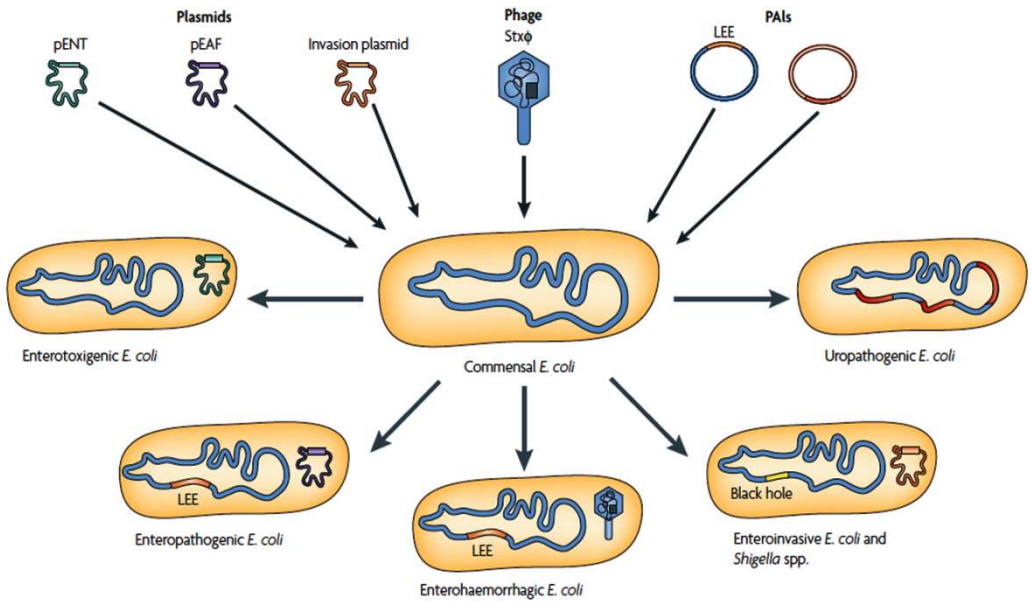

Figure 3.1 The evolution of pathotypes *E. coli* associated mobile genetic elements (19).

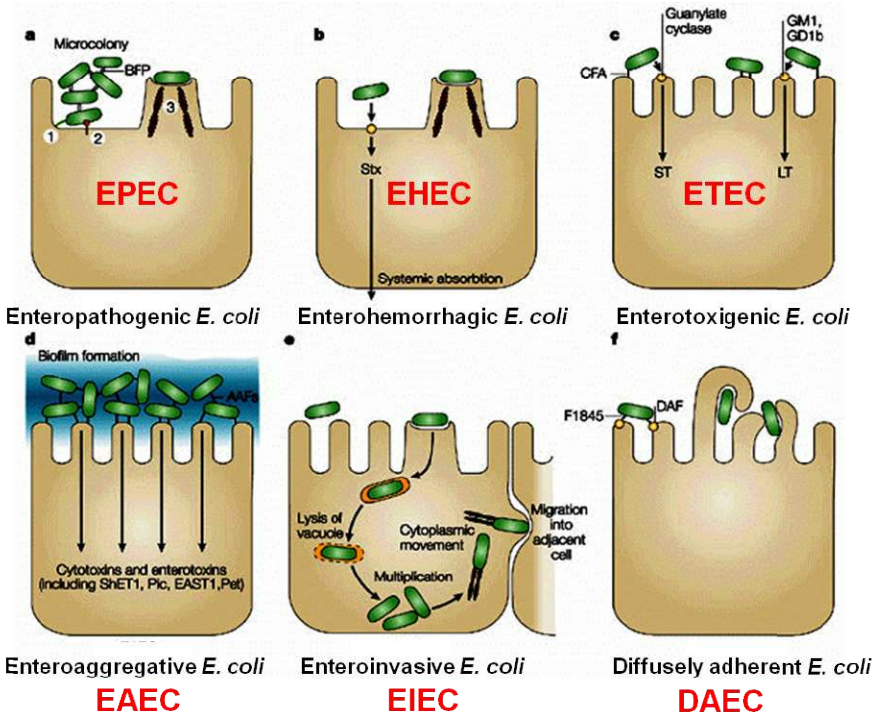

Figure 3.2 The six pathogenic *E. coli* and their interaction with target cell (modified from ref.16).

3.2 Foodborne diseases and antimicrobial resistance affect the global health problem

Foodborne disease is an infection of the gastrointestinal tract caused by ingestion of contaminated foods and water which contains bacteria, virus, parasites, or toxins. The clinical manifestation includes vomiting, diarrhea, abdominal pain, fever, and chill. Although most of them are self-limiting, they may cause more serious complications. Foodborne illness is a major public health problem, which largely impacts on social and economic cost worldwide. Even in the developed countries, it is a common phenomenon. In 2011, the US Centers for Control Disease and Prevention (US CDC) estimated 48 million foodborne illnesses in the United States, along with 128,000 hospitalizations, and 3,000 deaths each year (21). More than 15 billion USD was estimated for the annual cost of foodborne illnesses by United States Department

of Agriculture Economic Research Service (22, 23). In Thailand, approximately 1 million cases of diarrhea were reported by the Bureau of Epidemiology, Thailand (24).

Nowadays, there are more than 250 different foodborne diseases (25). Among all agents, bacteria are common causes of foodborne diseases. The three major pathogens are *Salmonella* spp., *Campylobacter* spp., and *E. coli* (26). Particularly, diarrheagenic *E. coli* is an important cause of diarrhea in children in developing countries and international travelers in those countries (2). These bacteria are naturally and harmlessly live as commensals in the intestinal tract of animals. When they are shed in their feces, they can survive for months to years and can cross-contaminate foods. Unfortunately, the prevalence of antimicrobial resistant bacteria has dramatically increased globally. Antimicrobial resistant bacteria can spread and cause the problem in human, animals, and environment. Furthermore, the globalization and global food trade provide opportunities for the distribution of antimicrobial resistant bacteria, which can lead to a wide spread outbreak. As a consequence, the critical treatment situation in both of human and animals is exacerbated.

3.3 Emergence of multidrug resistant bacteria link to human and animal health

Antimicrobial resistance remains the critical problem of human, animals, and environment. The usage of antimicrobials either appropriate or inappropriate use is the selective pressure of antimicrobial resistant organisms. Since the antimicrobial agents were discovered in the early 1900s until now, these products are widely used in human and animals.

In human, antimicrobial agents are used for the treatment and prevention of diseases. However, antimicrobial use, misuse, and abuse for long time periods are driving force of antimicrobial resistance, e.g., antimicrobials prescribed for non-bacterial infections such as colds and influenza, patients unable to take a full course of medication, and self-medication, are important causes of antimicrobial overuse/misuse. The overuse of antimicrobial agents as well as poor hygiene and infection control are important drivers for a wide spread of resistant bacteria in both community and healthcare settings.

Furthermore, antimicrobials are used widely as therapeutic and non-therapeutic purpose in animals including agriculture and aquaculture. Although therapeutic doses are mainly used to treat animal diseases, sub-therapeutic concentrations are added to animal feeds as supplements for growth promotion or increased feed efficiency in the food-producing animal industry. In 2009, 80% of all antimicrobials were estimated for using in food producing animals in United Stated (27). Moreover, those are important classes of antimicrobials also used in humans including penicillins, aminoglycosides, tetracyclines, sulfonamides, macrolides, and others (5). Antimicrobial growth promoters (AGPs) are considerable problem, because they are used without veterinary prescriptions or fed to herds of animals for long time periods, which promote the selection and spread of resistant bacteria among animals, to environment, e.g., food, water, soil, and plants, and then to human (28). Additionally, commensal flora, e.g., *E. coli* and *E. faecium*, that acquired resistance determinants may colonize in the gut and act as potential reservoirs of resistance genes for other pathogens such as *Salmonella* spp., *Campylobacter* spp., and pathogenic *E. coli*. Moodley and Guardabassi detected the transmission for CTX-M plasmids of IncN type between commensal *E. coli* in pigs, environment, and farm workers from the pig farms using prophylactic ceftiofur (8). Stecher et al. illustrated the horizontal gene transfer between *Salmonella* Typhimurium and commensal *E. coli* in mammalian gut model (29).

Even though most bacteria are killed by antimicrobials, minor populations of them are adaptable to resist, survive, multiply, and can transfer their drug-resistance to others (Figure 3.3). Antimicrobial resistant bacteria may be intrinsic resistance, e.g., *E. coli* resist to vancomycin; however they can acquire resistance from spontaneous mutations which occur in the chromosome and related to their exposure to drugs or acquire from horizontal gene transfer. Resistant bacteria utilize various routes to spread themselves or resistant determinants to human including consumption of contaminated food, direct contact with animals, and indirect contact with manures by two modes of their spread, vertical and horizontal gene transfers (Figure 3.4). Vertical gene transfer or clonal spread is the transmission of genetic materials from ancestors to descendants. For example, Aarestrup FM. and team reported the incidence of international spread of MDR *Salmonella* Schwarzengrund from imported Thai food

products (30). In contrast, horizontal gene transfer is the transfer of genetic materials between cells of the same generation via 3 mechanisms including conjugation, transformation, and transduction by mobile genetic elements, e.g., plasmids, transposons, integrons, and bacteriophages. Recently, there have been reported high incidence of MDR *S. enterica* and several resistance genes of different resistant mechanisms in meat and dairy products from Egypt (31).

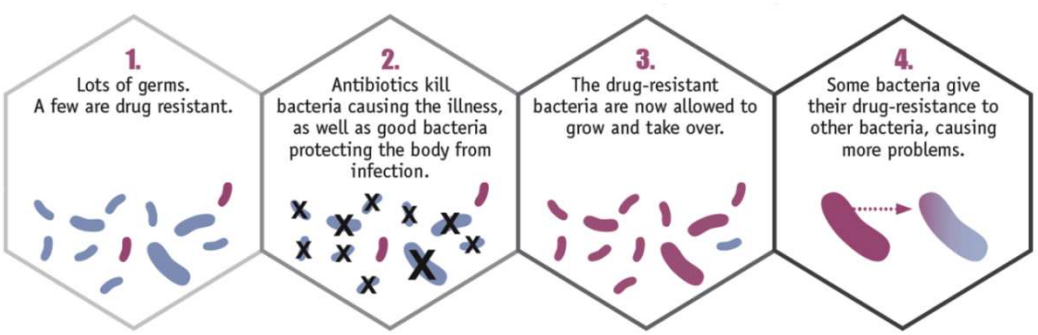

Figure3.3 Emergence of antimicrobial resistance bacteria (32)

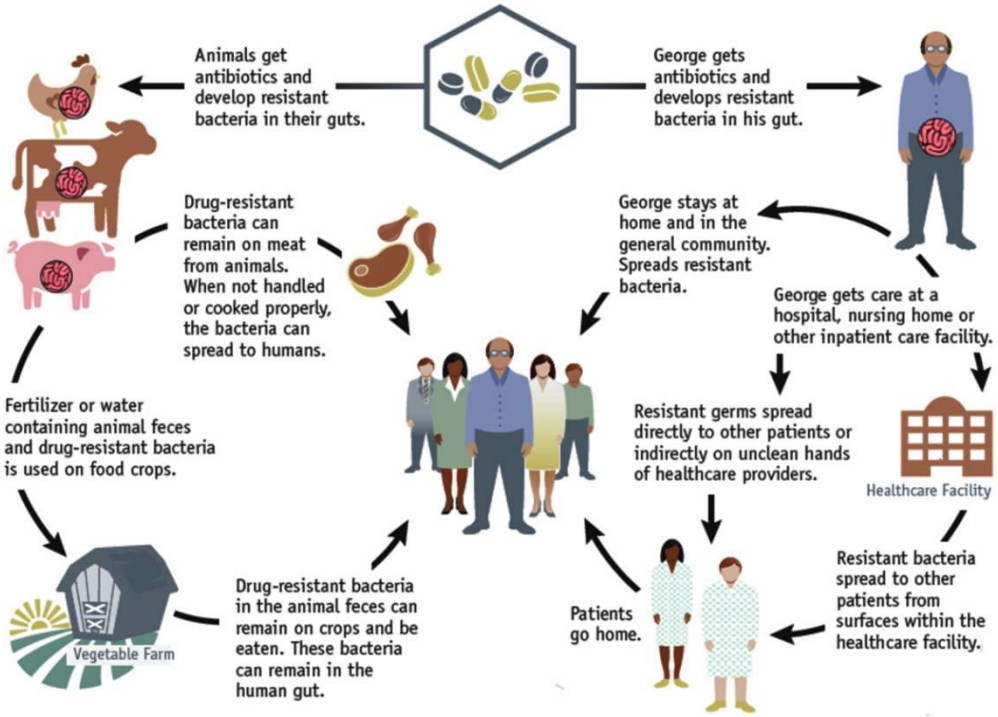

Figure3.4 Spread of antimicrobial resistance via various routes of transmission (32)

3.4 Antimicrobial resistance mechanisms of bacteria

Since the microbes including causative agents of infection were discovered leading to the efforts to eliminate pathogens and rescue the patients. In the early 1900s, anti-syphilitic agent named Salvasan, an arsenic arsphenamine was discovered by Paul Ehrlich. Later, sulfonamide named Prontosil was synthesized by Gerhard Domark in 1932 after that many sulfonamide derivatives were made for the treatment of various bacterial infections. Penicillin and streptomycin were discovered by Alexander Fleming and Selman Waksman, respectively. These agents were introduced in the early 1940s followed by introduction of chloramphenicol, erythromycin, and tetracycline in the early1950s. Antibacterial agents can be produced naturally from microorganisms, e.g., β-lactams, or synthetically, e.g., trimethoprim-sulfamethoxazole. Unfortunately, only a few years after introduction of penicillin, some strains of *S. aureus* developed resistance (33). Likewise, very soon after widely used of antimicrobials, MDR-*Shigella* has been reported as the first case of plasmid-determined resistance in 1955 (34). In fact, antimicrobials are miraculous drugs, the use of them saved several lives, and meanwhile the emergence of antimicrobial resistance is inevitable. Antimicrobial agents can be categorized into five major modes of actions, which are inhibition of bacterial cell wall synthesis (β-lactams and vancomycin), protein synthesis (tetracyclines, macrolides, and aminoglycosides), DNA and RNA synthesis (fluoroquinolones and rifamycins), folic acid synthesis (sulfonamides and trimethoprim), and cell membrane function (daptomycin) (35). However, bacteria responded to these selective pressures by developing resistance mechanisms. The four main mechanisms of antimicrobial resistance in bacteria are reduced drug accumulation, alteration of target sites, drug inactivation or modification, and alteration of metabolic pathways. In addition, single bacterial strain may resist to antimicrobials by several types of resistance mechanisms, or several different mechanisms may cooperate to resist to single antimicrobial agent.

3.4.1 Reduced drug accumulation

Unlike Gram-positive bacteria, Gram-negative species have an intrinsic resistance property to toxic substances including antimicrobials by their outer membrane. Porins are the outer membrane proteins which function as permeability

barrier to allow the passive penetration of small hydrophilic molecules, e.g., nutrition and antimicrobials into bacterial cell. Therefore, decrease or loss function of porins in resistant bacteria can lead to the entry prevention of antimicrobial agents. Clinical bacterial strains, e.g., *Escherichia*, *Enterobater*, *Citrobacter*, and *Klebsiella* have been reported to use non-specific porins, belong to the OmpC and OmpF family to resist the important antibacterial agents such as β-lactams, fluoroquinolones, chloramphenicols, as well as tetracyclines (36, 37). In contrast, OprD protein, which is a substrate-specific porin in *P. aeruginosa* that binds the basic amino acids and peptides, which have structure similar to the carbapenem molecule, e.g., imipenem (36). Thereby, mutation, deletion or decrease expression of OprD are associated with the carbapenem resistance in this species (36). Conversely, most bacterial species have efflux pump transporters that are the important mechanism of drug resistance by removing the toxic substances from inside the cells to external environment. Generally, efflux pumps are divided into two main types consist of five superfamilies. The first type is the primary active transport by using the hydrolysis of ATP, ATP-binding cassette (ABC) family. The secondary active transport is another type that uses the proton motive force to actively export substrates consists of four efflux pump superfamilies, the major facilitator superfamily (MFS), resistance nodulation cell division subfamily (RND), small multidrug regulator subfamily (SMR), and multidrug and toxic effects (MATE) family. The gene encoding this protein can be intrinsic or acquired that located on chromosomes or plasmids, respectively. QepA, a plasmid-encoded efflux pump belongs to MSF responsible for fluoroquinolones resistance and found in *E. coli* (38). Some efflux pumps are narrow substrate specificity such as Tet pumps (tetracycline efflux pumps), while others transport multiple substrates. The well-known examples for MDR efflux pumps are the RND superfamily members, e.g., AcrB-TolC in *E. coli* and MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN from *P. aeruginosa* which export many type of antimicrobial agents, e.g., β-lactams, quinolones, tetracycline, and chloramphenicol (39). Therefore, a single bacterial strain can utilize MDR efflux pumps from several types of the same family and/or more than one family to become more resistant to antimicrobial agents.

3.4.2 Alteration of target site

Most of antimicrobial agents need to specifically bind to their targets before they interact with target site according to their classes. Therefore, the modification of target sites can reduce the affinity binding for the antimicrobial agents. Target site alterations caused by chromosomal mutation and by enzymatic alteration. The example for chromosomal mutation is substitution in the quinolone-resistance determining region (QRDR) of DNA gyrase and topoisomerase IV which are targets of quinolone agents. These mutation lead to reduced efficient binding of quinolone, however this target still carries out its normal function. The single mutation at position 83 or 87 is the most frequent in fluoroquinolone resistant GyrA and provides high level resistance (40). Quinolone resistance is also caused by enzymatic alteration. The *qnr* families are plasmid-mediated quinolone resistance (PMQR), which encode the pentapeptide repeat proteins (PRPs) that prevent the action of quinolones on DNA gyrase and topoisomerase IV by interacting with topoisomerase-quinolone complex, result in the low-level of quinolone resistance (41). Enzymatic alteration of antimicrobial targets also includes erythromycin ribosome methylase (*erm*), which methylates 16S rRNA and alters drug-binding site resulting in resistant to macrolides, lincosamides, and streptogramins, chloramphenicol-florfenicol resistance (*cfr*) methyltransferase, which confer resistance to phenicols, pleuromutilins, streptogramins, lincosamides, and oxazolidinones. Both genes are found on plasmids which responsible to their spread (41).

3.4.3 Drug inactivation or modification

Enzyme-catalyzed inactivation is a major mechanism that bacteria use to survive from antimicrobial action. This resistance mode confers high level resistance to many classes of antimicrobial agents and spread widely. Resistance bacteria can inactivate the antimicrobials directly by enzymatic hydrolysis, e.g., β–lactamase enzymes and/or chemical group transfer, e.g., aminoglycoside-modifying enzymes and chloramphenicol acetyltransferases. The relevant example is the production of β–lactamase enzymes, which were discovered shortly after introduction of penicillin. They can inactivate β-lactam antimicrobial, e.g., penicillins, cephalosporins by hydrolyzing the β–lactam ring. β-lactamase enzymes can be classified into four

classes. Ambler class A, C, and D are serine active site enzymes, whereas class B contains metallo β-lactamase, which zinc-dependent enzymes (38). The discovery of β–lactamases began with the early β–lactamases, e.g., TEM-1, TEM-2, and SHV-1, which hydrolyze penicillins and narrow-spectrum cephalosporins. Then they were followed by extended-spectrum β-lactamases (ESBLs), which mutate from these enzymes (42). ESBLs enzymes can hydrolyze penicillins, narrow-spectrum and extended–spectrum cephalosporins including oxy-imino cephalosporins, e.g., cefotaxime and ceftazidime, fourth generation cephalosporins, e.g., cefepime, and monobactams, i.e., aztreonam, but sensitive to β-lactamase inhibitors, e.g., clavulanic acid, sulbactam, and tazobactam. Nowadays, CTX-M is the major ESBLs, which greater hydrolyze cefotaxime than other oxy-imino β-lactams (43). The emergence of CTX-M is the acquisition of plasmid-mediated β-lactamase derived from chromosome of *Kluyvera* spp. Among hundreds variants of CTX-M enzymes, CTX-M-15 is the most spread worldwide, especially in cephalosporin-resistant *E. coli* and *K. pneumoniae* isolates (41). Since carbapenem antimicrobials, e.g., imipenem are introduced to treat ESBLs-producing clinical isolates, the more use of them emerges carbapenem hydrolytic enzymes, carbapenemases, which hydrolyze the broad range of β-lactams including extended-spectrum cephalosporins and carbapenems. The prominent examples are the serine carbapenemase KPC that was first identified in *K. pneumoniae* and New Delhi metallo β-lactamase (NDM) that was initially reported in New Delhi, India. Both bla_{KPC} and bla_{NDM} genes are plasmid-borne and most widespread worldwide in Gram-negative bacteria including *E. coli*, *K. pneumoniae*, and *Acinobacetr baumannii* (41). Like β-lactamases, macrolide esterases confer resistance to macrolides that are produced by *Enterobacteriaceae* and fosfomycin epoxidases causing fosfomycin resistance (43). Chemical group transfer enzymes are the most diverse family of resistant enzymes that inactivate antimicrobials by transfer of a functional group to them. The production of aminoglycoside-modifying enzymes (AMEs) is the major mechanism conferring aminoglycoside resistance, which consists of three main classes: aminoglycoside acetyltransferases (AACs) transfer the acetyl group to the amino group, aminoglycoside nucleotidyltransferases (ANTs), and aminoglycoside phosphotransferases (APHs) transfer the nucleotide triphosphate and the phosphoryl group to the hydroxyl group, respectively (38). In addition, AAC(6′)-

Ib-cr is an aminoglycoside acetyltransferase that can inactivate the synthetic drug, ciprofloxacin and widely distributed across the globe (40). Other chemical group transfer examples include chloramphenicol acetyltransferases (CATs), streptogramin acetyltransferases (VATs), lincosamide nucleotidyltransferases (LINs) for lincomycin and clindamycin resistance, whereas macrolide resistance is mediated by macrolide phosphotransferases (MPHs) and glycosyltransferases (MGTs) (43).

3.4.4 Alteration of metabolic pathway

Some bacteria have resistance mode to specific antimicrobial agents such as trimethoprim-sulfamethoxazole (SXT) resistant bacteria. SXT is the synergy between trimethoprim and sulfamethoxazole (sulfonamide) that inhibits the folic acid synthesis pathway. Sulfonamides, a structure analog of para-aminobenzoic acid (PABA) interact with the dihydropteroate synthase (DHPS) to inhibit the dihydrofolate formation, whereas trimethoprim competitively interacts with the dihydrofolate reductase (DHFR) to inhibit the synthesis of tetrahydrofolic acid. SXT resistance is conferred by the overproduction of DHFR enzymes, which caused by promoter mutations and altered DHPS enzymes by genetic mutation or recombination to reduce sensitivity and low-affinity for thrimethoprim and sulfonamides (44).

3.5 The acquisition and dissemination of resistance genes

Antimicrobial resistance in bacteria can be occurred naturally (intrinsic resistance), e.g., Gram-negative bacteria resist vancomycin due to permeability barrier of outer membrane against antimicrobial agents, or it can be acquired by various ways. Susceptible bacteria can acquire resistance through spontaneous mutations, through the acquisition of resistance genes from other bacteria, or through a combination of these mechanisms. Likewise, the resistance genes can be spread by vertical gene transfer (clonal spread) and horizontal gene transfer (lateral gene transfer). Vertical gene transfer refers to the transmission of existing genes from parent to offspring. For instance, the clone of fluoroquinolone resistant *E. coli* O25-ST131 strains spread in Eastern Asia and emerge worldwide (45). In contrast, horizontal gene transfer is defined as a transfer of genetic material between organisms without reproduction

either within species (intraspecies) or different genera (interspecies). The resistance genes can be transfer via three main mechanisms, which are transformation, transduction, and conjugation. Transformation involves the direct uptake of extracellular DNA of the competent bacterial cells. Some bacteria become competent at a certain physiological stage of their life cycle, e.g., *Streptococcus spp*., *B. subtilis*, whereas other bacteria, such as *H. influenza*, *N. gonorrhoeae*, and *N. meningitidis* can only acquire DNA with specific sequence, known as the uptake signal sequence or USS, which specific for DNA from the same or related species (46, 47). Transduction is the process of DNA transferring from one bacterium to another by bacteriophages. By using bacterial virus as the vector, lytic phages can randomly pick up and transfer host DNA alone (generalized transduction), e.g., bacteriophage P1 whereas lysogenic phages can only transfer specific portions of host DNA, which adjacent to phage attachment site (specialized transduction), e.g., phage λ. Although phages are the most abundant organisms, they have a narrow host range that only infected bacteria are phage-mediated horizontal gene transfer (47). Unlike transformation and transduction, conjugation requires cell-to-cell contact for genetic material transferring between bacterial cells. DNA can be transferred by either self-transmissible (conjugative plasmid) or mobilizable plasmids that lack of *tra* genes for transmissibility. In case of conjugative plasmid integrated into chromosome, known as Hfr (High frequency of recombination) strain, consequence the chromosomal DNA can be transferred to recipient strain (47). In addition, chromosomal transfer can also mediate by integrative and conjugative elements (ICEs), e.g., conjugative transposon Tn*916* and ICE SXT. ICEs are self-transmissible elements that involve the integration into chromosome, excision from their chromosome, and subsequent conjugation into a new host (47). However, horizontal gene transfer plays a major role in bacterial evolution and dissemination of multidrug resistance because most of resistance genes are located on mobile genetic elements, e.g., plasmids, transposons, and integrons and can be prompted to transfer to others, especially; integrons are an important part in the wide spread of multidrug resistance in Gram-negative bacteria (11).

3.6 Integrons and their resistance gene cassettes

In 1989, integrons were firstly discovered in the plasmid-mediated multidrug resistant *S. dysenteriae* isolates from Japan in 1950s (40) as indicated by the involvement of transposon, Tn*21* in the resistance plasmid NR1 (R100) (48). This was an evidence of integrons involving in bacterial adaptation after a few years of introduction of antimicrobial agents.

Integrons are genetic elements able to incorporate open reading frames (ORFs) of mobile cassettes carrying antimicrobial resistance genes by site-specific recombination and convert them into functional genes as gene-capturing and expression systems (12).

Although integrons are not self-mobilized, they are located on other mobile genetic elements, e.g., transposons and plasmid, which promote their dissemination within bacterial communities (11). Integrons platform comprises three functional elements necessary for the systemic operation; an integrase encoded by *intI*, which encodes a site-specific tyrosine recombinase, a primary recombination site, *attI*, and a strong promoter, Pc, which can drive the expression of resistance genes (Figure 3.5). Gene cassettes are compact DNA elements, normally containing a single promoterless ORF and a recombination site, termed *attC* or formerly called 59 base elements, which is specifically recognized by IntI. Gene cassettes can exist in free circular DNA molecules, not a stable form. However, circular gene cassettes can be inserted into an integron as a linear form by site-specific recombination between *attI* and *attC*, which is mediated by an integrase. Conversely, linear gene cassettes can be excised as a free circular form by reversible process between two *attC* sites (49). Owing to gene cassettes carried on integrons lack a promoter, their expression is driven by Pc promoter, of which the closest to Pc promoter is the strongest expression. Moreover, Integrons can accumulate many gene cassettes, meanwhile they are prompt to transfer to others that contribute to rapid and wide spread of multidrug resistance. The *attC* sites are diverse and varies in length from 57 to 141 bp, which contain conserved sequences in binding domain called inverse core site (RYYYAAC, where R is a purine and Y is a pyrimidine) at the end closest to 3′ end of the gene coding sequences and core site (GTTRRRY) at the other end includes the cross-over point at between G and first T (48, 50).

All integrons can be divided into two categories; mobile integrons, usually contain antimicrobial resistance gene cassettes with diverse *attC* sites and are carried by transposons or plasmids, which promote their spread, and chromosomal superintegrons, which could contain hundred of gene cassettes and have homologous *attC* sites (14). More than 130 gene cassettes conferring resistance to most classes antimicrobial used in medicine and agriculture have been detected in mobile integrons, including β-lactam, all aminoglycosides, chloramphenicol, trimethoprim, rifampin, erythromycin, fosfomycin, lincomycin, quinolones, and antiseptics of the quaternary ammonium compound family (11, 12). Five different classes of mobile integrons are classified to date based on sequence homology of integrases and associated with antimicrobial resistance. Class 1, 2, and 3 integrons are the most commonly identified in clinical isolates. Class1 integrons are associated with transposon derived from Tn*402* that are frequently embedded in plasmid or larger transposons, e.g., Tn*3* family (Tn*21*, Tn*1696*). Class 2 integrons are typically associated with Tn*7* transposon and their derivatives, however they contain a defective integrase gene, *intI2*, resulting in stable inserted gene cassettes, including *dfrA1*, *sat2*, *aadA1* encoding for trimethoprim, streptothricin, and streptomycin resistance genes, respectively (11). Class 3 integrons are also found in Tn*402* transposon and are low prevalence in clinical isolates (14). Class 4 integrons have been found on the SXT element of *V. cholerae*, whereas class 5, found on the pRSV1 plasmid of *Alivibrio salmonicida* (11, 14).

Class 1 integrons are the most widespread and mostly found in both of clinical isolates and commensal flora from human and animals, especially in Gram-negative bacteria, and they are also been occasionally found in Gram-positive bacteria and plant pathogens (14). Most class 1 integrons comprise two conserved segments that flank either end of the variable gene cassette region. The 5′-conserved segment contains an integrase, *intI*, the integration site, *attI*, and a promoter, which is located within *intI*. The 3′-conserved segment usually contains a truncated of antiseptic resistance gene, *qacEΔ1*, a sulfonamide resistance gene, *sul1*, and an open reading frame 5, *orf5*, encoding a protein of unknown function (Figure 3.6). In addition, Mostly class 1 integrons contain at least one inserted gene cassette exception for In0, which found in plasmid pVS1, contains no integrated cassette (51).

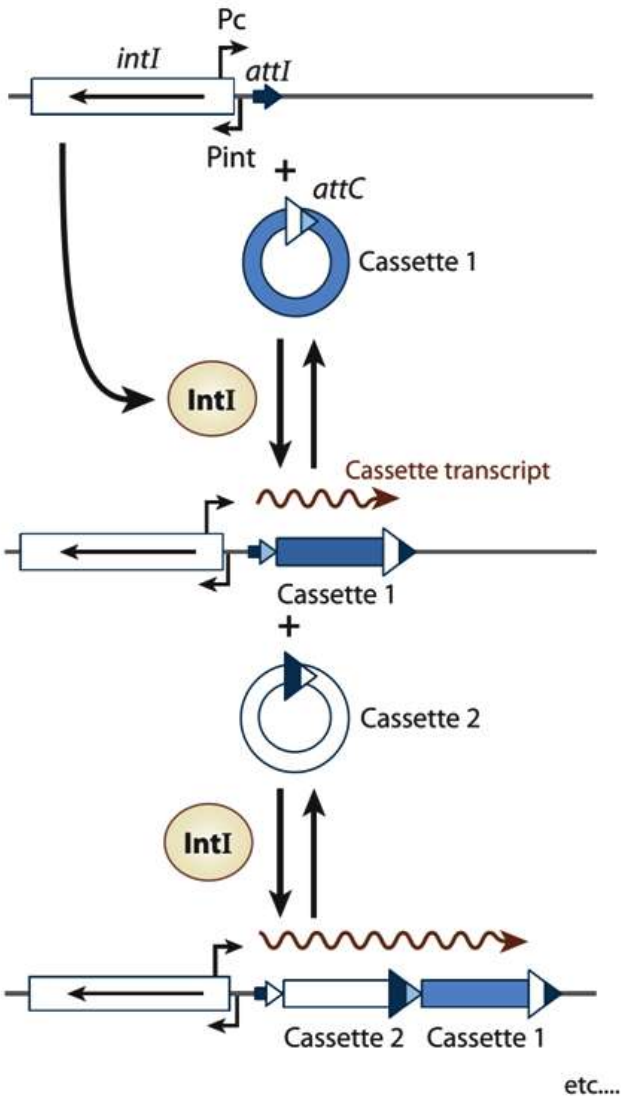

Figure 3.5 Integron-mediated gene capture and model for cassette exchange. Outline of the process by which circular gene cassettes are repeatedly inserted at the specific *attI* site in an integron downstream of the strong promoter Pc. *intI*, integrase encoding gene; IntI, integrase IntI (12).

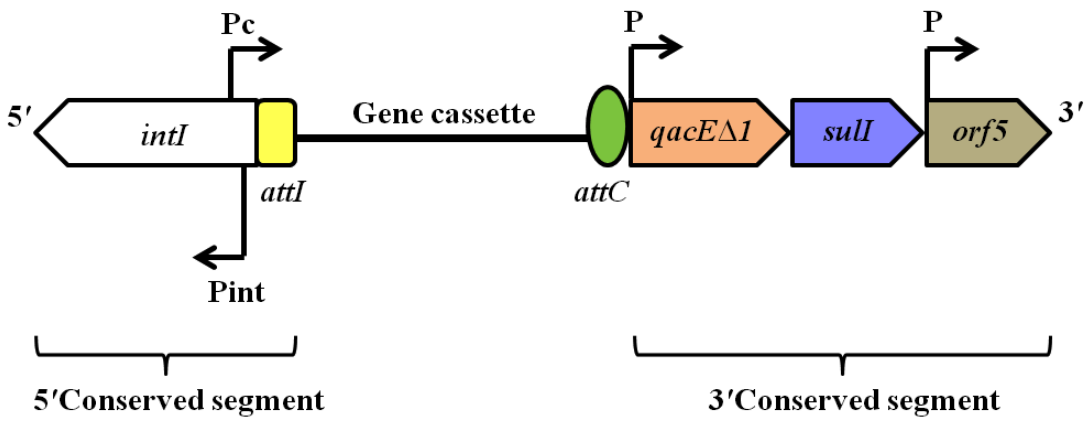

Figure 3.6 Basic structure of class 1 integron. *intI*, *attI* recombination site, and Pc promoter are located in the 5′CS, whereas the 3′CS comprise *qacEΔ1* gene encoding resistance to quaternary ammonium compounds, *sul1* gene encoding sulfonamide resistance, and *orf5* gene encoding protein of unknown function. The direction of gene transcription is indicated as arrows (modified from ref.13).

3.7 Molecular epidemiological typing

E. coli are one of the major public health issues, particularly diarrheagenic *E. coli*, which is the crucial cause of diarrheal disease in children under five years of age in developing countries and travelers to these setting. *E. coli* are the most common cause of foodborne pathogen. Moreover, they have become increasingly resistant to antimicrobial agents. In addition, the international travelers who visit in the developing countries might play a partial role in their spread worldwide, e.g., the international clone of CTX-M-15-producing *E. coli* ST131. Therefore, understanding of clonal relatedness between bacterial strains is essential for surveillance and control of their spread. Epidemiological study is important to determine the sources and routes of bacteria, investigate an outbreak situation, understand pathogenesis of an infection, recognize particularly virulent strains and evaluate the effectiveness of control measures. Accordingly, epidemiology also contributes to the effectiveness of monitoring and surveillance systems and provides significant data to public health control strategies (15). Conventional epidemiological typing methods, e.g.,

antibiogram, serotyping, and phage typing, are generally too variable, laborious, and time-consuming (15). Moreover, they are limiting to differentiate subtyping and viable but non-culturable bacteria. Consequently, molecular typing methods have become necessary in epidemiological investigation. The criteria to use typing methods are based on the performance variables, e.g., unequivocal interpretation of results, intra- and inter-laboratory reproducibility, interlaboratory portability, high throughput, and appropriateness, and the convenience variables, e.g., simplicity, cost, rapidity, and affordability (15). In addition, types of epidemiology including short term and long term epidemiology should be considered to select the suitable typing methods that can answer a specific epidemiological question and the combination of typing methods can be used for more comprehensive knowledge of epidemiology of bacterial pathogens (52).

3.7.1 Analysis of plasmid profiles

Plasmids are extrachromosomal DNA, which have important role in the horizontal gene transfer between bacteria, e.g., resistance gene. However, they are also distributed between the daughter cells in cell division. Therefore, they can be used as a marker for comparison among bacterial isolates. Plasmid profiling is one of the first molecular typing methods used for epidemiological studies. This technique is DNA-based typing method depending on number and molecular weight of different plasmids that carried by bacteria, which can be separated by agarose gel electrophoresis (53). Although plasmid profiles are relatively easy to compare and interpret, they have many drawbacks that cause the confusion in the attribution of genetic relatedness to bacterial isolates. For examples, this technique requires the presence of at least one plasmid in a bacterium (53), gain or loss of plasmids can affect the reproducibility (15), and conformational changes of plasmids may affect the migration property on agarose gel electrophoresis (54).

3.7.2 Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR

ERIC PCR is one of the amplification-based typing methods, which can be used to type a wide variety of bacterial pathogens (53). ERIC sequences are 126 bp, which located in noncoding transcribed regions of the chromosome and contain highly

conserved central inverted repeat sequences. Moreover, the position of ERIC sequences in bacterial genomes differs in different species that can be used as a target PCR to generate DNA fingerprints (55). Consensus primers are used to amplify DNA sequences located between successive repetitive elements by outward direction from the center of the ERIC elements (56). The different banding patterns on agarose gel electrophoresis are a result of the number and position of repetitive elements in bacterial genome that are compared to others to determine genetic relatedness (15).

3.7.3 Multi-locus sequence typing (MLST)

MLST is genotyping method based on the determination of DNA sequence variation of internal fragments of selected housekeeping genes, which present in all isolates within a species and their genetic changes are relatively slow process. This technique involves PCR amplification of internal fragments of housekeeping genes followed by nucleotide sequencing. Each of the gene sequence is compared with already known alleles at the locus on the MLST database and assigned as allele number. Subsequently, alleles at the loci are combined into an allelic profile and assigned a sequence type (ST). If the sequence is different at even a single nucleotide from database, it is assigned to new allele number, which leading to a unique allelic profile and new ST designation. The identical STs or STs with differences in few loci are considered to be closely related isolates, whereas unrelated isolates have STs that differ at many more loci. The results of MLST are highly unambiguous, portable, exchangeable, and comparable rather than images of agarose gel electrophoresis patterns. Moreover, MLST has been successfully used for global epidemiology, genetic evolution, genetic diversity and pathogenicity among bacteria (52). The major limitation of this method is relatively expensive technique due to the Sanger sequencing. Large-scale sequencing by using Sanger technique requires expensive infrastructure and laborious bench work. However, large scale projects can be easily done and by using the next-generation sequencing (NGS) on bench-top machine, which overcome the limitation of the classical MLST (57). Recently, high throughput MLST (HiMLST) was developed that employed NGS to generate the sequence data. MLST genes are amplified in a two-step PCR by using specific primers including universal tail sequences and multiplex identifier (MID) primers. After that, the

amplicons from multiple bacterial strains are pooled and simultaneously sequenced by the machine, allows the generation of MLST profiles in a single NGS-run. Therefore, HiMLST approach generates the robust MLST technique with the reduced cost that may become a more practical and economical method for genotyping of large number of bacterial isolates (58).

CHAPTER IV
MATERIALS AND METHODS

4.1 Source of isolates and sample collection

The bacterial isolates in this study were chosen 200 multidrug resistant (MDR) *E. coli* of 580 MDR pathogenic *E. coli* from the total of 3,834 pathogenic *E. coli*. They were isolated from rectal and stool samples of outpatients with or without acute diarrhea in Thailand and other countries including of Cambodia, Maldives, Nepal, and Vietnam from 2001 to 2010. Populations under study were 110 indigenous children less than 5 years old from Thailand, Cambodia, and Vietnam and 88 adults including 15 natives from Thailand, Nepal, and Maldives, 40 international travelers in Thailand and Nepal, and 33 U.S. soldiers on military maneuvers in Thailand with 2 more *E. coli* isolates from the same person at different times using as internal control. Due to limitation on the amount of samples from adults, all 90 adult isolates that matched with the criteria were included in this study, whereas 110 *E. coli* isolates from children were selected by using program SPSS version 12. All 200 MDR pathogenic *E. coli* isolates belong to 44 EAEC (7 adults, 35 children), 89 EPEC (53 adults, 36 children), 56 ETEC (22 adults, 34 children), 6 EIEC (4 adults, 2 children), and 5 STEC (2 adults, 3 children).

Pathogenic *E. coli* of this study had been identified by using colony blot hybridization with specific digoxigenin-labeled polynucleotide probes (Table 1) and subjected to antimicrobial susceptibility testing (AST) by disk diffusion method that interpreting results had obtained according to Clinical and Laboratory Standards Institute (CLSI) guidelines of each AFRIMS's year project. All pathogenic *E. coli* isolates with AST results and demographic data were kindly provided by Armed Forces Research Institute of Medical Sciences (AFRIMS).

MDR pathogenic *E. coli* from non-diarrhea patients who reflected the carrier were included in this study to determine the presence of class 1 integrons and investigate the possible clonal spread since this sample group is also responsible to the

dissemination of antimicrobial resistance bacteria in the community. MDR phenotype was considered as resistant to ≥3 drug groups of aminoglycoside, sulfonamide, tetracycline, chloramphenicol, quinolones, and β-lactams. All isolates were re-identified using biochemical test and API® 20E (bioMérieux®, France), and kept duplicated frozen at -70°C in Luria-Bertani broth containing 15% glycerol.

Table 4.1 Pathogenic *E. coli* by using colony blot hybridization. *lt* = heat labile toxin, *stIa* and *stIb* = heat stable toxin, *eae* = effacing attachment factor, *eaf* = enteroadherent factor, *bfpA* = bundle forming pilus A, *ial* = invasion-associated locus, *sltI* and *sltII* = shiga-like toxin, and pCVD432 = plasmid-derived enteroaggregative *E. coli*

Isolate	Diarrhea	Hybridization with specific probe										Pathotype
		lt	*stIa*	*stIb*	*eae*	*eaf*	*bfpA*	*ial*	*sltI*	*sltII*	pCVD432	
EA001	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EA002	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EA003	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EA004	No	-	-	-	-	-	-	-	-	-	+	EAEC
EA005	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EA006	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EA007	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EA008	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EA009	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA010	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA011	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA012	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA013	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA014	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA015	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA016	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA017	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA018	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA019	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA020	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA021	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA022	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA023	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA024	Yes	-	-	-	+	-	-	-	-	-	-	EPEC

Table 4.1 Pathogenic *E. coli* by using colony blot hybridization. *lt* = heat labile toxin, *stIa* and *stIb* = heat stable toxin, *eae* = effacing attachment factor, *eaf* = enteroadherent factor, *bfpA* = bundle forming pilus A, *ial* = invasion-associated locus, *sltI* and *sltII* = shiga-like toxin, and pCVD432 = plasmid-derived enteroaggregative *E. coli* (cont.)

Isolate	Diarrhea	Hybridization with specific probe										Pathotype
		lt	*stIa*	*stIb*	*eae*	*eaf*	*bfpA*	*ial*	*sltI*	*sltII*	pCVD432	
EA025	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA026	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA027	Yes	+	-	+	-	-	-	-	-	-	-	ETEC
EA028	Yes	+	-	+	-	-	-	-	-	-	-	ETEC
EA029	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EA030	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EA031	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EA032	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EA033	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EA034	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EA035	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
ET036	No	-	-	-	-	-	-	-	-	-	+	EAEC
ET037	Yes	-	-	-	-	-	-	+	-	-	-	EIEC
ET038	Yes	-	-	-	-	-	-	+	-	-	-	EIEC
ET039	Yes	-	-	-	-	-	-	+	-	-	-	EIEC
ET040	Yes	-	-	-	-	-	-	+	-	-	-	EIEC
ET041	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET042	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET043	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET044	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET045	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET046	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET047	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET048	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET049	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET050	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET051	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET052	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET053	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET054	Yes	-	-	-	+	-	-	-	-	-	-	EPEC

Table 4.1 Pathogenic *E. coli* by using colony blot hybridization. *lt* = heat labile toxin, *stIa* and *stIb* = heat stable toxin, *eae* = effacing attachment factor, *eaf* = enteroadherent factor, *bfpA* = bundle forming pilus A, *ial* = invasion-associated locus, *sltI* and *sltII* = shiga-like toxin, and pCVD432 = plasmid-derived enteroaggregative *E. coli* (cont.)

Isolate	Diarrhea	Hybridization with specific probe										Pathotype
		lt	*stIa*	*stIb*	*eae*	*eaf*	*bfpA*	*ial*	*sltI*	*sltII*	pCVD432	
ET055	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET056	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET057	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET058	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET059	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET060	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET061	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET062	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET063	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET064	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET065	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET066	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
ET067	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET068	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET069	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET070	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET071	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET072	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET073	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET074	No	-	-	-	+	-	-	-	-	-	-	EPEC
ET075	Yes	+	-	+	-	-	-	-	-	-	-	ETEC
ET076	Yes	+	-	+	-	-	-	-	-	-	-	ETEC
ET077	Yes	+	-	+	-	-	-	-	-	-	-	ETEC
ET078	Yes	+	-	-	-	-	-	-	-	-	-	ETEC
ET079	No	-	-	+	-	-	-	-	-	-	-	ETEC
ET080	Yes	+	-	+	-	-	-	-	-	-	-	ETEC
ET081	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
ET082	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
ET083	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
ET084	Yes	-	+	-	-	-	-	-	-	-	-	ETEC

Table 4.1 Pathogenic *E. coli* by using colony blot hybridization. *lt* = heat labile toxin, *stIa* and *stIb* = heat stable toxin, *eae* = effacing attachment factor, *eaf* = enteroadherent factor, *bfpA* = bundle forming pilus A, *ial* = invasion-associated locus, *sltI* and *sltII* = shiga-like toxin, and pCVD432 = plasmid-derived enteroaggregative *E. coli* (cont.)

Isolate	Diarrhea	Hybridization with specific probe										Pathotype
		lt	*stIa*	*stIb*	*eae*	*eaf*	*bfpA*	*ial*	*sltI*	*sltII*	pCVD432	
ET085	Yes	+	-	-	-	-	-	-	-	-	-	ETEC
ET086	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
ET087	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
ET088	No	+	-	+	-	-	-	-	-	-	-	ETEC
ET089	No	-	-	-	-	-	-	-	+	-	-	STEC
ET090	No	-	-	-	-	-	-	-	+	-	-	STEC
EC091	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC092	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC093	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC094	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC095	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC096	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC097	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC098	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC099	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EC100	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC101	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EC102	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC103	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC104	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC105	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC106	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC107	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC108	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EC109	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EC110	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EC111	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EC112	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EC113	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EC114	Yes	-	-	-	-	-	-	-	-	-	+	EAEC

Table 4.1 Pathogenic *E. coli* by using colony blot hybridization. *lt* = heat labile toxin, *stIa* and *stIb* = heat stable toxin, *eae* = effacing attachment factor, *eaf* = enteroadherent factor, *bfpA* = bundle forming pilus A, *ial* = invasion-associated locus, *sltI* and *sltII* = shiga-like toxin, and pCVD432 = plasmid-derived enteroaggregative *E. coli* (cont.)

Isolate	Diarrhea	Hybridization with specific probe										Pathotype
		lt	*stIa*	*stIb*	*eae*	*eaf*	*bfpA*	*ial*	*sltI*	*sltII*	pCVD432	
EC115	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EC116	Yes	-	-	-	-	-	-	-	-	-	+	EAEC
EC117	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC118	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC119	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC120	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC121	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC122	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC123	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC124	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC125	No	-	-	-	-	-	-	-	-	-	+	EAEC
EC126	Yes	-	-	-	-	-	-	+	-	-	-	EIEC
EC127	No	-	-	-	-	-	-	+	-	-	-	EIEC
EC128	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EC129	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EC130	Yes	-	-	-	+	+	+	-	-	-	-	EPEC
EC131	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EC132	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EC133	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EC134	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC135	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC136	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC137	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC138	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC139	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC140	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC141	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC142	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC143	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC144	No	-	-	-	+	-	-	-	-	-	-	EPEC

Table 4.1 Pathogenic *E. coli* by using colony blot hybridization. *lt* = heat labile toxin, *stIa* and *stIb* = heat stable toxin, *eae* = effacing attachment factor, *eaf* = enteroadherent factor, *bfpA* = bundle forming pilus A, *ial* = invasion-associated locus, *sltI* and *sltII* = shiga-like toxin, and pCVD432 = plasmid-derived enteroaggregative *E. coli* (cont.)

Isolate	Diarrhea	Hybridization with specific probe										Pathotype
		lt	*stIa*	*stIb*	*eae*	*eaf*	*bfpA*	*ial*	*sltI*	*sltII*	pCVD432	
EC145	No	-	-	-	+	+	+	-	-	-	-	EPEC
EC146	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC147	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC148	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC149	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC150	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EC151	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EC152	Yes	-	-	-	+	-	-	-	-	-	-	EPEC
EC153	No	-	-	-	+	+	+	-	-	-	-	EPEC
EC154	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC155	No	-	-	-	+	+	+	-	-	-	-	EPEC
EC156	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC157	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC158	No	-	-	-	-	-	+	-	-	-	-	EPEC
EC159	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC160	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC161	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC162	No	-	-	-	+	-	-	-	-	-	-	EPEC
EC163	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EC164	Yes	-	-	+	-	-	-	-	-	-	-	ETEC
EC165	Yes	+	-	+	-	-	-	-	-	-	-	ETEC
EC166	Yes	-	-	-	-	-	-	-	+	-	-	STEC
EC167	No	-	-	-	-	-	-	-	+	-	-	STEC
EC168	No	-	-	-	-	-	-	-	-	+	-	STEC
EC169	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EC170	No	-	-	+	-	-	-	-	-	-	-	ETEC
EC171	No	+	-	-	-	-	-	-	-	-	-	ETEC
EC172	No	-	+	-	-	-	-	-	-	-	-	ETEC
EC173	No	+	-	-	-	-	-	-	-	-	-	ETEC
EC174	No	-	-	+	-	-	-	-	-	-	-	ETEC

Table 4.1 Pathogenic *E. coli* by using colony blot hybridization. *lt* = heat labile toxin, *stIa* and *stIb* = heat stable toxin, *eae* = effacing attachment factor, *eaf* = enteroadherent factor, *bfpA* = bundle forming pilus A, *ial* = invasion-associated locus, *sltI* and *sltII* = shiga-like toxin, and pCVD432 = plasmid-derived enteroaggregative *E. coli* (cont.)

Isolate	Diarrhea	Hybridization with specific probe										Pathotype
		lt	*stIa*	*stIb*	*eae*	*eaf*	*bfpA*	*ial*	*sltI*	*sltII*	pCVD432	
EC175	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EC176	Yes	+	-	+	-	-	-	-	-	-	-	ETEC
EC177	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EC178	Yes	+	-	-	-	-	-	-	-	-	-	ETEC
EC179	No	+	-	-	-	-	-	-	-	-	-	ETEC
EC180	No	-	+	-	-	-	-	-	-	-	-	ETEC
EC181	No	+	-	-	-	-	-	-	-	-	-	ETEC
EC182	No	-	-	+	-	-	-	-	-	-	-	ETEC
EC183	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EC184	Yes	+	+	-	-	-	-	-	-	-	-	ETEC
EC185	Yes	+	-	-	-	-	-	-	-	-	-	ETEC
EC186	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EC187	Yes	+	-	-	-	-	-	-	-	-	-	ETEC
EC188	Yes	+	-	-	-	-	-	-	-	-	-	ETEC
EC189	Yes	+	-	-	-	-	-	-	-	-	-	ETEC
EC190	Yes	+	-	-	-	-	-	-	-	-	-	ETEC
EC191	Yes	-	-	+	-	-	-	-	-	-	-	ETEC
EC192	No	+	-	-	-	-	-	-	-	-	-	ETEC
EC193	No	-	-	+	-	-	-	-	-	-	-	ETEC
EC194	No	+	+	-	-	-	-	-	-	-	-	ETEC
EC195	No	-	-	+	-	-	-	-	-	-	-	ETEC
EC196	Yes	+	-	-	-	-	-	-	-	-	-	ETEC
EC197	No	+	-	-	-	-	-	-	-	-	-	ETEC
EC198	No	+	-	+	-	-	-	-	-	-	-	ETEC
EC199	Yes	-	+	-	-	-	-	-	-	-	-	ETEC
EC200	Yes	-	-	-	+	-	-	-	-	-	-	EPEC

4.2. Antimicrobial susceptibility testing (AST) by disk diffusion method

Additional antimicrobial susceptibility testing (AST) was performed for screening and phenotypic confirmatory test for Extended Spectrum ß-Lactamases (ESBLs)-producing isolates by Kirby-Bauer disk diffusion method according to the Performance Standards for Antimicrobial Disk Susceptibility Tests (CLSI, M02-A11) (59) for cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg), ceftriaxone (CRO; 30 µg), Cefotaxime-clavulanic acid (CTX/CLA; 30/10 µg), and ceftazidime-clavulanic acid (CAZ/CLA; 30/10 µg). In brief, selected colonies from an overnight Tryptic soy agar plate were directly suspended in the Muller Hinton broth and adjusted to 0.5 McFarland Standard. Within 15 minutes after adjusting the turbidity of the inoculum suspension, the sterile swab was dipped into the inoculum, pressed, and rotated several times to remove excess inoculum. The swab was evenly streaked on the Muller Hinton agar plate and rotated the plates 60º two more times to ensure an even distribution of inoculum. Within 15 minutes after inoculation, antimicrobial disks were placed on surface of the inoculated plates by using sterile forceps. The plates were inverted and placed in 35±2°C incubator for 16-18 hours within 15 minutes after the disks were applied. The results were interpreted the size of the inhibition zone by referring to the Performance Standards for Antimicrobial Disk Susceptibility Tests (CLSI, M100-S23) (Table 4.2). Quality control strains, *E. coli* ATCC® 25922 and *K. pneumoniae* ATCC®700603 were performed each time AST was done. All isolates were detected by screening for ESBL-phenotypes, and then positive strains were performed ESBLs confirmatory test by using combination disk method. The positive strains for ESBLs in the confirmatory test were subjected to polymerase chain reaction (PCR) with specific primers for $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} antimicrobial resistance genes.

Table 4.2 Disk diffusion: Quality control ranges for nonfastidious organisms according to CLSI 2013 (60)

Quality control strain	Acceptable quality control ranges (mm)				
	CTX (30 µg)	CTX/CLA (30 µg /10 µg)	CAZ (30 µg)	CAZ/CLA (30 µg /10 µg)	CRO (30 µg)
E. coli ATCC® 25922	29-35	≤2-mm increase in zone diameter compare to test agent alone	25-32	≤2-mm increase in zone diameter compare to test agent alone	29-35
K. pneumoniae ATCC®700603	17-25	≥3-mm increase in zone diameter compare to test agent alone	10-18	≥5-mm increase in zone diameter compare to test agent alone	16-24

CTX; cefotaxime, CTX/CLA; cefotaxime-clavulonic acid, CAZ; ceftazidime; CAZ/CLA; ceftazidime-clavulonic acid, and CRO; ceftriaxone

Table 4.3 Zone diameter interpretative standard for *Enterobacteriaceae* and screening and confirmatory tests for ESBLs in *E. coli* according to CLSI 2013 (60)

Antimicrobial agents	Disk content (µg)	Diameter of inhibition zone (mm)			Diameter of inhibition zone for ESBLs (mm)	
		S	I	R	ESBLs screening	ESBLs confirmatory test
Cefotaxime (CTX)	30	≥ 26	23-25	≤ 22	≤ 27	-
Ceftazidime (CAZ)	30	≥ 21	18-20	17	≤ 22	-
Ceftriaxone (CRO)	30	≥ 23	20-22	≤ 19	≤ 25	-
Cefotaxime-clavulonic acid (CTX/CLA)	30/10	-	-	-	-	≥5-mm increase in zone diameter compare to test agent alone
Ceftazidime-clavulonic acid (CAZ/CLA)	30/10	-	-	-	-	≥5-mm increase in zone diameter compare to test agent alone

S; susceptible, I; intermediate, and R; resistant

4.3 Genomic DNA extraction

Genomic DNA of 200 *E. coli* isolates was extracted by Gentra® Puregene® Yeast/Bact. kit (Qiagen, USA) according to manufacturer's instructions. Briefly, the overnight bacterial colonies were directly suspended in 300 µl of Cell Lysis Solution, mixed by pipetting, and incubated at 80°C for 5 minutes. RNase A solution was added into cell lysate tube for elimination of RNA, mixed by inverting 25 times, and incubated at 37°C for 15-60 minutes. After sample was cooled to room temperature, 100 µl of Protein Precipitation Solution was added and vigorously mixed by vortex. The sample was centrifuged at 15,000 x g for 3 minutes. The precipitated protein was formed as a tight pellet. Supernatant was transferred into 1.5 ml microcentrifuged tube containing 300 µl of isopropanol to precipitate DNA. The DNA should be visible as a small white pellet. The supernatant was carefully discarded, then 300 µl of 70% ethanol was added into the pellet and inverted several times to wash the DNA pellet. The sample was centrifuged at 13,000-16,000 x g for 1 minute, carefully discarded supernatant, and allowed air dry for 10-15 minutes. DNA Hydration Solution was added and incubated at 65°C for 1 hour or overnight at room temperature. Extracted DNA was analyzed for their purity and quantity by using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA) for further analysis and kept at -20°C until used.

4.4 The presence of class 1 integrons in multidrug pathogenic *E. coli* isolates from human

4.4.1 Class 1 integrons determination by polymerase chain reaction (PCR)

Genomic DNA was subjected to PCR with specific primers for the presence of integrase gene (*intI1*) and 5′-3′ conserved sequence (5′CS-3′CS) for internal segment of integron element. The primer sequences used for amplification of *intI1* and 5′CS-3′CS are shown in the Table 4.4. PCR reagents and conditions for *intI1* are shown in Table 4.5-4.6 and 5′CS-3′CS in Table 4.7-4.8.

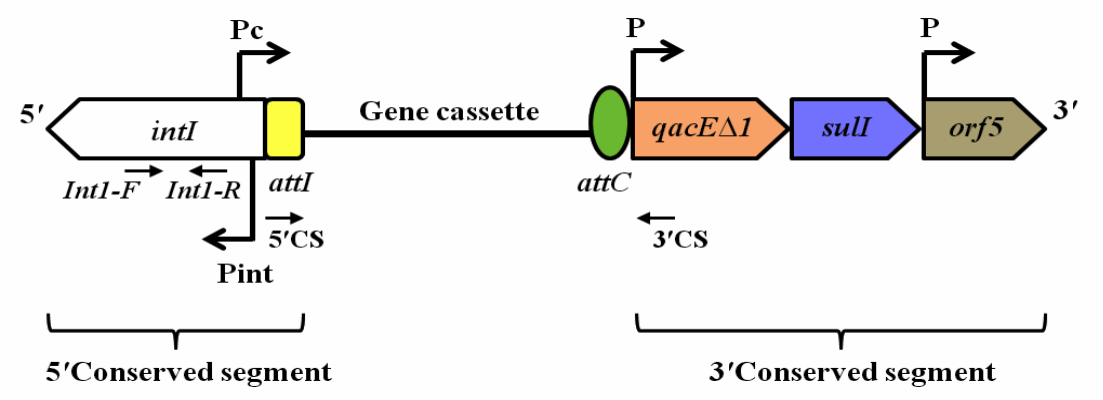

Figure 4.1 Schematic representation of the molecular marker for class 1 integrons in this study. *intI1* is located in 5′-Conserved segment whereas an inserted resistance gene is presented between internal segments of 5′CS and 3′CS.

Table 4.4 Primers used for amplification of *intI1* and 5′CS-3′CS.

Primer	Oligonucleotide sequence (5′- 3′)	Product size (bp)	Reference
intI1-F	GCCTTGCTGTTCTTCTACGG	558	(61)
intI1-R	GATGCCTGCTTGTTCTACGG		
5′CS	GGCATCCAAGCAGCAAG	Variable	(61)
3′CS	AAGCAGACTTGACCTGA		

Table 4.5 PCR components for amplification of *intI1* in total volume 20 µl

Reagents	Volume (µl)	Final concentration
MilliQ water	14.4	-
10x standard Taq reaction buffer (with $MgCl_2$)	2	1x
10 mM dNTPs	0.4	0.2 mM
10 µM Forward primer (*intI1*-F)	1	0.5 µM
10 µM Reversed primer (*intI1*-R)	1	0.5 µM
NEB Taq polymerase (5U/µl)	0.2	1U
Template DNA	1	1-10 ng/µl

Table 4.6 PCR conditions for amplification of *intI1*

Step	Temp	Time	Cycles
Initial denaturation	95ºC	5 min	1
Denaturation	95ºC	30 sec	
Annealing	58ºC	30 sec	30
Extension	68ºC	1 min	
Final Extension	68ºC	5 min	1
Hold	4ºC	∞	

Table 4.7 PCR components for amplification of 5′CS-3′CS in total volume 20 µl

Reagents	Volume (µl)	Final concentration
MilliQ water	10.4	-
10x ThermoPol buffer (with $MgCl_2$)	2	1x
Betain (5M)	4	1 M
10 mM dNTPs	0.4	0.2 mM
10 µM 5'CS primer	1	0.5 µM
10 µM 3'CS primer	1	0.5 µM
NEB Taq polymerase (5U/µl)	0.2	1U
Template DNA	1	1-10 ng/µl

Table 4.8 PCR conditions for amplification of 5′CS-3′CS

Step	Temp	Time	Cycles
Initial denaturation	95ºC	5 min	1
Denaturation	95ºC	30 sec	
Annealing	58ºC	1 min	30
Extension	68ºC	3 min	
Final Extension	68ºC	5 min	1
Hold	4ºC	∞	

All PCR products were determined by electrophoresis in 1.5% agarose gel containing 1X TBE buffer. GeneRuler 1 Kb DNA ladder was used as a marker. *E. coli*

EC114, an integron containing *dfrA17* and *aadA5* strain and *E. coli* Top10 were run in parallel with samples for positive and negative control, respectively.

4.4.2 Class 1 integrons determination by dot blot hybridization

Dot blot hybridization was performed for confirmation of class 1 integrase gene (*intI1*). PCR product with specific primer to *intI1* was used to synthesize and label for *intI1* probe by using DIG High Prime DNA Labeling kit and Detection Starter I (Roche Applied Science, Germany). The *intI1*-DIG-labeled probes were hybridized to a membrane-bound nucleic acid through dot blotting. These hybridized probes were immunodetected with alkaline phosphatase-conjugated anti-digoxigenin antibody and then visualized with colorimetric substrates (NBT and X-Phosphate) as shown in Figure 4.2

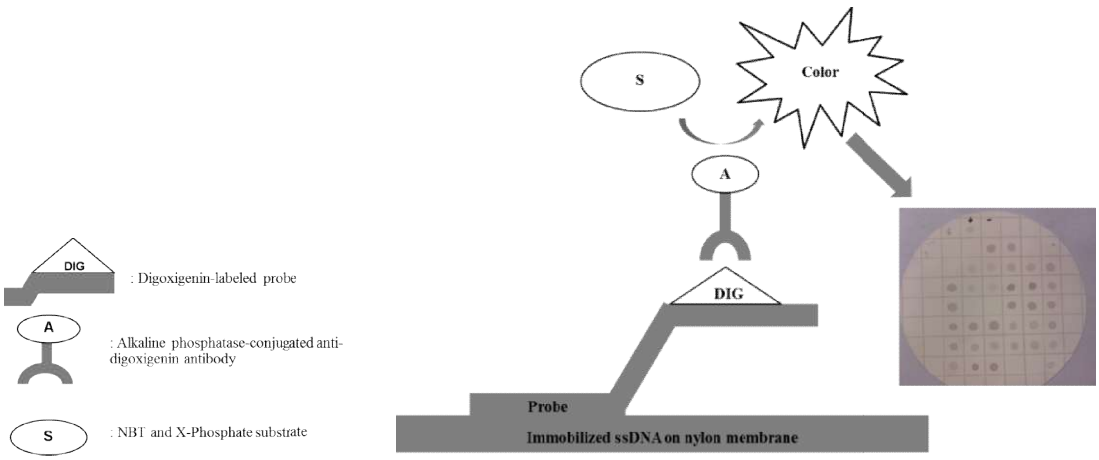

Figure 4.2 Schematic overview of dot blot hybridization for *intI1* gene

4.4.2.1 Random primed DNA probe labeling

Digoxigenin (DIG), a steroid hapten, was used to generate DIG-labeled DNA probes which are based on the hybridization of random oligonucleotides to the denatured DNA template. The complementary DNA strand was synthesized by Klenow enzyme in the presence of the 3′OH termini of random hexanucleotide primers and a dNTP mix containing alkali-labile DIG-11-dUTP. DIG dUTP was incorporated every 20-25 nucleotides into the newly synthesized DNA.

This density of haptens in the DNA yielded the highest sensitivity in the detection reaction. Reagents used for *intI1*-DIG-labeled probes are shown in Table 4.9.

Table 4.9 Reagents for *intI1*-DIG-labeled probes for dot blot hybridization

Reagent	Volume	Final concentration
Sterile distilled water	55 µl	-
10x Hexanucleotide mix	10 µl	1x
10x dNTP labeling mix	10 µl	1x
Klenow enzyme, labeling grade	5 µl	100 U/ml
DNA template (*intI1* PCR product)	20 µl	500 ng
Total volume	100 µl	

Briefly, DNA template was heated to denature for 10 minutes and quickly chilled on ice for 5 minutes. All reagents were added to DNA template (on ice) and incubated overnight at 37 °C. After that, 0.1 volumes of 0.2 M EDTA were added to stop the reaction. 1 µl of glycogen solution, 0.1 volumes of 3M NaOAc, pH 5.2, and 375 µl (3 volumes) of chilled ethanol were added into the reaction tube to precipitate the labeled DNA and incubated at -70 °C for 30 minutes. The sample was centrifuged at 13,000 rpm for 20 minutes, carefully discarded supernatant, and 100 µl of 70% ethanol was added to wash the pellet. The sample was centrifuged at 13,000 rpm for 7 minutes, and took off ethanol solution. The pellet was air dry and dissolved in 50 µl of TE/SDS buffer. The DIG-*intI1* labeled probes were stored at -20°C until used.

4.4.2.2 Evaluation of probe labeling efficiency

The yield of DIG-labeled DNA was estimated to confirm the success of the labeling reaction and prepare the correct amount of probe for hybridization. Labeled control DNA was prepared serial dilutions from 1:5 (1 ng/µl) to 1:50,000 (0.1 pg/µl), labeled A-E, and 1µl of dilution B-E were spotted onto a positively charged nylon membrane. The *intI1* labeled probe was made serial ten-fold dilutions from 10^{-1} to 10^{-4} dilutions and 1 µl of each dilution was applied onto the same nylon membrane. The DNA were fixed to membrane by cross-linking with UV

light and transferred to Genius buffer 1 (washing buffer) for 3 minutes. The membrane was incubated in Genius buffer 2 (Blocking solution) for 5 minutes, transferred in anti-DIG-alkaline phosphatase for 5 minutes on rocking shaker, washed twice in Genius buffer 1, 15 minutes per wash on horizontal shaker to remove unbound antibody, and then equilibrated in Genius buffer 3 (Detection buffer) for 5 minutes. Color substrate solution was added to the membrane and incubated in dark room for 20 minutes to develop color. The membrane was rinsed in trap water to stop reaction and allowed to dry. The spot intensities of the control and the *intI1* labeled probe dilutions were compared to estimate the concentration of *intI1* labeled probe.

4.4.2.3 DNA blotting

Zero point one volume of denaturing solution was added into the sample and incubated at room temperature for 10 minutes to denature double stranded DNA and degrade any contaminating RNA in the sample. Two microliter of the single stranded DNA sample was spotted onto positively charged nylon membrane which each sample was duplicated blot. The DNA was fixed to the membrane by UV cross-linking twice.

4.4.2.4 Prehybridization

The membrane was placed in a hybridization bottle containing 10 ml standard hybridization solution and rotated at 65°C for 2 hours.

4.4.2.5 Hybridization

The DIG *intI1*-labeled probe was heated in a boiling water bath for 10 minutes to denature the DNA and added 10 µl in 10 ml of standard hybridization solution, and then replaced the standard hybridization solution in the hybridization bottle. The bottle was rotated at 65°C for overnight. After that, the membrane was washed twice, 5 minutes per wash, in 200 ml of wash solution 1 (2X SSC, 0.1% SDS) at room temperature on horizontal shaker and washed twice, 15 minutes per wash, in 200 ml of wash solution 2 (0.5X SSC, 0.1% SDS) at 65°C. The hybridization solution was kept at -20°C for reuse.

4.4.2.6 Colorimetric detection

After post-hybridization washes, the membrane was equilibrated in 100 ml of Genius buffer 1 for 5 minutes on rocking shaker and blocked in 5 ml of Genius buffer 2 for 60 minutes by gentle agitating. The membrane was transferred in the anti-DIG-alkaline phosphatase for 30 minutes on rocking shaker, washed twice, 15 minutes per wash in 150 ml of Genius buffer 1 on horizontal shaker to remove unbound antibody, and then equilibrated in 100 ml of Genius buffer 3 (Detection buffer) for 5 minutes. Color substrate solution was added to the membrane and incubated in dark room for 20 minutes to develop color. The membrane was rinsed in trap water to stop reaction and allowed to dry at room temperature.

4.5 Characterization of class 1 integrons

4.5.1 Cloning experiment

PCR product of 5′CS-3′CS was inserted into pCR®2.1 plasmid vector by using TA Cloning Kit (Invitrogen, USA). Reagents and condition for ligation are shown in Table 4.10.

Table 4.10 Reagents and condition for pCR®2.1 ligation reaction in total volume 5µl

Reagent	Volume (µl)
MilliQ water	1.5
10x ligation buffer	0.5
pCR®2.1 vector	1
Fresh PCR product	1.5
T4 DNA ligase	0.5
Reaction mixture was incubated at 14°C for overnight.	

After incubation and 50 µl of TOP10 *E. coli* competent cells was thawed on ice, 2 µl of ligation product was added, gently mixed and incubated on ice for 30 minutes. Then cell in the reaction tube was heat-shocked at 42°C for 45 seconds, incubated on ice for 2 minutes follow by adding of 250 µl of room temperature LB

broth and incubation at 37°C for 3 hour with agitation. After that, 100 µl of transformation mixture was spread on LB agar plate containing X-Gal and 100 µg of ampicillin and incubated overnight at 37°C. At least 10 white colonies were selected to grow on 3 ml of LB broth containing 100 µg of ampicillin and incubated overnight at 37°C for transformants analysis.

4.5.2 Transformants analysis

4.5.2.1 Recombinant plasmid extraction

Recombinant plasmids were extracted from transformant clones using alkaline lysis miniprep (62). Briefly, 1.5 ml of bacterial overnight culture was centrifuged at 15,000 x g for 20 seconds. The pellet was resuspended in 100 µl of Alkaline lysis buffer I with RNaseA and transferred into a microcentrifuge tube. Two hundred microliter of Alkaline buffer II was added and mixed by inverting the tube 10 times. The tube was let stand at room temperature for 5 minutes and incubated on ice for 5 minutes, the lysate should be viscous and slightly clear. Then, 150 µl of Alkaline lysis buffer III was added, immediately mixed by inverting the tube 10 times, and centrifuged at 15,000 x g, 4°C for 10 minutes. Supernatant was transferred into new 1.5 ml microcentrifuged tube containing 450 µl of isopropanol to precipitate DNA, mixed, centrifuged at 15,000 x g for 10 minutes, and supernatant was discarded. One hundred microliter of 70% ethanol was added into the pellet, mixed, centrifuged at 15,000 x g for 5 minute, discarded supernatant, and allowed air dry for 15 minutes. The pellet was resuspended in 50 µl of milliQ water and kept at -20°C before used.

4.5.2.2 Restriction enzyme analysis

The pCR®2.1 plasmid vector contains *Eco*RI flanked PCR product insertion site (Figure 4.3). The extracted recombinant plasmid was digested by *Eco*RI restriction enzyme to confirm the presence of PCR product insertion. Reagents and conditions for digestion are shown in Table 4.11.

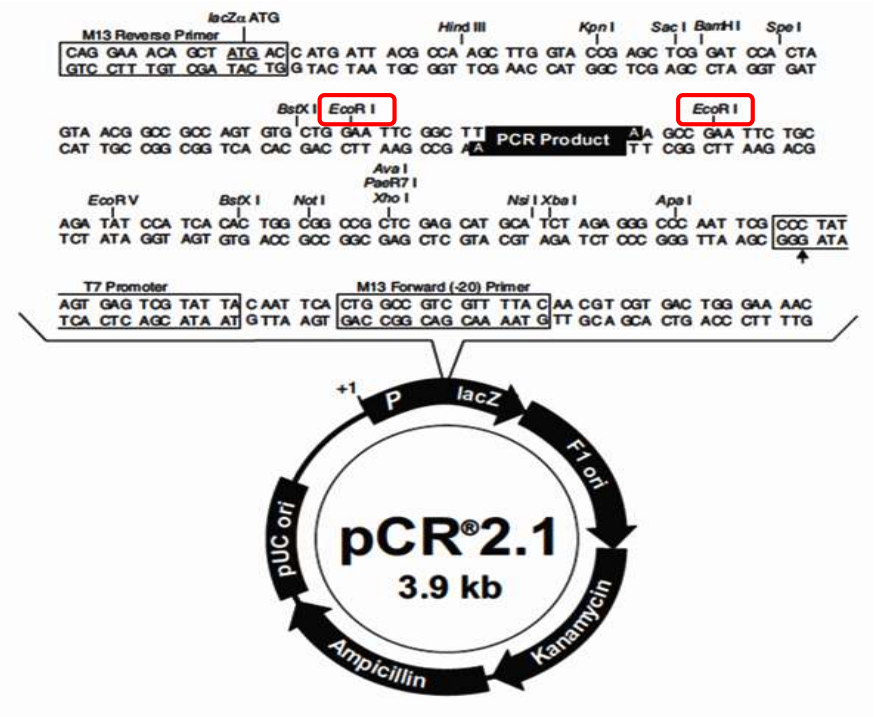

Figure 4.3 The map of pCR®2.1 plasmid vector

Table 4.11 Reagents and conditions for *Eco*RI digestion in total volume 20 µl

Reagent	Volume
MilliQ water	12
10x H buffer	2
Recombinant plasmid	5
*Eco*RI (15U/µl)	1
Digestion reaction was incubated at 37°C for 3 hrs. and stopped at 65°C for 20 mins.	

The digestion products were checked fragment sizes by agarose gel electrophoresis. Representative recombinant plasmid was extracted by using Nucleospin® Plasmid DNA Purification kit for DNA sequencing (protocol was described in Plasmid profiling part).

4.5.2.3 Sequencing of gene cassette region

The purified recombinant plasmid containing gene cassette was sequenced by using Sanger method with M13 forward and reverse sequencing primers. Nucleotide sequences were compared online at the National Center for Biotechnology Information (NCBI) website.

4.6. Class A β-lactamases-producing isolates determination by PCR

The genomic DNA of positive samples for ESBLs in the phenotypic confirmatory test were examined PCR for class A β-lactamase genes using specific primers, including *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, and *bla*$_{\text{SHV}}$ genes. The primer sequences are shown in the Table 4.12. PCR reagents for *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$ are shown in Table 4.13, and *bla*$_{\text{TEM}}$ in Table 4.14. PCR conditions for *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$ are shown in Table 4.15, and *bla*$_{\text{CTX-M}}$ in Table 4.16.

Table 4.12 Specific primers used for amplification of *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{CTX-M}}$ genes.

Primer	Oligonucleotide sequence (5′- 3′)	Product size (bp)	Reference
TEM-F	ATGAGTATTCAACATTTCCG	868	(63)
TEM-R	CTGACAGTTACCAATGCTTA		
SHV-F	GGTTATGCGTTATATTCGCC	868	(63)
SHV-R	TTAGCGTTGCCAGTGCTC		
CTX-M MA1	SCSATGTGCAGYACCAGTAA	544	(64)
CTX-M MA2	CCGCRATATGRTTGGTGGTG		

S= G or C, Y= C or T, and R= A or G

Table 4.13 PCR components for amplification of *bla*$_{CTX-M}$ *and bla*$_{SHV}$ in total volume 20 µl

Reagents	Volume (µl)	Final concentration
MilliQ water	14.3	-
10x standard Taq reaction buffer (with $MgCl_2$)	2	1x
10 mM dNTPs	0.5	0.25 mM
10 µM Forward primer	1	0.5 µM
10 µM Reversed primer	1	0.5 µM
NEB Taq polymerase (5U/µl)	0.2	1U
Template DNA	1	1-10 ng/µl

Table 4.14 PCR components for amplification of *bla*$_{TEM}$ in total volume 20 µl

Reagents	Volume (µl)	Final concentration
MilliQ water	14.1	-
10x standard Taq reaction buffer (with $MgCl_2$)	2	1x
50 mM $MgCl_2$	0.2	0.5 mM
10 mM dNTPs	0.5	0.25 mM
10 µM Forward primer (TEM-F)	1	0.5 µM
10 µM Reversed primer (TEM-R)	1	0.5 µM
NEB Taq polymerase (5U/µl)	0.2	1U
Template DNA	1	1-10 ng/µl

Table 4.15 PCR conditions for amplification of *bla*$_{TEM}$ and *bla*$_{SHV}$

Step	Temp	Time	Cycles
Initial denaturation	95ºC	5 min	1
Denaturation	95ºC	30 sec	
Annealing	55ºC	1 min	30
Extension	68ºC	1 min	
Final Extension	68ºC	5 min	1
Hold	4ºC	∞	

Table 4.16 PCR conditions for amplification of bla_{CTX-M}

Step	Temp	Time	Cycles
Initial denaturation	95ºC	5 min	1
Denaturation	95ºC	30 sec	
Annealing	58ºC	1 min	30
Extension	68ºC	1 min	
Final Extension	68ºC	5 min	1
Hold	4ºC	∞	

All PCR products were determined by agarose gel electrophoresis. *Salmonella* Sal22, *Salmonella* Sal1, and *K. pneumoniae* KPN91, laboratory strains positive for bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes, respectively were used as positive control and *E. coli* Top10 as negative control

4.7. Molecular typing

4.7.1 Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR

ERIC PCR was performed to amplify between copies of the ERIC sequence in genomic DNA using ERICIR and ERIC2 primers as shown in Table 4.17. The specific band pattern of PCR products were used for bacterial DNA fingerprinting. The PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide and visualized by UV-transillumination. The dendrogram was constructed by using Bionumerics version 6.5. Reagents and condition of PCR are shown in Table 4.18-4.19.

Table 4.17 Primers used for amplification of ERIC sequence

Primer	Oligonucleotide sequence (5′- 3′)	Product size (bp)	Reference
ERICIR	ATGTAAGCTCCTGGGGATTCAC	Variable	(56)
ERIC2	AAGTAAGTGACTGGGGTGAGCG		

Table 4.18 PCR components for ERIC PCR in total volume 20 µl

Reagents	Volume (µl)	Final concentration
MilliQ water	12.8	-
10X standard buffer (without $MgCl_2$)	2	1X
50 mM MgCl2	1	2.5 mM
10 mM dNTPs	0.4	0.2 mM
10 µM ERIC1R	0.8	0.4 µM
10 µM ERIC2	0.8	0.4 µM
Vivantis Taq polymerase (5U/µl)	0.2	1U
Template DNA	2	1-10 ng/µl

Table 4.19 PCR conditions for ERIC PCR

Step	Temp	Time	Cycles
Initial denaturation	95ºC	5 min	1
Denaturation	95ºC	50 sec	35
Annealing	46ºC	30 sec	
	49ºC	30 sec	
Extension	72ºC	3 min	
Final Extension	72ºC	10 min	1
Hold	4ºC	∞	

4.7.2 Plasmid profiling

Plasmid DNA was extracted by using Nucleospin® Plasmid DNA Purification kit (Machery-Nagel, Germany) according to manufacturer's instructions. Briefly, 3 ml of bacterial overnight culture in LB broth containing 100 µg/ml of ampicillin was harvested by centrifuge at 11,000 x g for 30 seconds. The pellet was resuspended in 250 µl of Buffer A1 and transferred into a microcentrifuge tube. Then 250 µl of Buffer A2 was added and mixed by inverting the tube 8 times to avoid shearing of genomic DNA, incubated at room temperature for up to 5 minutes, the lysate should be viscous and slightly clear. Three hundred µl of Buffer A3 was added and immediately mixed by inverting the tube 8 times and then microcentrifuged at 11,000 x g for 15 minutes. The supernatant was transferred into the Nucleospin®

Plasmid Column, centrifuged at 11,000 x g for 1 minute, and the flow-through was discarded. After that, the column was washed by adding 500 µl of Buffer AW preheated to 50ºC and was centrifuged at 11,000 x g for 1 minute, the flow-through was discarded, and then added 600 µl of Buffer A4, centrifuged for 1 minute and the flow-through was discarded. The column was centrifuged at 11,000 x g for 2 minutes to dry silica membrane and placed in a new microcentrifuge tube. Fifty µl of Buffer AE (elution buffer) was added into the center of the column, incubated for 1 minute at room temperature, centrifuged for 1 minute at 11,000 x g, and the column was discarded. The purified plasmids were run on 0.8% agarose gel at 100 Volts for 2 hours to compare number and size of plasmids, and then the dendrogram was constructed by using Bionumerics version 6.5.

4.7.3 High throughput multi-locus sequence typing (HiMLST)

E. coli isolates in this study were typed by using HiMLST with 454 sequencing and multiplex identifier (MID), internal fragment of seven housekeeping genes of 96 isolates were simultaneously sequenced and MLST profile of each individual isolate was generated by its unique MID. Briefly, The target genes were amplified by two-step PCR using primer sequence of target genes includes universal tail sequence primers at 5′ end in the first step PCR and MID sequence primers with 454 sequencing-specific nucleotides in the second round PCR. The PCR products were pooled, clonally amplified by emulsion PCR (emPCR) and sequencing using GS junior. Allele and sequence type (ST) assignments were made at the publicly accessible *E. coli* MLST database at http://mlst.warwick.ac.uk/mlst/dbs/Ecoli.

4.7.3.1 Seven housekeeping genes used for *E. coli* HiMLST scheme

Seven housekeeping genes were used in this study according to Achtman MLST typing scheme:

adk (adenylate kinase)

fumC (fumarate hydratase)

gyrB (DNA gyrase)

icd (isocitrate/isopropylmalate dehydrogenase)

mdh (malate dehydrogenase)
purA (adenylosuccinate dehydrogenase)
recA (ATP/GTP binding motif)

4.7.3.2 PCR round 1 for MLST target gene adding universal tails

The seven target genes were amplified by PCRs in 96 well plates with 10 µl reaction volumes using the FastStart High Fidelity Reaction Kit (Roche, Germany) and 3 sets of specific primer including universal tail sequences at 5′ end, primers set 1 for Penta-plex PCR; *adk*, *fumC*, *gyrB*, *icd*, and *mdh*, primers set 2 for *purA*, and primers set 3 for *recA* gene. The specific PCR primers to each allele were designed from the Achtman MLST typing scheme (65) by using Primer3 program excepting *adk*, to obtain the optimal amplicon sizes for *454* sequencing system. The primer sequences are shown in the Table 4.20. PCR reagents for penta-plex PCR are shown in Table 4.21, *purA* and *recA* in Table 4.22. PCR conditions for penta-plex PCR and *purA* are shown in Table 4.23, and *recA* in Table 4.24.

Table 4.20 *E. coli* MLST target gene-specific primers used in this study

Primers	Sequence (5′- 3′)	Amplicon size (bp)	References
adk_eF_univ_F	GACACTATAGATTCTGCTTGGCGCTCCGGG	604	(65)
adk_eR_univ_R	CACTATAGGGCCGTCAACTTTCGCGTATTT		
fumC_eF_univ_F	GACACTATAGGCGGCAAAAGTTAATGAAGA	580	This study
fumC_eR_univ_R	CACTATAGGGTCCGGATGGGTATTTAGTCC		
gyrB_eF_univ_F	GACACTATAGAGTGATCATGACCGTTCTGC	567	This study
gyrB_eR_univ_R	CACTATAGGGCGGAATGTTGTTGGTAAAGC		
icd_eF_univ_F	GACACTATAGAAGGTGATGGAATCGGTGTA	627	This study
icd_eR_univ_R	CACTATAGGGTTCATGATGTTGCCTTTGTG		
mdh_eF_univ_F	GACACTATAGGCGCAGATGTCGTTCTTATC	526	This study
mdh_eR_univ_R	CACTATAGGGAGACAGACCAAAACGTGCAG		
purA_eF_univ_F	GACACTATAGATGTCCGCTGATCCTTGAT	605	This study
purA_eR_univ_R	CACTATAGGGAATTCGTTACCCTGCTTGC		
recA_eF_univ_F	GACACTATAGGTTTATCGATGCTGAACACG	594	This study
recA_eR_univ_R	CACTATAGGGCTTCTCTTTTACGCCCAGGT		

Nucleotide in black represent the gene-specific part and universal tails are shown in blue or red.

Table 4.21 PCR components for first round penta-plex PCR; *adk*, *fumC*, *gyrB*, *icd*, and *mdh* in total volume 10 µl

Reagents	Volume (µl)	Final concentration
MilliQ water	3.1	-
10X Faststart High Fidelity	1	1X
25 mM MgCl2	0.6	1.5 mM
10 mM dNTPs	0.2	0.2 mM
10 µM adk_eF_univ_F	0.4	0.4 µM
10 µM adk_eR_univ_R	0.4	0.4 µM
10 µM fumC_eF_univ_F	0.4	0.4 µM
10 µM fumC_eR_univ_R	0.4	0.4 µM
10 µM gyrB_eF_univ_F	0.4	0.4 µM
10 µM gyrB_eR_univ_R	0.4	0.4 µM
10 µM icd_eF_univ_F	0.4	0.4 µM
10 µM icd_eR_univ_R	0.4	0.4 µM
10 µM mdh_eF_univ_F	0.4	0.4 µM
10 µM mdh_eR_univ_R	0.4	0.4 µM
Faststart High Fidelity enzyme	0.1	0.5 U
Template DNA	1	1-10 ng/µl

Table 4.22 PCR components of first round PCR; *purA* or *recA* in total volume 10 µl

Reagents	Volume (µl)	Final concentration
MilliQ water	6.6	-
10X Faststart High Fidelity Reaction buffer	1	1X
25 mM $MgCl_2$	0.6	1.5 mM
10 mM dNTPs	0.2	0.2 mM
10 µM purA_eF_univ_F or recA_eF_univ_F	0.5	0.5 µM
10 µM purA_eR_univ_R or recA_eR_univ_R	0.5	0.5 µM
Faststart High Fidelity enzyme blend (5U/µl)	0.1	0.5 U
Template DNA	0.5	1-10 ng/µl

Table 4.23 PCR conditions for first round penta-plex PCR; *adk*, *fumC*, *gyrB*, *icd*, and *mdh* and simplex PCR; *purA*

Step	Temp	Time	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	1 min	
Annealing	54°C	30 sec	30
Extension	72°C	1 min	
Final Extension	72°C	10 min	1
Hold	4°C	∞	

Table 4.24 PCR conditions for first round simplex PCR; *recA*

Step	Temp	Time	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	1 min	
Annealing	59°C	30 sec	30
Extension	72°C	1 min	
Final Extension	72°C	10 min	1
Hold	4°C	∞	

All PCR products were purified by using magnetic AMPure XP beads (Agencourt® AMPure® XP, Beckman Coulter, USA) to removed unincorporated dNTPs, primers, primer dimers, salts, and other unwanted fragments. Then the purified amplicons were analyzed by electrophoresis in 2% agarose gel containing 1X TBE buffer and diluted to 1:100 for the second-step PCR.

4.7.3.3 PCR round 2 for barcode incorporation

The purified MLST amplicons were re-amplified to incorporate 454 sequencing-specific nucleotides and isolate-specific nucleotides (MIDs) by using 454 Universal Tailed-directed Primers A and B fusion primers targeting the universal tails of the first round PCR product. The fusion primer sequences are shown in Appendix B. PCR reagents and conditions of PCR are shown in Table 4.25-4.26.

Table 4.25 PCR components of second round PCR in total volume 10 µl

Reagents	Volume (µl)	Final concentration
MilliQ water	6.6	-
10X Faststart High Fidelity Reaction buffer	1	1X
25 mM $MgCl_2$	0.6	1.5 mM
10 mM dNTPs	0.2	0.2 mM
10 µM fusion forward primer	0.5	0.5 µM
10 µM fusion reverse primer	0.5	0.5 µM
Faststart High Fidelity enzyme blend (5U/µl)	0.1	0.5 U
Template DNA	0.5	1-10 ng/µl

Table 4.26 PCR conditions for second round PCR

Step	Temp	Time	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	1 min	10 (increase 0.5°C/cycle)
Annealing	45°C	30 sec	
Extension	72°C	1 min	
Denaturation	95°C	1 min	25
Annealing	50°C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	10 min	1
Hold	4°C	∞	

The PCR products were purified by using magnetic AMPure XP beads and analyzed by electrophoresis in 2% agarose gel containing 1X TBE.

4.7.3.4 Sample pooling and quantification

All purified products of *purA* and *recA* amplicons from second round PCR were transferred into 96 well plate containing second round PCR of 5-plex of each sample and measured fluorescence intensity by using Quan-it™ PicoGreen® dsDNA Assay kit (Invitrogen, USA) on a LightCycler 480 instrument. Then each sample was calculated DNA concentrations and diluted to $1X10^9$ DNA molecules/µl.

All samples were pooled in equimolar concentration and purified twice by using magnetic AMPure XP beads. Amplicon pooling was finally quantified and prepared to $1X10^5$ DNA molecules/µl as the DNA library concentration.

4.7.3.5 Emulsion PCR (emPCR)

Briefly, 10 µl of DNA library sample was added into capture Beads (A and B), then mixed in PCR reagents and emulsion oil in water-in-oil mixture to amplified clonal DNA fragments by PCR Thermal Cycler (Esco Healthcare, Singapore) using GS Junior Titanium emPCR Kit (Lib-A) according to emPCR Amplification Method Manual-Lib-A, GS Junior Titanium Series (454 Life Sciences, Roche, USA), March 2012. Emulsion PCR was amplified by using amplification cycles consist of initial denaturation at 94°C for 4 minutes followed by 50 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 4.5 minutes, and extension at 68°C for 30 seconds, and hold on at 10°C for up to 16 hours before further process. After that, the PCR reaction wells were checked for emulsion breakage as shown in Figure 4.4. Then the amplification beads were recovered and enriched the DNA beads.

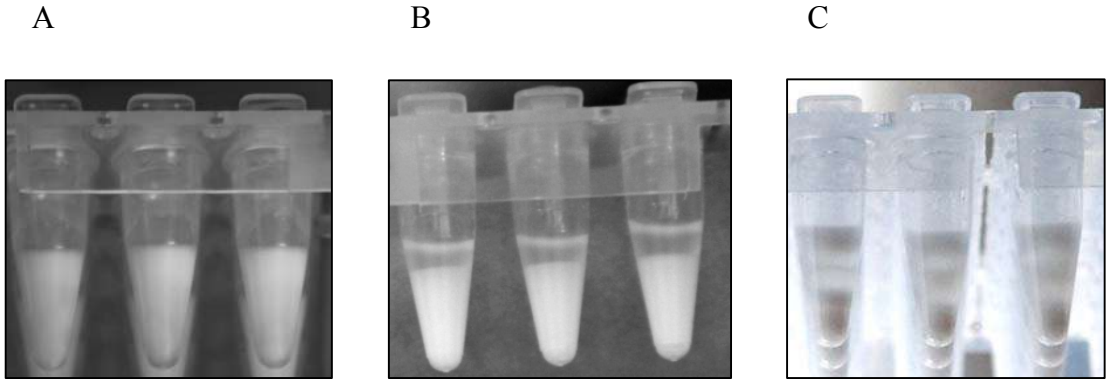

Figure 4.4 The emulsion PCR. Intact emulsion is a homogenous suspension before amplification (A), and after amplification with a clear phase (B). The multiple bands after amplification are a sign of a broken emulsion, should be discarded (C).

The enriched DNA beads were evaluated by using the GS Junior Bead Counter as shown in Figure 4.5. The recommended input beads number for a GS Junior Sequencing Run is 500,000 enriched beads (5% bead enrichment).

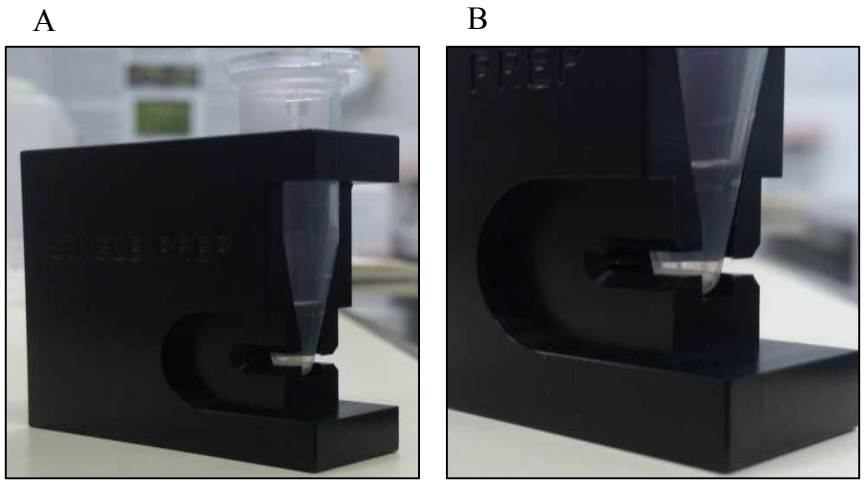

Figure 4.5 The Single Prep side view of GS Junior Bead Counter V2 (A) and (B). The top of beads pellet is lower edge of the window, defined 500,000 beads (5% bead enrichment) while the upper edge of window is defined 2,000,000 beads (20% bead enrichment). If the beads pellet is more than 20% bead enrichment, the preparation has failed and should be discarded.

4.7.3.6 DNA sequencing

The enriched DNA beads were annealed with the sequence primer (Seq Primer A and B) and loaded into PicoTiterPlate (PTP) device in specific layer: layer 1 is Enzyme Beads Pre-layer; layer 2 is DNA and Packing Beads; layer 3 is Enzyme Beads Post-layer; and layer 4 is PPiase Beads. Then the DNA was simultaneously sequenced by GS Junior Titanium instrument using 454 pyrosequencing with GS Junior Titanium Sequencing Kit according to the Sequencing Method Manual, GS Junior Titanium Series (454 Life Sciences, Roche, USA), January 2013.

4.7.3.7 Data analysis

The processing data from GS Run Processor application were extracted into Standard Flowgram Format (SFF). The reads and flowgrams of SFF file were used as input data for the GS Amplicon Variant Analyzer (AVA) application (Roche), which were processed to trim reads, demultiplex data sets for identification

of seven alleles from each sample, map each sample read to reference FASTA sequences of *E. coli* strain MG1655. The output data was exported to FASTA file for further analysis with BioEdit software version 7.1.11. The sequences of seven housekeeping genes of each sample were submitted to *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) for allele and sequence type (ST) assignments. The Minimum spanning tree (MS_{TREE}) of *E. coli* sequence type was constructed by using Bionumerics version 6.5.

4.7.3.8 Sanger sequencing

Sanger sequencing was carried to validate *E. coli* isolates that failed to get the STs from HiMLST protocol and verify the novel allele variant by using specific primer according to described in the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). The primer sequences are shown in the Table 4.27. PCR reagents and conditions for 7 housekeeping genes are shown in Table 4.28-4.30.

Table 4.27 Primer sequence and an expected product size of 7 housekeeping genes used for validation and verification.

Name	Sequence (5′-3′)	Product size (bp)
adk_P'1	TCATCATCTGCACTTTCCGC	766
adk_P'2	CCAGATCAGCGCGAACTTCA	
fumC_P1	TCACAGGTCGCCAGCGCTTC	806
fumC_P2	GTACGCAGCGAAAAAGATTC	
gyrB_P1	TCGGCGACACGGATGACGGC	880
gyrB_P2	ATCAGGCCTTCACGCGCATC	
icd_P1	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	878
icd_P2	GGACGCAGCAGGATCTGTT	
mdh_P1	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	932
mdh_P2	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT	
purA_P1	CGCGCTGATGAAAGAGATGA	817
purA_P2	CATACGGTAAGCCACGCAGA	
recA_P1	CGCATTCGCTTTACCCTGACC	734
recA_P2	TCGTCGAAATCTACGGACCGGA	

Table 4.28 PCR components for amplification of 7 housekeeping genes of *E. coli* in total volume 50 µl.

Reagents	Volume (µl)	Final concentration
MilliQ water	29.8	-
10X Faststart High Fidelity Reaction buffer	5	1X
25 mM $MgCl_2$	3	1.5 mM
10 mM dNTPs	1	0.2 mM
10 µM forward primer	5	1µM
10 µM reverse primer	5	1µM
Faststart High Fidelity enzyme blend (5U/µl)	0.2	1U
Template DNA	1	1-10 ng/µl

Table 4.29 PCR conditions for amplification of 6 housekeeping genes including *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*.

Step	Temp	Time	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	1 min	
Annealing	56°C	30 sec	30
Extension	72°C	1 min	
Final Extension	72°C	10 min	1
Hold	4°C	∞	

Table 4.30 PCR conditions for *adk* amplification.

Step	Temp	Time	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	1 min	
Annealing	54°C	30 sec	30
Extension	72°C	1 min	
Final Extension	72°C	10 min	1
Hold	4°C	∞	

The PCR products were determined by electrophoresis in 1.5% agarose gel containing 1X TBE buffer. GeneRuler 1 Kb DNA ladder was used as a marker. Then PCR products were purified by Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan) before submitting for Sanger sequencing. The sequencing data was analyzed by BioEdit software version 7.1.11 and submitted to *E. coli* MLST database.

CHAPTER V
RESULTS

5.1 Antimicrobial drug resistance patterns of *E. coli* isolates during 2001-2010

Of 198 multidrug resistant *E. coli* isolates, 88 isolates from adults, most of them were resistant to tetracycline (97.7%), sulfisoxazole (95.5%), ampicillin (83.0%), streptomycin (83.0%), and trimethoprim-sulfamethoxazole (79.5%) (Table 5.1). One hundred and ten *E. coli* isolates from children, most of them displayed resistant to sulfisoxazole (99.1%), tetracycline (92.7%), ampicillin (89.1%), trimethoprim-sulfamethoxazole (87.3%), and streptomycin (86.4%) (Table 5.1). Antimicrobial drug resistance patterns among *E. coli* isolates between adults and children was shown in Figure 5.1. The most frequent phenotype was resistant to 5 antimicrobial agents (20 and 25 isolates from adults and children, respectively); consist of ampicillin, streptomycin, sulfisoxazole, trimethroprim-sulfamethoxazole, and tetracycline, followed by resistance pattern against 6 antimicrobial agents (11 and 12 isolates from adults and children, respectively), ampicillin, chloramphenicol, streptomycin, sulfisoxazole, trimethroprim-sulfamethoxazole, and tetracycline (Table 5.2). Only 5% (2 and 8 isolates from adults and children respectively) of the isolates were positive for screening and confirmatory extended spectrum ß–lactamases (ESBLs) phenotypes.

Table 5.1 Antimicrobial susceptibility of 198 pathogenic *E. coli* isolates by disk diffusion method

Antimicrobial agents	No. of resistance strains					
	Adults (n=88)		Children (n=110)		Total (n=198)	
	n	%	n	%	n	%
Quinolones						
Nalidixic acid	32	36.4	37	33.6	69	34.8
Ciprofloxacin	9	10.2	8	7.3	17	8.6
Penicillins						
Ampicillin	73	83.0	98	89.1	171	86.4
Phenicols						
Chloramphenicol	38	43.2	49	44.5	87	43.9
Aminoglycosides						
Gentamycin	11	12.5	17	15.5	28	14.1
Kanamycin	11	12.5	21	19.1	32	16.2
Neomycin	8	9.1	5	4.5	13	6.6
Streptomycin	73	83.0	95	86.4	168	84.8
Sulfonamides						
sulfisoxazole	84	95.5	109	99.1	193	97.5
Trimethoprim-sulfamethoxazole	70	79.5	96	87.3	166	83.8
Tetracyclines						
Tetracycline	86	97.7	102	92.7	188	94.9
3rd Cephalosporins						
Cefotaxime	3	3.4	8	7.3	11	5.6
Ceftriaxone	3	3.4	7	6.4	10	5.1

Table 5.2 Antimicrobial resistance profile of 198 pathogenic *E. coli* isolates

No. of antimicrobial resistance	Resistance pattern	No. of isolates
3	S,G,TE	3
4	AM,C,GM,G	1
4	AM,G,SXT,TE	1
4	AM,C,S,TE	1
4	AM,S,G,SXT	3
4	AM,S,G,TE	13
4	C,G,SXT,TE	5
4	C,S,G,SXT	1
4	NA,AM,C,TE	1
4	NA,N,G,TE	1
4	NA,G,SXT,TE	1
4	S,G,SXT,TE	5
5	AM,C,G,SXT,TE	2
5	AM,C,S,G,SXT	1
5	AM,C,S,G,TE	2
5	AM,GM,G,SXT,TE	2
5	AM,K,G,SXT,TE	2
5	AM,S,G,STX,TE	45
5	C,GM,K,G,SXT	1
5	C,S,G,SXT,TE	4
5	NA,AM,C,GM,G	1
5	NA,AM,C,G,TE	1
5	NA,AM,S,G,SXT	1
5	NA,AM,C,S,TE	1
5	NA,AM,S,G,TE	2
5	NA,S,G,STX,TE	1
6	AM,C,GM,S,G,SXT	1
6	AM,C,GM,G,SXT,TE	1
6	AM,C,S,G,SXT,TE	23
6	AM,K,N,S,G,TE	1
6	AM,K,S,G,SXT,TE	2
6	C,K,N,G,SXT,TE	1
6	AM,N,S,G,SXT,TE	1
6	NA,AM,C,G,SXT,TE	2

Table 5.2 Antimicrobial resistance profile of 198 pathogenic *E. coli* isolates (cont.)

No. of antimicrobial resistance	Resistance pattern	No. of isolates
6	NA,AM,C,S,G,TE	1
6	NA,AM,GM,G,SXT,TE	3
6	NA,AM,S,G,SXT,TE	16
6	NA,CIP,S,G,SXT,TE	1
6	NA,CIP,GM,G,SXT,TE	1
7	AM,C,GM,S,G,SXT,TE	2
7	AM,C,K,S,G,SXT,TE	1
7	NA,AM,C,S,G,SXT,TE	5
7	NA,AM,GM,S,G,SXT,TE	1
7	NA,C,K,N,G,SXT,TE	1
7	NA,CIP,AM,S,G,SXT,TE	1
7	NA,CIP,AM,GM,G,SXT,TE	1
8	AM,C,K,N,S,G,SXT,TE	3
8	NA,AM,C,GM,S,G,SXT,TE	2
8	NA,AM,C,K,S,G,SXT,TE	3
8	NA,CIP,AM,C,K,TE,CTX,CRO	1
8	NA,CIP,AM,C,S,G,SXT,TE	2
8	NA,CIP,C,GM,S,G,SXT,TE	1
9	AM,C,GM,K,S,G,SXT,TE,CTX	1
9	NA,AM,C,GM,K,S,G,SXT,TE	1
9	NA,AM,C,S,G,SXT,TE,CTX,CRO	1
9	NA,AM,C,K,N,S,G,SXT,TE	3
9	NA,CIP,AM,GM,K,N,S,G,TE	1
9	NA,CIP,AM,C,K,S,G,SXT,TE	1
10	NA,AM,C,K,S,G,SXT,TE,CTX,CRO	1
10	NA,AM,GM,K,S,G,SXT,TE,CTX,CRO	2
10	NA,CIP,AM,C,GM,K,S,G,SXT,TE,CRO	1
10	NA,CIP,AM,C,GM,S,SXT,TE,CTX,CRO	1
10	NA,CIP,AM,C,K,N,S,G,SXT,TE	1
11	NA,CIP,AM,C,K,S,G,SXT,TE,CTX,CRO	1
11	NA,CIP,AM,GM,K,S,G,SXT,TE,CTX,CRO	1
12	NA,CIP,AM,C,GM,K,S,G,SXT,TE,CTX,CRO	2

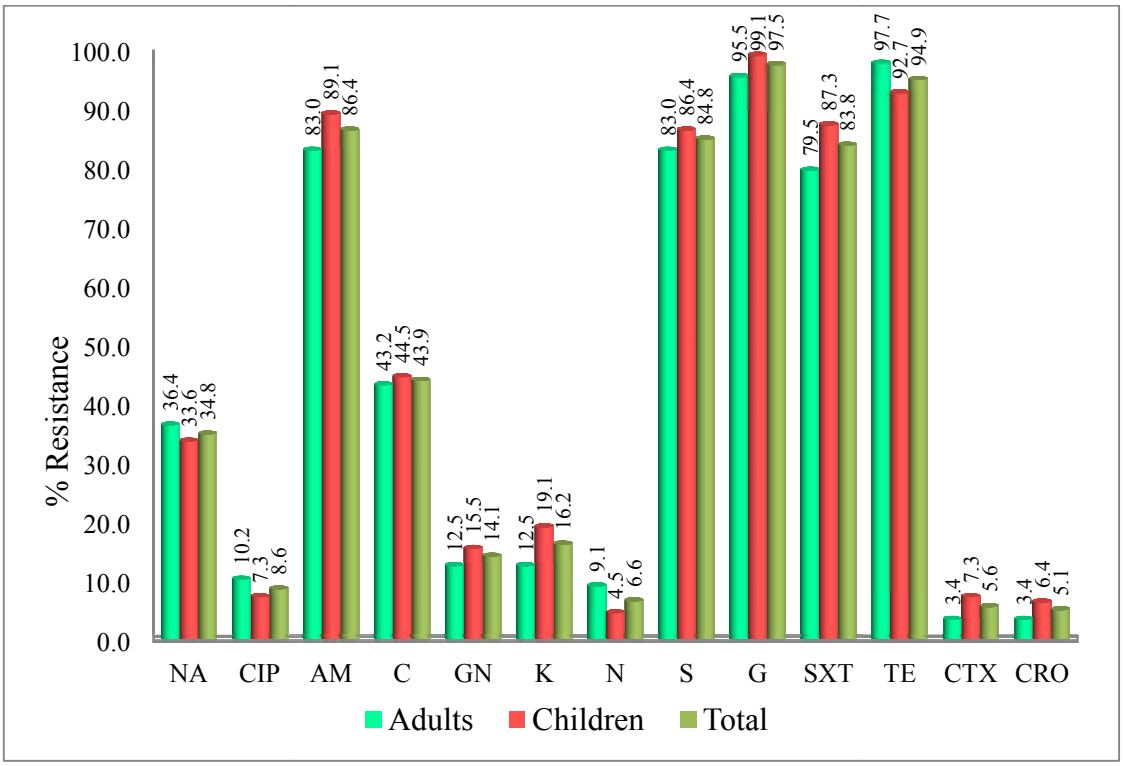

Figure 5.1 Antimicrobial drug resistance patterns among *E. coli* isolates between adults and children from 2001-2010

NA = nalidixic acid, CIP = ciprofloxacin, AM = ampicillin, C = chloramphenicol, GN = gentamycin, K = kanamycin, N = neomycin, S = streptomycin, G = sulfisoxazole, SXT = trimethoprim-sulfamethoxazole, TE = tetracycline, CTX = cefotaxime, CRO = ceftriaxone

5.2 Presence of class 1 integrons in multidrug pathogenic *E. coli* isolates from human

5.2.1 Class 1 integrons determination by polymerase chain reaction (PCR)

PCR with specific primers for *intI1* and 5′CS-3′CS was performed for the presence of integrase gene and internal segment of integron element. Of 198 MDR *E. coli* isolates, 62.5% (55/88) and 66.4% (73/110) were *intI1* positive for adults and children, respectively. 5′CS-3′CS products were found in 53.4% (47/88) of adults and 59.1% (65/110) of children. PCR products showed expected size of 558 bp for *intI1* (Figure5.2) and variable size for 5′CS-3′CS (Figure 5.3).

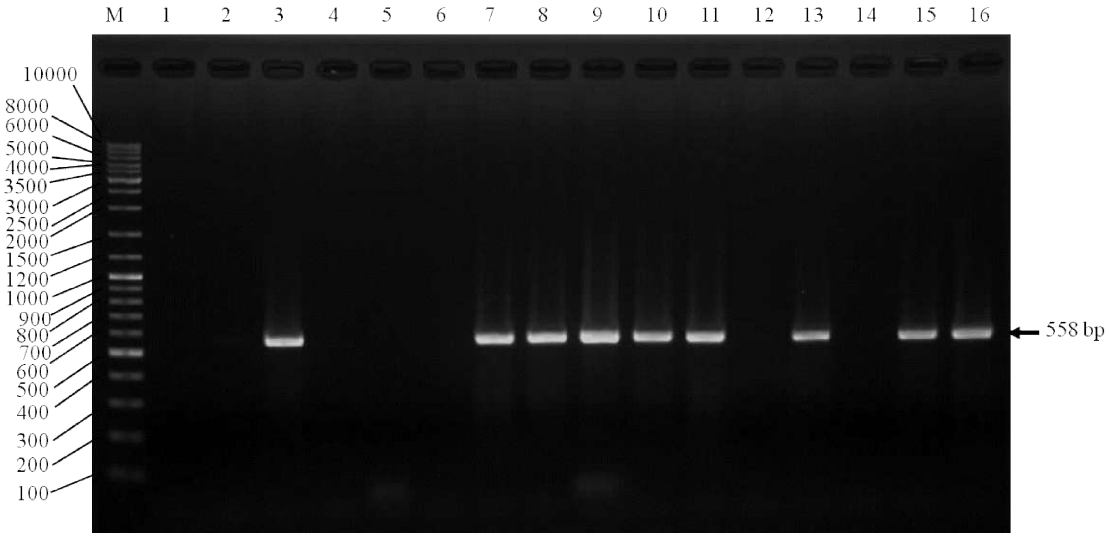

Figure 5.2 PCR using specific primers for *intI1*. Lane M, GeneRuler^{TM} 1 kb DNA Ladder; Lane 1, negative control, *E. coli* Top10; Lane 2-15, samples; Lane 16, positive control, EC114 *E. coli* carrying *dfrA17* and *aadA5*.

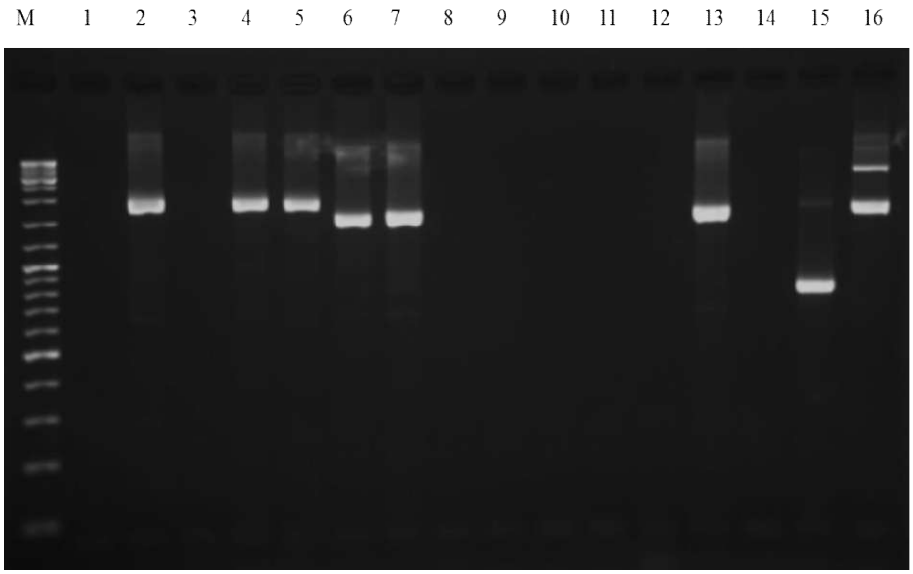

Figure 5.3 PCR amplification for variable size of 5′CS-3′CS. Lane M, GeneRuler^{TM} 1 kb DNA Ladder; Lane 1, negative control, *E. coli* Top10; Lane 2-15, samples; Lane 16, positive control, EC114 *E. coli* carrying *dfrA17* and *aadA5*.

5.2.2 Class 1 integrons determination by dot blot hybridization

Dot blot hybridization was carried out to confirm the presence of class 1 integrase gene by using *intI1* specific probe. The positive results showed purple dot for each sample (Figure 5.4). By using hybridization method, the presence of class 1 integrase was increased to 65.9% (58/88) and 72.7% (80/110) in adults and children, respectively (Table 5.3). However, internal segment of integron element with specific 5′CS-3′CS primers was detected for 8 integrase negative *E. coli* isolates, i.e., EA010, EC079, EC087, EC089, EC093, EC115, EC142, and EC167, whereas, 29 integrase positive isolates didn't present product of 5′CS-3′CS. Comparison of class 1 integrons determination between by using PCR for *intI1*, 5′CS-3′CS and dot blot hybridization was shown in Table 5.5.

Overall, class 1 integrons was detected 146 from 198 of MDR *E. coli* isolates (73.7%) by using combination of all detection method. The presence of class 1 integrons was classified according to diarrheagenic pathotype, EAEC, EPEC, ETEC, STEC, and EIEC were 81%, 80.9%, 53.6%, 100%, and 83.3%, respectively (Table 5.4).

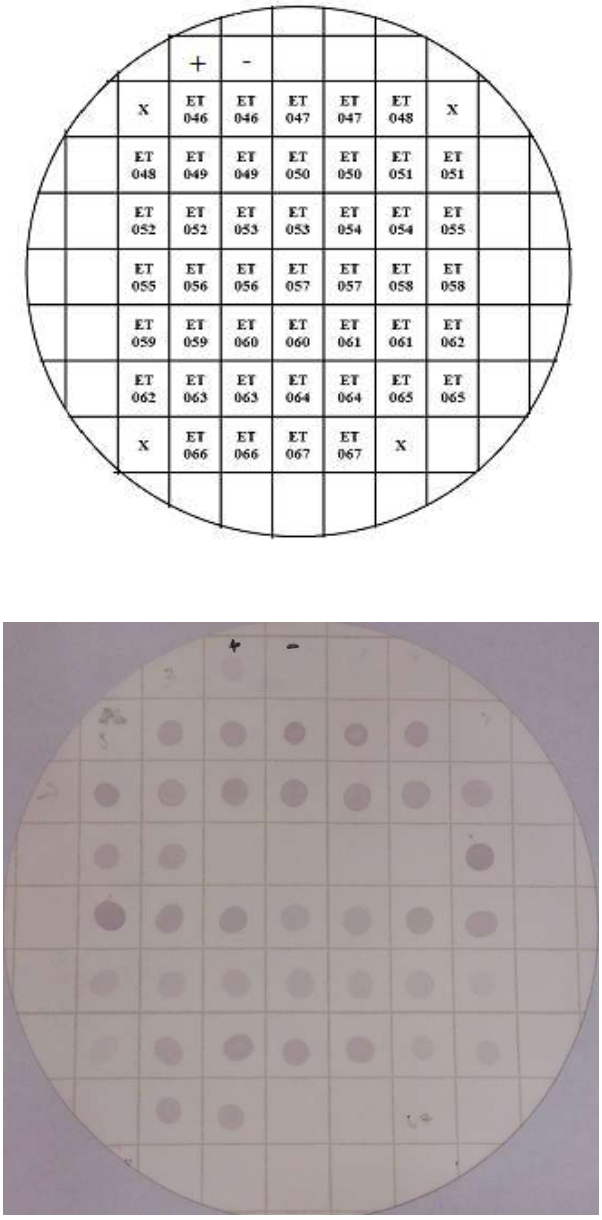

Figure 5.4 Dot blot hybridization in duplicates of *E. coli* isolates by using *intI1* specific probe. "+", positive control, EC114 *E. coli* carrying *dfrA17* and *aadA5*; "-"; negative control, *E. coli* Top10; and "X", no blotted DNA sample.

Table 5.3 Class 1 integrons determination by PCR and dot blot hybridization

Sample	Class 1 integrons by PCR		Class 1 integrons by dot blot hybridization (%)	Presence of class1 integrons (combined methods) (%)
	intI1 (%)	5′CS-3′CS (%)		
Adult (88)	55 (62.5)	47 (53.4)	58 (65.9)	62 (70.5)
Children (110)	73 (66.4)	65 (59.1)	80 (72.7)	84 (76.4)
Total (198)	128 (64.6)	112 (56.6)	138 (69.7)	146 (73.7)

Table 5.4 The presence of class 1 integrons in pathogenic *E. coli* in this study.

Pathotype	Presence of Class 1 integrons					
	Adult (n)	%	Children (n)	%	Total (n)	%
EAEC	5 (7)	71.4	29 (35)	82.9	34 (42)	81.0
EPEC	41 (53)	77.4	35 (36)	97.2	72 (89)	80.9
ETEC	10 (22)	45.5	20 (34)	58.8	30 (56)	53.6
STEC	2 (2)	100.0	3 (3)	100.0	5 (5)	100.0
EIEC	4 (4)	100.0	1 (2)	50.0	5 (6)	83.3

Table 5.5 Comparison of class 1 integrons detection between PCR for *intI1*, 5′CS-3′CS and dot blot hybridization. Pos = positive and Neg = negative

Sample	PCR		Dot blot hybridization	Sample	PCR		Dot blot hybridization
	intI1	5′CS-3′CS			*intI1*	5′CS-3′CS	
EA001	Pos	Pos	Pos	ET036	Neg	Neg	Neg
EA002	Pos	Pos	Pos	ET037	Pos	Pos	Pos
EA003	Pos	Pos	Pos	ET038	Pos	Neg	Pos
EA004	Pos	Pos	Pos	ET039	Pos	Pos	Pos
EA005	Neg	Neg	Neg	ET040	Neg	Neg	Pos
EA006	Pos	Pos	Pos	ET041	Neg	Neg	Neg
EA007	Pos	Pos	Pos	ET042	Neg	Neg	Neg
EA008	Pos	Pos	Pos	ET043	Neg	Neg	Neg
EA009	Neg	Neg	Neg	ET044	Pos	Pos	Pos
EA010	Neg	Pos	Neg	ET045	Pos	Pos	Pos
EA011	Pos	Pos	Pos	ET046	Pos	Pos	Pos
EA012	Pos	Pos	Pos	ET047	Pos	Pos	Pos
EA013	Pos	Pos	Pos	ET048	Pos	Neg	Pos
EA014	Neg	Neg	Neg	ET049	Pos	Pos	Pos
EA015	Pos	Neg	Pos	ET050	Neg	Neg	Pos
EA016	Pos	Pos	Pos	ET051	Pos	Neg	Pos
EA017	Pos	Pos	Pos	ET052	Neg	Neg	Pos
EA018	Pos	Neg	Pos	ET053	Neg	Neg	Neg
EA019	Pos	Pos	Pos	ET054	Neg	Neg	Neg
EA020	Pos	Neg	Pos	ET055	Pos	Pos	Pos
EA021	Pos	Pos	Pos	ET056	Pos	Neg	Pos
EA022	Pos	Pos	Pos	ET057	Pos	Neg	Pos
EA023	Pos	Neg	Pos	ET058	Pos	Pos	Pos
EA024	Neg	Neg	Neg	ET059	Pos	Pos	Pos
EA025	Pos	Pos	Pos	ET060	Pos	Pos	Pos
EA026	Pos	Pos	Pos	ET061	Pos	Pos	Pos
EA027	Pos	Neg	Pos	ET062	Pos	Pos	Pos
EA028	Pos	Pos	Pos	ET063	Pos	Pos	Pos
EA029	Pos	Pos	Pos	ET064	Pos	Pos	Pos
EA030	Neg	Neg	Neg	ET065	Pos	Pos	Pos
EA031	Neg	Neg	Neg	ET066	Pos	Pos	Pos
EA032	Neg	Neg	Neg	ET067	Neg	Neg	Neg
EA033	Neg	Neg	Neg	ET068	Neg	Neg	Neg
EA034	Neg	Neg	Neg	ET069	Pos	Neg	Pos
EA035	Neg	Neg	Neg	ET070	Neg	Neg	Neg

Table 5.5 Comparison of class 1 integrons detection between PCR for *intI1*, 5′CS-3′CS and dot blot hybridization. Pos = positive and Neg = negative (cont.)

Sample	PCR for		Dot blot hybridization
	intI1	5′CS-3′CS	
ET071	Pos	Neg	Pos
ET072	Neg	Neg	Neg
ET073	Pos	Pos	Pos
ET074	Pos	Pos	Pos
ET075	Pos	Pos	Pos
ET076	Pos	Pos	Pos
ET077	Pos	Pos	Pos
ET078	Pos	Pos	Pos
ET079	Neg	Pos	Neg
ET080	Pos	Pos	Pos
ET081	Neg	Neg	Neg
ET082	Neg	Neg	Neg
ET083	Neg	Neg	Neg
ET084	Neg	Neg	Neg
ET085	Neg	Neg	Neg
ET086	Neg	Neg	Neg
ET087	Neg	Pos	Neg
ET088	Pos	Pos	Pos
ET089	Neg	Pos	Neg
ET090	Pos	Pos	Pos
EC091	Neg	Neg	Neg
EC092	Pos	Pos	Pos
EC093	Neg	Pos	Neg
EC094	Neg	Pos	Pos
EC095	Neg	Pos	Pos
EC096	Pos	Pos	Pos
EC097	Pos	Pos	Pos
EC098	Pos	Pos	Pos
EC099	Pos	Pos	Pos
EC100	Pos	Pos	Pos
EC101	Neg	Neg	Neg
EC102	Pos	Pos	Pos
EC103	Neg	Neg	Neg
EC104	Pos	Pos	Pos
EC105	Neg	Neg	Neg

Sample	PCR for		Dot blot hybridization
	intI1	5′CS-3′CS	
EC106	Pos	Pos	Pos
EC107	Neg	Neg	Neg
EC108	Pos	Pos	Pos
EC109	Pos	Pos	Pos
EC110	Pos	Pos	Pos
EC111	Pos	Neg	Pos
EC112	Pos	Pos	Pos
EC113	Pos	Pos	Pos
EC114	Pos	Pos	Pos
EC115	Neg	Pos	Neg
EC116	Pos	Pos	Pos
EC117	Pos	Neg	Pos
EC118	Pos	Pos	Pos
EC119	Pos	Pos	Pos
EC120	Pos	Pos	Pos
EC121	Pos	Pos	Pos
EC122	Pos	Pos	Pos
EC123	Pos	Neg	Pos
EC124	Pos	Pos	Pos
EC125	Neg	Neg	Neg
EC126	Pos	Pos	Pos
EC127	Neg	Neg	Neg
EC128	Pos	Pos	Pos
EC129	Pos	Pos	Pos
EC130	Pos	Pos	Pos
EC131	Pos	Pos	Pos
EC132	Pos	Neg	Pos
EC133	Neg	Pos	Pos
EC134	Pos	Pos	Pos
EC135	Neg	Neg	Neg
EC136	Pos	Pos	Pos
EC137	Pos	Pos	Pos
EC138	Pos	Pos	Pos
EC139	Neg	Neg	Neg
EC140	Pos	Pos	Pos

Table 5.5 Comparison of class 1 integrons detection between PCR for *intI1*, 5′CS-3′CS and dot blot hybridization. Pos = positive and Neg = negative (cont.)

Sample	PCR for		Dot blot hybridization
	intI1	5′CS-3′CS	
EC141	Pos	Pos	Pos
EC142	Neg	Pos	Neg
EC143	Neg	Pos	Pos
EC144	Pos	Pos	Pos
EC145	Pos	Neg	Pos
EC146	Pos	Neg	Pos
EC147	Pos	Neg	Pos
EC148	Neg	Pos	Pos
EC149	Pos	Pos	Pos
EC150	Pos	Neg	Pos
EC151	Pos	Pos	Pos
EC152	Pos	Pos	Pos
EC153	Pos	Pos	Pos
EC154	Neg	Neg	Neg
EC155	Pos	Pos	Pos
EC156	Pos	Pos	Pos
EC157	Pos	Pos	Pos
EC158	Neg	Neg	Neg
EC159	Pos	Pos	Pos
EC160	Pos	Pos	Pos
EC161	Neg	Neg	Neg
EC162	Pos	Neg	Pos
EC163	Pos	Pos	Pos
EC164	Pos	Pos	Pos
EC165	Neg	Neg	Neg
EC166	Pos	Pos	Pos
EC167	Neg	Pos	Neg
EC168	Neg	Neg	Pos
EC169	Neg	Neg	Neg
EC170	Pos	Pos	Pos
EC171	Pos	Neg	Pos
EC172	Neg	Neg	Neg
EC173	Pos	Neg	Pos
EC174	Neg	Neg	Pos
EC175	Neg	Neg	Neg
EC176	Pos	Neg	Pos
EC177	Neg	Neg	Neg
EC178	Pos	Neg	Pos
EC179	Neg	Neg	Neg
EC180	Neg	Neg	Neg
EC181	Neg	Neg	Neg
EC182	Pos	Pos	Pos
EC183	Neg	Neg	Neg
EC184	Neg	Neg	Neg
EC185	Pos	Pos	Pos
EC186	Neg	Neg	Neg
EC187	Pos	Pos	Pos
EC188	Pos	Neg	Pos
EC189	Pos	Pos	Pos
EC190	Pos	Pos	Pos
EC191	Pos	Pos	Pos
EC192	Pos	Pos	Pos
EC193	Pos	Neg	Pos
EC194	Neg	Neg	Neg
EC195	Neg	Neg	Neg
EC196	Pos	Neg	Pos
EC197	Pos	Neg	Pos
EC198	Pos	Pos	Pos
EC199	Neg	Neg	Neg
EC200	Pos	Pos	Pos

5.3 Characterization of class 1 integrons

E. coli containing 5′CS-3′CS amplicons isolates were characterized for resistance gene cassettes. Based on antimicrobial resistance pattern, 8 representative isolates from the most frequent- and broad- resistance phenotypes were selected. Transformants *E. coli* were screened for the presence of 5′CS-3′CS PCR products by plasmid extraction and digestion by *Eco*RI restriction endonuclease (Figure 5.5). The Representative recombinant plasmids were sequenced by DNA Sanger sequencing and compared at the National Center for Biotechnology Information (NCBI) website to identify their resistance gene. Sequence analysis of this study indicated the presence of *aadA1*, *aadA2*, *aadA5* for resistant to streptomycin and spectinomycin, *dfrA12* and *dfrA17* are refer to trimethoprim resistance, and *linF*, conferring resistant to lincosamide (table 5.6). The nucleotide sequence alignment of *dfrA17* and *aadA5* resistance gene cassette was shown in Figure 5.6.

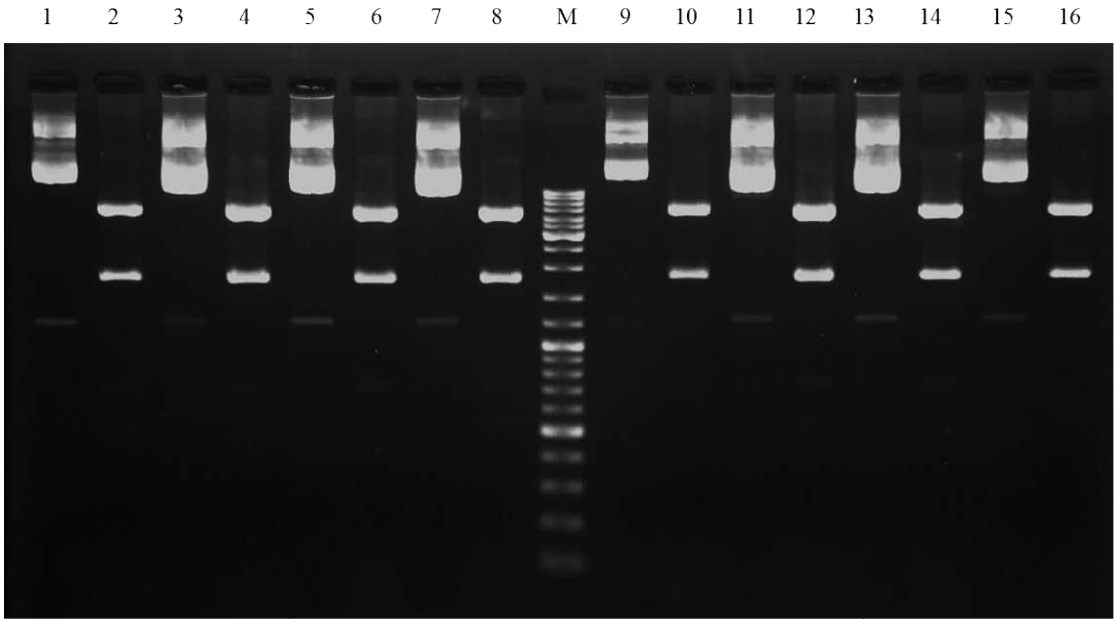

Figure 5.5 Transformantion analysis of *E. coli*, EC143 containing 5′CS-3′CS amplicons. The recombinant plasmids were digested by *Eco*RI restriction enzyme. Lane M, GeneRuler^{TM} 1 kb DNA Ladder; Lane 1, 3, 5, 7, 9, 11, 13, and 15, undigested plasmids; Lane 2, 4, 6, 8, 10, 12, 14, and 16, digested plasmids.

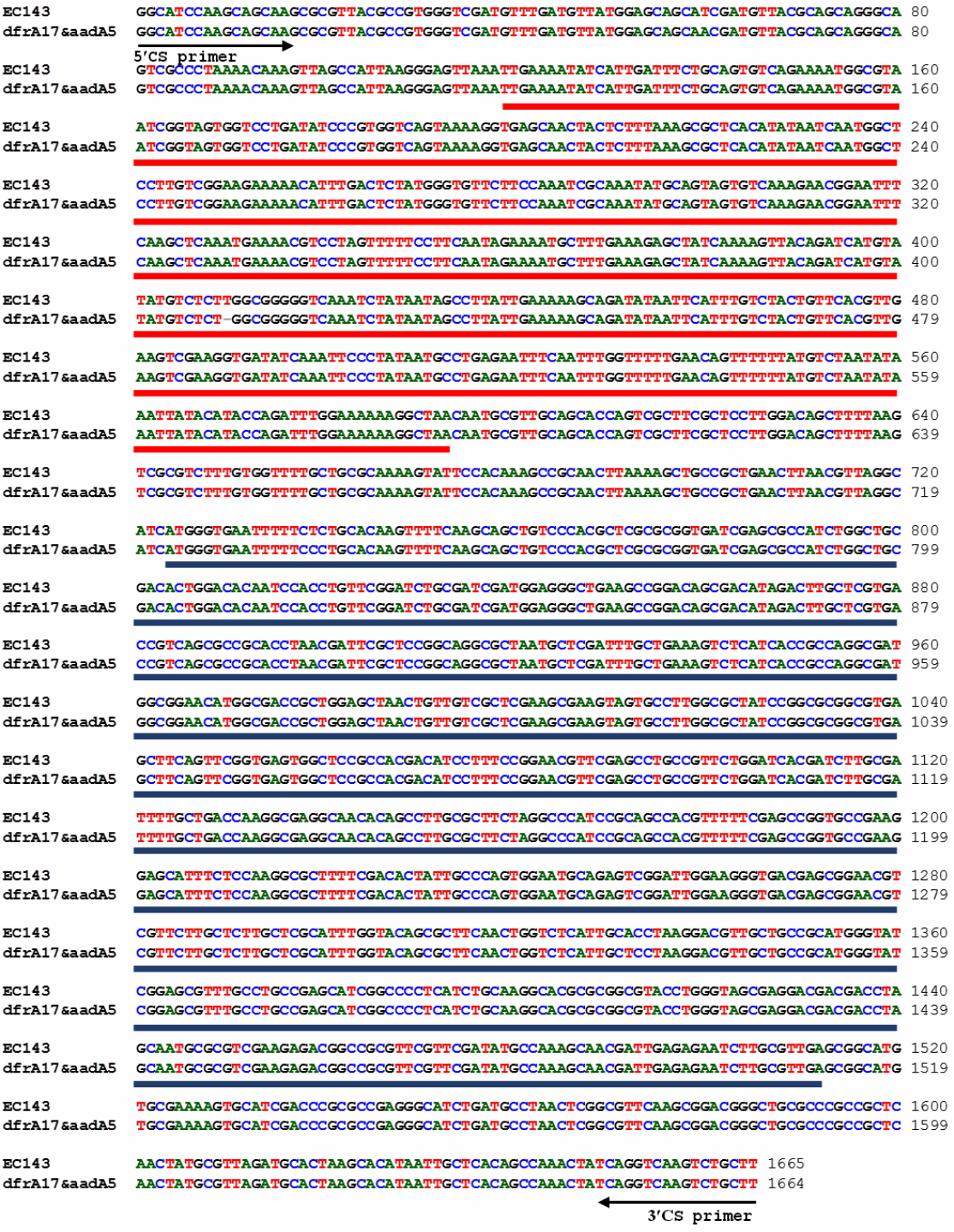

Figure 5.6 Nucleotide sequence alignment of class 1 integrons containing *dfrA17* (474 bp) and *aadA5* (789 bp) in EC143. An open reading frame of *dfrA17* and *aadA5* were shown in red and blue bar, respectively.

Table 5.6 Class 1 integrons resistance gene cassettes and antimicrobial resistance phenotype of multidrug resistant pathogenic *E. coli* in this study.

Sample	Presence of *intI1*		5′CS-3′CS (bp)	Resistance cassettes	Resistance phenotype	Accession No.
	PCR	Dot-blot				
EA011	+	+	1,947	*aadA2*, *linF*	NA, AM, C, K, N, S, G, SXT, TE	JQ414042.1
EA016	+	+	1,011	*aadA1*	NA, CIP,AM, C, GN, K, S, G, SXT, TE	FJ855126.1
ET055	+	+	1,664	*dfrA17*, *aadA5*	NA, CIP,AM, GN, K, S, G, SXT, TE, CTX, CRO	GU055937.1
EC100	+	+	1,016	*aadA2*	NA, CIP, AM, C, GN, K, S, G, SXT, TE, CTX, CRO	DQ133165.1
EC114	+	+	1,664	*dfrA17*, *aadA5*	NA, AM, C, K, S, G, SXT,TE, CTX, CRO	GU055937.1
EC119	+	+	1,664	*dfrA17*, *aadA5*	NA, AM, C, K, S, G, SXT, TE	GU055937.1
EC143	-	+	1,664	*dfrA17*, *aadA5*	NA, AM, GN, K, S, G, SXT,TE, CTX, CRO	GU055937.1
EC190	+	+	2,012	*dfrA12*, *orfF*, *aadA2*	AM, S, G, SXT, TE	KM278182.1

NA = nalidixic acid, CIP = ciprofloxacin, AM = ampicillin, C = chloramphenicol, GN = gentamycin, K = kanamycin, N = neomycin, S = streptomycin, G = sulfisoxazole, SXT = trimethoprim-sulfamethoxazole, TE = tetracycline, CTX = cefotaxime, CRO = ceftriaxone

5.4 Class A β-lactamases-producing isolates determination by PCR

Ten positive isolates for ESBLs in phenotypic confirmatory test were identified type-specific class A β-lactamases genes by PCR using 3 sets of specific primers, including *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$. The result was shown in Table 5.7. Either integrons- or non-integrons-harboring isolates have been found to carry *bla*$_{CTX-M}$ (90%), *bla*$_{TEM}$ (80%), and *bla*$_{SHV}$ (10%), respectively. Most of them produced multiple β-lactamases which CTX-M with TEM genotype was the most prevalent (7/10) in this study. Surprisingly, class A β-lactamases-producing *E. coli*, which responsible for ESBL production were detected in children less than 1 year old, i.e., EC097, EC100, and EC114.

Table 5.7 Genotypes of class A β-lactamases-producing *E. coli* in this study.

Sample	Year	Age		Integrons	ESBLs phenotype	ESBLs genotype		
		Year	Month			*bla*$_{CTX-M}$	*bla*$_{TEM}$	*bla*$_{SHV}$
ET036	2009	21	-	Neg	Pos	Pos	Pos	Neg
ET055	2002	23	-	Pos	Pos	Pos	Pos	Neg
EC091	2008	2	0	Neg	Pos	Pos	Pos	Neg
EC092	2008	2	0	Pos	Pos	Pos	Neg	Neg
EC097	2006	0	3	Pos	Pos	Neg	Pos	Pos
EC100	2008	0	5	Pos	Pos	Pos	Pos	Neg
EC114	2006	0	6	Pos	Pos	Pos	Pos	Neg
EC133	2008	2	0	Pos	Pos	Pos	Pos	Neg
EC141	2006	3	2	Pos	Pos	Pos	Pos	Neg
EC143	2007	2	6	Pos	Pos	Pos	Neg	Neg

Pos = positive and Neg = negative

5.5 Molecular typing

5.5.1 Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR

E. coli isolates were subjected to ERIC PCR for genotyping on the basis of copies and distances of ERIC sequences in chromosomal DNA which generated the

differences banding patterns on agarose gel. The number of DNA bands observed on agarose gel was between 5 and 20, average 13 bands ranging from 150 bp to 3.5 kb (Figure 5.7). At ≥ 80% similarity, 60 different patterns were determined among 198 *E. coli* isolates with the discriminatory index (DI) of 0.973. The most frequent ERIC type contained 15 isolates. The same ERIC types were found in either adult or children while 17 ERIC patterns were unique (Figure 5.8). The dendrogram of 60 ERIC PCR patterns was shown in Figure 5.9.

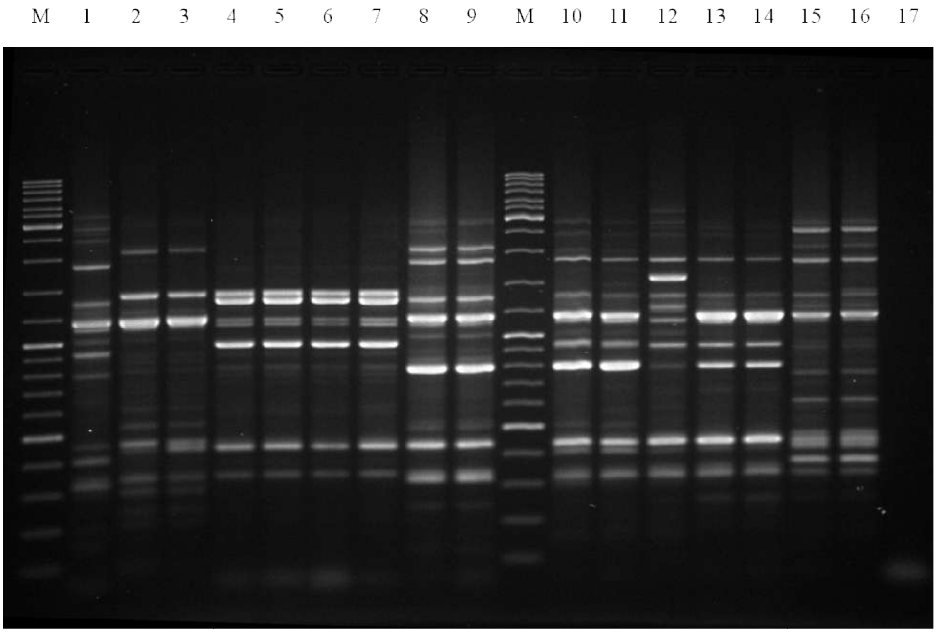

Figure 5.7 ERIC PCR DNA fingerprint of *E. coli* isolates. M, GeneRuler^{TM} 1 kb DNA Ladder; Lane 1, *E. coli* Top10; Lane 2-16; samples; Lane 17; negative control (water).

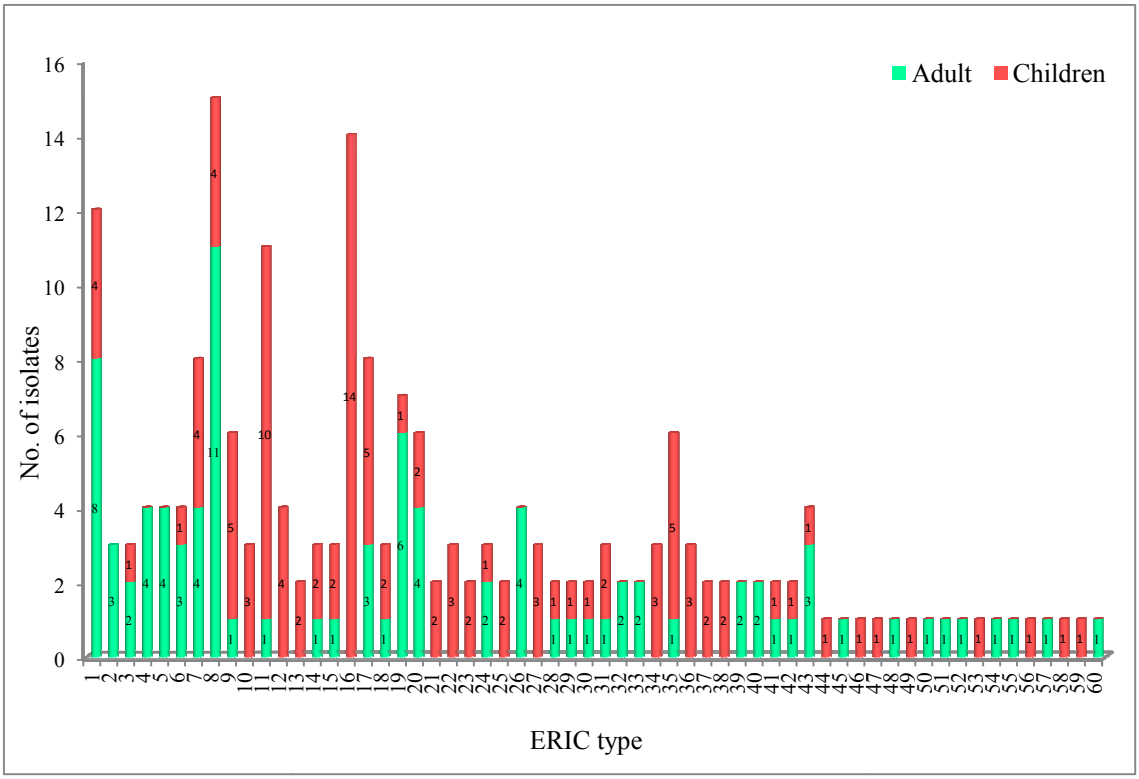

Figure 5.8 Frequency distribution of ERIC types among 200 *E. coli* isolates including internal control.

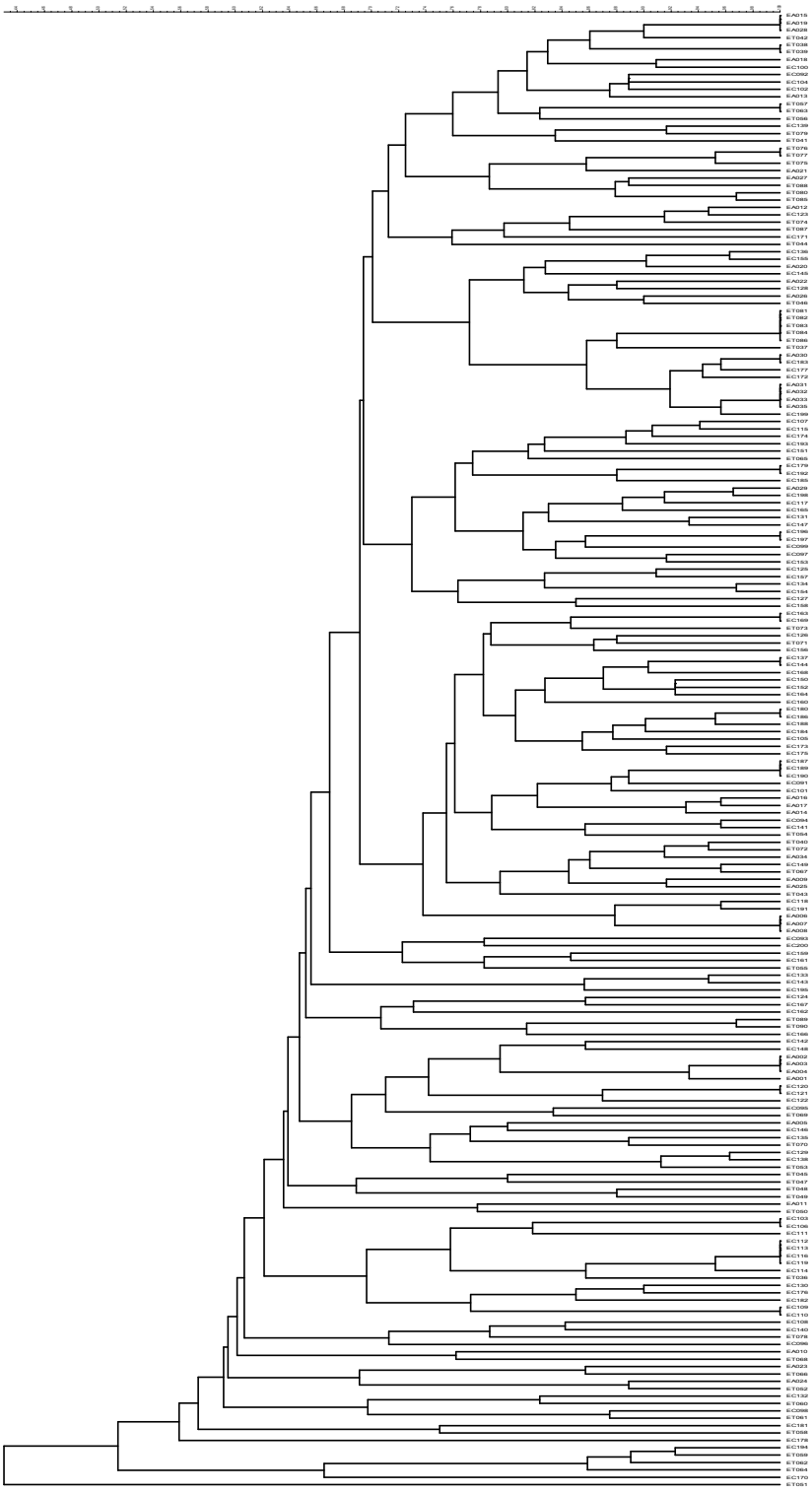

Figure 5.9 Dendrogram of 200 *E. coli* isolates including internal control by ERIC PCR.

5.5.2 Plasmid profiling

Plasmid profile study was carried out to support the other molecular typing. Plasmids were characterized for numbers and molecular weights by using electrophoresis on agarose gel. Of the 198 *E. coli* isolates, 160 (81%) harbored plasmids with molecular sizes ranging from 800 bp to >10 kb between 1 to 14 bands (Figure 5.10). Most of them (83.1%) carried plasmids with molecular sizes more than 10 kb. The same plasmid patterns were found in both adult and children, e.g., P1, P3, P5, and P8, etc. (Table 5.8). At ≥90% similarity, 118 different plasmid profiles were obtained with discriminatory index of 0.956. The dendrogram of 118 plasmid profiling was shown in Figure 5.11.

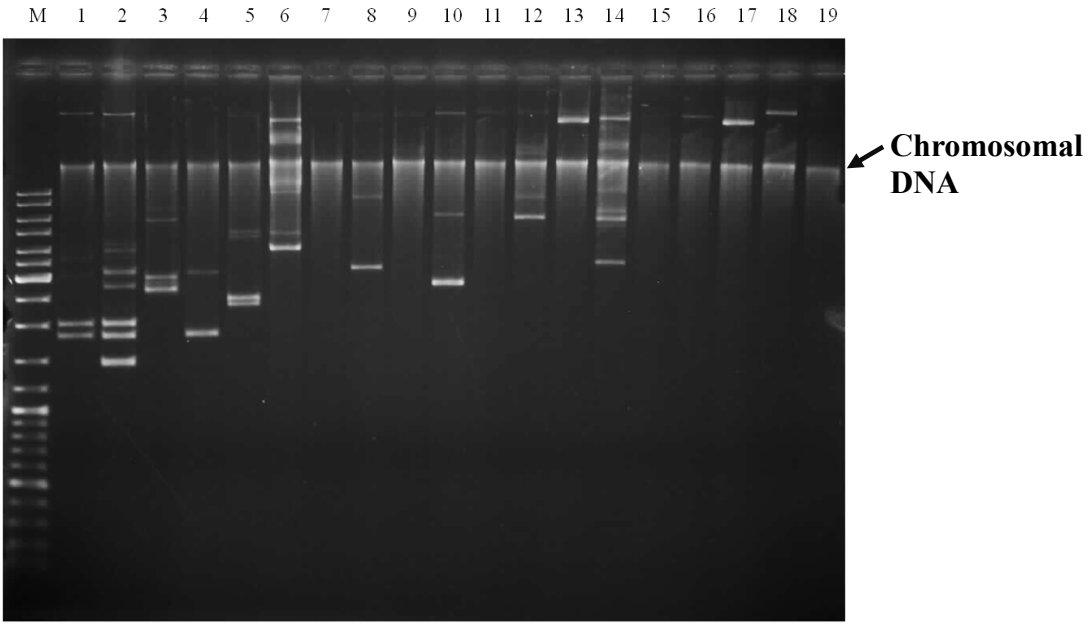

Figure 5.10 Plasmid DNA of *E. coli* isolates. M, GeneRuler^{TM} 1 kb DNA Ladder; Lane 1-19, samples.

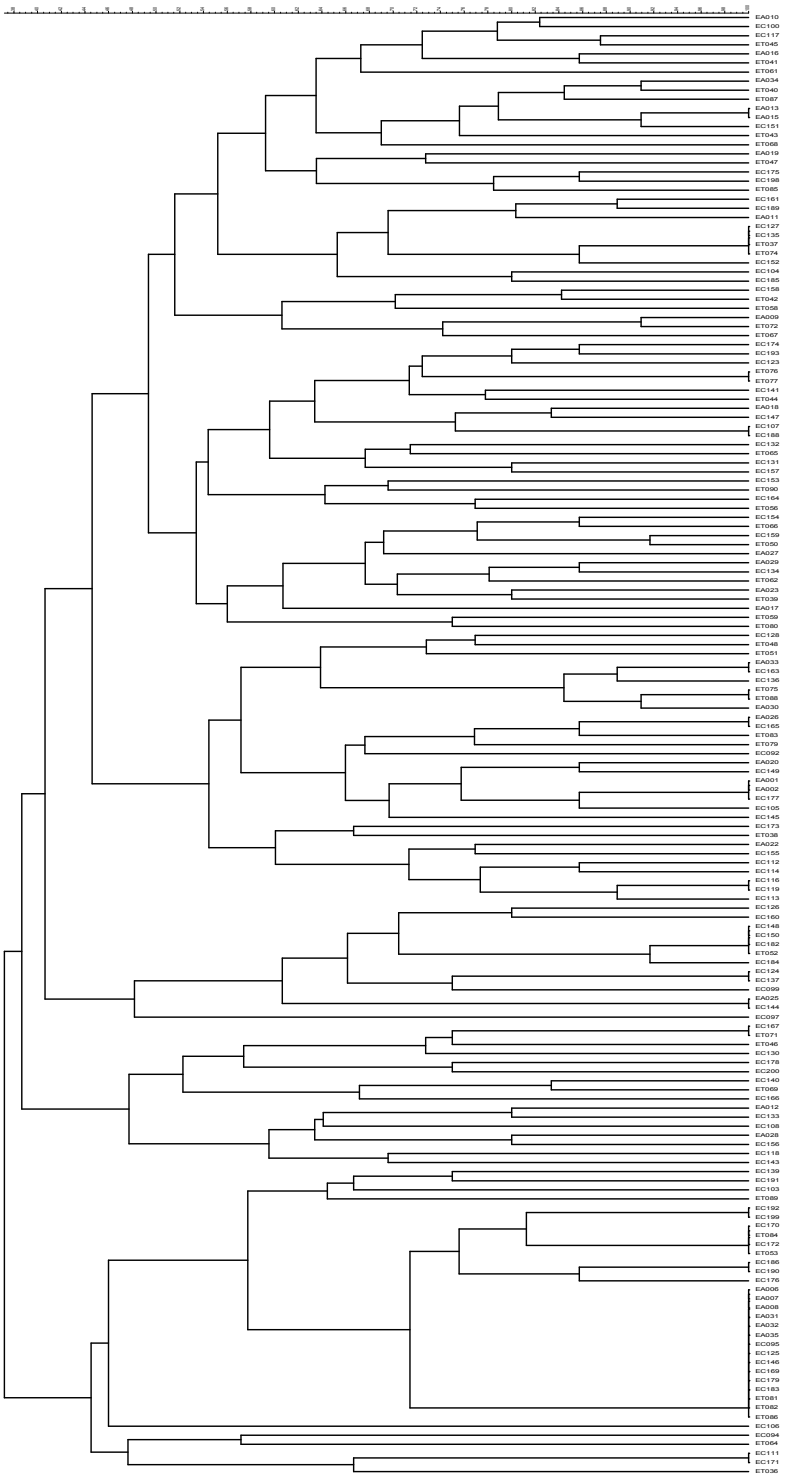

Figure 5.11 Dendrogram of 200 *E. coli* isolates including internal control by plasmid profiling.

Table 5.8 Plasmid profile and antimicrobial resistance profile of 200 pathogenic *E. coli* isolates including internal control

Plasmid profile	Sample	Plasmids		Antimicrobial resistance pattern
		No. of bands	Size (kb)	
P1	EC148	2	3,7	S,G,TE
P1	EC150	2	3,6.5	AM,S,G,STX,TE
P1	EC182	2	3,7	AM,S,G,STX,TE
P1	EC184	2	3.4,8	AM,S,G,STX,TE
P1	ET052	2	3,7	AM,C,S,G,SXT,TE
P2	EC124	3	2.5,2.7,6	AM,C,G,SXT,TE
P2	EC137	3	2.8,3,6	AM,S,G,STX,TE
P3	EA025	2	2,3	AM,S,G,STX,TE
P3	EC144	2	1.9,3	AM,S,G,STX,TE
P4	EC188	3	2.9,6,>10	AM,S,G,STX,TE
P4	EC107	3	3,5,>10	NA,AM,S,G,SXT,TE
P5	EC159	11	1.4,1.6,1.8,2,3,3.5,6,8, >10	AM,C,S,G,TE
P5	ET050	11	1.3,1.4,1.7,2,2.7,3.5,6,8,>10	AM,C,GM,G,SXT,TE
P6	ET076	8	2.6,3,5,6,10,>10	NA,AM,S,G,SXT,TE
P6	ET077	8	2.6,3,5,6,10,>10	NA,AM,S,G,SXT,TE
P7	EA009	4	1.2,1.6,10,>10	S,G,SXT,TE
P7	ET072	4	1.2,1.7,10,>10	S,G,SXT,TE
P8	EC127	3	7,>10	AM,S,G,TE
P8	ET037	3	7,>10	AM,S,G,STX,TE
P8	ET074	3	7,>10	AM,C,S,G,SXT,TE
P8	EC135	3	7,>10	AM,K,N,S,G,TE
P9	EC195	3	9,>10	NA,AM,S,G,SXT,TE
P10	EA034	5	>10	AM,S,G,STX,TE
P10	ET040	4	10,>10	C,S,G,SXT,TE
P11	EA013	4	3,8,>10	C,S,G,SXT,TE
P11	EA015	4	3,8,>10	C,S,G,SXT,TE
P11	EC151	3	3,8,10	NA,CIP,AM,C,K,N,S,G, SXT, TE
P12	EA030	4	3.5,4.5,10,>10	AM,S,G,STX,TE
P12	ET075	5	4,4.5,>10	AM,S,G,STX,TE
P12	ET088	5	4,4.5,>10	AM,S,G,STX,TE
P13	EA033	4	3.5,4.5,10,>10	AM,S,G,STX,TE
P13	EC163	3	3.5,8,>10	AM,S,G,STX,TE
P14	EC177	3	3.7,>10	AM,S,G,TE
P14	EA001	3	3.7,>10	NA,AM,C,G,SXT,TE
P14	EA002	3	3.7,>10	NA,AM,C,G,SXT,TE

Table 5.8 Plasmid profile and antimicrobial resistance profile of 200 pathogenic *E. coli* isolates including internal control (cont.)

Plasmid profile	Sample	Plasmids		Antimicrobial resistance pattern
		No. of bands	Size (kb)	
P15	EC165	3	4.5,5,>10	AM,S,G,SXT
P15	EA026	3	4.5,5,>10	NA,AM,GM,G,SXT,TE
P16	EC116	4	2.4,4.1,4.9,5.5	NA,AM,C,K,S,G,SXT,TE
P16	EC119	4	2.4,4.1,4.9,5.5	NA,AM,C,K,S,G,SXT,TE
P17	EC167	3	1.6,2.5,>10	AM,S,G,TE
P17	ET071	3	1.5,2.5,>10	NA,CIP,GM,G,SXT,TE
P18	EC171	1	1.3	C,G,SXT,TE
P18	EC111	1	1.1	AM,K,S,G,SXT,TE
P19	EC192	1	>10	AM,S,G,STX,TE
P19	EC199	1	>10	AM,S,G,STX,TE
P20	ET084	1	>10	AM,S,G,STX,TE
P20	EC170	1	>10	AM,K,S,G,SXT,TE
P20	EC172	1	>10	NA,AM,S,G,SXT,TE
P20	ET053	1	>10	NA,AM,S,G,SXT,TE
P21	EC095	1	>10	AM,C,GM,G
P21	EA031	1	>10	AM,S,G,TE
P21	ET081	1	>10	AM,S,G,TE
P21	ET082	1	>10	AM,S,G,TE
P21	EA006	1	>10	AM,GM,G,SXT,TE
P21	EA007	1	>10	AM,GM,G,SXT,TE
P21	EA008	1	>10	AM,S,G,STX,TE
P21	EA032	1	>10	AM,S,G,STX,TE
P21	EA035	1	>10	AM,S,G,STX,TE
P21	EC125	1	>10	AM,S,G,STX,TE
P21	EC169	1	>10	AM,S,G,STX,TE
P21	EC179	1	>10	AM,S,G,STX,TE
P21	ET086	1	>10	AM,S,G,STX,TE
P21	EC183	1	>10	NA,AM,S,G,SXT,TE
P21	EC146	1	>10	NA,AM,C,GM,K,S,G, SXT,TE
P22	EA017	6	1.7,2,3,4,>10	NA,AM,C,K,N,S,G,SXT, TE
P23	EC097	4	2.2,3.2,4.5,8	AM,C,GM,K,S,G,SXT, TE,CTX
P24	ET064	4	1,1.4,>10	NA,CIP,AM,GM,G,SXT, TE
P25	EC126	3	2.9,6,7	AM,S,G,STX,TE

Table 5.8 Plasmid profile and antimicrobial resistance profile of 200 pathogenic *E. coli* isolates including internal control (cont.)

Plasmid profile	Sample	Plasmids		Antimicrobial resistance pattern
		No. of bands	Size (kb)	
P26	EC160	5	3,4,6,8,10	AM,S,G,STX,TE
P27	EA018	4	3,6.5,>10	AM,S,G,STX,TE
P28	EC147	7	2.7,3,3.5,5.5,6,>10	C,G,SXT,TE
P29	ET065	7	1.2,1.5,3,5.5,7,>10	AM,S,G,STX,TE
P30	EC130	6	1,1.5,1.7,2.7,6,>10	AM,C,S,G,SXT,TE
P31	EC132	6	1.7,2.7,4,4.1,6,>10	AM,C,G,SXT,TE
P32	EC154	14	1.2,1.5,1.7,2,2.1,2.4, 2.9,3,3.5,6,7,8,>10	NA,CIP,AM,C,S,G,SXT,TE
P33	ET066	11	1.9,2,2.2,3,3.5,6,8,10, >10	C,S,G,SXT,TE
P34	EA027	13	1.8,2,2.1,2.7,4.5,6,7, 10,>10	AM,S,G,STX,TE
P35	EA029	6	2,3.5,5,6.5,>10	NA,CIP,AM,C,S,G,SXT,TE
P36	EC134	9	3,3.2,4,5.5,7,7.5,>10	NA,AM,C,GM,S,G,SXT,TE
P37	ET062	8	1.7,2.5,3,3.7,6,10>10	AM,N,S,G,SXT,TE
P38	EA023	7	2,2.4,3,4,5,>10	NA,AM,C,S,G,SXT,TE
P39	ET039	9	2,3,3.2,4,5.5,7,10,>10	AM,C,S,G,SXT,TE
P40	EC141	6	2,2.7,3,6,8,>10	NA,CIP,AM,C,GM,S,SXT, TE,CTX,CRO
P41	ET044	7	2.9,3,6,8,10,>10	AM,C,K,N,S,G,SXT,TE
P42	EC174	5	2.5,4.5,7,>10	AM,S,G,STX,TE
P43	EC193	5	2.5,5,6,>10	AM,C,S,G,SXT
P44	EC123	7	2.5,2.7,4.9,6,7,>10	NA,CIP,AM,GM,K,N,S,G,TE
P45	ET051	6	2.4,2.6,3.4,4,4.5,>10	NA,AM,S,G,SXT,TE
P46	EC153	11	1.1,1.2,1.5,2.6,4,6,7, 10,>10	AM,C,S,G,SXT,TE
P47	ET090	7	1.5,6,>10	AM,S,G,STX,TE
P48	ET059	6	1,1.1,2.7,3,3.5,6	NA,CIP,AM,S,G,SXT,TE
P49	ET080	6	2.4,3,3.5,4.1,5.5,10	NA,G,SXT,TE
P50	EC131	8	3,3.5,4,4.9,6,10,>10	S,G,SXT,TE
P51	EC157	9	0.9,1.1,3.9,4,5,6,8, >10	AM,S,G,STX,TE
P52	EC158	9	1.6,2.3,2.4,5,8,>10	AM,S,G,TE
P53	ET042	9	1.5,2.5,3,6,6.1,>10	NA,S,G,STX,TE
P54	ET058	9	1,1.1,1.5,2.1,3,4,8, >10	NA,AM,C,G,SXT,TE
P55	ET067	7	1.2,1.7,2,3.2,10,>10	S,G,SXT,TE

Table 5.8 Plasmid profile and antimicrobial resistance profile of 200 pathogenic *E. coli* isolates including internal control (cont.)

Plasmid profile	Sample	Plasmids		Antimicrobial resistance pattern
		No. of bands	Size (kb)	
P56	EA011	2	>10	NA,AM,C,K,N,S,G,SXT,TE
P57	EC161	2	>10	S,G,SXT,TE
P58	EC189	4	9,>10	AM,S,G,STX,TE
P59	EC104	3	>10	AM,C,GM,S,G,SXT,TE
P60	EC185	1	>10	AM,K,G,SXT,TE
P61	EC196	6	4,5.9,6,8,>10	NA,AM,C,S,G,SXT,TE
P62	ET085	5	5.9,6,>10	AM,S,G,SXT
P63	ET079	4	4.9,6.5,7,>10	AM,S,G,STX,TE
P64	EA019	4	7,>10	NA,AM,S,G,SXT,TE
P65	ET047	7	1.1,1.7,5.5,10	NA,CIP,C,GM,S,G,SXT,TE
P66	EA010	6	6,8,10,>10	NA,AM,S,G,TE
P67	EC100	7	1.4,2,6,9,>10	NA,CIP,AM,C,GM,K,S,G, SXT,TE,CTX,CRO
P68	EC117	5	5,7,9,10,>10	NA,AM,S,G,SXT,TE
P69	ET045	7	5,6,6.1,9,10,>10	NA,AM,C,S,G,SXT,TE, CTX, CRO
P70	EA016	5	6,8,>10	NA,CIP,AM,C,GM,K,S,G, SXT,TE
P71	ET041	6	4.9,5,6,>10	NA,N,G,TE
P72	ET061	11	1,1.4,1.5,2.2,2.5,5.5,6, 7,8,>10	NA,CIP,AM,C,K,S,G, SXT,TE
P73	ET087	3	10,>10	AM,S,G,TE
P74	ET043	6	1.1,10,>10	AM,S,G,TE
P75	ET068	4	3.2,7,8,>10	S,G,TE
P76	EC164	4	6,10,>10	AM,S,G,STX,TE
P77	ET056	3	4,7,>10	AM,C,S,G,SXT,TE
P78	EC145	2	4.5,6.5	AM,S,G,STX,TE
P79	EC152	2	6,>10	C,K,N,G,SXT,TE
P80	EC136	4	4,5,>10	NA,AM,GM,S,G,SXT,TE
P81	EC198	6	3.5,4,5,8,>10	AM,K,G,SXT,TE
P82	ET048	5	3.5,4.9,5,10,>10	AM,C,S,G,SXT,TE
P83	EC128	8	0.8,1,3.5,3.6,4,4.9,10, >10	NA,AM,GM,G,SXT,TE
P84	EA020	2	4,>10	AM,C,S,G,SXT,TE
P85	EC105	2	3.5,>10	S,G,TE
P86	EC092	1	4	NA,CIP,AM,C,K,S,G,SXT, TE,CTX,CRO

Table 5.8 Plasmid profile and antimicrobial resistance profile of 200 pathogenic *E. coli* isolates including internal control (cont.)

Plasmid profile	Sample	Plasmids		Antimicrobial resistance pattern
		No. of bands	Size (kb)	
P87	EC149	2	6,>10	AM,C,S,G,SXT,TE
P88	EC175	5	4.5,5,>10	AM,S,G,SXT
P89	ET083	2	4.5,>10	AM,S,G,TE
P90	EC173	6	2.4,2.5,4.5,5,>10	AM,G,SXT,TE
P91	ET038	7	1.7,2.5,2.6,4,4.5,4.6, >10	AM,C,S,G,SXT,TE
P92	EA022	6	1.7,2.5,3,4,4.5,>10	AM,C,K,N,S,G,SXT,TE
P93	EC155	5	1.2,2.2,4,4.5,>10	NA,AM,C,S,G,SXT,TE
P94	EC112	5	2.4,3.5,4.1,4.9,>10	AM,C,K,S,G,SXT,TE
P95	EC114	6	2.4,4.1,4.9,5.1,>10	NA,AM,C,K,S,G,SXT, TE,CTX,CRO
P96	EC113	3	2.4,4.1,4.9,	NA,AM,C,K,S,G,SXT, TE
P97	EA012	3	2,2.5,>10	AM,C,S,G,TE
P98	EC133	5	1.9,2,3,3.5,>10	NA,AM,GM,K,S,G,SXT, TE,CTX,CRO
P99	EC108	6	1.6,1.8,2,2.1,4,>10	AM,C,S,G,SXT,TE
P100	EA028	7	2,2.4,3.5,4,5,>10	AM,S,G,STX,TE
P101	EC156	9	1.3,1.9,2,2.5,3,3.2,5.5, 8,>10	AM,S,G,STX,TE
P102	EC118	9	1.1,1.3,1.5,2,2.5,3,3.5, 4,5,7	AM,C,S,G,SXT,TE
P103	EC143	7	1.5,1.9,2,2.9,3,4,>10	NA,AM,GM,K,S,G,SXT, TE, CTX,CRO
P104	EC140	5	1.1,1.5,2.1,4,>10	NA,CIP,S,G,SXT,TE
P105	ET069	6	1,1.5,2,3.5,5,>10	C,GM,K,G,SXT
P106	EC166	4	1.1,1.3,2,2.1	C,S,G,SXT
P107	EC200	3	1.5,2,3,	NA,AM,S,G,SXT,TE
P108	ET089	4	1.5,>10	AM,S,G,STX,TE
P109	EC178	3	1.5,2.5,>10	C,G,SXT,TE
P110	ET046	3	1.6,1.9,5	NA,AM,GM,G,SXT,TE
P111	EC103	4	2.9,3,>10	AM,C,K,N,S,G,SXT,TE
P112	EC191	3	4.5,>10	NA,AM,S,G,SXT,TE
P113	EC139	3	1.2,>10	NA,AM,S,G,SXT,TE
P114	EC190	2	>10	AM,S,G,STX,TE
P114	EC186	2	>10	NA,AM,S,G,SXT
P115	EC099	4	2.5,2.9,3.5,5	NA,AM,C,GM,G
P116	EC176	3	>10	C,G,SXT,TE

Table 5.8 Plasmid profile and antimicrobial resistance profile of 200 pathogenic *E. coli* isolates including internal control (cont.)

Plasmid profile	Sample	Plasmids		Antimicrobial resistance pattern
		No. of bands	Size (kb)	
P117	EC094	1	>10	AM,C,GM,S,G,SXT
P118	EC106	1	3.5	AM,S,G,STX,TE
none	ET060	none	none	AM,C,S,TE
none	EA024	none	none	AM,S,G,TE
none	EC181	none	none	AM,S,G,TE
none	EC194	none	none	AM,S,G,TE
none	EC168	none	none	C,G,SXT,TE
none	ET057	none	none	NA,AM,C,TE
none	EC101	none	none	AM,S,G,STX,TE
none	EC102	none	none	AM,S,G,STX,TE
none	EC187	none	none	AM,S,G,STX,TE
none	ET078	none	none	AM,S,G,STX,TE
none	EC142	none	none	NA,AM,C,G,TE
none	ET063	none	none	NA,AM,C,S,TE
none	ET054	none	none	NA,AM,S,G,TE
none	EA003	none	none	AM,C,S,G,SXT,TE
none	EA004	none	none	AM,C,S,G,SXT,TE
none	EA005	none	none	AM,C,S,G,SXT,TE
none	EA014	none	none	AM,C,S,G,SXT,TE
none	EC096	none	none	AM,C,S,G,SXT,TE
none	EC098	none	none	AM,C,S,G,SXT,TE
none	EC109	none	none	AM,C,S,G,SXT,TE
none	EC120	none	none	AM,C,S,G,SXT,TE
none	EC121	none	none	AM,C,S,G,SXT,TE
none	EC122	none	none	AM,C,S,G,SXT,TE
none	EC162	none	none	AM,C,S,G,SXT,TE
none	ET070	none	none	NA,AM,C,G,SXT,TE
none	ET049	none	none	NA,AM,C,S,G,TE
none	EC093	none	none	NA,AM,S,G,SXT,TE
none	EC115	none	none	NA,AM,S,G,SXT,TE
none	EC180	none	none	NA,AM,S,G,SXT,TE
none	EC138	none	none	AM,C,GM,S,G,SXT,TE
none	EC110	none	none	NA,AM,C,S,G,SXT,TE
none	EC197	none	none	NA,AM,C,S,G,SXT,TE
none	ET073	none	none	NA,C,K,N,G,SXT,TE

Table 5.8 Plasmid profile and antimicrobial resistance profile of 200 pathogenic *E. coli* isolates including internal control (cont.)

Plasmid profile	Sample	Plasmids		Antimicrobial resistance pattern
		No. of bands	Size (kb)	
none	EC129	none	none	NA,AM,C,GM,S,G,SXT, TE
none	ET036	none	none	NA,CIP,AM,C,K,TE, CTX,CRO
none	EA021	none	none	NA,AM,C,K,N,S,G,SXT, TE
none	ET055	none	none	NA,CIP,AM,GM,K,S,G, SXT, TE,CTX,CRO
none	EC091	none	none	NA,CIP,AM,C,GM,K,S,G, SXT,TE,CTX,CRO

5.5.3 High-throughput Mul-tilocus Sequence Typing (HiMLST)

All *E. coli* isolates were genotyped by high-throughput MLST (HiMLST) with 454 sequencing technology instead of traditional MLST to generate sequences of large numbers of bacterial isolates at low cost. PCR products of seven housekeeping genes were shown in Figure 5.12. One hundred and ninety eight isolates were divided into 82 difference sequence types (STs) with discriminatory index (DI) of 0.979; however, 8 and 22 isolates were obtained from Sanger sequencing due to failure to get PCR product from HiMLST and verify new allele variants, respectively. Twenty-six new alleles, *adk*; 385, 386, 387, 393, 394, 395, *fumC*; 587, 588, 589, 590, 591, 596, 597, *gyrB*; 408, 412, *icd*; 481, 482, 483, 484, 485, 489, *purA*352, and *recA*; 318, 319, 320, and 321 were accepted by Warwick *E. coli* database curator and permanently accepted in database. Twenty novel sequence types consist of ST; 4386, 4581, 4587, 4588, 4589, 4590, 4591, 4592, 4593, 4594, 4595, 4596, 4597, 4598, 4606, 4617, 4619, 4622, 4625, and 4627. ST4588 and 4589 are closely related with ST795, which differing only in *fumC* (*fumC*588 vs *fumC*23 and *fumC*589 vs *fumC*23, respectively). ST4590 is a single locus variant (SLV) of ST301(*fumC*590 vs *fumC*27), ST4593 is SLV of ST1040 (*fumC*591 vs *fumC*194), ST4581 is SLV of ST4102 (*icd*481 vs *icd*440), ST4595 is SLV of ST641 (*icd*482 vs *icd*131), ST4591 is SLV of ST562 (*adk*386 vs *adk*15), ST4592 is SLV of ST381 (*recA*319 vs *recA*7), ST4594 is SLV of ST316 (*recA*320 vs *recA*6), and ST4598 is SLV of ST1771 (*recA*6 vs *recA*7) (Figure

5.13). The most frequent MLST types were clonal complex CC10 (ST4, ST10, ST34, and ST48), CC38 (ST38 and ST315), ST182, ST501, and ST517. The same clones were identified in both of adult and children e.g., ST4, ST10, ST34, ST38, ST182, ST517, etc (Figure 5.14). By using Bionumerics V. 6.5, the Minimum spanning tree (MS_{TREE}) of *E. coli* sequence types was constructed. At least 5 identical loci are assigned into a cluster, 82 STs were grouped into 14 clusters and 42 singletons (Figure 5.13).

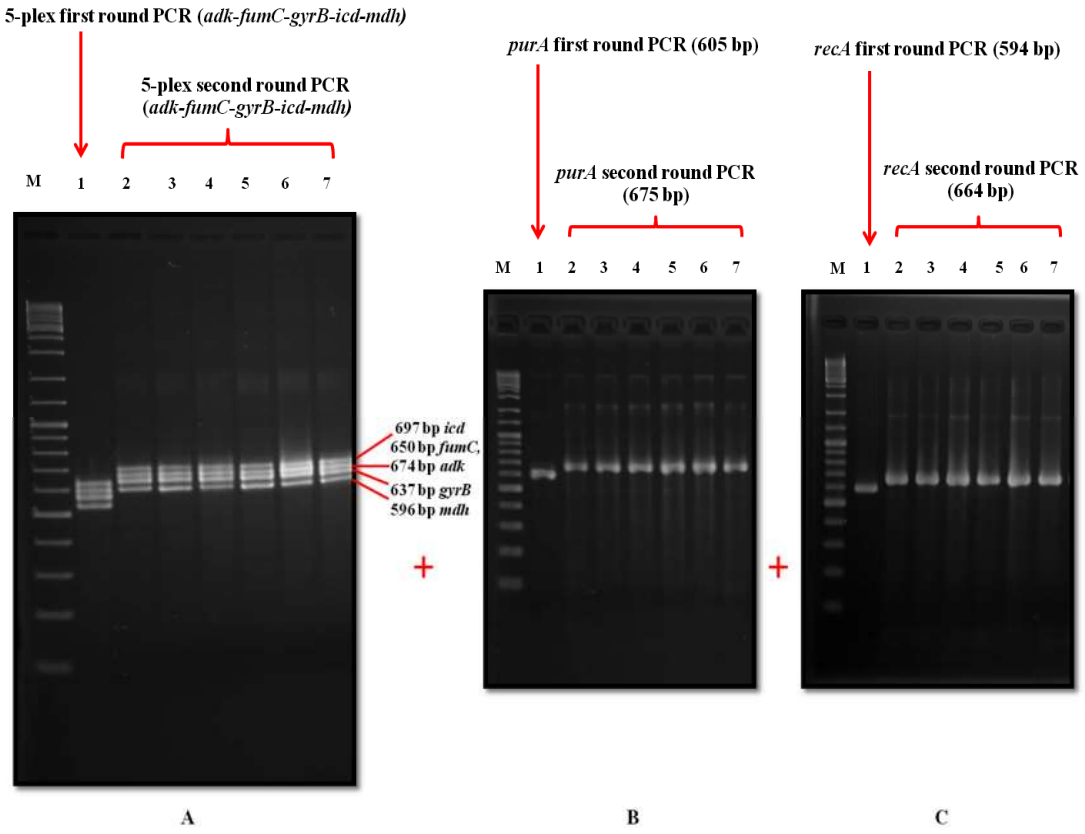

Figure 5.12 PCR product of two-step PCR of seven housekeeping genes used for HiMLST protocol for *E. coli* isolates. M, GeneRuler^{TM} 1 kb DNA Ladder; A, amplicons of first round and second round 5-plex PCR; B, amplicons of first round and second round PCR of *purA*; and C, amplicons of first round and second round PCR of *recA*.

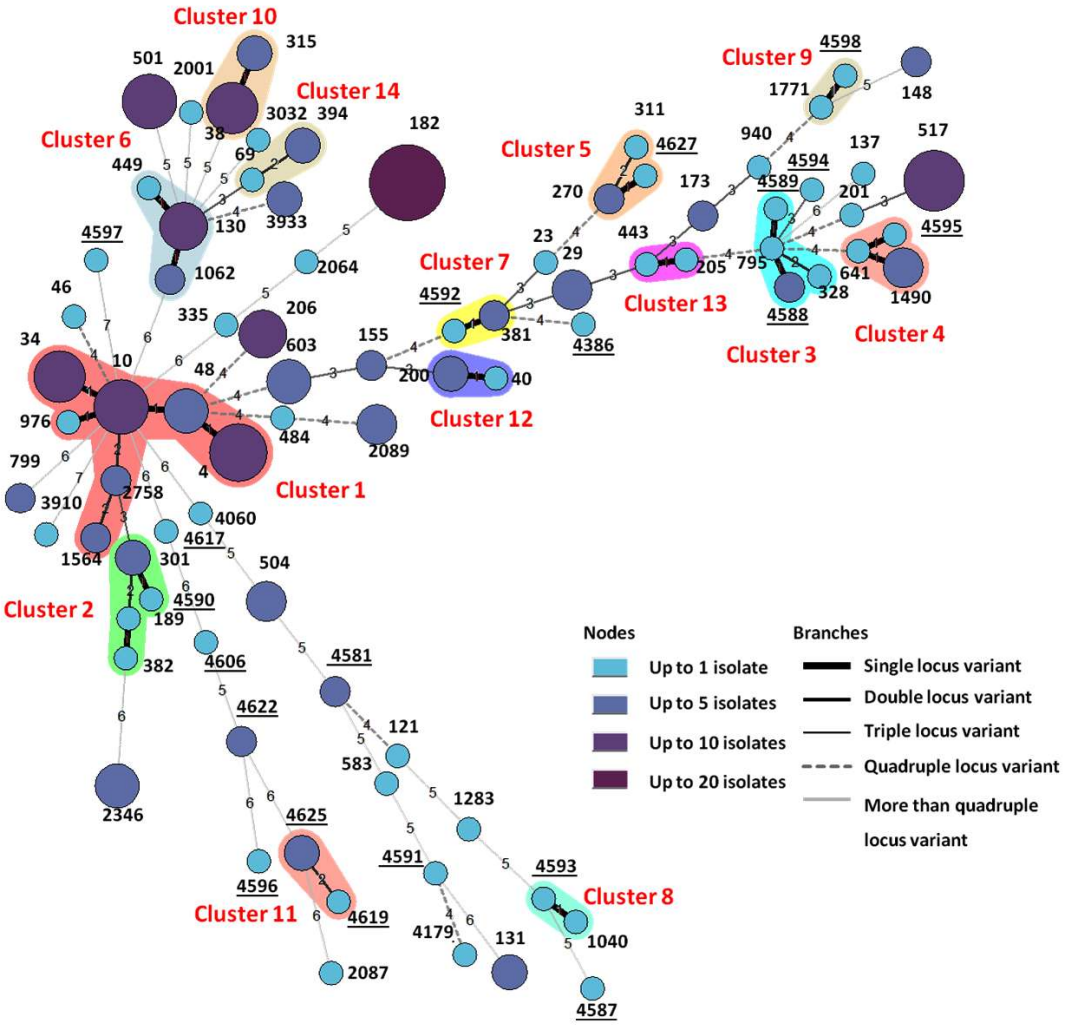

Figure 5.13 Minimum spanning tree (MS_{TREE}) of 200 *E. coli* isolates. Each ST is represented by a circle. Color shading indicates number of isolates. At least 5 identical loci are assigned into a cluster. Strains within the same ST possess seven identical alleles, whereas a single locus variant possesses one different allele to the other STs and a double locus variant differs by two alleles. Novel STs from this study are underlined.

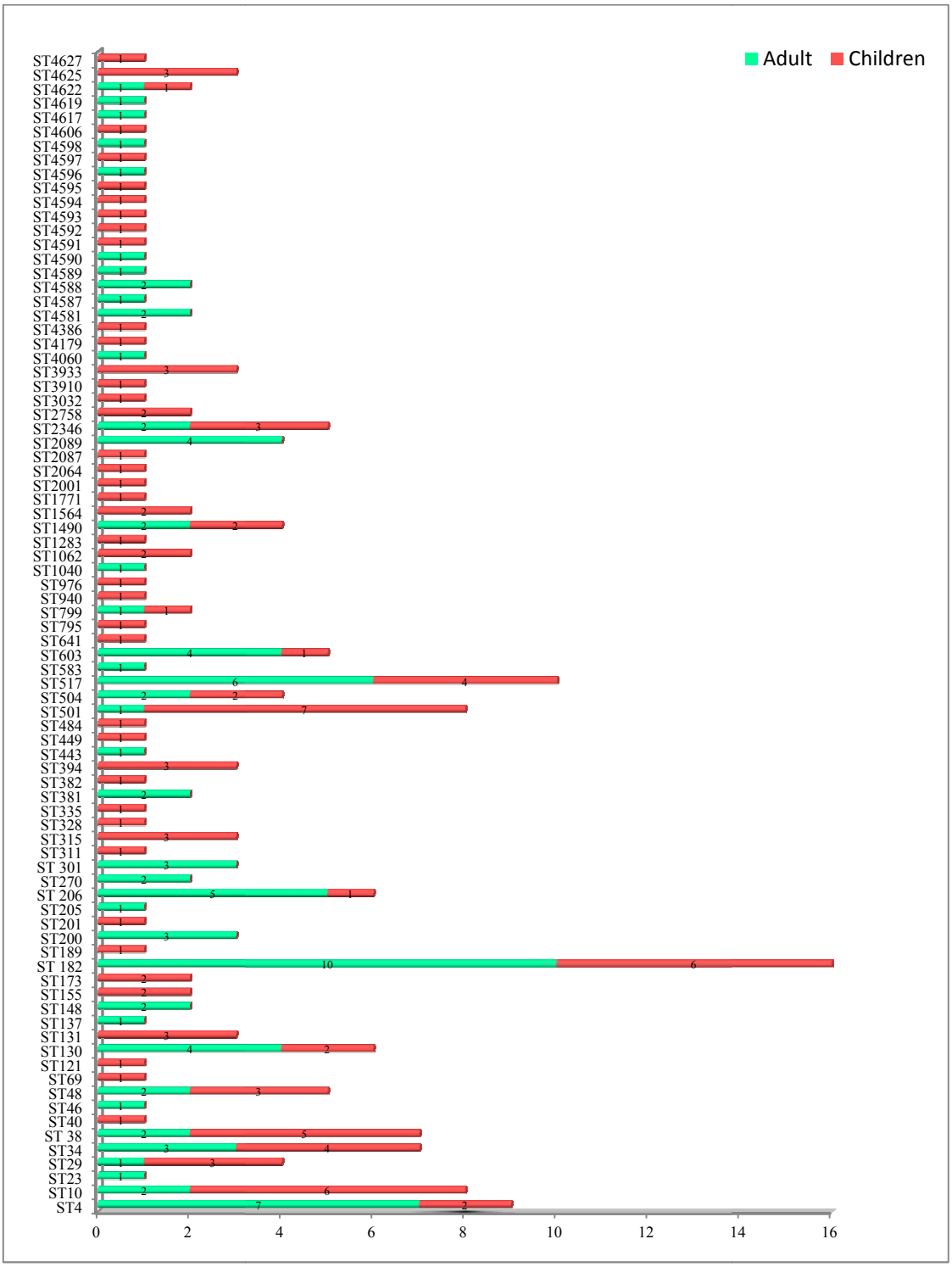

Figure 5.14 Frequencies of Multi-Locus Sequence Types (MLST) among 200 *E. coli* isolates including internal control.

5.5.4 Discrimination of multidrug resistant pathogenic *E. coli*.

By using all typing techniques, there were 118 different patterns of plasmid profile, 60 different types of ERIC PCR, and 82 sequence types (STs) of MLST with discriminatory index (DI) of 0.956, 0.973, and 0.979, respectively. Among three typing techniques, plasmid profiling gave the lowest discriminatory power whereas ERIC PCR is very similar to MLST. The discrimination of 198 *E. coli* was shown in Table 5.9.

5.6 Investigation of possible clonal spread of community-acquired multidrug resistant pathogenic *E. coli*.

By using all typing technique and space-time disease surveillance, some repetitive clones were identified in the same space and time clusters indicating possible outbreak situations, i.e., ET076 and ET077 (ST4); EA030, EA031, EA032, and EA033 (ST182); ET081 and ET082 (ST182); EA006 and EA007 (ST200); EA013 and EA015 (ST206); EA018 and EA019 (ST206); EC187, EC189, and EC190 (ST315); EC111 and EC112 (ST501); EC113, EC114, and EC119 (ST501); EA009 and EA014 (ST517); EC137 and EC139 (ST517); and EA017 and EA021 (ST4588) (Table 5.10). The same clones were present in travelers and indigenous population in Thailand and Nepal during the past ten years suggesting time-lag outbreaks or persistence clones, i.e., ST4, ST10, ST29, ST34, ST48, ST130, ST182, ST517, ST603, ST799, ST1490, ST2346, and ST4622. Moreover, the repetitive clones were shared in among Thailand, Vietnam, Cambodia, or Nepal indicating the clonal spread in countries of Asia, i.e., ST4, ST10, ST29, ST34, ST38, ST48, ST131, ST155, ST173, ST182, ST206, ST501, ST504, ST517, ST1490, ST1564, ST2346, and ST2758. Furthermore, STs in this study were matched with strains in Warwick *E. coli* database especially, strains from food-producing animals, e.g., ST10, ST23, ST29, ST38, ST48, ST69, ST131, ST137, ST155, ST206, ST301, ST394, ST443, and ST1564 (Table 5.11).

Table 5.9 Discrimination of MDR pathogenic *E. coli* by MLST, ERIC PCR, and plasmid profiling

MLST	ERIC type	Plasmid profiling	No. of isolates
ST4	E1,4,5,11	P6,12,15,35,49,81,100	9
ST10	E10,18,22,28,34,40	P1,9,21,60,111,117,118,none	8
ST23	E3	P63	1
ST29	E1,11	P28,50,51,53	4
ST34	E7,9,11,22,32,42	P4,68,69,72,92,98,103	7
ST 38	E9,17,29,33,37	none	7
ST40	E16	P85	1
ST46	E48	none	1
ST48	E5,12,13,32	P32,36,52,62,65	5
ST69	E46	none	1
ST121	E47	P107	1
ST130	E26,27	none	4
ST131	E1,11	P23,59,115	3
ST137	E50	P56	1
ST148	E1	P39,91	2
ST155	E16,53	P90,none	2
ST173	E16,20	P76112	2
ST 182	E8,14	P12,13,14,19,20,21,89	16
ST189	E38	P104	1
ST200	E20	P21	3
ST201	E16	none	1
ST205	E52	none	1
ST 206	E1,5,16	P1,11,27,34,64	6
ST270	E8,19	P8,10	2
ST 301	E7,39	P33,38,84	3
ST311	E15	P25	1
ST315	E17	P58,114,none	3
ST328	E16	P26	1
ST335	E21	P57	1

Table 5.9 Discrimination of MDR pathogenic *E. coli* by MLST, ERIC PCR, and plasmid profiling (cont.)

MLST	ERIC type	Plasmid profiling	No. of isolates
ST381	E43	P37,48	2
ST382	E41	P31	1
ST394	E36,59	P1,20,116	3
ST443	E43	P24	1
ST449	E1	P67	1
ST484	E12	P21	1
ST501	E1,34,35	P16,18,86,94,95,96,none	8
ST504	E23,24	P17,47,106,108	4
ST517	E3,16,17,19,20	P2,3,7,55,74,87,113,none	10
ST583	E3	P71	1
ST603	E6,17,51	P5,8,44,70,97	5
ST641	E16	P4	1
ST795	E16	P79	1
ST799	E45,49	P41,none	2
ST940	E18	P40	1
ST976	E9	P11	1
ST1040	E28	P105	1
ST1062	E27,42	none	2
ST1283	E11	P46	1
ST1490	E6,16,19	P10,73,114,none	4
ST1564	E9	P42,43	2
ST1771	E43	none	1
ST2001	E1	none	1
ST2064	E44	P18	1
ST2087	E30	P8	1
ST2089	E2,18,41	none	4
ST2346	E7	P15,80,83,93,110	5
ST2758	E10	P19,21	2
ST3032	E23	P2	1

Table 5.9 Discrimination of MDR pathogenic *E. coli* by MLST, ERIC PCR, and plasmid profiling (cont.)

MLST	ERIC type	Plasmid profiling	No. of isolates
ST3910	E38	P99	1
ST3933	E11,58	P61,109,none	3
ST4060	E57	P54	1
ST4179	E7	P78	1
ST4386	E20	P102	1
ST4581	E54,55	P66,75	2
ST4587	E9	P29	1
ST4588	E4,17	P22,none	2
ST4589	E33	P82	1
ST4590	E2	P77	1
ST4591	E36	P30	1
ST4592	E15	P101	1
ST4593	E21	P15	1
ST4594	E16	P88	1
ST4595	E16	P1	1
ST4596	E60	P45	1
ST4597	E56	none	1
ST4598	E25	P1	1
ST4606	E15	P17	1
ST4617	E14	none	1
ST4619	E31	P20	1
ST4622	E25,30	none	2
ST4625	E29,31	P21,none	3
ST4627	E13	P8	1

Table 5.10 Comparison of MDR pathogenic *E. coli* isolates by using all molecular technique and demographic information

Sample	Date	Pathogen	Status	Diarrhea	Nationality	Location	Integron	ST	ERIC	Plasmid	Resistance pattern
ET076	13/3/2002	ETEC	Adult	Yes	Japanese	Bumrungrad Hosp. (Thailand)	pos	4	4	6	NA,AM,S,G,SXT,TE
ET077	13/3/2002	ETEC	Adult	Yes	Japanese	Bumrungrad Hosp. (Thailand)	pos	4	4	6	NA,AM,S,G,SXT,TE
ET075	31/7/2001	ETEC	Adult	Yes	American	Bumrungrad Hosp. (Thailand)	pos	4	4	12	AM,S,G,STX,TE
ET080	3/5/2001	ETEC	Adult	Yes	European	Nepal	pos	4	5	49	NA,G,SXT,TE
ET088	19/4/2001	ETEC	Adult	No	Thai	Bumrungrad Hosp. (Thailand)	pos	4	5	12	AM,S,G,STX,TE
EA029	6/5/2002	EPEC	Adult	Yes	American	Sakaeo (Thailand)	pos	4	11	35	NA,CIP,AM,C,S,G,SXT,TE
EC165	3/8/2001	ETEC	Children	Yes	Vietnamese	Vietnam	neg	4	11	15	AM,S,G,SXT
EC198	23/1/2006	ETEC	Children	No	Thai	Trang (Thailand)	pos	4	11	81	AM,K,G,SXT,TE
EA028	21/5/2001	ETEC	Adult	Yes	American	Pitsanulok (Thailand)	pos	4	1	100	AM,S,G,STX,TE
EC103	3/11/2004	EAEC	Children	No	Thai	Ubon (Thailand)	neg	10	34	111	AM,C,K,N,S,G,SXT,TE
EC106	30/6/2006	EAEC	Children	No	Thai	Ubon (Thailand)	pos	10	34	118	AM,S,G,STX,TE
EA024	12/5/2004	EPEC	Adult	Yes	American	Korat (Thailand)	neg	10	40	none	AM,S,G,TE
ET052	10/1/2003	EPEC	Adult	No	Canadian	Bumrungrad Hosp. (Thailand)	pos	10	40	1	AM,C,S,G,SXT,TE
EC094	28/12/2004	EAEC	Children	No	Thai	Children Hosp. (Thailand)	pos	10	18	117	AM,C,GM,S,G,SXT
EC095	4/1/2005	EAEC	Children	No	Thai	Children Hosp. (Thailand)	pos	10	28	21	AM,C,GM,G
EC185	21/6/2005	ETEC	Children	Yes	Cambodian	Cambodia	pos	10	10	60	AM,K,G,SXT,TE
EC195	18/10/2006	ETEC	Children	No	Cambodian	Cambodia	neg	10	22	9	NA,AM,S,G,SXT,TE
EC131	14/8/2001	EPEC	Children	Yes	Vietnamese	Vietnam	pos	29	11	50	S,G,SXT,TE
EC147	14/12/2004	EPEC	Children	No	Thai	Ubon (Thailand)	pos	29	11	28	C,G,SXT,TE
EC157	21/3/2006	EPEC	Children	No	Cambodian	Cambodia	pos	29	12	51	AM,S,G,STX,TE

Table 5.10 Comparison of MDR pathogenic *E. coli* isolates by using all molecular technique and demographic information (cont.)

Sample	Date	Pathogen	Status	Diarrhea	Nationality	Location	Integron	ST	ERIC	Plasmid	Resistance pattern
ET042	23/3/2001	EPEC	Adult	Yes	Japanese	Bumrungrad Hosp. (Thailand)	neg	29	1	53	NA,S,G,STX,TE
EA022	14/5/2003	EPEC	Adult	Yes	American	Pranburi (Thailand)	pos	34	7	92	AM,C,K,N,S,G,SXT,TE
EC107	23/8/2006	EAEC	Children	No	Thai	Ubon (Thailand)	neg	34	9	4	NA,AM,S,G,SXT,TE
EC117	15/2/2005	EAEC	Children	No	Cambodian	Cambodia	pos	34	11	68	NA,AM,S,G,SXT,TE
EC133	9/7/2008	EPEC	Children	Yes	Nepalese	Nepal	pos	34	22	98	NA,AM,GM,K,S,G,SXT,TE, CTX,CRO
EC143	25/5/2007	EPEC	Children	No	Nepalese	Nepal	pos	34	22	103	NA,AM,GM,K,S,G,SXT,TE, CTX,CRO
ET045	21/12/2001	EPEC	Adult	Yes	Japanese	Bumrungrad Hosp. (Thailand)	pos	34	32	69	NA,AM,C,S,G,SXT, TE,CTX,CRO
ET061	25/12/2002	EPEC	Adult	Yes	European	Nepal	pos	34	42	72	NA,CIP,AM,C,K,S,G,SXT,TE
EA005	14/5/2004	EAEC	Adult	Yes	American	Samae-san (Thailand)	neg	38	29	none	AM,C,S,G,SXT,TE
EC091	21/1/2008	EAEC	Children	No	Nepalese	Nepal	neg	38	17	none	NA,CIP,AM,C,GM,K,S,G, SXT,TE,CTX,CRO
EC101	3/12/2008	EAEC	Children	Yes	Nepalese	Nepal	neg	38	17	none	AM,S,G,STX,TE
EC109	9/12/2004	EAEC	Children	Yes	Cambodian	Cambodia	pos	38	37	none	AM,C,S,G,SXT,TE
EC110	25/5/2005	EAEC	Children	Yes	Cambodian	Cambodia	pos	38	37	none	NA,AM,C,S,G,SXT,TE
EC115	16/8/2006	EAEC	Children	Yes	Cambodian	Cambodia	pos	38	9	none	NA,AM,S,G,SXT,TE
ET049	18/4/2001	EPEC	Adult	No	Japanese	Bumrungrad Hosp. (Thailand)	pos	38	33	none	NA,AM,C,S,G,TE
EC134	29/3/2005	EPEC	Children	No	Thai	Samutsongkhram (Thailand)	pos	48	12	36	NA,AM,C,GM,S,G,SXT,TE
EC154	14/3/2005	EPEC	Children	No	Cambodian	Cambodia	neg	48	12	32	NA,CIP,AM,C,S,G,SXT,TE
EC158	17/3/2008	EPEC	Children	No	Thai	Phramongkut Hosp. (Thailand)	neg	48	13	52	AM,S,G,TE

Table 5.10 Comparison of MDR pathogenic *E. coli* isolates by using all molecular technique and demographic information (cont.)

Sample	Date	Pathogen	Status	Diarrhea	Nationality	Location	Integron	ST	ERIC	Plasmid	Resistance pattern
ET047	29/11/2002	EPEC	Adult	Yes	American	Bumrungrad Hosp. (Thailand)	pos	48	32	65	NA,CIP,C,GM,S,G,SXT,TE
ET085	31/10/2002	ETEC	Adult	Yes	European	Nepal	neg	48	5	62	AM,S,G,SXT
EA001	19/5/2004	EAEC	Adult	Yes	American	Korat (Thailand)	pos	130	26	14	NA,AM,C,G,SXT,TE
EA002	24/5/2004	EAEC	Adult	Yes	American	Korat (Thailand)	pos	130	26	14	NA,AM,C,G,SXT,TE
EA003	15/5/2004	EAEC	Adult	Yes	American	Korat (Thailand)	pos	130	26	none	AM,C,S,G,SXT,TE
EA004	10/5/2004	EAEC	Adult	No	American	Samae-san (Thailand)	pos	130	26	none	AM,C,S,G,SXT,TE
EC120	13/3/2009	EAEC	Children	No	Thai	Phramongkut Hosp. (Thailand)	pos	130	27	none	AM,C,S,G,SXT,TE
EC121	4/11/2004	EAEC	Children	No	Thai	Trang (Thailand)	pos	130	27	none	AM,C,S,G,SXT,TE
EC097	11/5/2006	EAEC	Children	No	Thai	Children Hosp. (Thailand)	pos	131	11	23	AM,C,GM,K,S,G,SXT,TE, CTX
EC099	20/6/2007	EAEC	Children	Yes	Nepalese	Nepal	pos	131	11	115	NA,AM,C,GM,G
EC104	29/11/2004	EAEC	Children	No	Thai	Ubon (Thailand)	pos	131	1	59	AM,C,GM,S,G,SXT,TE
ET038	11/7/2002	EIEC	Adult	Yes	European	Nepal	pos	148	1	91	AM,C,S,G,SXT,TE
ET039	22/11/2002	EIEC	Adult	Yes	American	Nepal	pos	148	1	39	AM,C,S,G,SXT,TE
EC096	4/5/2005	EAEC	Children	No	Thai	Children Hosp. (Thailand)	pos	155	53	none	AM,C,S,G,SXT,TE
EC173	22/5/2008	ETEC	Children	No	Nepalese	Nepal	pos	155	16	90	AM,G,SXT,TE
EC164	8/7/2002	ETEC	Children	Yes	Thai	Sankhlaburi (Thailand)	pos	173	16	76	AM,S,G,STX,TE
EC191	13/12/2004	ETEC	Children	Yes	Cambodian	Cambodia	pos	173	20	112	NA,AM,S,G,SXT,TE
EA030	13/5/2002	ETEC	Adult	Yes	American	Sakaeo (Thailand)	neg	182	8	12	AM,S,G,STX,TE
EA031	14/5/2002	ETEC	Adult	Yes	American	Sakaeo (Thailand)	neg	182	8	21	AM,S,G,TE
EA032	14/5/2002	ETEC	Adult	Yes	American	Sakaeo (Thailand)	neg	182	8	21	AM,S,G,STX,TE
EA033	16/5/2002	ETEC	Adult	Yes	American	Sakaeo (Thailand)	neg	182	8	13	AM,S,G,STX,TE
EA035	13/5/2004	ETEC	Adult	Yes	American	Korat (Thailand)	neg	182	8	21	AM,S,G,STX,TE
EC199	12/3/2002	ETEC	Children	Yes	Vietnamese	Vietnam	neg	182	8	19	AM,S,G,STX,TE

Table 5.10 Comparison of MDR pathogenic *E. coli* isolates by using all molecular technique and demographic information (cont.)

Sample	Date	Pathogen	Status	Diarrhea	Nationality	Location	Integron	ST	ERIC	Plasmid	Resistance pattern
ET084	13/9/2002	ETEC	Adult	Yes	European	Nepal	neg	182	8	20	AM,S,G,STX,TE
ET086	24/2/2003	ETEC	Adult	Yes	Japanese	Nepal	neg	182	8	21	AM,S,G,STX,TE
EC177	19/6/2002	ETEC	Children	Yes	Thai	Sankhlaburi (Thailand)	neg	182	8	14	AM,S,G,TE
ET081	3/5/2001	ETEC	Adult	Yes	American	Nepal	neg	182	8	21	AM,S,G,TE
ET082	7/5/2001	ETEC	Adult	Yes	European	Nepal	neg	182	8	21	AM,S,G,TE
ET083	23/5/2002	ETEC	Adult	Yes	New Zealander	Nepal	neg	182	8	89	AM,S,G,TE
EC172	31/3/2008	ETEC	Children	No	Nepalese	Nepal	neg	182	8	20	NA,AM,S,G,SXT,TE
EC183	7/12/2004	ETEC	Children	Yes	Cambodian	Cambodia	neg	182	8	21	NA,AM,S,G,SXT,TE
EC163	8/7/2002	ETEC	Children	Yes	Thai	Sankhlaburi (Thailand)	pos	182	14	13	AM,S,G,STX,TE
EC169	28/3/2007	ETEC	Children	Yes	Nepalese	Nepal	neg	182	14	21	AM,S,G,STX,TE
EA006	27/5/2004	EAEC	Adult	Yes	American	Samae-san (Thailand)	pos	200	20	21	AM,GM,G,SXT,TE
EA007	27/5/2004	EAEC	Adult	Yes	American	Samae-san (Thailand)	pos	200	20	21	AM,GM,G,SXT,TE
EA008	5/2/2010	EAEC	Adult	Yes	American	Samae-san (Thailand)	pos	200	20	21	AM,S,G,STX,TE
EA013	17/5/2001	EPEC	Adult	Yes	American	Pitsanulok (Thailand)	pos	206	1	11	C,S,G,SXT,TE
EA015	22/5/2001	EPEC	Adult	Yes	American	Pitsanulok (Thailand)	pos	206	1	11	C,S,G,SXT,TE
EA018	13/5/2002	EPEC	Adult	Yes	American	Sakaeo (Thailand)	pos	206	1	27	AM,S,G,STX,TE
EA019	13/5/2002	EPEC	Adult	Yes	American	Sakaeo (Thailand)	pos	206	1	64	NA,AM,S,G,SXT,TE
EA027	15/5/2001	ETEC	Adult	Yes	American	Pitsanulok (Thailand)	pos	206	5	34	AM,S,G,STX,TE
EC150	24/5/2005	EPEC	Children	Yes	Cambodian	Cambodia	pos	206	16	1	AM,S,G,STX,TE
ET037	1/5/2001	EIEC	Adult	Yes	Japanese	Bumrungrad Hosp. (Thailand)	pos	270	8	8	AM,S,G,STX,TE
ET040	1/2/2001	EIEC	Adult	Yes	Thai	Bumrungrad Hosp. (Thailand)	pos	270	19	10	C,S,G,SXT,TE
EA020	14/5/2002	EPEC	Adult	Yes	American	Sakaeo (Thailand)	pos	301	7	84	AM,C,S,G,SXT,TE
EA023	20/5/2003	EPEC	Adult	Yes	American	Pranburi (Thailand)	pos	301	39	38	NA,AM,C,S,G,SXT,TE
ET066	19/12/2001	EPEC	Adult	Yes	Thai	Bumrungrad Hosp. (Thailand)	pos	301	39	33	C, S,G,SXT,TE

Table 5.10 Comparison of MDR pathogenic *E. coli* isolates by using all molecular technique and demographic information (cont.)

Sample	Date	Pathogen	Status	Diarrhea	Nationality	Location	Integron	ST	ERIC	Plasmid	Resistance pattern
EC187	26/4/2006	ETEC	Children	Yes	Cambodian	Cambodia	pos	315	17	none	AM,S,G,STX,TE
EC189	17/7/2006	ETEC	Children	Yes	Cambodian	Cambodia	pos	315	17	58	AM,S,G,STX,TE
EC190	12/9/2006	ETEC	Children	Yes	Cambodian	Cambodia	pos	315	17	114	AM,S,G,STX,TE
ET059	20/11/2002	EPEC	Adult	Yes	European	Nepal	pos	381	43	48	NA,CIP,AM,S,G,SXT,TE
ET062	18/2/2003	EPEC	Adult	Yes	American	Nepal	pos	381	43	37	AM, N, S,G,SXT,TE
EC170	25/5/2005	ETEC	Children	No	Thai	Children Hosp. (Thailand)	pos	394	59	20	AM, K, S,G,SXT,TE
EC176	6/6/2002	ETEC	Children	Yes	Thai	Sankhlaburi (Thailand)	pos	394	36	116	C,G,SXT,TE
EC182	23/11/2006	ETEC	Children	No	Thai	Mae Hong Son (Thailand)	pos	394	36	1	AM,S,G,STX,TE
EC092	28/4/2008	EAEC	Children	No	Nepalese	Nepal	pos	501	1	86	NA,CIP,AM,C,K,S,G,SXT, TE,CTX,CRO
EC111	17/6/2005	EAEC	Children	Yes	Cambodian	Cambodia	pos	501	34	18	AM, K, S,G,SXT,TE
EC112	11/7/2005	EAEC	Children	Yes	Cambodian	Cambodia	pos	501	35	94	AM,C,K,S,G,SXT,TE
EC113	17/1/2006	EAEC	Children	Yes	Cambodian	Cambodia	pos	501	35	96	NA,AM,C,K,S,G,SXT,TE
EC114	16/2/2006	EAEC	Children	Yes	Cambodian	Cambodia	pos	501	35	95	NA,AM,C,K,S,G,SXT,TE, CTX,CRO
EC119	16/1/2006	EAEC	Children	No	Cambodian	Cambodia	pos	501	35	16	NA,AM,C,K,S,G,SXT,TE
EC116	11/9/2006	EAEC	Children	Yes	Cambodian	Cambodia	pos	501	35	16	NA,AM,C,K,S,G,SXT,TE
ET036	27/2/2009	EAEC	Adult	No	Nepalese	Nepal	neg	501	35	none	NA,CIP,AM,C,K,TE, CTX,CRO
EC166	18/7/2006	STEC	Children	Yes	Cambodian	Cambodia	pos	504	24	106	C,S,G,SXT
EC167	17/6/2005	STEC	Children	No	Cambodian	Cambodia	pos	504	23	17	AM,S,G,TE
ET089	13/8/2002	STEC	Adult	No	American	Bumrungrad Hosp. (Thailand)	pos	504	24	108	AM,S,G,STX,TE
ET090	14/4/2002	STEC	Adult	No	American	Nepal	pos	504	24	47	AM,S,G,STX,TE
EA009	8/5/2001	EPEC	Adult	Yes	American	Pitsanulok (Thailand)	neg	517	19	7	S,G,SXT,TE
EA014	19/5/2001	EPEC	Adult	Yes	American	Pitsanulok (Thailand)	neg	517	17	none	AM,C,S,G,SXT,TE

Table 5.10 Comparison of MDR pathogenic *E. coli* isolates by using all molecular technique and demographic information (cont.)

Sample	Date	Pathogen	Status	Diarrhea	Nationality	Location	Integron	ST	ERIC	Plasmid	Resistance pattern
EA025	24/5/2004	EPEC	Adult	Yes	American	Korat (Thailand)	pos	517	19	3	AM,S,G,STX,TE
EC137	19/5/2005	EPEC	Children	No	Thai	Children Hosp. (Thailand)	pos	517	16	2	AM,S,G,STX,TE
EC139	13/6/2005	EPEC	Children	No	Thai	Children Hosp. (Thailand)	neg	517	3	113	NA,AM,S,G,SXT,TE
EC144	1/4/2008	EPEC	Children	No	Nepalese	Nepal	pos	517	16	3	AM,S,G,STX,TE
EC149	15/3/2006	EPEC	Children	No	Thai	Ubon (Thailand)	pos	517	19	87	AM,C,S,G,SXT,TE
ET043	30/3/2001	EPEC	Adult	Yes	English	Bumrungrad Hosp. (Thailand)	neg	517	20	74	AM,S,G,TE
ET067	19/1/2001	EPEC	Adult	No	Thai	Bumrungrad Hosp. (Thailand)	neg	517	19	55	S,G,SXT,TE
ET072	6/3/2002	EPEC	Adult	No	Thai	Bumrungrad Hosp. (Thailand)	neg	517	19	7	S,G,SXT,TE
EA012	15/5/2001	EPEC	Adult	Yes	American	Pitsanulok (Thailand)	pos	603	6	97	AM,C,S,G,TE
EA016	9/5/2002	EPEC	Adult	Yes	American	Sakaeo (Thailand)	pos	603	17	70	NA,CIP,AM,C,GM,K, S,G,SXT,TE
EC123	9/2/2005	EAEC	Children	No	Thai	Trang (Thailand)	pos	603	6	44	NA,CIP,AM,GM,K,N,S,G,TE
ET050	21/6/2001	EPEC	Adult	No	Japanese	Bumrungrad Hosp. (Thailand)	pos	603	51	5	AM,C,GM,G,SXT,TE
ET074	21/11/2002	EPEC	Adult	No	Thai	Bumrungrad Hosp. (Thailand)	pos	603	6	8	AM,C,S,G,SXT,TE
EC162	4/1/2006	EPEC	Children	No	Thai	Trang (Thailand)	pos	799	49	none	AM,C,S,G,SXT,TE
ET044	26/11/2001	EPEC	Adult	Yes	English	Bumrungrad Hosp.(Thailand)	pos	799	45	41	AM,C,K,N,S,G,SXT,TE
EC098	20/10/2006	EAEC	Children	No	Thai	Children Hosp. (Thailand)	pos	1062	42	none	AM,C,S,G,SXT,TE
EC122	21/12/2004	EAEC	Children	No	Thai	Trang (Thailand)	pos	1062	27	none	AM,C,S,G,SXT,TE

Table 5.10 Comparison of MDR pathogenic *E. coli* isolates by using all molecular technique and demographic information (cont.)

Sample	Date	Pathogen	Status	Diarrhea	Nationality	Location	Integron	ST	ERIC	Plasmid	Resistance pattern
EA034	19/5/2002	ETEC	Adult	Yes	American	Sakaeo (Thailand)	neg	1490	19	10	AM,S,G,STX,TE
EC180	12/7/2006	ETEC	Children	No	Thai	Mae Hong Son (Thailand)	neg	1490	16	none	NA,AM,S,G,SXT,TE
EC186	6/9/2005	ETEC	Children	Yes	Cambodian	Cambodia	neg	1490	16	114	NA, AM,S,G,SXT
ET087	2/5/2001	ETEC	Adult	Yes	Thai	Bumrungrad Hosp. (Thailand)	pos	1490	6	73	AM,S,G,TE
EC174	6/7/2008	ETEC	Children	No	Nepalese	Nepal	pos	1564	9	42	AM,S,G,STX,TE
EC193	21/3/2006	ETEC	Children	No	Cambodian	Cambodia	pos	1564	9	43	AM,C,S,G,SXT
ET054	3/4/2001	EPEC	Adult	Yes	European	Nepal	neg	2089	18	none	NA,AM,S,G,TE
ET057	11/2/2002	EPEC	Adult	Yes	European	Nepal	pos	2089	2	none	NA,AM,C,TE
ET060	6/12/2002	EPEC	Adult	Yes	European	Nepal	pos	2089	41	none	AM,C,S,TE
ET063	15/10/2001	EPEC	Adult	No	American	Nepal	pos	2089	2	none	NA,AM,C,S,TE
EA026	19/5/2004	EPEC	Adult	Yes	American	Korat (Thailand)	pos	2346	7	15	NA,AM,GM,G,SXT,TE
EC128	10/1/2005	EPEC	Children	Yes	Cambodian	Cambodia	pos	2346	7	83	NA,AM,GM,G,SXT,TE
EC136	14/3/2005	EPEC	Children	No	Thai	Children Hosp. (Thailand)	pos	2346	7	80	NA,AM,GM,S,G,SXT,TE
EC155	21/4/2005	EPEC	Children	No	Cambodian	Cambodia	pos	2346	7	93	NA,AM,C,S,G,SXT,TE
ET046	11/3/2002	EPEC	Adult	Yes	Japanese	Bumrungrad Hosp. (Thailand)	pos	2346	7	110	NA,AM,GM,G,SXT,TE
EC179	13/9/2005	ETEC	Children	No	Thai	Mae Hong Son (Thailand)	neg	2758	10	21	AM,S,G,STX,TE
EC192	8/7/2005	ETEC	Children	No	Cambodian	Cambodia	pos	2758	10	19	AM,S,G,STX,TE
EC178	13/12/2001	ETEC	Children	Yes	Thai	Sankhlaburi (Thailand)	pos	3933	58	109	C,G,SXT,TE
EC196	23/8/2007	ETEC	Children	Yes	Thai	Phramongkut Hosp. (Thailand)	pos	3933	11	61	NA,AM,C,S,G,SXT,TE
EC197	21/10/2005	ETEC	Children	No	Thai	Trang (Thailand)	pos	3933	11	none	NA,AM,C,S,G,SXT,TE
EA010	9/5/2001	EPEC	Adult	Yes	American	Pitsanulok (Thailand)	pos	4581	54	66	NA,AM,S,G,TE

Table 5.10 Comparison of MDR pathogenic *E. coli* isolates by using all molecular technique and demographic information (cont.)

Sample	Date	Pathogen	Status	Diarrhea	Nationality	Location	Integron	ST	ERIC	Plasmid	Resistance pattern
ET068	25/4/2001	EPEC	Adult	No	Thai	Bumrungrad Hosp. (Thailand)	neg	4581	55	75	S,G,TE
EA017	10/5/2002	EPEC	Adult	Yes	American	Sakaeo (Thailand)	pos	4588	17	22	NA,AM,C,K,N,S,G,SXT,TE
EA021	14/5/2002	EPEC	Adult	Yes	American	Sakaeo (Thailand)	pos	4588	4	none	NA,AM,C,K,N,S,G,SXT,TE
EC142	20/11/2006	EPEC	Children	No	Thai	Children Hosp. (Thailand)	pos	4622	25	none	NA,AM,C,G,TE
ET070	17/7/2001	EPEC	Adult	No	Thai	Bumrungrad Hosp. (Thailand)	neg	4622	30	none	NA,AM,C,G,SXT,TE
EC129	8/4/2002	EPEC	Children	Yes	Thai	Sankhlaburi (Thailand)	pos	4625	31	none	NA,AM,C,GM,S,G,SXT,TE
EC138	25/5/2005	EPEC	Children	No	Thai	Children Hosp. (Thailand)	pos	4625	31	none	AM,C,GM,S,G,SXT,TE
EC146	10/7/2006	EPEC	Children	No	Thai	Mae Hong Son (Thailand)	pos	4625	29	21	NA,AM,C,GM,K,S,G,SXT,TE
ET079	24/5/2001	ETEC	Adult	No	Japanese	Bumrungrad Hosp. (Thailand)	pos	23	3	63	AM,S,G,STX,TE
EC105	19/5/2005	EAEC	Children	No	Thai	Ubon (Thailand)	neg	40	16	85	S,G,TE
ET055	25/1/2002	EPEC	Adult	Yes	Japanese	Nepal	pos	46	48	none	NA,CIP,AM,GM,K,S,G,SXT, TE,CTX,CRO
EC093	30/7/2008	EAEC	Children	No	Nepalese	Nepal	pos	69	46	none	NA,AM,S,G,SXT,TE
EC200	1/12/2008	EPEC	Children	Yes	Nepalese	Nepal	pos	121	47	107	NA,AM,S,G,SXT,TE
EA011	15/5/2001	EPEC	Adult	Yes	American	Pitsanulok (Thailand)	pos	137	50	56	NA,AM,C,K,N,S,G,SXT,TE
EC140	27/7/2005	EPEC	Children	No	Thai	Children Hosp. (Thailand)	pos	189	38	104	NA,CIP,S,G,SXT,TE
EC168	19/1/2006	STEC	Children	No	Thai	Trang (Thailand)	pos	201	16	none	C,G,SXT,TE
ET078	23/12/2002	ETEC	Adult	Yes	Japanese	Bumrungrad Hosp. (Thailand)	pos	205	52	none	AM,S,G,STX,TE
EC126	14/8/2001	EIEC	Children	Yes	Vietnamese	Vietnam	pos	311	15	25	AM,S,G,STX,TE

Table 5.10 Comparison of MDR pathogenic *E. coli* isolates by using all molecular technique and demographic information (cont.)

Sample	Date	Pathogen	Status	Diarrhea	Nationality	Location	Integron	ST	ERIC	Plasmid	Resistance pattern
EC160	19/5/2005	EPEC	Children	No	Thai	Trang (Thailand)	pos	328	16	26	AM,S,G,STX,TE
EC161	28/11/2005	EPEC	Children	No	Thai	Trang (Thailand)	neg	335	21	57	S,G,SXT,TE
EC132	20/9/2001	EPEC	Children	Yes	Vietnamese	Vietnam	pos	382	41	31	AM,C,G,SXT,TE
ET064	20/9/2007	EPEC	Adult	Yes	Maldivian	Maldives	pos	443	43	24	NA,CIP,AM,GM,G,SXT,TE
EC100	24/3/2008	EAEC	Children	No	Nepalese	Nepal	pos	449	1	67	NA,CIP,AM,C,GM, K,
EC125	1/9/2006	EAEC	Children	No	Thai	Trang (Thailand)	neg	484	12	21	AM,S,G,STX,TE
ET041	16/1/2001	EPEC	Adult	Yes	Thai	Bumrungrad Hosp. (Thailand)	neg	583	3	71	NA,N,G,TE
EC188	26/5/2006	ETEC	Children	Yes	Cambodian	Cambodia	pos	641	16	4	AM,S,G,STX,TE
EC152	18/7/2006	EPEC	Children	Yes	Cambodian	Cambodia	pos	795	16	79	C,K,N,G,SXT,TE
EC141	15/2/2006	EPEC	Children	No	Thai	Children Hosp. (Thailand)	pos	940	18	40	NA,CIP,AM,C,GM,S,SXT, TE, CTX,CRO
EC151	16/8/2005	EPEC	Children	Yes	Cambodian	Cambodia	pos	976	9	11	NA,CIP,AM,C,K,N,S,G,SXT, TE
ET069	1/6/2001	EPEC	Adult	No	Thai	Bumrungrad Hosp. (Thailand)	pos	1040	28	105	C,GM,K,G,SXT
EC153	7/12/2004	EPEC	Children	No	Cambodian	Cambodia	pos	1283	11	46	AM,C,S,G,SXT,TE
EC194	24/3/2006	ETEC	Children	No	Cambodian	Cambodia	neg	1771	43	none	AM,S,G,TE
EC102	21/7/2006	EAEC	Children	No	Thai	Mae Hong Son (Thailand)	pos	2001	1	none	AM,S,G,STX,TE
EC171	12/4/2006	ETEC	Children	No	Thai	Children Hosp. (Thailand)	pos	2064	44	18	C,G,SXT,TE
EC135	10/11/2004	EPEC	Children	No	Thai	Children Hosp. (Thailand)	neg	2087	30	8	AM,K,N,S,G,TE
EC108	22/11/2004	EAEC	Children	Yes	Cambodian	Cambodia	pos	3910	38	99	AM,C,S,G,SXT,TE
EC124	26/8/2005	EAEC	Children	No	Thai	Trang (Thailand)	pos	3032	23	2	AM,C,G,SXT,TE
ET058	14/2/2002	EPEC	Adult	Yes	American	Nepal	pos	4060	57	54	NA,AM,C,G,SXT,TE

Table 5.10 Comparison of MDR pathogenic *E. coli* isolates by using all molecular technique and demographic information (cont.)

Sample	Date	Pathogen	Status	Diarrhea	Nationality	Location	Integron	ST	ERIC	Plasmid	Resistance pattern
EC145	4/6/2008	EPEC	Children	No	Nepalese	Nepal	pos	4179	7	78	AM,S,G,STX,TE
EC118	24/5/2005	EAEC	Children	No	Cambodian	Cambodia	pos	4386	20	102	AM,C,S,G,SXT,TE
ET065	5/2/2001	EPEC	Adult	Yes	Thai	Bumrungrad Hosp. (Thailand)	pos	4587	9	29	AM,S,G,STX,TE
ET048	8/3/2001	EPEC	Adult	No	English	Bumrungrad Hosp. (Thailand)	pos	4589	33	82	AM,C,S,G,SXT,TE
ET056	6/2/2002	EPEC	Adult	Yes	European	Nepal	pos	4590	2	77	AM,C,S,G,SXT,TE
EC130	27/6/2002	EPEC	Children	Yes	Thai	Sankhlaburi (Thailand)	pos	4591	36	30	AM,C,S,G,SXT,TE
EC156	21/6/2005	EPEC	Children	No	Cambodian	Cambodia	pos	4592	15	101	AM,S,G,STX,TE
EC159	25/11/2004	EPEC	Children	No	Thai	Trang (Thailand)	pos	4593	21	5	AM,C,S,G,TE
EC175	6/7/2008	ETEC	Children	Yes	Nepalese	Nepal	neg	4594	16	88	AM,S,G,SXT
EC184	16/6/2005	ETEC	Children	Yes	Cambodian	Cambodia	neg	4595	16	1	AM,S,G,STX,TE
ET051	23/7/2001	EPEC	Adult	No	Japanese	Bumrungrad Hosp. (Thailand)	pos	4596	60	45	NA,AM,S,G,SXT,TE
EC181	22/11/2006	ETEC	Children	No	Thai	Mae Hong Son (Thailand)	neg	4597	56	none	AM,S,G,TE
ET071	4/1/2002	EPEC	Adult	No	Thai	Bumrungrad Hosp. (Thailand)	pos	4598	15	17	NA,CIP,GM,G,SXT,TE
EC148	16/9/2005	EPEC	Children	No	Thai	Ubon (Thailand)	pos	4606	25	1	S,G,TE
ET073	12/11/2002	EPEC	Adult	No	Thai	Bumrungrad Hosp. (Thailand)	pos	4617	14	none	NA,C,K,N,G,SXT,TE
ET053	19/3/2001	EPEC	Adult	Yes	European	Nepal	neg	4619	31	20	NA,AM,S,G,SXT,TE
EC127	13/3/2008	EIEC	Children	No	Nepalese	Nepal	neg	4627	13	8	AM,S,G,TE

Table 5.11 strains information of Warwick *E. coli* database associated with *E. coli* strains in this study

ST	Host species	Country
4	Human	Russia, Germany
10	Bovine, camel, cattle, chicken, dog, horse, human, parrot, pig, pigeon, rabbit, sea lion, soil	Australia, Brazil, Canada, Ecuador, Egypt, England, France, Germany, Ghana, Hungary, Ireland, Italy, Malaysia, Mexico, Nigeria, Norway, Peru, Scotland, Spain, Sri Lanka, Sweden, USA, UK
23	chicken, dog, duck, human, pig, poultry, sheep, soil, turkey	Brazil, Canada, England, France, Germany, Greece, Morocco, Nigeria
29	Bovine, human, sheep	Belgium, Brazil, Germany, Norway, Scotland, Sweden, USA, UK
34	Human	Germany, Mexico, Nigeria, Peru
38	Barley, chicken, dog, Human, *M*alurus cyaneus (bird), pigeon, sea lion, turkey	Australia, Brazil, Canada, Denmark, Ecuador, France, Germany, Japan, Nigeria, UK
40	Human, pigeon	England, Germany, Italy, Japan, Norway, Thailand, USA
46	Human, dog, Leopard	Angola, Brazil, Spain, Egypt, Nigeria, Germany, USA
48	Bovine, ape, cheese, Human, chicken, pig	Bali, Bangladesh, Brazil, Ecuador, France, Germany, Ghana, Nigeria, Spain , Sri Lanka, USA, UK
69	Cattle, chicken, dog, horse, human, sheep, turkey	Brazil, France, Germany, Ireland, Papua-New Guinea, Portugal, Spain, USA, UK
121	parrot	Germany
130	Dog, human	Germany, Nigeria
131	Avian, cat, chicken, dog, horse, human, pig, poultry	Australia, Brazil, Canada, Denmark, Ecuador, France, Germany, Ghana, Ireland, Italy, Japan, Kuwait, Portugal, Spain, Switzerland, Netherlands, USA, UK
137	Bovine	Germany
148	Human	Germany
155	Bovine, chicken, duck, human, musk ox, sea lion, soil	Ecuador, Egypt, France, Germany, Ghana, Nigeria, South Africa, Sri Lanka, Thailand
173	Human, soil	Ghana
182	Human	Ghana, India

Table 5.11 strains information of Warwick *E. coli* database associated with *E. coli* strains in this study (cont.)

ST	Host species	Country
189	Human	Ghana
200	Human, marmot	China, England, Germany, Ghana, Thailand
201	Human	Ghana
205	Human	Brazil, Ghana, UK
206	Bovine, chicken, human	England, Ghana, Norway, Sri Lanka
270	Human	Unknown
301	Bovine, cattle	Germany, Ireland
311	Human	Unknown
315	Human	Canada, India
328	Human, rabbit	England, Germany
335	Fox, human	England, Norway
381	Human	Brazil
382	Human	Brazil
394	Human, pig	Brazil, Nigeria, Thailand, USA
443	Bovine, human	Brazil, Ghana, Scotland
449	Human	Nigeria, Peru
484	Human	Nigeria
501	Human	Nigeria
504	Human	Nigeria
517	Human	Nigeria
583	Dog, human	Germany, Norway
603	Horse, human	Germany, Spain
641	Human, marine intertidal sediment, Pig	Canada, Germany, Hong Kong, Portugal
795	Human	Germany
799	Human	Germany
940	Dog, unknown	Germany, France
976	Human	Norway
1040	Human	Germany

Table 5.11 strains information of Warwick *E. coli* database associated with *E. coli* strains in this study (cont.)

ST	Host species	Country
1062	Human	Germany
1283	Human	Germany
1490	Human	Egypt
1564	Turkey	UK
1771	Human	Brazil
2001	Human	Singapore
2064	Human	Netherlands
2087	Human	Spain
2089	Human	Spain
2346	Demoiselle Crane(bird), human	Mongolia, Mexico
2758	Human	China
3032	Human	Netherlands
3910	Human	Brazil
3933	Human	China
4060	Unknown	China
4179	Human	Peru

CHAPTER VI
DISCUSSION

The emergence and spread of antimicrobial resistance bacteria are the critical concern worldwide, particularly; *E. coli* has the potential roles as commensal flora that can be a reservoir for resistance genes and clinical pathogen including DEC. MDR diarrheagenic *E. coli* have been reported in many developing countries (66-70). In this study, DEC isolates more than 80% were resistant to tetracycline, sulfisoxazole, ampicillin, streptomycin, and trimethoprim-sulfamethoxazole. This similar resistance pattern was reported in Cambodia, which was resistant to ampicillin (90.7%), trimethoprim-sulfamethoxazole (88.3%), and tetracycline (87.0%), respectively (68), high percentage of resistance against ampicillin and trimethoprim-sulfamethoxazole was reported in Vietnam (66), Peru (67), and high resistance rate against ampicillin, trimethoprim, streptomycin, and kanamycin was observed in Iran (70). Interestingly, the resistance pattern in this study was similar in both of children and adults, which most of them are international travelers. In developing countries these antimicrobials are widely used in human and animals due to low cost and ready availability (5, 66), which select the antimicrobial resistance bacteria and followed by their dissemination. To better understand genetic mechanism conferring antimicrobial resistance, class 1 integrons were detected by PCR using specific primers for class1 integrase (*intI1*) gene, 5′-3′ conserved segment (5′CS-3′CS), and dot-blot hybridization. The high prevalence of class 1 integrons in MDR diarrheagenic *E. coli* in this study was evidenced in both adults (70.5%) and children (76.4%), which was similar to that reported in Iran (78%) (70). The lower rates of class 1 integrons were reported in Libya (50%) (71) and Peru (9%) (67), of which high antimicrobial resistance may occurred by other resistance mechanisms. Class 1 integrons were detected in 100% of STEC, 81% of EPEC and EAEC, in contrast to the other studies that reported in Brazil (28.6% of STEC) (72), Iran (31.4% of EPEC) (73), and Libya (44% of EPEC and 22% of EAEC) (74). The higher rate of class 1 integrons was found in dot-blot

hybridization technique, when compared with PCR, suggesting higher sensitivity of the homologous probe in dot-blot hybridization. However, 8 integrase negative isolates, which were positive for 5′CS-3′CS may indicate the possible *intI1* gene truncated. On the other hand, 29 *int11*-positive isolates which had no amplicon of 5′CS-3′CS can be explained by the long arrays containing many gene cassettes or the deletion of 3′CS caused by insertion elements (75). Various resistance genes identified in this study were *aadA1*, *aadA2*, *aadA5*, *dfrA12*, *dfrA17*, and *linF*. The most cassette arrays are *aadA* and *dfrA*, encoding resistance to streptomycin and trimethoprim, respectively. This result is agreed with the previous study that these cassettes reflected the stability and persistence in class 1 integrons (73, 76, 77). Gene cassettes containing *dfrA17-aadA5* and *dfrA12-orfF-aadA2*, which seemed to be global widespread (7, 73, 76, 78), were found in this study. Interestingly, the rare cassette type of *aadA2-linF* conferring streptomycin and lincosamide resistance was detected in EA011 isolated from American traveler in 2001. This similar cassette previously has reported in Norway from clinical specimen collection during 1995-1996 (79), in Malaysia, 2009-2010 (80), and in Thailand from chicken meat, 2010-2011 (81). This finding indicated an occurrence of resistance gene cassettes might transfers between different bacterial species in different hosts under selective pressure, which happened more than 10 years ago. Although, only 10 (5.1%) isolates were class A β-lactamases producers, 80% of them carried class 1 integrons, similar in Nepal (82) and Thailand (83). However, the association between class A β-lactamases gene and class 1 integrons should be further analyzed. Most of class A β-lactamase producers were indigenous children from Thailand, Cambodia, and Nepal. Furthermore, this study found EAEC harboring bla_{TEM} and bla_{SHV} belonged to ST131 that carried class 1 integrons, which isolated from only 3-months of age with asymptomatic children in Thailand (EC097 in Table 5.7 and 5.9). These results emphasize an important role of class 1 integrons dissemination among MDR pathogenic *E. coli* and might be associated with ESBLs-producers in the community.

To understand the genetic relatedness and investigate the possible clonal spread of community-acquired multidrug resistant pathogenic *E. coli* with long term period, the combination of various typing methods and space-time disease surveillance were analyzed. The results showed high diversity in each group of *E. coli* pathotype,

which agrees with the previous study (77, 84, 85), especially, clonal complex (CC)10 is one of the most common STs in the *E. coli* database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and known to be heterogeneous. CC10 was the most prevalent in this study that was presented by four STs including ST10, ST34 (single locus *icd*), ST48 (single locus *adk*), and ST4 (double locus *adk* and *fumC*) variant of ST10. In contrast to previous study reported ST10 belonged to *E. coli* pathotypes except for EPEC, EIEC, EHEC, and MNEC (84), ST10 in this study found in EAEC, EPEC, and ETEC. Not surprisingly, the same ST could be detected in different *E. coli* either pathotype or non-pathotype due to MLST technique is based on DNA sequence of housekeeping genes, which relatively conserved, whereas, the pathogenic *E. coli* is associated with acquired virulence determinants. However, ST182 was predominant as ETEC strain expressing the heat-stable enterotoxin p-variant (STp or STIa), which found in indigenous children, native adults and travelers, suggesting some ETEC strains were clonal expansion. Most of strains with novel sequence types were clonally closely related with STs, e.g., ET048 with ST4589, which isolated in 2001 and EA017 and EA021 with ST4588 which isolated in 2002, were a single locus variant in *fumC* of EC152 with ST795, which recovered in 2006 by only one base and two bases substitution, respectively. Furthermore, all of them belonged to EPEC indicating the genetic drifting in the clonal group. Some isolates were indistinguishable by all high discriminatory typing techniques and identified in the same space and time clusters indicating possible outbreak situations, e.g., EA006 and EA007 (ST200), EA013 and EA015 (ST206), and ET076 and ET077 (ST4) (Table 5.3). During the past ten years, the same clones were present in the same space, e.g., ST4, ST10, ST29, ST34, ST48, ST130, and ST182, suggesting the persistence clones; moreover, the repetitive clones were shared in among these countries indicating the international spread of these clones. Although specimens of this study were collected from human, many STs were matched with strains in *E. coli* database especially, strains from food-producing animals, e.g., ST10, ST29, and ST131, etc (Table 5.4). These findings highlight the role of clonal expansion among these bacteria.

Among three different based molecular typing techniques were applied in this study, plasmid profiling gave the lowest discriminatory power whereas ERIC PCR and MLST had the similar discriminatory power. Although plasmid profiling is

simplicity and low cost technique, the limitations of this technique can affect the reproducibility and stability due to the intrinsic properties of plasmids, e.g., gain or loss and conformational changes of plasmids. In addition, the results of this technique cannot be predicted for the true genetic pathway. Therefore, plasmid profiling should be only used to support the other typing methods. *E. coli* in this study were successfully typed with ERIC-PCR, similar to previous study (86). This technique is simplicity, easy to perform, rapid, low cost, accessibility, reproducibility, and relative stability, which can be applied for small laboratory setting. However, a drawback of this technique is that it is limited to portability of data, inter-laboratory reproducibility and comparison. Meanwhile, results from MLST are highly unambiguous due to an international standardized nomenclature, reproducible, portable, exchangeable, and easily comparable since allele sequences and ST profiles are available in the online database. Not surprisingly, this method has become a very popular tool for global epidemiology and genetic evolution studies (52). This study, HiMLST was carried out to overcome the drawback of classical MLST in point of high cost of DNA sequencing. Recently, Boers SA and team introduced an efficient two-step of 7-plex PCR approach for DNA sequencing in a single next generation sequencing (NGS)-run of 96 isolates (58). This strategy used 14 gene-specific primers for 7 alleles and 192 universal multiplex identifier (MID) primers for 96 bacterial isolates, which each carrying unique MID and 454-specific sequences. The usefulness of this approach is the universal MID primers can be used for new HiMLST experiment even if new bacterial species, which reduce cost of labor and reagents when comparable with classical MLST. In contrast to Sanger sequencing, the optimal size of amplicons (400-500 bp) is an essential for HiMLST protocol, since the efficiency of the emulsion-PCR depend on the lengths of amplicons (58). Therefore, this study had designed the specific PCR primers to each allele from the Achtman MLST typing scheme (65) excepting *adk*, to reduce the amplicon sizes but still covers the informative sequences. Seven housekeeping genes were separately amplified in 3 sets of PCR in both first- and second-round PCR to solve the problem of unequal efficiency of each primer in the multiplex PCR including penta-plex of *adk*, *fumC*, *gyrB*, *icd*, and *mdh*, and simplex PCR of *purA* and *recA*, respectively. However, 8 *E. coli* isolates failed to obtain STs which caused by missing PCR products of some genes, i.e., *fumC*, *gyrB*, and *mdh* due

to allelic sequence variation of that locus, indicating the PCR process is the most critical part of HiMLST, which should be further optimized. In part of sequencing data, the limitation of pyrosequencing, which is the 454-technology, is the low performance for homo-polymeric stretches that are abundant in the MLST target genes of *E. coli* leading to error insertions of bases, followed by deletions. Nonetheless, this technique is absent the substitutions, which are the most common error type of some techniques that can be caused the frame-shift mutation (87). Consequently, the more attention and the manual correction of contigs are required for analysis of these homo-polymeric repeats even if it is a laborious. The cost of HiMLST in this study is approximately 800 baht per bacterial isolate when compared to traditional MLST including PCR reagents that is about 3,000 baht per bacterial strain. By using HiMLST protocol, the cost is apparently reduced more than 3 times compared to MLST with Sanger sequencing. Therefore, HiMLST approach absolutely generates the robust MLST on a large-scale sequencing that can be utilized for new affordable applications or creating new strategies without the obstacle anymore.

CHAPTER VII
CONCLUSION

The emergence of multidrug resistance (MDR) in diarrheagenic *E. coli* is becoming a public health concern, especially for children under five years old in developing countries and international travelers who visit those countries. Although, multidrug resistant *E. coli* has been increasingly reported during the last decade in both the hospital setting and the community, there are few studies on MDR diarrhegenic *E. coli* in community setting, especially about genetic mechanism conferring antimicrobial resistance and their genetic relatedness. Therefore, the study on prevalence, molecular characterization and epidemiology of community-acquired MDR diarrheagenic *E. coli* isolates were conducted.

In this study, 200 community-acquired MDR diarrheagenic *E. coli* isolates from children and adults including natives and travelers among Asian countries including Thailand, Cambodia, Maldives, Nepal, and Vietnam from 2001 to 2010 were subjected to determine the prevalence of class 1 integrons. The presence of class 1 integrons was demonstrated by PCR using specific primers for class1 integrase (*intI1*) gene, 5′-3′ conserved segment (5′CS-3′CS), and dot-blot hybridization. The results exhibited the high prevalence of class 1 integrons in both adults (70.5%) and children (76.4%), which most of them were resistant to 5 antimicrobials including ampicillin, streptomycin, sulfisoxazole, trimethoprim-sulfamethoxazole, and tetracycline. Resistance gene cassettes identified in this study were *aadA1*, *aadA2*, and *aadA5*, which refer to streptomycin and spectinomycin resistance; *dfrA12* and *dfrA17*, which refer to trimethoprim resistance; and *linF* encoding lincosamide resistance. Even though few class A β-lactamases-producing isolates were found in this study (5.1%), most of them (80%) were detected in class 1 integrons-harboring isolates from indigenous children at least 3-months of age. These findings emphasize an important role of class 1 integrons dissemination among MDR diarrheagenic *E. coli* and might be associated with ESBLs-producers in the community. Although, dot-blot

hybridization technique was higher sensitivity than PCR, which can restore 10 integrase negative *E. coli* isolates, this technique has more complicated process than PCR technique. Therefore, dot-blot hybridization should be used to confirm the PCR result. Epidemiological study was performed to understand genetic relatedness and investigate the possible clonal spread among these bacteria by using various typing methods and space-time disease surveillance. Beside high diversity in each group of *E. coli* pathotypes, ST182 was predominant as the ETEC, suggesting some ETEC strains were clonal expansion. The clonal spread in this study can be divided into 3 types: the possible outbreaks, which were indistinguishable by all typing techniques even if space and time clusters; the persistence clones, which were recurrence in the same space during the past ten years; and the international spread clones, which were shared in among Asian countries. Moreover, many STs in this study were matched with strains in *E. coli* database especially, strains from food-producing animals. These findings highlight the role of clonal expansion among these bacteria in human and animals.

In conclusion, this study revealed the long-term genetic relatedness among community-acquired MDR diarrheagenic *E. coli* isolates harboring class 1 integrons, and suggested the circulation of these resistance elements in the community. These results provide crucial public health information for surveillance and control of antimicrobial resistance pathogens in this part of Asia. A cost-effective high-throughput MLST (HiMLST), which coupling next generation sequencing (NGS) to MLST cans overcome the major drawback of classical MLST. This approach absolutely generates the robust MLST on a large-scale sequencing, which can be applied for new strategies in MLST studies using NGS or new affordable applications.

REFERENCES

1. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev. 1998;11(1):142-201.
2. Farthing M, Salam MA, Lindberg G, Dite P, Khalif I, Salazar-Lindo E, et al. Acute diarrhea in adults and children: a global perspective. J Clin Gastroenterol. 2013;47(1):12-20.
3. Giske CG, Monnet DL, Cars O, Carmeli Y. Clinical and economic impact of common multidrug-resistant gram-negative bacilli. Antimicrob Agents Chemother. 2008;52(3):813-21.
4. Brusselaers N, Vogelaers D, Blot S. The rising problem of antimicrobial resistance in the intensive care unit. Ann Intensive Care. 2011;1:47.
5. Tadesse DA, Zhao S, Tong E, Ayers S, Singh A, Bartholomew MJ, et al. Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950-2002. Emerg Infect Dis. 2012;18(5):741-9.
6. Carlet J, Jarlier V, Harbarth S, Voss A, Goossens H, Pittet D, et al. Ready for a world without antibiotics? The Pensieres Antibiotic Resistance Call to Action. Antimicrob Resist Infect Control. 2012;1(1):11.
7. Ajiboye RM, Solberg OD, Lee BM, Raphael E, Debroy C, Riley LW. Global spread of mobile antimicrobial drug resistance determinants in human and animal *Escherichia coli* and *Salmonella* strains causing community-acquired infections. Clin Infect Dis. 2009;49(3):365-71.
8. Moodley A, Guardabassi L. Transmission of IncN plasmids carrying $bla_{\text{CTX-M-1}}$ between commensal *Escherichia coli* in pigs and farm workers. Antimicrob Agents Chemother. 2009;53(4):1709-11.
9. Asai T, Kojima A, Harada K, Ishihara K, Takahashi T, Tamura Y. Correlation between the usage volume of veterinary therapeutic antimicrobials and resistance in *Escherichia coli* isolated from the feces of food-producing animals in Japan. Jpn J Infect Dis. 2005;58(6):369-72.

10. Cocchi S, Grasselli E, Gutacker M, Benagli C, Convert M, Piffaretti JC. Distribution and characterization of integrons in *Escherichia coli* strains of animal and human origin. FEMS Immunol Med Microbiol. 2007;50(1):126-32.
11. Stalder T, Barraud O, Casellas M, Dagot C, Ploy MC. Integron involvement in environmental spread of antibiotic resistance. Front Microbiol. 2012;3:119.
12. Cambray G, Guerout AM, Mazel D. Integrons. Annu Rev Genet. 2010;44:141-66.
13. Levesque C, Piche L, Larose C, Roy PH. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob Agents Chemother. 1995;39(1):185-91.
14. Gillings MR. Integrons: past, present, and future. Microbiol Mol Biol Rev. 2014;78(2):257-77.
15. Ranjbar R, Karami A, Farshad S, Giammanco GM, Mammina C. Typing methods used in the molecular epidemiology of microbial pathogens: a how-to guide. New Microbiol. 2014;37(1):1-15.
16. Todar K. Pathogenic *E. coli*. Todar's Online Textbook of Bacteriology [Internet]. [cited 2014 Nov 15]. Available from: http://textbookofbacteriology. net/e.coli.html.
17. Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. Nat Rev Microbiol. 2004;2(2):123-40.
18. Croxen MA, Finlay BB. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Microbiol. 2010;8(1):26-38.
19. Ahmed N, Dobrindt U, Hacker J, Hasnain SE. Genomic fluidity and pathogenic bacteria: applications in diagnostics, epidemiology and intervention. Nat Rev Microbiol. 2008;6(5):387-94.
20. Cennimo DJ, Koo H, Mohamed JA, Huang DB, Chiang T. Enteroaggregative *Escherichia coli* : A review of trends, diagnosis, and treatment. Infect Med. 2007;24:100-10.
21. Center for Disease Control and Prevention [Internet]. Estimating foodborne illness: an overview. [cited 2014 December 9]. Available from: http://www.cdc.gov/food borneburden/attribution/overview.html.

22. Flynn D. USDA: U.S. foodborne illnesses cost more than $15.6 billion annually. Food Safety News [Internet]. 2014. [cited 2015 Jun 7]. Available from: http://www.foodsafetynews.com/2014/10/foodborne-illnesses-cost-usa-15-6-billion-annually/#.VXQHU 1KHVpm.
23. United States Department of Agriculture Economic Research Service [Internet]. Cost estimates of foodborne illnesses. [updated 2014 Nov 12; cited 2015 Jun 7]. Available from: http://www.ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses.aspx# 48972.
24. Bureau of Epidemiology, MoPH, Thailand [Internet]. Diarrhoea. [cited 2014 Dec 16]. Available from: http://www.boe.moph.go.th/boedb/surdata/506wk/y57/en/d02_4957_en. pdf.
25. National Institute of Allergy and Infectious Diseases [Internet]. Foodborne diseases. [updated 2014 Jun 25; cited 2014 Dec 9]. Available from: http://www.niaid.nih.gov/topics/foodborne/Pages/Default.aspx.
26. Newell DG, Koopmans M, Verhoef L, Duizer E, Aidara-Kane A, Sprong H, et al. Food-borne diseases - the challenges of 20 years ago still persist while new ones continue to emerge. Int J Food Microbiol.2010;139 Suppl 1:S3-15.
27. Loglisci R. Animals consume lion's share of antibiotics. Food Safety News [Internet]. 2010 [cited 2015 Jan 27]. Available from: http://www.foodsafetynews.com/2010/12/1nimals-consume-lions-share-of-antibiotics/#.VMcRX2 isXkU.
28. Alliance for the Prudent Use of Antibiotics (APUA) [Internet]. Consequences of antibiotic misuse in food animals and interventions: APUA background paper.[cited 2015 Jan 26]. Available from: http://www.tufts.edu/med/apua/news/press_release_2010-6.shtml.
29. Stecher B, Denzler R, Maier L, Bernet F, Sanders MJ, Pickard DJ, et al. Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. Proc Natl Acad Sci U S A. 2012;109(4):1269-74.
30. Aarestrup FM, Hendriksen RS, Lockett J, Gay K, Teates K, McDermott PF, et al. International spread of multidrug-resistant *Salmonella* Schwarzengrund in food products. Emerg Infect Dis. 2007;13(5):726-31.

31. Ahmed AM, Shimamoto T, Shimamoto T. Characterization of integrons and resistance genes in multidrug-resistant *Salmonella enterica* isolated from meat and dairy products in Egypt. Int J Food Microbiol. 2014;189:39-44.
32. Center for Disease Control and Prevention [Internet]. Antibiotic resistance threats in the United States, 2013. [cited 2015 Jan 12]. Available from: http://www.cdc. gov/drugresistance/pdf/ar-threats-2013-508.pdf.
33. Nikaido H. Multidrug resistance in bacteria. Annu Rev Biochem. 2009;78:119-46.
34. Davies J. Microbes have the last word. A drastic re-evaluation of antimicrobial treatment is needed to overcome the threat of antibiotic-resistant bacteria. EMBO Rep. 2007;8(7):616-21.
35. Wright GD. Q&A: Antibiotic resistance: where does it come from and what can we do about it? BMC Biol. 2010;8:123.
36. Delcour AH. Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta. 2009;1794(5):808-16.
37. Pages JM, James CE, Winterhalter M. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat Rev Microbiol. 2008;6(12):893-903.
38. Debabov D. Antibiotic resistance: Origins, mechanisms, approaches to counter. Appl Biochem Microbiol. 2013;49(8):665-71.
39. Piddock LJ. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol. 2006;4(8):629-36.
40. Wright GD. Molecular mechanisms of antibiotic resistance. Chem Commun (Camb). 2011;47(14):4055-61.
41. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol. 2015;13(1):42-51.
42. Livermore DM. Beta-Lactamases in laboratory and clinical resistance. Clin Microbiol Rev. 1995;8(4):557-84.
43. Kumar S, Varela MF. Molecular mechanisms of bacterial resistance to antimicrobial agents In: Méndez-Vilas A, editor. Microbial pathogens and strategies for combating them: science, technology and education. vol. 1. Badajoz: Formatex Research Center; 2013. p. 522-34.

44. Huovinen P. Resistance to trimethoprim-sulfamethoxazole. Clinical infectious diseases: Clin Infect Dis. 2001;32(11):1608-14.
45. Uchida Y, Mochimaru T, Morokuma Y, Kiyosuke M, Fujise M, Eto F, et al. Clonal spread in Eastern Asia of ciprofloxacin-resistant Escherichia coli serogroup O25 strains, and associated virulence factors. Int J Antimicrob Agents. 2010;35(5):444-50.
46. Ochman H, Lawrence JG, Groisman EA. Lateral gene transfer and the nature of bacterial innovation. Nature. 2000;405(6784):299-304.
47. Blakely GW. Mechanisms of horizontal gene transfer and DNA recombination. In: Tang,YW, Sussman M, Liu D, Poxton IR, Schwartzman J, editors Molecular medical microbiology. 2nd ed. London: Academic Press; 2015. p. 291-302.
48. Mazel D. Integrons: agents of bacterial evolution. Nat Rev Microbiol. 2006;4(8):608-20.
49. Labbate M, Case RJ, Stokes HW. The integron/gene cassette system: an active player in bacterial adaptation. Methods Mol Biol. 2009;532:103-25.
50. Recchia GD, Hall RM. Gene cassettes: a new class of mobile element. Microbiology. 1995;141 (Pt 12):3015-27.
51. Brown HJ, Stokes HW, Hall RM. The integrons In0, In2, and In5 are defective transposon derivatives. J Bacteriol. 1996;178(15):4429-37.
52. Bonofiglio L, Gardella N, Mollerach M. Application of molecular typing methods to the study of medically relevant gram-positive cocci. In: Magdeldin S, editor. Gel electrophoresis - advanced techniques: InTech; 2012. p. 113-38.
53. Riley LW. Laboratory methods used for strain typing of pathogens: conventional and molecular techniques. Molecular epidemiology of infectious diseases: principles and practices. USA: ASM Press; 2004. p. 29-62.
54. Threlfall EJ, Woodford N. Plasmid profile typing and plasmid fingerprinting. Methods Mol Biol. 1995;46:225-36.
55. Lupski JR, Weinstock GM. Short, interspersed repetitive DNA sequences in prokaryotic genomes. J Bacteriol. 1992;174(14):4525-9.

56. Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 1991;19(24):6823-31.

57. Perez-Losada M, Cabezas P, Castro-Nallar E, Crandall KA. Pathogen typing in the genomics era: MLST and the future of molecular epidemiology. Infect Genet Evol. 2013;16:38-53.

58. Boers SA, van der Reijden WA, Jansen R. High-throughput multilocus sequence typing: bringing molecular typing to the next level. PloS one. 2012;7(7):e39630.

59. CLSI. Performance standard for antimicrobial disk susceptibility tests; approved standard-eleventh edition. CLSI document M02-A11. vol. 32. 11th ed. Clinical and Laboratory Standadrs Institute: Wayne, PA; 2012.

60. CLSI. Performance standards for antimicrobial susceptibility testing; twenty-third information supplement. CLSI document M100-S23. vol. 33. 23th ed. Wayne, PA: Clinical and Laboratory Standard Institute; 2013.

61. Ng LK, Mulvey MR, Martin I, Peters GA, Johnson W. Genetic characterization of antimicrobial resistance in Canadian isolates of *Salmonella* serovar Typhimurium DT104. Antimicrob Agents Chemother. 1999;43(12):3018-21.

62. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Short protocols in molecular biology: a compendium of methods from current protocols in molecular biology. vol. 1. 5th ed. United Statesof America: Wiley; 2002.

63. Rasheed JK, Jay C, Metchock B, Berkowitz F, Weigel L, Crellin J, et al. Evolution of extended-spectrum beta-lactam resistance (SHV-8) in a strain of *Escherichia coli* during multiple episodes of bacteremia. Antimicrob Agents Chemother. 1997;41(3):647-53.

64. Saladin M, Cao VT, Lambert T, Donay JL, Herrmann JL, Ould-Hocine Z, et al. Diversity of CTX-M β-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. FEMS Microbiol Lett. 2002;209(2):161-8.

65. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol. 2006;60(5):1136-51.
66. Nguyen TV, Le PV, Le CH, Weintraub A. Antibiotic resistance in diarrheagenic *Escherichia coli* and *Shigella* strains isolated from children in Hanoi, Vietnam. Antimicrob Agents Chemother. 2005;49(2):816-9.
67. Mosquito S, Ruiz J, Pons MJ, Durand D, Barletta F, Ochoa TJ. Molecular mechanisms of antibiotic resistance in diarrhoeagenic *Escherichia coli* isolated from children. Int J Antimicrob Agents 2012;40(6):544-8.
68. Meng CY, Smith BL, Bodhidatta L, Richard SA, Vansith K, Thy B, et al. Etiology of diarrhea in young children and patterns of antibiotic resistance in Cambodia. Pediatr Infect Dis J. 2011;30(4):331-5.
69. Kalantar E, Soheili F, Salimi H, Soltan Dallal MM. Frequency, antimicrobial susceptibility and plasmid profiles of *Escherichia coli* pathotypes obtained from children with acute diarrhea. Jundishapur J Microbiol. 2011;4(1):23-8.
70. Kargar M, Mohammadalipour Z, Doosti A, Lorzadeh S, Japoni-Nejad A. High Prevalence of Class 1 to 3 Integrons Among Multidrug-Resistant Diarrheagenic *Escherichia coli* in Southwest of Iran. Osong Public Health Res Perspect. 2014;5(4):193-8.
71. Omran EA, Mahafzah AM, Shehabi AA. Antimicrobial resistance patterns of diarrheagenic and nondiarrheagenic *Escherichia coli* isolates from Libyan children. Int Arab J Antimicrob Agents. 2014;4(1):1-8.
72. Cergole-Novella MC, Pignatari AC, Castanheira M, Guth BE. Molecular typing of antimicrobial-resistant Shiga-toxin-producing *Escherichia coli* strains (STEC) in Brazil. Res Microbiol. 2011;162(2):117-23.
73. Shahcheraghi F, Rahmati Ghezelgeh F, Nobari S, Torabi E, Mousavi SF, Aslani MM, et al. Identification and characterization of class 1 integrons among atypical enteropathogenic *Escherichia coli* isolated from children under 5 years of age. Iran J Microbiol. 2014;6(3):156-62.
74. Dow MA, Toth I, Malik A, Herpay M, Nogrady N, Ghenghesh KS, et al. Phenotypic and genetic characterization of enteropathogenic *Escherichia*

coli (EPEC) and entero-aggregative *E. coli* (EAEC) from diarrhoeal and non-diarrhoeal children in Libya. Comp Immunol Microbiol Infect Dis. 2006;29(2-3):100-13.

75. Dawes FE, Kuzevski A, Bettelheim KA, Hornitzky MA, Djordjevic SP, Walker MJ. Distribution of class 1 integrons with IS26-mediated deletions in their 3'-conserved segments in *Escherichia coli* of human and animal origin. PloS one. 2010;5(9):e12754.
76. Chang LL, Chang TM, Chang CY. Variable gene cassette patterns of class 1 integron-associated drug-resistant Escherichia coli in Taiwan. Kaohsiung J Med Sci. 2007;23(6):273-80.
77. Najibi S, Bakhshi B, Fallahzad S, Pourshafie MR, Katouli M, Sattari M, et al. Distribution of class 1 integrons among enteropathogenic *Escherichia coli*. Can J Microbiol. 2012;58(5):637-43.
78. Krauland MG, Marsh JW, Paterson DL, Harrison LH. Integron-mediated multidrug resistance in a global collection of nontyphoidal *Salmonella enterica* isolates. Emerg Infect Dis. 2009;15(3):388-96.
79. Heir E, Lindstedt BA, Leegaard TM, Gjernes E, Kapperud G. Prevalence and characterization of integrons in blood culture *Enterobacteriaceae* and gastrointestinal *Escherichia coli* in Norway and reporting of a novel class 1 integron-located lincosamide resistance gene. Ann Clin Microbiol Antimicrob. 2004;3:12.
80. Kor SB, Choo QC, Chew CH. New integron gene arrays from multiresistant clinical isolates of members of the *Enterobacteriaceae* and *Pseudomonas aeruginosa* from hospitals in Malaysia. J Med Microbiol. 2013;62(Pt 3):412-20.
81. Chaisatit C, Tribuddharat C, Pulsrikarn C, Dejsirilert S. Molecular characterization of antibiotic-resistant bacteria in contaminated chicken meat sold at supermarkets in Bangkok, Thailand. Jpn J Infect Dis. 2012;65(6):527-34.
82. Pokhrel RH, Thapa B, Kafle R, Shah PK, Tribuddharat C. Co-existence of β-lactamases in clinical isolates of *Escherichia coli* from Kathmandu, Nepal. BMC Res Notes. 2014;7:694.

83. Phongpaichit S, Tunyapanit W, Pruekprasert P. Antimicrobial resistance, class 1 integrons and extended-spectrum β-lactamases in *Escherichia coli* clinical isolates from patients in South Thailand. J Health Sci. 2011;57(3):281-8.
84. Okeke IN, Wallace-Gadsden F, Simons HR, Matthews N, Labar AS, Hwang J, et al. Multi-locus sequence typing of enteroaggregative *Escherichia coli* isolates from Nigerian children uncovers multiple lineages. PloS one. 2010;5(11):e14093.
85. Pan H, Zhang J, Kuang D, Yang X, Ju W, Huang Z, et al. Molecular analysis and antimicrobial susceptibility of enterotoxigenic *Escherichia coli* from diarrheal patients. Diagn Microbiol Infect Dis. 2015;81(2):126-31.
86. Kosek M, Yori PP, Gilman RH, Vela H, Olortegui MP, Chavez CB, et al. Facilitated molecular typing of *Shigella* isolates using ERIC-PCR. Am J Trop Med Hyg. 2012;86(6):1018-25.
87. Metzker ML. Sequencing technologies - the next generation. Nat Rev Genet. 2010;11(1):31-46.

APPENDICES

APPENDIX A

1. Reagents for Alkaline lysis miniprep

1.1 Alkaline buffer I

Glucose	0.9	g
1 M Tris-cl, pH 8.0	2.5	ml
0.5 M EDTA, pH 8.0	2	ml
Distilled water to	100	ml

Buffer was mixed, autoclaved at 121˚C for 15 minutes, and kept at 4ºC.

1.2 1 M Tris-Cl, pH 8.0 (or 7.5, 9.5)

Tris base	121.1	g
Distilled water	800	ml

All ingredients were mixed, adjusted pH to 8.0 (or 7.5, 9.5), and added distilled water to 1 L. Solution was autoclaved at 121˚C for 15 minutes, and kept at room temperature.

1.3 0.5 M EDTA, pH 8.0

$Na_2EDTA.2H_2O$	186.1	g
Distilled water	800	ml

All ingredients were mixed, adjusted pH to 8.0 by using NaOH pellet, and added distilled water to 1 L. Solution was autoclaved at 121˚C for 15 minutes, and kept at room temperature.

1.4 Alkaline lysis buffer II

0.2 M NaOH

1% SDS

A desired volume of 0.2 M NaOH/1% SDS solution was freshly prepared from 10 M NaOH and 10% SDS.

1.5 10 M NaOH

NaOH	40	g
Distilled water to	100	ml

Solution was mixed and kept at room temperature.

1.6 10% SDS

SDS powder	10	g
Distilled water	80	ml

Solution was mixed, added distilled water to 1L, and kept at room temperature.

1.7 Alkaline lysis buffer III

Potassium acetate	29.4	g
Glacial acetic acid	11.5	ml
Distilled water to	100	ml

Solution was mixed and kept at room temperature.

2. Reagent for dot blot hybridization

2.1 Genius buffer 1

1 M Tris-Cl, pH 7.5	100	ml
5 M NaCl	30	ml
Distilled water to	1000	ml

Solution was mixed and kept at room temperature.

2.2 5 M NaCl

NaCl	292.2	g
Distilled water	800	ml

Solution was mixed, added distilled water to 1 L, autoclaved at 121˚C for 15 minutes, and kept at room temperature.

2.3 Genius buffer 2

Genius buffer 1	400	ml
Blocking reagent	8	g
(Roche Applied Science)		

Solution was mixed and kept at -20ºC. The solution was warmed before used.

2.4 Genius buffer 3

1 M Tris-Cl, pH 9.5	100	ml
5 M NaCl	20	ml
1 M $MgCl_2$	50	ml
Distilled water to	1000	ml

Solution was mixed and kept at room temperature.

2.5 1 M $MgCl_2$

$MgCl_2.6H_2O$	20.33	g
Distilled water to	100	ml

Solution was mixed, autoclaved at 121˚C for 15 minutes, and kept at room temperature.

2.6 Standard hybridization solution

20X SSC	125	ml
N- Lauryl Sarcosine	0.5	g
Blocking reagent	5	g

All ingredients was mixed and heated. One ml of 10% SDS was added in solution, and then brought up volume to 500 ml with distilled water. Solution was mixed and kept at -20ºC. The solution was warmed before used.

2.7 20X SSC

NaCl	175.3	g
Sodium citrate	88.2	g
Distilled water	800	ml

All ingredients were mixed, adjusted pH to 7.0, brought up volume to 1 L with distilled water, and then autoclaved at 121˚C for 15 minutes. Solution was kept at room temperature.

2.8 Wash solution 1 (2X SSC, 0.1% SDS)

20X SSC	100	ml
10% SDS	10	ml
Distilled water to	1000	ml

Solution was mixed and kept at room temperature.

2.9 Wash solution 2 (0.5 X SSC, 0.1 % SDS)

20X SSC	25	ml
10% SDS	10	ml
Distilled water to	1000	ml

Solution was mixed and kept at room temperature.

2.10 TE/SDS (10mM Tris, pH 8.0; 1mM EDTA; 0.1 % SDS)

1 M Tris-Cl, pH 8.0	100	µl
0.5 M EDTA, pH 8.0	20	µl
Distilled water	780	µl

Solution was mixed, 100 µl of 10% SDS was added into the solution and brought up volume to 10 ml with distilled water.

2.11 3M NaOAc

Sodium acetate anhydrous ($C_2H_3O_2Na$)	2.46	g
Distilled water	80	ml

Solution was mixed, adjusted pH to 5.2, and brought up volume to 100 ml with distilled water. The solution was autoclaved at 121˚C for 15 minutes. Solution was kept at room temperature.

3. 10X TBE buffer

Tris base	108	g
Boric acid	55	g
Distilled water	700	ml

All ingredients were thoroughly mixed. Forty ml of 0.5 M EDTA, pH 8.0 was added, brought up volume to 1 L with distilled water, and mixed. Solution was kept at room temperature.

APPENDIX B

Data Suppl. The fusion primer sequences for *E. coli* HiMLST protocol**.** 5′-portion is a 25 whose sequence is dictated by the requirements of the 454 Sequencing System for binding to the DNA, adaptor sequences are shown in purple or yellow and sequencing key is shown in green. 3′-portion is universal tail as shown in blue or red. Multiplex Identifiers (MIDs) are used to barcode of each sample and placed immediately after the sequencing key as shown in black.

Primer	Oligonucleotide sequence (5′- 3′)
PrimA-MID001-FORW	CGTATCGCCTCCCTCGCGCCATCAGACGAGTGCGTGACACTATAG
PrimB-MID001-REV	CTATGCGCCTTGCCAGCCCGCTCAGACGAGTGCGTCACTATAGGG
PrimA-MID002-FORW	CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACAGACACTATAG
PrimB-MID002-REV	CTATGCGCCTTGCCAGCCCGCTCAGACGCTCGACACACTATAGGG
PrimA-MID003-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCGACACTATAG
PrimB-MID003-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGACGCACTCCACTATAGGG
PrimA-MID004-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGCACTGTAGGACACTATAG
PrimB-MID004-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGCACTGTAGCACTATAGGG
PrimA-MID005-FORW	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGGACACTATAG
PrimB-MID005-REV	CTATGCGCCTTGCCAGCCCGCTCAGATCAGACACGCACTATAGGG
PrimA-MID006-FORW	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGGACACTATAG
PrimB-MID006-REV	CTATGCGCCTTGCCAGCCCGCTCAGATATCGCGAGCACTATAGGG
PrimA-MID007-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGTGTCTCTAGACACTATAG
PrimB-MID007-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGTGTCTCTACACTATAGGG
PrimA-MID008-FORW	CGTATCGCCTCCCTCGCGCCATCAGCTCGCGTGTCGACACTATAG
PrimB-MID008-REV	CTATGCGCCTTGCCAGCCCGCTCAGCTCGCGTGTCCACTATAGGG
PrimA-MID010-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCTCTATGCGGACACTATAG
PrimB-MID010-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCTCTATGCGCACTATAGGG
PrimA-MID011-FORW	CGTATCGCCTCCCTCGCGCCATCAGTGATACGTCTGACACTATAG
PrimB-MID011-REV	CTATGCGCCTTGCCAGCCCGCTCAGTGATACGTCTCACTATAGGG

Data Suppl. The fusion primer sequences for *E. coli* HiMLST protocol**.** 5′-portion is a 25 whose sequence is dictated by the requirements of the 454 Sequencing System for binding to the DNA, adaptor sequences are shown in purple or yellow and sequencing key is shown in green. 3′-portion is universal tail as shown in blue or red. Multiplex Identifiers (MIDs) are used to barcode of each sample and placed immediately after the sequencing key as shown in black (cont.).

Primer	Oligonucleotide sequence (5′- 3′)
PrimA-MID013-FORW	CGTATCGCCTCCCTCGCGCCATCAGCATAGTAGTGGACACTATAG
PrimB-MID013-REV	CTATGCGCCTTGCCAGCCCGCTCAGCATAGTAGTGCACTATAGGG
PrimA-MID014-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGAGAGATACGACACTATAG
PrimB-MID014-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGAGAGATACCACTATAGGG
PrimA-MID015-FORW	CGTATCGCCTCCCTCGCGCCATCAGATACGACGTAGACACTATAG
PrimB-MID015-REV	CTATGCGCCTTGCCAGCCCGCTCAGATACGACGTACACTATAGGG
PrimA-MID016-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCACGTACTAGACACTATAG
PrimB-MID016-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCACGTACTACACTATAGGG
PrimA-MID017-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGTCTAGTACGACACTATAG
PrimB-MID017-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGTCTAGTACCACTATAGGG
PrimA-MID018-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCTACGTAGCGACACTATAG
PrimB-MID018-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCTACGTAGCCACTATAGGG
PrimA-MID019-FORW	CGTATCGCCTCCCTCGCGCCATCAGTGTACTACTCGACACTATAG
PrimB-MID019-REV	CTATGCGCCTTGCCAGCCCGCTCAGTGTACTACTCCACTATAGGG
PrimA-MID020-FORW	CGTATCGCCTCCCTCGCGCCATCAGACGACTACAGGACACTATAG
PrimB-MID020-REV	CTATGCGCCTTGCCAGCCCGCTCAGACGACTACAGCACTATAGGG
PrimA-MID021-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGTAGACTAGGACACTATAG
PrimB-MID021-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGTAGACTAGCACTATAGGG
PrimA-MID022-FORW	CGTATCGCCTCCCTCGCGCCATCAGTACGAGTATGGACACTATAG
PrimB-MID022-REV	CTATGCGCCTTGCCAGCCCGCTCAGTACGAGTATGCACTATAGGG
PrimA-MID023-FORW	CGTATCGCCTCCCTCGCGCCATCAGTACTCTCGTGGACACTATAG
PrimB-MID023-REV	CTATGCGCCTTGCCAGCCCGCTCAGTACTCTCGTGCACTATAGGG
PrimA-MID024-FORW	CGTATCGCCTCCCTCGCGCCATCAGTAGAGACGAGGACACTATAG
PrimB-MID024-REV	CTATGCGCCTTGCCAGCCCGCTCAGTAGAGACGAGCACTATAGGG

Data Suppl. The fusion primer sequences for *E. coli* HiMLST protocol**.** 5′-portion is a 25 whose sequence is dictated by the requirements of the 454 Sequencing System for binding to the DNA, adaptor sequences are shown in purple or yellow and sequencing key is shown in green. 3′-portion is universal tail as shown in blue or red. Multiplex Identifiers (MIDs) are used to barcode of each sample and placed immediately after the sequencing key as shown in black (cont.).

Primer	Oligonucleotide sequence (5′- 3′)
PrimA-MID025-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCGTCGCTCGGACACTATAG
PrimB-MID025-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCGTCGCTCGCACTATAGGG
PrimA-MID026-FORW	CGTATCGCCTCCCTCGCGCCATCAGACATACGCGTGACACTATAG
PrimB-MID026-REV	CTATGCGCCTTGCCAGCCCGCTCAGACATACGCGTCACTATAGGG
PrimA-MID027-FORW	CGTATCGCCTCCCTCGCGCCATCAGACGCGAGTATGACACTATAG
PrimB-MID027-REV	CTATGCGCCTTGCCAGCCCGCTCAGACGCGAGTATCACTATAGGG
PrimA-MID028-FORW	CGTATCGCCTCCCTCGCGCCATCAGACTACTATGTGACACTATAG
PrimB-MID028-REV	CTATGCGCCTTGCCAGCCCGCTCAGACTACTATGTCACTATAGGG
PrimA-MID029-FORW	CGTATCGCCTCCCTCGCGCCATCAGACTGTACAGTGACACTATAG
PrimB-MID029-REV	CTATGCGCCTTGCCAGCCCGCTCAGACTGTACAGTCACTATAGGG
PrimA-MID030-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGACTATACTGACACTATAG
PrimB-MID030-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGACTATACTCACTATAGGG
PrimA-MID031-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGCGTCGTCTGACACTATAG
PrimB-MID031-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGCGTCGTCTCACTATAGGG
PrimA-MID032-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGTACGCTATGACACTATAG
PrimB-MID032-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGTACGCTATCACTATAGGG
PrimA-MID033-FORW	CGTATCGCCTCCCTCGCGCCATCAGATAGAGTACTGACACTATAG
PrimB-MID033-REV	CTATGCGCCTTGCCAGCCCGCTCAGATAGAGTACTCACTATAGGG
PrimA-MID034-FORW	CGTATCGCCTCCCTCGCGCCATCAGCACGCTACGTGACACTATAG
PrimB-MID034-REV	CTATGCGCCTTGCCAGCCCGCTCAGCACGCTACGTCACTATAGGG
PrimA-MID035-FORW	CGTATCGCCTCCCTCGCGCCATCAGCAGTAGACGTGACACTATAG
PrimB-MID035-REV	CTATGCGCCTTGCCAGCCCGCTCAGCAGTAGACGTCACTATAGGG
PrimA-MID036-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGACGTGACTGACACTATAG
PrimB-MID036-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGACGTGACTCACTATAGGG

Data Suppl. The fusion primer sequences for *E. coli* HiMLST protocol**.** 5′-portion is a 25 whose sequence is dictated by the requirements of the 454 Sequencing System for binding to the DNA, adaptor sequences are shown in purple or yellow and sequencing key is shown in green. 3′-portion is universal tail as shown in blue or red. Multiplex Identifiers (MIDs) are used to barcode of each sample and placed immediately after the sequencing key as shown in black (cont.).

Primer	Oligonucleotide sequence (5′- 3′)
PrimA-MID037-FORW	CGTATCGCCTCCCTCGCGCCATCAGTACACACACTGACACTATAG
PrimB-MID037-REV	CTATGCGCCTTGCCAGCCCGCTCAGTACACACACTCACTATAGGG
PrimA-MID038-FORW	CGTATCGCCTCCCTCGCGCCATCAGTACACGTGATGACACTATAG
PrimB-MID038-REV	CTATGCGCCTTGCCAGCCCGCTCAGTACACGTGATCACTATAGGG
PrimA-MID039-FORW	CGTATCGCCTCCCTCGCGCCATCAGTACAGATCGTGACACTATAG
PrimB-MID039-REV	CTATGCGCCTTGCCAGCCCGCTCAGTACAGATCGTCACTATAGGG
PrimA-MID040-FORW	CGTATCGCCTCCCTCGCGCCATCAGTACGCTGTCTGACACTATAG
PrimB-MID040-REV	CTATGCGCCTTGCCAGCCCGCTCAGTACGCTGTCTCACTATAGGG
PrimA-MID041-FORW	CGTATCGCCTCCCTCGCGCCATCAGTAGTGTAGATGACACTATAG
PrimB-MID041-REV	CTATGCGCCTTGCCAGCCCGCTCAGTAGTGTAGATCACTATAGGG
PrimA-MID042-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCGATCACGTGACACTATAG
PrimB-MID042-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCGATCACGTCACTATAGGG
PrimA-MID043-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCGCACTAGTGACACTATAG
PrimB-MID043-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCGCACTAGTCACTATAGGG
PrimA-MID044-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCTAGCGACTGACACTATAG
PrimB-MID044-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCTAGCGACTCACTATAGGG
PrimA-MID045-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCTATACTATGACACTATAG
PrimB-MID045-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCTATACTATCACTATAGGG
PrimA-MID046-FORW	CGTATCGCCTCCCTCGCGCCATCAGTGACGTATGTGACACTATAG
PrimB-MID046-REV	CTATGCGCCTTGCCAGCCCGCTCAGTGACGTATGTCACTATAGGG
PrimA-MID047-FORW	CGTATCGCCTCCCTCGCGCCATCAGTGTGAGTAGTGACACTATAG
PrimB-MID047-REV	CTATGCGCCTTGCCAGCCCGCTCAGTGTGAGTAGTCACTATAGGG
PrimA-MID048-FORW	CGTATCGCCTCCCTCGCGCCATCAGACAGTATATAGACACTATAG
PrimB-MID048-REV	CTATGCGCCTTGCCAGCCCGCTCAGACAGTATATACACTATAGGG

Data Suppl. The fusion primer sequences for *E. coli* HiMLST protocol**.** 5′-portion is a 25 whose sequence is dictated by the requirements of the 454 Sequencing System for binding to the DNA, adaptor sequences are shown in purple or yellow and sequencing key is shown in green. 3′-portion is universal tail as shown in blue or red. Multiplex Identifiers (MIDs) are used to barcode of each sample and placed immediately after the sequencing key as shown in black (cont.).

Primer	Oligonucleotide sequence (5′- 3′)
PrimA-MID049-FORW	CGTATCGCCTCCCTCGCGCCATCAGACGCGATCGAGACACTATAG
PrimB-MID049-REV	CTATGCGCCTTGCCAGCCCGCTCAGACGCGATCGACACTATAGGG
PrimA-MID050-FORW	CGTATCGCCTCCCTCGCGCCATCAGACTAGCAGTAGACACTATAG
PrimB-MID050-REV	CTATGCGCCTTGCCAGCCCGCTCAGACTAGCAGTACACTATAGGG
PrimA-MID051-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGCTCACGTAGACACTATAG
PrimB-MID051-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGCTCACGTACACTATAGGG
PrimA-MID052-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGTATACATAGACACTATAG
PrimB-MID052-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGTATACATACACTATAGGG
PrimA-MID053-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGTCGAGAGAGACACTATAG
PrimB-MID053-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGTCGAGAGACACTATAGGG
PrimA-MID054-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGTGCTACGAGACACTATAG
PrimB-MID054-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGTGCTACGACACTATAGGG
PrimA-MID055-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGATCGTATAGACACTATAG
PrimB-MID055-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGATCGTATACACTATAGGG
PrimA-MID056-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGCAGTACGAGACACTATAG
PrimB-MID056-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGCAGTACGACACTATAGGG
PrimA-MID057-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGCGTATACAGACACTATAG
PrimB-MID057-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGCGTATACACACTATAGGG
PrimA-MID058-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGTACAGTCAGACACTATAG
PrimB-MID058-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGTACAGTCACACTATAGGG
PrimA-MID059-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGTACTCAGAGACACTATAG
PrimB-MID059-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGTACTCAGACACTATAGGG
PrimA-MID060-FORW	CGTATCGCCTCCCTCGCGCCATCAGCTACGCTCTAGACACTATAG
PrimB-MID060-REV	CTATGCGCCTTGCCAGCCCGCTCAGCTACGCTCTACACTATAGGG

Data Suppl. The fusion primer sequences for *E. coli* HiMLST protocol**.** 5′-portion is a 25 whose sequence is dictated by the requirements of the 454 Sequencing System for binding to the DNA, adaptor sequences are shown in purple or yellow and sequencing key is shown in green. 3′-portion is universal tail as shown in blue or red. Multiplex Identifiers (MIDs) are used to barcode of each sample and placed immediately after the sequencing key as shown in black (cont.).

Primer	Oligonucleotide sequence (5′- 3′)
PrimA-MID061-FORW	CGTATCGCCTCCCTCGCGCCATCAGCTATAGCGTAGACACTATAG
PrimB-MID061-REV	CTATGCGCCTTGCCAGCCCGCTCAGCTATAGCGTACACTATAGGG
PrimA-MID062-FORW	CGTATCGCCTCCCTCGCGCCATCAGTACGTCATCAGACACTATAG
PrimB-MID062-REV	CTATGCGCCTTGCCAGCCCGCTCAGTACGTCATCACACTATAGGG
PrimA-MID063-FORW	CGTATCGCCTCCCTCGCGCCATCAGTAGTCGCATAGACACTATAG
PrimB-MID063-REV	CTATGCGCCTTGCCAGCCCGCTCAGTAGTCGCATACACTATAGGG
PrimA-MID064-FORW	CGTATCGCCTCCCTCGCGCCATCAGTATATATACAGACACTATAG
PrimB-MID064-REV	CTATGCGCCTTGCCAGCCCGCTCAGTATATATACACACTATAGGG
PrimA-MID065-FORW	CGTATCGCCTCCCTCGCGCCATCAGTATGCTAGTAGACACTATAG
PrimB-MID065-REV	CTATGCGCCTTGCCAGCCCGCTCAGTATGCTAGTACACTATAGGG
PrimA-MID066-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCACGCGAGAGACACTATAG
PrimB-MID066-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCACGCGAGACACTATAGGG
PrimA-MID067-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCGATAGTGAGACACTATAG
PrimB-MID067-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCGATAGTGACACTATAGGG
PrimA-MID068-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCGCTGCGTAGACACTATAG
PrimB-MID068-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCGCTGCGTACACTATAGGG
PrimA-MID069-FORW	CGTATCGCCTCCCTCGCGCCATCAGTCTGACGTCAGACACTATAG
PrimB-MID069-REV	CTATGCGCCTTGCCAGCCCGCTCAGTCTGACGTCACACTATAGGG
PrimA-MID070-FORW	CGTATCGCCTCCCTCGCGCCATCAGTGAGTCAGTAGACACTATAG
PrimB-MID070-REV	CTATGCGCCTTGCCAGCCCGCTCAGTGAGTCAGTACACTATAGGG
PrimA-MID071-FORW	CGTATCGCCTCCCTCGCGCCATCAGTGTAGTGTGAGACACTATAG
PrimB-MID071-REV	CTATGCGCCTTGCCAGCCCGCTCAGTGTAGTGTGACACTATAGGG
PrimA-MID072-FORW	CGTATCGCCTCCCTCGCGCCATCAGTGTCACACGAGACACTATAG
PrimB-MID072-REV	CTATGCGCCTTGCCAGCCCGCTCAGTGTCACACGACACTATAGGG

Data Suppl. The fusion primer sequences for *E. coli* HiMLST protocol**.** 5′-portion is a 25 whose sequence is dictated by the requirements of the 454 Sequencing System for binding to the DNA, adaptor sequences are shown in purple or yellow and sequencing key is shown in green. 3′-portion is universal tail as shown in blue or red. Multiplex Identifiers (MIDs) are used to barcode of each sample and placed immediately after the sequencing key as shown in black (cont.).

Primer	Oligonucleotide sequence (5′- 3′)
PrimA-MID073-FORW	CGTATCGCCTCCCTCGCGCCATCAGTGTCGTCGCAGACACTATAG
PrimB-MID073-REV	CTATGCGCCTTGCCAGCCCGCTCAGTGTCGTCGCACACTATAGGG
PrimA-MID074-FORW	CGTATCGCCTCCCTCGCGCCATCAGACACATACGCGACACTATAG
PrimB-MID074-REV	CTATGCGCCTTGCCAGCCCGCTCAGACACATACGCCACTATAGGG
PrimA-MID075-FORW	CGTATCGCCTCCCTCGCGCCATCAGACAGTCGTGCGACACTATAG
PrimB-MID075-REV	CTATGCGCCTTGCCAGCCCGCTCAGACAGTCGTGCCACTATAGGG
PrimA-MID076-FORW	CGTATCGCCTCCCTCGCGCCATCAGACATGACGACGACACTATAG
PrimB-MID076-REV	CTATGCGCCTTGCCAGCCCGCTCAGACATGACGACCACTATAGGG
PrimA-MID077-FORW	CGTATCGCCTCCCTCGCGCCATCAGACGACAGCTCGACACTATAG
PrimB-MID077-REV	CTATGCGCCTTGCCAGCCCGCTCAGACGACAGCTCCACTATAGGG
PrimA-MID078-FORW	CGTATCGCCTCCCTCGCGCCATCAGACGTCTCATCGACACTATAG
PrimB-MID078-REV	CTATGCGCCTTGCCAGCCCGCTCAGACGTCTCATCCACTATAGGG
PrimA-MID079-FORW	CGTATCGCCTCCCTCGCGCCATCAGACTCATCTACGACACTATAG
PrimB-MID079-REV	CTATGCGCCTTGCCAGCCCGCTCAGACTCATCTACCACTATAGGG
PrimA-MID080-FORW	CGTATCGCCTCCCTCGCGCCATCAGACTCGCGCACGACACTATAG
PrimB-MID080-REV	CTATGCGCCTTGCCAGCCCGCTCAGACTCGCGCACCACTATAGGG
PrimA-MID081-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGAGCGTCACGACACTATAG
PrimB-MID081-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGAGCGTCACCACTATAGGG
PrimA-MID082-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGCGACTAGCGACACTATAG
PrimB-MID082-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGCGACTAGCCACTATAGGG
PrimA-MID083-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGTAGTGATCGACACTATAG
PrimB-MID083-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGTAGTGATCCACTATAGGG
PrimA-MID084-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGTGACACACGACACTATAG
PrimB-MID084-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGTGACACACCACTATAGGG

Data Suppl. The fusion primer sequences for *E. coli* HiMLST protocol**.** 5′-portion is a 25 whose sequence is dictated by the requirements of the 454 Sequencing System for binding to the DNA, adaptor sequences are shown in purple or yellow and sequencing key is shown in green. 3′-portion is universal tail as shown in blue or red. Multiplex Identifiers (MIDs) are used to barcode of each sample and placed immediately after the sequencing key as shown in black (cont.).

Primer	Oligonucleotide sequence (5′- 3′)
PrimA-MID085-FORW	CGTATCGCCTCCCTCGCGCCATCAGAGTGTATGTCGACACTATAG
PrimB-MID085-REV	CTATGCGCCTTGCCAGCCCGCTCAGAGTGTATGTCCACTATAGGG
PrimA-MID086-FORW	CGTATCGCCTCCCTCGCGCCATCAGATAGATAGACGACACTATAG
PrimB-MID086-REV	CTATGCGCCTTGCCAGCCCGCTCAGATAGATAGACCACTATAGGG
PrimA-MID087-FORW	CGTATCGCCTCCCTCGCGCCATCAGATATAGTCGCGACACTATAG
PrimB-MID087-REV	CTATGCGCCTTGCCAGCCCGCTCAGATATAGTCGCCACTATAGGG
PrimA-MID088-FORW	CGTATCGCCTCCCTCGCGCCATCAGATCTACTGACGACACTATAG
PrimB-MID088-REV	CTATGCGCCTTGCCAGCCCGCTCAGATCTACTGACCACTATAGGG
PrimA-MID089-FORW	CGTATCGCCTCCCTCGCGCCATCAGCACGTAGATCGACACTATAG
PrimB-MID089-REV	CTATGCGCCTTGCCAGCCCGCTCAGCACGTAGATCCACTATAGGG
PrimA-MID090-FORW	CGTATCGCCTCCCTCGCGCCATCAGCACGTGTCGCGACACTATAG
PrimB-MID090-REV	CTATGCGCCTTGCCAGCCCGCTCAGCACGTGTCGCCACTATAGGG
PrimA-MID091-FORW	CGTATCGCCTCCCTCGCGCCATCAGCATACTCTACGACACTATAG
PrimB-MID091-REV	CTATGCGCCTTGCCAGCCCGCTCAGCATACTCTACCACTATAGGG
PrimA-MID092-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGACACTATCGACACTATAG
PrimB-MID092-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGACACTATCCACTATAGGG
PrimA-MID093-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGAGACGCGCGACACTATAG
PrimB-MID093-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGAGACGCGCCACTATAGGG
PrimA-MID094-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGTATGCGACGACACTATAG
PrimB-MID094-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGTATGCGACCACTATAGGG
PrimA-MID095-FORW	CGTATCGCCTCCCTCGCGCCATCAGCGTCGATCTCGACACTATAG
PrimB-MID095-REV	CTATGCGCCTTGCCAGCCCGCTCAGCGTCGATCTCCACTATAGGG
PrimA-MID096-FORW	CGTATCGCCTCCCTCGCGCCATCAGCTACGACTGCGACACTATAG
PrimB-MID096-REV	CTATGCGCCTTGCCAGCCCGCTCAGCTACGACTGCCACTATAGGG

Data Suppl. The fusion primer sequences for *E. coli* HiMLST protocol**.** 5′-portion is a 25 whose sequence is dictated by the requirements of the 454 Sequencing System for binding to the DNA, adaptor sequences are shown in purple or yellow and sequencing key is shown in green. 3′-portion is universal tail as shown in blue or red. Multiplex Identifiers (MIDs) are used to barcode of each sample and placed immediately after the sequencing key as shown in black (cont.).

Primer	**Oligonucleotide sequence (5′- 3′)**
PrimA-MID097-FORW	CGTATCGCCTCCCTCGCGCCATCAGCTAGTCACTCGACACTATAG
PrimB-MID097-REV	CTATGCGCCTTGCCAGCCCGCTCAGCTAGTCACTCCACTATAGGG
PrimA-MID098-FORW	CGTATCGCCTCCCTCGCGCCATCAGCTCTACGCTCGACACTATAG
PrimB-MID098-REV	CTATGCGCCTTGCCAGCCCGCTCAGCTCTACGCTCCACTATAGGG

BIOGRAPHY

NAME	Miss Supaporn Ruksasiri
DATE OF BIRTH	15 June 1981
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Chulalongorn University, 1999-2002: Bachelor of Science (Medical Technology) Mahidol University, 2011-2015: Master of Science (Microbiology)
RESEARCH GRANT	Siriraj Graduate Thesis scholarship from Faculty of Medicine Siriraj Hospital, Mahidol University Armed Forces Health Surveillance Center-Global Emerging Infections Surveillance (AFHSC-GEIS), Washington, DC
HOME ADDRESS	55/13 Leab Klong Sam Rd, Tambon Prachathipat, Amphoe Thanyaburi, Pathum thani 12130 Tel. 0816434402 E-mail: smile_again24@hotmail.com
PUBLICATION / PRESENTATION	"Molecular characterization and long-term epidemiological study of multidrug resistant community-acquired diarrheagenic *Escherichia coli* by HiMLST" NGS2014, Winsor Suites Hotel, Bangkok, Thailand, 29-30 July 2014.