PREVALENCE AND MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANT *SALMONELLA* SPP. AND *ESCHERICHIA COLI* IN RAW CHICKEN MEAT FROM SUPERMARKETS IN BANGKOK

CHAIYAPORN CHAISATIT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE (MICROBIOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2012

COPYRIGHT OF MAHIDOL UNIVERSITY

Thesis
entitled

PREVALENCE AND MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANT *SALMONELLA* SPP. AND *ESCHERICHIA COLI* IN RAW CHICKEN MEAT FROM SUPERMARKETS IN BANGKOK

……………………………………..
Mr. Chaiyaporn Chaisatit
Candidate

……………………………………..
Assist. Prof. Chanwit Tribuddharat,
M.D., Ph.D.
Major advisor

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

…………………………………….
Assoc. Prof. Somporn Srifuengfung,
Ph.D.
Co-advisor

……………………………………..
Mr. Iyarit Thaipisuttikul,
M.D., Ph.D.
Co-advisor

……………………………………..
Prof. Banchong Mahaisavariya,
M.D., Dip.Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

……………………………………..
Prof. Ruengpung Sutthent,
M.D., Ph.D.
Program Director
Master of Science Program
in Microbiology
Faculty of Medicine Siriraj Hospital
Mahidol University

Thesis
entitled

PREVALENCE AND MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANT *SALMONELLA* SPP. AND *ESCHERICHIA COLI* IN RAW CHICKEN MEAT FROM SUPERMARKETS IN BANGKOK

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Microbiology)
on
April 24, 2012

……………………………………..
Mr. Chaiyaporn Chaisatit
Candidate

……………………………………..
Assoc. Prof. Pintip Pongpech,
Ph.D.
Chair

……………………………………..
Assist. Prof. Chanwit Tribuddharat,
M.D., Ph.D.
Member

……………………………………..
Assoc. Prof. Chertsak Dhiraputra,
M.D.
Member

…………………………………….
Assoc. Prof. Somporn Srifuengfung,
Ph.D.
Member

……………………………………..
Mr. Iyarit Thaipisuttikul,
M.D., Ph.D.
Member

……………………………………..
Prof. Banchong Mahaisavariya,
M.D., Dip.Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

……………………………………..
Clin. Prof. Udom Kachintorn,
M.D., M.R.C.P.T.
Dean
Faculty of Medicine Siriraj Hospital
Mahidol University

ACKNOWLEDGEMENTS

First of all, I would like to express a gratitude to my advisor Assist. Prof. Chanwit Tribuddharat for his valuable guidance, advice, support, and helpful comments throughout this study. I am lucky to be his student and have a chance to work with him. This thesis cannot be succeeded without him. I also thank him for being a role model for me and other students in the CT's family.

My special gratefulness is expressed to my thesis committee; Assoc. Prof. Chertsak Dhiraputra, Assoc. Prof. Somporn Srifuengfung, Ajarn Iyarit Thaipisuttikul, and Assoc. Prof. Pintip Pongpech, for their comments, and helpful suggestion of this thesis.

I would like to thank Apirada Punyadee and Thonchaya Nitcharat for their help and support in antibiotic susceptibility testing in this study. I have learned many good things from them during 3 years in Phyathai 2 Hospital.

I would like to thank Mrs. Kesanee Kanchanalai for a donation for graduate students to do the research for their studies in CT research laboratory.

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

I also thank all CT's family members; Paweena Kongsanan, Tossawan Jitwasinkul, Sasiprapa Prombhul, Benjaporn Phusri, Paweena Naksena, Sirirat Chuanphung for their friendship and helpful suggestion throughout my study.

My special gratitude is expressed to my family for their support, belief, and confidence in me to select my own destiny.

PREVALENCE AND MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANT *SALMONELLA* SPP. AND *ESCHERICHIA COLI* IN RAW CHICKEN MEAT FROM SUPERMARKETS IN BANGKOK

CHAIYAPORN CHAISATIT 5236043 SIMI/M

M.Sc. (MICROBIOLOGY)

THESIS ADVISORY COMMITTEE : CHANWIT TRIBUDDHARAT, M.D., Ph.D., CHERTSAK DHIRAPUTRA, M.D., M.Sc., SOMPORN SRIFUENGFUNG, Ph.D., IYARIT THAIPISUTTIKUL, M.D., Ph.D.

ABSTRACT

Antibiotic resistant bacteria that contaminate food pose a major health risk and possibly a large economic loss worldwide. In Thailand, even though prevalence data are available, little is known about molecular characteristics of these bacteria. In this study, 200 sealed packages of fresh chicken meat were purchased from supermarkets in Bangkok, Thailand, and the prevalence of *Salmonella* spp. and *Escherichia coli* were determined according to standard methods. The prevalence of *Salmonella* spp. and *E. coli* were 18.67% (14/75) and 53% (106/200), respectively. Most probable number (MPN) analysis showed that 56.66% (34/60) of the samples violated the limit of allowable coliform bacteria in raw chicken meat, which had the highest value of 46,000. Multidrug resistant phenotypes were found in both *Salmonella* spp. and *E. coli*, and demonstrated high resistance to ampicillin, gentamicin, and tetracycline. The presence of class 1 integron was investigated by polymerase chain reaction (PCR), and dot blot hybridization with probe specific to *intI1*, and confirmed by DNA sequencing. The PCR showed that class 1 integron was found in 42.86% (6/14), and 37.74% (40/106) of *Salmonella* spp. and *E. coli*, respectively. Resistance genes identified in this study were *aadA2*, *aadA4*, *aadA22*, and *aadA23* (for aminoglycoside resistance); *dfrA5* (for trimethoprim resistance); and *lnuF* (for lincosamide resistance). Genetic relatedness among these bacteria was determined by plasmid profile study, randomly amplified polymorphic DNA PCR (RAPD-PCR), and multilocus sequence typing (MLST). The spread of resistant bacteria among supermarkets was evidenced from typing data incorporated with demographic data. Four *Salmonella* isolates were subjected to MLST analysis. MLST results were ST 50, ST 96, ST 1543, and ST 1549, which matched well with strains from many countries, e.g., Vietnam, Denmark, Chile, and USA, reflecting an international spread. In conclusion, this study revealed that both class 1 integrons and their bacterial carriers now have been spread into community sources, and clonal spread played an important role contributing to antibiotic resistance gene spread. Antibiotic usage in animals should be tightly monitored in order to limit an emergence and a spread of resistant bacteria.

KEY WORDS: INTEGRON/ MOLECULAR EPIDEMIOLOGY/ FOOD SAFETY/ CLONAL SPREAD

126 pages

ความชุกและการตรวจระดับอณูชีวโมเลกุลของเชื้อ *Salmonella* spp. และ *Escherichia coli* ที่ดื้อต่อยาต้านจุลชีพหลายขนาน จากเนื้อไก่สดใน supermarket ในกรุงเทพมหานคร

PREVALENCE AND MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANT *SALMONELLA* SPP. AND *ESCHERICHIA COLI* IN RAW CHICKEN MEAT FROM SUPERMARKETS IN BANGKOK

ไชยพร ชัยสถิตย์ 5236043 SIMI/M

วท.ม. (จุลชีววิทยา)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ : ชาญวิทย์ ตรีพุทธรัตน์, M.D., Ph.D., เชิดศักดิ์ ธีระบุตร, M.D., M.Sc., สมพร ศรีเฟื่องฟุ้ง, Ph.D., ไอยฤทธิ์ ไทยพิสุทธิกุล, M.D., Ph.D.

บทคัดย่อ

การปนเปื้อนของเชื้อแบคทีเรียดื้อยาในอาหารนับว่าเป็นปัญหาทางสาธารณสุขที่สำคัญและอาจรวมถึงปัญหาทางเศรษฐกิจที่มีผลกระทบทั่วโลก ในประเทศไทยถึงแม้ว่าจะมีรายงานความชุกของการปนเปื้อนของแบคทีเรียดื้อยาในอาหาร แต่ข้อมูลทางด้านลักษณะทางอณูชีวโมเลกุลของเชื้อเหล่านี้ยังมีน้อย การศึกษาครั้งนี้ได้ทำการทดสอบความชุกของเชื้อ *Salmonella* spp. และ *Escherichia coli* จากแพ็คเนื้อไก่สดจำนวน 200 ตัวอย่างจากซุปเปอร์มาร์เก็ตในกรุงเทพมหานคร ความชุกของเชื้อ *Salmonella* spp. และ *E. coli* คือ 18.67% (14/75) และ 53% (106/200) ตามลำดับ นอกจากนั้นได้ทำการทดสอบการปนเปื้อนของเชื้อโคลิฟอร์มแบคทีเรียโดยวิธี most probable number analysis (MPN) พบว่า 56.66% (34/60) ของเนื้อไก่สดมีการปนเปื้อนของเชื้อโคลิฟอร์มแบคทีเรียเกินเกณฑ์ที่ยอมรับได้ โดยมีค่า MPN สูงสุดคือ 46,000 ส่วนการทดสอบการดื้อยาต้านจุลชีพของเชื้อเหล่านี้โดยวิธี disk diffusion method พบลักษณะการดื้อต่อยาต้านจุลชีพอย่างน้อย 3 กลุ่ม (multi-drug resistance) ทั้งในเชื้อ *Salmonella* spp. และ *E. coli* โดยทั้ง 2 เชื้อส่วนมากให้ผลดื้อต่อยาแอมพิซิลิน เจนต้ามัยซินและเตตร้าซัยคลิน การตรวจหา class 1 integron ได้ใช้วิธีเพิ่มจำนวนสารพันธุกรรมแบบลูกโซ่ (PCR) และวิธี dot blot hybridization โดยใช้ยีนเป้าหมายคือ *intI1* และทำการยืนยันด้วยวิธี DNA sequencing ผลการตรวจโดยวิธีเพิ่มจำนวนสารพันธุกรรมแบบลูกโซ่ (PCR) พบ class 1 integron 42.86% (6/14) และ 37.74% (40/106) ใน *Salmonella* spp. และ *E. coli* ตามลำดับ ยีนดื้อยาที่พบในการศึกษาครั้งนี้ได้แก่ *aadA2*, *aadA4*, *aadA22* และ *aadA23* (ดื้อต่อยาในกลุ่มอะมิโนไกลโคไซด์) *dfrA5* (ดื้อต่อยาไตรเมโทพริม) และ *lnuF* (ดื้อต่อยาในกลุ่มลินโคซาไมด์) ความสัมพันธ์ทางพันธุกรรมของแบคทีเรียเหล่านี้ได้ถูกทดสอบโดยใช้วิธี plasmid profile study, randomly amplified polymorphic DNA PCR (RAPD-PCR) และ multilocus sequence typing (MLST) ซึ่งจากผลการทดสอบความสัมพันธ์ทางพันธุกรรมเหล่านี้ประกอบกับข้อมูลทางด้านระบาดวิทยาทางกายภาพแสดงให้เห็นว่ามีการระบาดของเชื้อแบคทีเรียดื้อยาระหว่างซุปเปอร์มาร์เก็ตที่ทำการทดสอบในครั้งนี้ สุดท้ายเชื้อ *Salmonella* spp. จำนวน 4 ไอโซเลตได้ถูกนำไปทดสอบลักษณะทางพันธุกรรมโดยวิธี MLST ซึ่งให้ผลการทดสอบเป็น ST 50, ST 96, ST 1543 และ ST 1546 ซึ่งมีลักษณะทางพันธุกรรมเหมือนกับเชื้อที่เคยถูกรายงานจากการศึกษาของประเทศต่าง ๆ เช่น เวียดนาม ออสเตรเลีย เดนมาร์ก ชิลีและสหรัฐอเมริกาและสะท้อนให้เห็นว่าแบคทีเรียที่แยกได้จากไก่นี้มีการแพร่ระบาดระหว่างประเทศอีกด้วย การศึกษาครั้งนี้ได้แสดงให้เห็นว่าทั้ง class 1 integron และแบคทีเรียที่มี integron ในขณะนี้ได้มีการแพร่กระจายไปสู่ชุมชนและมีการแพร่กระจายทั้งแบบแนวตั้งตามชนิดของสายพันธุ์ (clonal spread) และแบบแนวนอนด้วยยีนดื้อยาเดียวกันในแบคทีเรียคนละสายพันธุ์ที่มีบทบาทสำคัญที่ทำให้เกิดการแพร่กระจายเชื้อดื้อยา การควบคุมการใช้ยาปฏิชีวนะในสัตว์จึงเป็นเรื่องที่จำเป็นอย่างยิ่งในการที่จะป้องกันการอุบัติใหม่และการแพร่ระบาดของเชื้อแบคทีเรียดื้อยา

126 หน้า

CONTENTS

Page

ACKNOWLEDGEMENTS **iii**

ABSTRACT (ENGLISH) **iv**

ABSTRACT (THAI) **v**

LIST OF TABLES **ix**

LIST OF FIGURES **xi**

LIST OF ABBRIVIATIONS **xiii**

CHAPTER I INTRODUCTION **1**

CHAPTER II OBJECTIVES **4**

CHAPTER III LITERATURE REVIEW **5**

Food safety and foodborne diseases 5

Salmonella 7

1. History 7
2. Evolution 7
3. Characteristics 8
4. Identification 8
5. Nomenclature 9
6. Pathogenicity and clinical manifestation 11
7. Treatment 14

Distribution of *Salmonella* in chicken 15

Escherichia coli 17

1. History and evolution 17
2. Characteristics 17
3. Identification 17
4. Pathogenesis 17

Distribution of *E. coli* in chicken 19

CONTENTS (cont.)

	Page
Antibiotics used in food animals links to an emergence of antibiotic resistant strains in animal and human	20
Mechanism of antimicrobial resistance in bacteria	24
Mode of resistance genes dissemination	29
Integron element	30
Typing methods of *Salmonella* and *E. coli*	34
1. Phenotypic method	34
2. Genotypic method	35
CHAPTER IV MATERIALS AND METHODS	**38**
Prevalence of *Salmonella* spp. and *E. coli* in raw chicken meat	38
1. Sample collection	38
2. Bacterial isolation	39
3. *Salmonella* serogrouping and serotyping	40
Determination of coliform bacteria by most probable number	42
Antibiotic Susceptibility Testing by disk diffusion method	44
Investigation the presence of class 1 integron and their antibiotic resistance genes	46
1. Total DNA extraction	46
2. Class 1 integron determination	47
3. Cloning experiment	51
4. Transformant clones analysis	52
Plasmid profile study	54
Multilocus sequence typing for characterization of *Salmonella*	55
Dot blot hybridization	58
Randomly amplified polymorphic DNA PCR	61

CONTENTS (cont.)

Page

Conjugation experiment 62

CHAPTER V RESULTS 64

Prevalence of *Salmonella* spp. and *E. coli* in raw chicken meat 64

Serotyping and Serogrouping of *Salmonella* isolates 66

Determination of coliform bacteria by most probable number 67

Antibiotic resistance profile of *Salmonella* and *E. coli* 67

Prevalence of *intI1* by using PCR amplification 71

Identification of resistance cassettes inserted in class 1 integron 71

Dot blot hybridization 82

Plasmid profile study 86

RAPD-PCR of *Salmonella* isolates 89

MLST of *Salmonella* isolates 92

Conjugation experiment 95

CHAPTER VI DISCUSSION 96

CHAPTER VII CONCLUSION 103

REFERENCES 106

APPENDIX 121

BIOGRAPHY 126

LIST OF TABLES

Table		Page
3.1	*Salmonella* nomenclature in use at CDC, 2000	10
3.2	Global prevalence of *Salmonella* in chicken	15
3.3	Top ten of prevalent *Salmonella* serotype from animals during 2003 to 2006 in Thailand	16
4.1	Biochemical test for *Salmonella* and *E. coli* identification Used in this study	40
4.2	Most Probable Number (MPN) estimates for three fermentation tubes	43
4.3	Zone diameter interpretative standard for Enterobacteriaceae according to CLSI 2010	45
4.4	Primer sequences used to detect class 1 integron	47
4.5	Recipe of PCR reaction for detecting integrase gene	49
4.6	Recipe for PCR reaction for 5'CS-3'CS	49
4.7	Recipe for StrataClone^{TM} PCR cloning reaction	51
4.8	Recipe for digestion reaction for *Eco*RI enzyme	53
4.9	Recipe of PCR reaction of MLST for *Salmonella*	56
4.10	Primer sequences and an expected PCR product size of 7 housekeeping genes used for perform MLST of *Salmonella*	56
4.11	Recipe for RAPD-PCR by using R018 primer	61
4.12	MIC interpretation of *Enterobacteriaceae*	63
5.1	Prevalence of *E. coli* and *Salmonella* spp. in sealed packages of chicken meat from supermarkets in Bangkok, Thailand	65
5.2	Antibiotic susceptibility of 14 *Salmonella* spp. isolates	68
5.3	Antibiotic susceptibility of 46 *E. coli* isolates	69
5.4	Summary features of characterized *Salmonella* and *E. coli* carrying class 1 integron in this study	81
5.5	Comparison of *intI1* detection between PCR and dot blot hybridization	84

LIST OF TABLES (cont.)

Table **Page**

5.6 Relationship of *Salmonella* isolates demonstrated by typing methods 91

5.7 Allelic profile of 4 *Salmonella* isolates in this study 92

5.8 Strain information of four *Salmonella* sequence types in this study 94

5.9 MIC determination by microbroth dilution method of E60, *E. coli* JM109, and *E. coli* DH5α to norfloxacin and ampicillin by microdilution broth. 95

LIST OF FIGURES

Figure		Page
3.1	Chicken production systems in Thailand	6
3.2	Model for the evolution of the genus *Salmonella*	8
3.3	Hydrogen sulfide detection of *Salmonella* on SS agar and DHL agar	9
3.4	Selected events in pathogenesis and associated virulence genes	12
3.5	Various sources of transmission routes of antibiotic resistance from animal to human	22
3.6	Several scenarios represent the genetic transport that occurs as bacteria migrate from animal to human environments	23
3.7	General structure of class 1 integron	32
3.8	Integron-mediated gene capture and model for cassette exchange	33
4.1	Flow chart of *Salmonella* and *E. coli* isolation from chicken meats	41
4.2	PCR schematic for class 1 integron detection in this study	48
4.3	The binding site of PCR primers for internal segment of 5'CS-3'CS	50
4.4	pSC-A-amp/kan PCR cloning vector used in cloning experiment	53
5.1	Prevalence of *Salmonella* spp. and *E. coli* from raw chicken meats according to distributors	65
5.2	Prevalence of *Salmonella* spp. and *E. coli* from raw chicken meats according to collection places	66
5.3	A Comparison of percentage of antibiotic susceptibility between *Salmonella* spp. and *E. coli*	70
5.4	PCR with specific primers to *intI1*	72
5.5	PCR using 5' CS and 3' CS primers of two *E. coli* isolates, E9 and E12	73
5.6	PCR using 5' CS and 3' CS primers of *E. coli* and *Salmonella*	74
5.7	PCR with specific primer to 5'CS-3'CS and digested PCR products pattern with *Afa*I enzyme	75

LIST OF FIGURES (cont.)

Figure		Page
5.8	PCR with specific primers to 5' CS-3' CS of 6 integron carrying *Salmonella* isolates	76
5.9	Identification of tranformants, which carried recombinant plasmids by *Eco*RI digestion analysis	77
5.10	Nucleotide sequence analysis of class 1 integron containing *aadA23* in E9	78
5.11	Nucleotide sequence analysis of class 1 integron containing *dfrA5* in E9	79
5.12	Nucleotide sequence analysis of class 1 integron containing *aadA22* in Sal162	80
5.13	Dot blot hybridization of *Salmonella* and *E. coli* by using *intI1* specific probe	83
5.14	Plasmid profile of *E. coli* carrying integron isolated from chicken meats	86
5.15	Plasmid profile of other *E. coli* carrying integron isolates	87
5.16	Plasmid profile of 6 *Salmonella* carrying integron isolates and their restriction enzyme analysis	88
5.17	RAPD-PCR of *Salmonella* isolates	90
5.18	PCR products of seven housekeeping genes used for MLST for *Salmonella*	93

LIST OF ABBREVIATIONS

ABBREVIATION	TERM
AST	Antibiotic susceptibility testing
BGLB	Brilliant green lactose broth
BPW	Buffered peptone water
CLSI	Clinical and Laboratory Standards Institute
°C	Celsius
CS	Conserved segment
DHL	Deoxycholate cholate hydrogen sulfide Lactose
ESBL	Extended spectrum β-lactamase
int	Integrase
LST	Lauryl sulphate tryptose
MDR	Multi drug resistance
MIC	Minimun inhibitory concentration
MLST	Multilocus sequence typing
MPN	Most probable number
OD	Ornithine decarboxylase
RAPD-PCR	Randomly amplified polymorphic DNA-polymerase chain reaction
RV	Rappaport-Vassiliadis
SPI	*Salmonella* pathogenicity island
SS agar	Salmonella-Shigella agar
ST	Sequence type
TSI	Triple sugar iron
TT broth	Tetrathionate broth
XLD	Xylose lysine deoxycholate

CHAPTER I
INTRODUCTION

Foodborne diseases are defined as diseases, usually either infectious or toxic in nature, caused by agents that enter the body through food ingestion (1). It was estimated by World Health Organization (WHO) that about 2.2 million of people are suffered from foodborne diseases annually (2). Symptoms of foodborne diseases range from mild to serious forms including nausea, vomiting, diarrhea, abdominal cramps, fever, and septicemia. Severity of the disease depends on an infectious dose, which differs in each agent and patient's immunity. Among all agents, bacteria are the most common causative agents of foodborne diseases. The examples of bacteria involved in such diseases are *Salmonella* spp., *Escherichia coli*, *Campylobacter* spp., *Listeria monocytogenes*, and *Vibrio cholerae*. Moreover, antibiotic resistant bacteria are now becoming a major public health concern from their effects on treatment outcomes, medical cost, and also economy. Thailand is a major exporter that exports many products including chicken meat. Therefore, antibiotic resistant bacteria contaminated in foods will be absolutely banned and causes a great impact on Thai economy.

There are multifactors involving in an emergence of antibiotic resistant strains, in particular, the misuse of antibiotics in human and animals (3). The correlation between the misuse of antibiotics in animals and an emergence of antibiotic resistant bacteria were well documented (4-6). Food producing animals including pigs, poultry, cattle account for reservoirs of antibiotic resistant bacteria especially *Salmonella* spp. and *E. coli*, and play an important role in spreading of the antibiotic resistant bacteria via undercooked foods. The contamination of antibiotic resistant *Salmonella* spp. and *E. coli* in food producing animals had been reported worldwide (7-12). *Salmonella* spp. and *E. coli*, which normally colonized in gastrointestinal tract of poultry, e.g., chicken, are found at the high rate of contamination in many food products. Interestingly, these bacteria exhibited resistance to many classes of antibiotics, which is likely the result of an overuse of antibiotics in

animals. The contamination of these pathogens can occur in all steps throughout food production process, e.g., poor processing, sterilization, handling and preparation, production, distribution, and retail marketing. The contaminated foods not only cause worse effects on consumers, but also act as vehicles for the spread of resistant bacteria. There are now multi-drug resistant (MDR) bacteria, which show resistance to a various classes of antibiotics.

The MDR bacteria have abilities to counteract with antibiotics by acquiring resistance genes, spontaneous mutations, and disseminating resistance genes via mobile genetic elements, i.e., plasmid, transposon, and insertion sequences (13). Subsequently, these bacteria developed a unique element called integron element, to increase the efficiency of capturing and spreading their resistance determinants to other bacteria (14). Integrons are defined as an assembly platform that incorporate open reading frames (ORFs) of resistance genes in form of gene cassettes, which integrated by site-specific recombination mediated by an integrase (15, 16). The principle components of integrons comprise three major key elements necessary for capturing resistance genes: integrase encoded by *intI* that belongs to tyrosine recombinase family, a primary recombination site called *attI*, and a strong promoter called Pc (16). Five classes of integrons are classified based on sequences of integrases, which share 40-58% identity (16, 17). Class 1 integron was found extensively in clinically important bacteria. Although integrons are not classified as a mobile genetic element, they have been shown to be associated with other mobile genetic elements, e.g., plasmids, and lead to a rapid spread among pathogenic and commensal bacteria (18). The contamination of class 1 integron bearing *Salmonella* spp. and *E. coli* in food producing animals was demonstrated worldwide (8, 19, 20). Additionally, clonal spread also plays an important role and is responsible for the spread of antibiotic resistant bacteria. The well-known example is *Salmonella* Typhimurium, definitive phage type 104 (DT 104), which emerged in cattle in the 1990s and spread globally (21).

In Thailand, there were many studies on the prevalence of antibiotic resistant bacteria in food producing animals (10, 12), but little is known about the genetic mechanism conferring antibiotic resistance and its molecular characteristics. Therefore, this study was conducted to determine the prevalence of antibiotic resistant

Salmonella spp. and *E. coli* in raw chicken meat collected from various supermarkets in Bangkok and to provide information on molecular characteristics of antibiotic resistant *Salmonella* spp. and *E. coli*. *Salmonella* spp. is a true pathogen and its presence in chicken meat is unacceptable, in contrast to *E. coli*, which can be both normal flora and pathogen. This study did not focus on pathogenic *E. coli*, but focus on non-pathogenic *E. coli*, which represents normal flora and can act as a vehicle for the spread of resistance genes. Class 1 integron was characterized by PCR with specific primers to *intI1* and dot blot hybridization. An internal segment of 5'CS and 3'CS was characterized by PCR and DNA sequencing. Antibiotic susceptibility testing was determined by disk diffusion according to Clinical and Laboratory Standards Institute 2010 (CLSI 2010). A presence of coliform bacteria in chicken meat was determined by most probable number (MPN). A transferability of resistance determinants was determined by conjugation experiment. In addition, plasmid profile study, serogrouping, serotyping, randomly amplified polymorphic DNA PCR (RAPD-PCR), and multilocus sequence typing (MLST) were used to determine genetic relatedness among *Salmonella* spp.

CHAPTER II
OBJECTIVES

The objectives of this study are as followed

1. To determine the prevalence of *Salmonella* spp. and *E. coli* in raw chicken meat from supermarkets in Bangkok.
2. To investigate the presence of class 1 integron and their antibiotic resistance genes.
3. To investigate the horizontal gene transfer of antibiotic resistance cassettes.
4. To perform molecular typing of antibiotic resistant *Salmonella* spp. and *E. coli.*

CHAPTER III
LITERATURE REVIEW

Food safety and foodborne diseases

Food safety is becoming a major public health concern due to a great impact on social health and economy. It has been estimated that foodborne diseases affect approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year (22). It caused about 1.8 million people death globally from a report of the world health organization (WHO) in 2005. In Thailand, it was estimated from the Bureau of Epidemiology, Thailand that about million cases of acute diarrhea were reported each year. Moreover, foodborne diseases also affect economy including medical costs and foods exportation. It was estimated in 2007 that foodborne diseases cost US 357 billion to US 1.4 trillion annually (23). The causative agents of foodborne diseases, diseases caused by ingestion of contaminated foods, can be bacteria, virus, and toxin with involving 200 known diseases (24).

Bacteria are common cause of foodborne diseases among all agents. The most common pathogens are *Salmonella* spp., *E. coli*, *Campylobacter*, *Staphylococcus aureus*, *Bacillus cereus*. In the United States, the highest incidence of diseases transmitted through foods in 2008-2009 was caused by *Salmonella* and *Campylobacter* spp. (25, 26). These bacteria are commonly found in nature and animals, but they can cross-contaminate in foods by closed contact with animals or poor personal hygiene. Many products, especially food products, have been demonstrated of contamination with foodborne bacteria. Chicken is an important product, which Thailand is a major exporter to other countries. Japan and EU are major importers for Thailand. Chicken production system in Thailand is divided into three sectors (Fig. 3.1) (27). The sector 3, company farms and their contracting farms, produce chicken for both export and slaughterhouse, whereas other two sectors use slaughterhouses for processing.

Contamination of chicken with these bacteria can occur in any steps throughout a production system either distribution, packaging, or sterilization. *Salmonella* and *E. coli*, which colonize in gastrointestinal tract of poultry, e.g., chicken, are an important pathogen contaminated in food products.

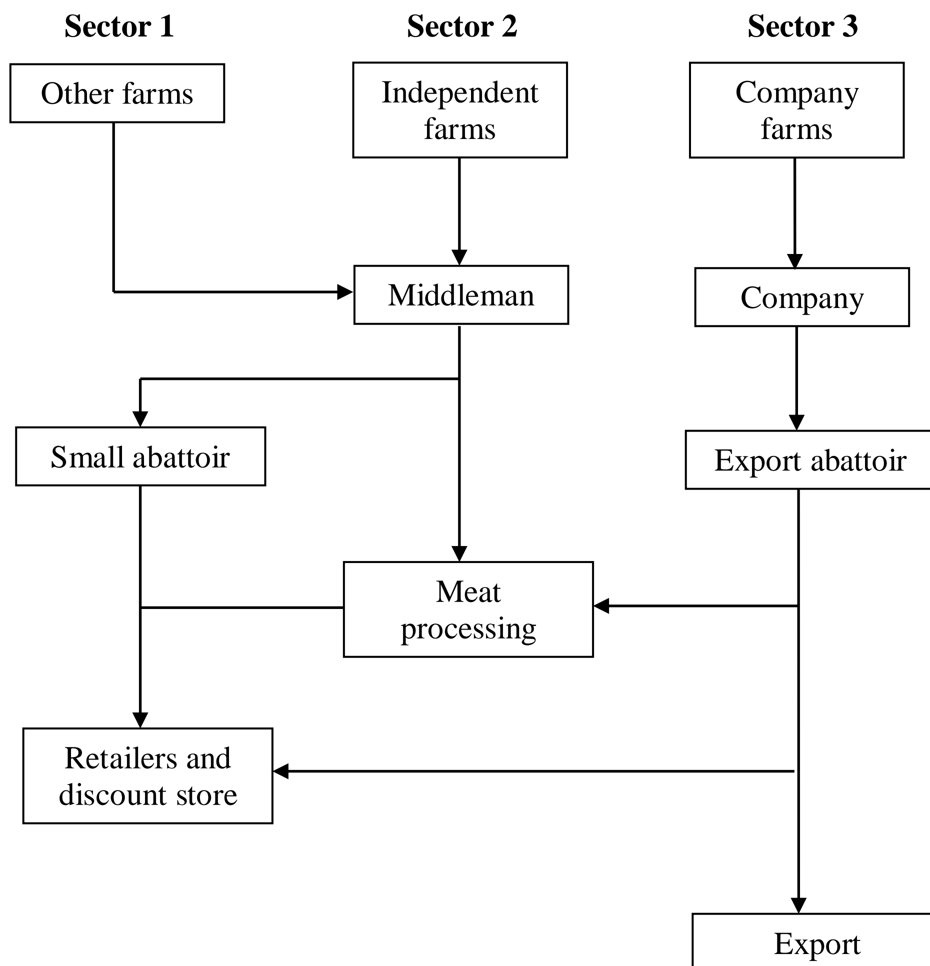


Figure 3.1 Chicken production systems in Thailand (27)

Salmonella

1. History

Salmonella was first isolated in 1880 by Karl J. Erberth who observed bacillus in tissue section from a patient died from typhoid. The causative agent, *Salmonella* Typhi was then successfully isolated in 1884. The first isolation of *Salmonella* in animals was in 1885 by two researchers, Daniel E. Salmon and Theobald Smith. Theobald Smith, who worked at Bureau of Animal Industry, discovered *Salmonella* Choleraesuis from pigs and he thought that this agent was a causative of "hog cholera" (fever of swine) at that period. The causative agent of hog cholera was then identified that it was caused from viral infection and *Salmonella* was a second invader (28). The name of *Salmonella* was credited to Daniel E. Salmon, Theobald Smith's chief, although it was first discovered by Theobald Smith (28).

2. Evolution

Salmonella and *Escherichia coli* were considered to be diverged from the common ancestor. *Salmonella* was demonstrated that it was evolved from *E. coli* in three phases (Fig. 3.2) (29). The first phase was the acquisition of *Salmonella* pathogenicity island 1 (SPI 1) by plasmid-or-phage mediated horizontal gene transfer, which is present in all *Salmonella* lineages, but absent in *E. coli*. SPI 1 encodes virulence factors that mediate mechanisms used by *Salmonella* during an intestinal phase of infection. The results of multilocus enzyme electrophoresis and DNA-DNA hybridization studies, revealed that *Salmonella* lineages represent two distinct species, designated *Salmonella enterica* and *Salmonella bongori* and could be considered as second phase. *S. enterica* possess SPI 2 that is not present in *S. bongeri*. SPI 2 was acquired after *S. enterica* branched from the *S. bongeri* lineage. The third phase is also important for host adapted ability. *Salmonella enterica* subspecies I (*enterica*) expanded host range to warm-blooded animals.

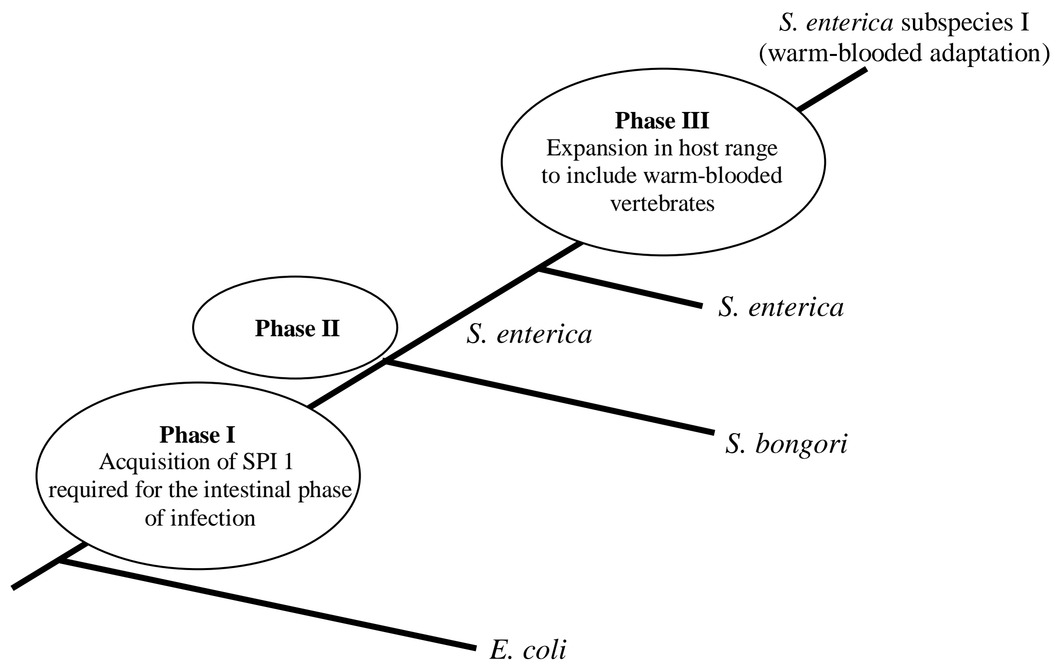


Figure 3.2 Model for the evolution of the genus *Salmonella* (modified from ref. 29)

3. Characteristics

Salmonella are Gram-negative facultative anaerobic bacteria, which belong to family Enterobacteriaceae. They are non-fastidious bacteria as they can grow in various conditions, i.e., temperature range from 5-47 °C with optimum temperature at 35-37°C (30), pH range from 4-9. All members of this genus are motile using peritrichous flagella except *Salmonella* Gallinarum and *Salmonella* Pullorum, which are non-motile host-specific avian pathogens.

4. Identification

Salmonella can be identified by using phenotypic properties, e.g., Gram staining, oxidase test, ability to grow in selective medium, and biochemical identification test. *Salmonella* are oxidase negative and catalase positive, which can catabolize glucose but cannot metabolize lactose and sucrose. They can also use citrate as a carbon source, produce hydrogen sulfide, but cannot utilize urea. In cultivation process, enrichments media, e.g., Tetrathionate broth (TT broth), Selenite F broth,

Rappaport-Vassiliadis broth (RV broth) were used prior to selection on selective media. Selective medium, e.g., Salmonella-Shigella agar (SS agar) contains bile salt, brilliant green, and citrate to inhibit Gram-positive bacteria and also sodium thiosulfate and ferric citrate, which allow hydrogen sulfide detection. Deoxycholate Hydrogen Sulfide Lactose Agar (DHL agar) or Xylose lysine deoxycholate agar (XLD agar) can also be used. Hydrogen sulfide detection on SS agar and DHL agar were shown in Fig. 3.3. The black color results from the formation of ferrous sulfides.

www.kohjin-bio.co.jp/products/up_img/1270279149-805525_2.jpg

www.kohjin-bio.co.jp/products/up_img/1270278582-890107_2.jpg

Figure 3.3 Hydrogen sulfide detection on SS agar (left) and DHL agar (right)

5. Nomenclature

Salmonella share a high degree of DNA similarity and used to be classified as only one species, *Salmonella choleraesuis* (31). It was then subsequently divided into seven groups, which group I is the major cause of diseases in humans. The species *choleraesuis* was changed to neospecies, *enterica*, because its name lead to confusion with serotypes or serovars (32). *Salmonella bongori* was a different species from other closely related species as demonstrated by multilocus enzyme electrophoresis, which was concordant with DNA-DNA hybridization experiment, and classified to new species apart from *S. enterica* (33).

Salmonella now comprises two species, *S. enterica* and *S. bongori*, which was formerly subspecies V (33), according to Centers for Disease Control and prevention (CDC) system (34). *S. enterica* is divided into six subspecies; *S. enterica* subspecies *enterica* (I), *S. enterica* subspecies *salamae* (II), *S. enterica* subspecies *arizonae* (IIIa), *S. enterica* subspecies *diarizonae* (IIIb), *S. enterica* subspecies *houtenae* (IV), and *S. enterica* subspecies *indica* (VI). *S. enterica* subspecies *enterica* (I) is the only one subspecies that expanded host range to warm-blooded animals including human and most pathogenic *Salmonella* belongs to this subspecies. All species are subdivided into serogroups based on variations in a somatic O antigen and serotypes based on O antigen and flagella H antigen. According to the CDC system, 2,463 serotypes were characterized, which subspecies I contains highest serotypes. Serotype names were designated as follow; subspecies designation, somatic O antigen followed by a colon, flagellar H antigen phase I followed by a colon, flagellar H antigen phase II, if present, e.g., *Salmonella* serotype IV 45:g,z_{51}:-. *Salmonella* nomenclature according to CDC is shown in Table 3.1.

Table 3.1 *Salmonella* nomenclature in use by CDC, 2000 (34)

Taxonomic position	Nomenclature
Genus (italics)	*Salmonella*
Species (italics)	- *enterica*, which includes subspecies I, II, IIIa, IIIb, IV, and VI - *bongori* (formerly subspecies V)
Serotype (capitalized, not italized)	- The first time a serotype mention in the text; the name should be preceded by the word “serotype” or “ser” - Serotypes are named in subspecies I and designated by antigenic formulae in subspecies II to IV, and VI and *S. bongori* - Members of subspecies II, IV, and VI and *S. bongori* retain their names if named before 1996.

6. Pathogenicity and clinical manifestation

Salmonellosis refers to an infection caused by *Salmonella*, which can be classified into four groups: enteric fever, gastroenteritis, bacteremia, and carrier state (30). *Salmonella* infection can cause infection through ingestion of contaminated foods or water and it can survive by adaptive response to an acidic pH in stomach (35). It enters to a small bowel and crosses an intestinal mucus layer before adhering to intestinal mucus membrane, which preferentially binds to microfold cell (M cell). *Salmonella* induces cytoskeleton rearrangement and disrupting membrane brush border with results in bacterial-mediated endocytosis (36, 37). *Salmonella* containing vesicles then moves to basolateral membrane and activates immune cells. The selected events in the pathogenesis of gastroenteritis and enteric fever, along with associated virulence genes, are shown in Fig. 3.4.

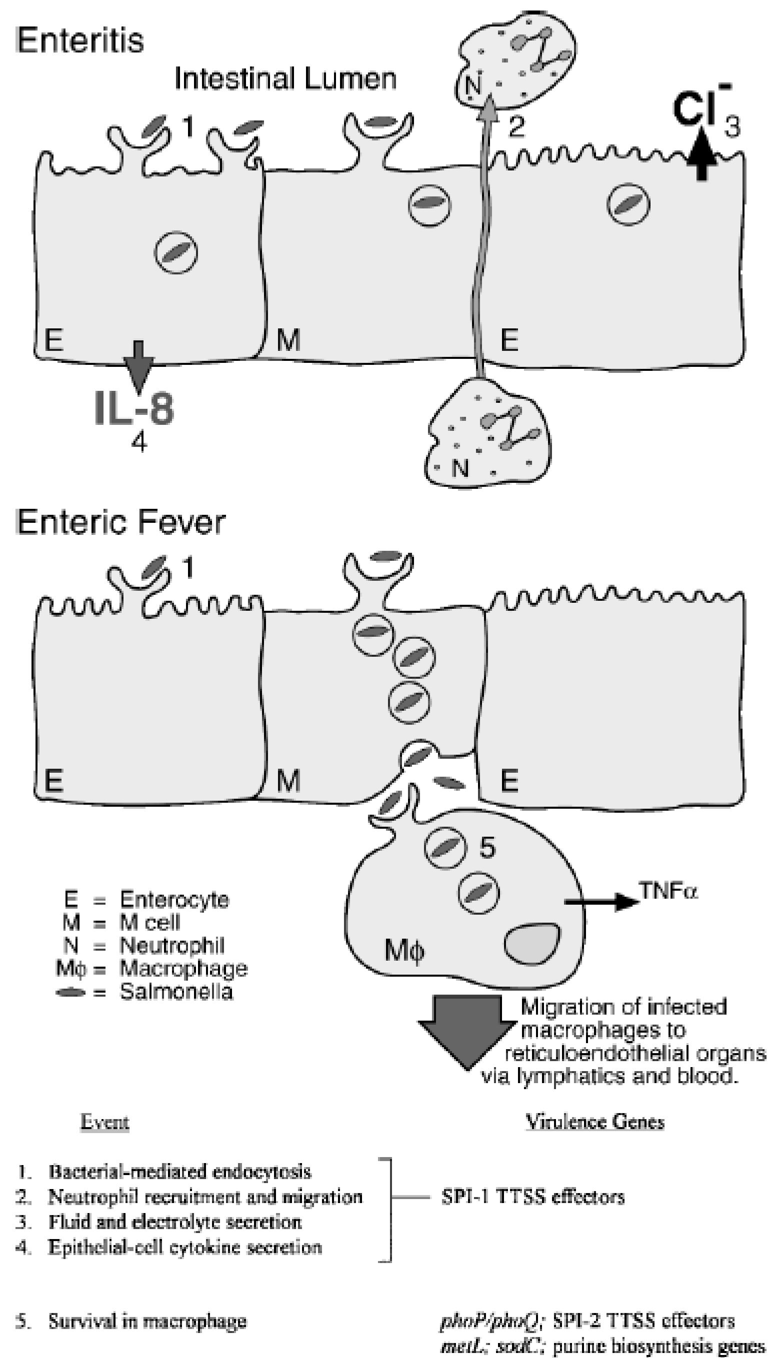


Figure 3.4 Selected events in pathogenesis and associated virulence genes (36)

6.1 Disease caused by typhoidal *Salmonella*

6.1.1 Enteric fever

Enteric fever comprises typhoid fever (caused by *S.* Typhi) and paratyphoid fever (caused by *S.* Paratyphi A, B, and D). In 2000, typhoid fever was estimated to cause 21,650,974 illnesses and 216,510 deaths, while paratyphoid fever was estimated to cause 5,412,744 illnesses (38). Transmission route is through ingestion of foods or water contaminated with such bacteria. The incubation period of typhoid fever is 7-14 days (39) and causes vary symptoms including fever, malaise, myalgia, anorexia, and dry cough. Paratyphoid fever takes a shorter incubation period, but exhibits less severity of diseases than typhoid fever (40).

6.2 Diseases caused by non-typhoidal *Salmonella*

Non-typhoidal *Salmonella* (NTS) are important agents that had been reported to cause foodborne illness (29, 30). Humans are not a reservoir for NTS, but they can be transmitted via ingestion of foods contaminated with these agents. There are evidences supporting the contamination of NTS in food products (7, 10, 11), and may consequently lead to health problem.

6.2.1 Gastroenteritis

This disease caused by NTS, serotypes other than Typhi and Paratyphi. *S.* Typhimurium and *S.* Enteritidis are the most frequent serotypes reported in the United States (43). In Thailand, *S.* Stanley, *S.* Rissen, *S.* Enteritidis, *S.* Typhimurium, *S.* Choleraesuis, were the most frequent serotypes during 2003-2005 (44-46). Clinical symptoms present as nausea, vomiting, diarrhea, and cannot be differentiated from diseases caused by other agents, e.g., *Shigella*. Diseases actually are self-limiting, but antibiotics, e.g., fluoroquinilones should be used in severe cases.

6.2.2 Bacteremia

NTS bacteremia depends on age, sex, serotypes, continents, and immunity, which *S.* Choleraesuis is the most frequent serotype involving this disease. High incidence of this disease was reported in african HIV patients (47, 48) and in patients with malarial infection (48).

6.2.3 Carrier state

Salmonella can be spread via a carrier, who gets infected with no symptoms. Carrier state does not only occur for NTS, but also for typhoid *Salmonella*. Mary Mallon, also known as “Typhoid Mary”, was the first reported person of typhoid fever carrier state who spread this agent to others via cooking.

7. Treatment

Diseases caused by *Salmonella* infection are usually self limiting diseases and require no treatment except patients who suffered from severe dehydration may require fluid and electrolyte replacement. Antibiotic treatment sometimes is recommended for shortening duration of infection and preventing an extraintestinal infection, but it is still controversy. It was demonstrated that antibiotic treatment could prolong fecal shedding of these organisms rather than limit shedding (43). Ampicillin and trimethoprim-sulfamethoxazole are drugs of choice for empirical treatment (49), but an increase of resistant strains to one or both of these drugs forced doctors to change to other drugs. Fluoroquinolones and third generation cephalosporins are now used instead. Nevertheless, fluoroquinolone resistant strains mediated by mutations in drug targets (50), quinolone resistance determining region (QRDR) of gyrase (51-54), and plasmid mediated quinolone resistance (PMQR) (50) have been reported (55-57). The common mechanism of third generation cephalosporins resistance is a production of extended spectrum β-lactamase (ESBL) enzymes.

Distribution of *Salmonella* in chicken

Salmonella colonizes in gastrointestinal tract of wide range of animals. Salmonellosis is a foodborne disease, which mainly caused by consumption of contaminated foods and closely contact with infected animals. Chickens are important food producing animals, which act as vehicles for animal-to-human transmission. Chicken meat product can be classified into two types, pre-packaging and post-packaging types. Pre-packaging type is sealed and kept in refrigerator and sold at supermarket, in contrast to post-packaging type, which is exposed to an environment, kept at ambient condition, and sold at open market. Prevalence of *Salmonella* in post-packaged chicken meat had been reported worldwide and demonstrated a great variation in each country (Table 3.2). In contrast, there are few reports focusing on prevalence of *Salmonella* in pre-packaged chicken meat that sold at supermarket. In Thailand, *Salmonella* contamination in chicken meat collected from supermarket was reported at the rate of 57% (12). The lower rate of contamination (4.2%) was reported in U.S.A. (11).

The most frequent serotypes of *Salmonella* found in animals in Thailand from 2003-2006 reported by Department of Medical Sciences, Ministry of Public Health, Thailand, were Enteritidis, Weltevreden, Rissen, and Anatum (Table 3.3).

Table 3.2 Global prevalence of *Salmonella* in post-packaged chicken

Country	Prevalence (percentage)	References
U.S.A.	4.2, 35, 35	11, 65, 66
Japan	14.3	67
Spain	35.83	68
Vietnam	48.9, 53.3	69, 70
UK	29	71
Cameroon	60	72
Thailand	1, 48, 75	73, 12, 10

Table 3.3 Top ten of prevalent *Salmonella* serotypes from animals during 2003 to 2006 in Thailand (44-46, 74)

2003	2004	2005	2006
S. Enteritidis (55.56%)	*S.* Stanley (14.81%)	*S.* Rissen (25.34%)	*S.* Weltevreden (19.12%)
S. Stanley (4.76%)	*S.* Rissen (8.64%)	*S.* Anatum (12.38%)	*S.* Corvallis (13.15%)
S. Rissen (4.76%)	*S.* Albany (7.41%)	*S.* Stanley (11.59%)	*S.* Enteritidis (12.35%)
S. Weltevreden (3.97%)	*S.* Anatum (6.17%)	*S.* Amsterdam (9.63%)	*S.* Newport (6.17%)
S. Lexington (3.17%)	*S.* Weltevreden (4.94%)	*S.* Weltevreden (8.06%)	*S.* Stanley (5.58%)
S. Yoruba (3.17%)	*S.* Corvallis (3.70%)	*S.* Enteritidis (4.13%)	*S.* Brunei (4.38%)
S. Augustenborg (2.38%)	*S.* Enteritidis (3.70%)	*S.* I 4,12:i:- (3.54%)	*S.* Typhimurium (3.59%)
S. Anatum (1.59%)	*S.* Agona (3.70%)	*S.* Schwarzengrund (3.14%)	*S.* Virchow (3.19%)
S. Derby (1.59%)	*S.* Panama (3.70%)	*S.* Altona (1.77%)	*S.* Javiana (3.19%)
S. Schwarzengrund (1.59%)	*S.* Schwarzengrund (3.70%)	*S.* Krefeld (1.57%)	*S.* Amsterdam (3.19%)

The most frequent serotypes in human are similar to those found in animals, which are Enteritidis, Weltevreden, Rissen except Choleraesuis. Similar trend of serotypes found in both human and animals indicates a possible route of transmission from animals to human.

Escherichia coli

1. History and evolution

Escherichia coli was named for Theodor Escherich, the German pediatrician who isolated an intestinal bacteria from feces and term it as "bacterium coli commune". *E. coli*, *Salmonella*, and *Shigella* are considered as closely related species, which *E. coli* and *Shigella* were considered to be a single species for some researchers.

2. Characteristics

E. coli are facultative anaerobic Gram-negative bacteria that belong to family Enterobacteriaceae. The optimal temperature for growth is 35-45°C, and pH ranges from 4-10. Most *E. coli* are motile by using flagella and ferment glucose, lactose, arabinose, maltose. *E. coli* have an ability to adapt and colonize to a various conditions, e.g., gastrointestinal tract of animals and open-air environment.

3. Identification

E. coli can be identified using phenotypic properties and biochemical tests. *E. coli* are an oxidase negative and catalase posive bacteria. By using MacConkey agar, *E. coli* show pink colonies from lactose fermention, with pitted appearance in the center of the colony. Most *E. coli* are indole positive and motile, but negative for citrate, urea, Voges-Proskaeur (VP) tests.

4. Pathogenesis

Most strains of *E. coli* are normal flora in human and animals, but they can also be pathogenic strains that cause three major diseases in human; neonatal meningitis, chronic urinary tract infection, and gastroenteritis. The six classes of

diarrheagenic *E. coli*, which associated with gastroenteritis are enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and diffusely adherent *E. coli* (DAEC). Four groups of diarrheagenic *E. coli* related to foods and water are ETEC, EPEC, EIEC, and EHEC.

4.1 ETEC

Pathogenesis of ETEC is due to the production of enterotoxins. ETEC may produce a heat-labile enterotoxin (LT), which functions similar to the cholera toxin (CT) of *Vibrio cholerae*. ETEC adheres to intestinal epithelial cell by colonization factors, e.g., fimbriae. LT comprises A and B subunits, which single A subunit binds to pentamers molecules of B (59). LT subunit A targets alpha subunit of G protein leading to adenosine diphosphate (ADP) ribosylation. This will activate guanosine triphosphate (GTP) leading to elevation level of cyclic adenosine monophosphate (cAMP). ETEC may also produce a heat stable toxin (ST) that is resistant to boiling for 30 minutes.

4.2 EPEC

The characteristic of infection from EPEC is attaching-and-effacing (A/E) histopathology (59). Pathogenesis of EPEC has three main stages; localized adherence, signal transduction, and intimate adherence. Intimin protein encoded by *eae* gene is required for attachment and effacing formation. Illnesses caused by EPEC including severe diarrhea, nausea, vomiting, abdominal cramps, headache, fever, and chills.

4.3 EIEC

EIEC is similar to *Shigella*, which is non-motile, non-lactose fermenter, and causes diseases similar to shigellosis. Diseases are caused by bacterial penetration and destruction of intestinal mucosa with the process comprises of epithelial cell penetration, lysis of the endocytic vacuole, intracellular multiplication, directional movement through the cytoplasm, and extension into adjacent epithelial

cells (59). Symptoms include chills, fever, headache, muscle pain, abdominal cramps, and profuse diarrhea.

4.4 EHEC

Pathogenesis of EHEC similar to EPEC, but it can produce Shiga toxin. EHEC produces Shiga toxins (Stx), which are encoded by phage, and Stx1 and Stx2 are most often implicated in serious illness in human. The important serotype is O157:H7, which contaminated in foods and caused many outbreaks in the United States (60-63). *E. coli* O157:H7 can be separated from other serotypes by the properties of delayed sorbitol fermentation, and inability to produce β-glucuronidase (64). This serotype or strain can also cause Hemolytic Uremic Syndrome (HUS) leading to kidney failure and death.

Distribution of *E. coli* in chicken

E. coli often contaminated in many kinds of foods especially poultry products. The contamination can occur in all steps through food processing, e.g., packaging, transportation, and surprisingly decontamination. In contrast to *Salmonella*, *E. coli* can be categorized into pathogenic *E. coli* and nonpathogenic *E. coli* (or normal flora). Although, flora *E. coli* contamination in food cannot cause diseases directly, but its role in reservoir of resistance genes is considered as an impact issue. High rate of *E. coli* contamination in chicken (72.3-84.0%) was reported from National Antimicrobial Resistance Monitoring System (NARMS) during 2002 to 2005. Zhao C et al. reported chicken contaminated with *E. coli* at the rate of 38.7% and nearly 60% of samples collected from supermarket were contaminated (11). The high rate of *E. coli* contamination (100%) was also reported in Cameroon, and 11.3% of these were EPEC (72). Moreover, extended-spectrum β-lactamase (ESBL) genes were detected at the highest rate in chicken meat (79.8%) compared with beef and pork (75). Interestingly, ESBL genes were also demonstrated a similar trend between chicken and human (75).

Antibiotics used in food animals link to an emergence of antibiotic resistant strains in animals and human

Antibiotics are used in human mainly for therapeutic purpose, which are treatment and prevention of diseases. However, an overuse of antibiotics in human, e.g., over-prescribing of antibiotics by doctors can promote an emergence of antibiotic resistant strains. The failure of patients to complete dose of prescription also leads to a survival of resistant strains. Moreover, poor hygiene and infection control are important factors driving a spread of these resistant bacteria in clinical setting.

Nevertheless, the overuse of antibiotics in food animals as therapeutic and non-therapeutic purpose is an alternative way conferring an emergence of resistant bacteria in human. Therapeutic purpose is used only for diseased animals, whereas prophylactic and subtherapeutic uses are for disease prevention, growth promotion, and feed efficiency (76). Many of antibiotics used in animals are identical to human, e.g., fluoroquinolones, penicillin, neomycin, and tetracycline (76, 77). Antibiotic resistant strains occur from spontaneous mutations or acquired resistance traits by other bacteria. These resistant bacteria in food animals can be transmitted to human by close contact with infected animals or consumption of contaminated products (Fig. 3.5). Resistant bacteria from animals spread to an environment, e.g., food, water, soil, and plant, and then spread to human. The spread of resistant bacteria can be categorized into two modes; vertical transfer and horizontal transfer. Vertical transfer or clonal spread is the spread of resistance trait from parent to progeny, whereas horizontal transfer is the spread of resistance elements, mostly, extrachromosomal part from one bacterium to others. Horizontal transfer comprises three major processes, conjugation, transformation, and transduction. The resistance element from one bacterium can be transferred to the same or new bacteria in human with 100% match or some modifications (Fig 3.6). For example, an emergence of fluoroquinolone resistant *Campylobacter* spp. correlated with fluoroquinolone usage in animals (4, 5). The proportion of quinolone resistant *Campylobacter jejuni* in human increased from 1992-1998 in Minnesota was due to an introduction of quinolone in animals (78). Molecular subtyping indicated that chicken product was a potential source of *C. jejuni* resistant strains (78). The use of glycopeptide avoparcin as a growth promoter enhanced an emergence of vancomycin-resistant *Enterococcus* (VRE) in Norway (79).

A decline of percentage of VRE in poultry meat after avoparcin ban was demonstrated (80).

An alternative way to trace back of resistance bacteria is a characterization of antibiotic resistance determinants. Apart from clonal spread, horizontal gene transfer driven by mobile genetic elements is a process of transferring of resistance determinants from one to other bacteria. Molecular typing methods, e.g., sequencing of resistance cassettes have been used to demonstrate a link between resistant strains in animals and human. Resistance cassettes contained in class 1 integron demonstrated an identical sequence and orientation in human and animal *E. coli* isolates, as well as other bacterial species (81). Moreover, it was demonstrated that these resistance cassettes were spread globally (81). Detection of *aac(3)-IV*, apramycin resistance gene, in human *E. coli* isolates with 99.3% identity to animal origin was also demonstrated (82). These resistance determinants not only be transferred within species (intraspecies fasion), but also be transferred to different species (interspecies fasion). Winokur PL et al. demonstrated that a transferring of CMY-2 AmpC β-lactamase plasmids between *E. coli* and *Salmonella* isolates came from animal to human (83).

The use of antibiotics in animals increases the number of resistant bacteria not only in pathogenic strains, but also in commensal flora. Antibiotic resistant bacteria transfer their resistance elements to commensal flora via horizontal gene transfer and make them as potential reservoirs for resistance genes. In human, intestinal flora was found to be reservoirs for resistance genes. The possible ways of acquisition of resistance genes are acquisition of resistance genes from other bacteria in intestine, or acquisition from contaminated bacteria transmitted via fecal oral route (84). Besides the issue of reservoir of resistance genes, commensal flora was considered to be an indicator for antibiotic used in that environment, and prediction of resistance problem caused by pathogenic bacteria (85).

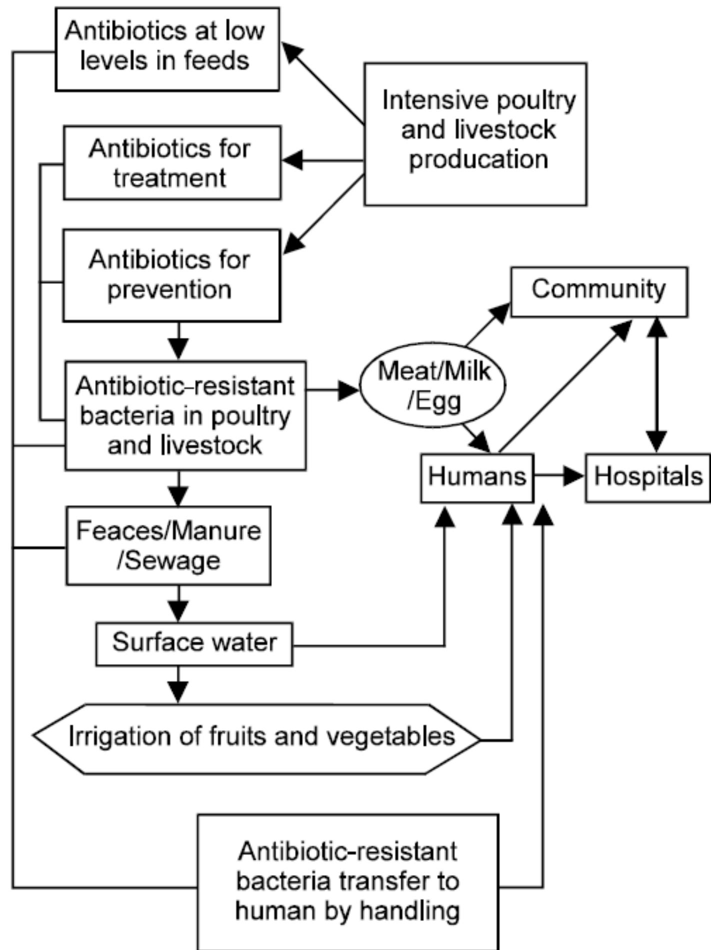


Figure 3.5 Various sources of transmission routes of antibiotic resistance from animal to human (77)

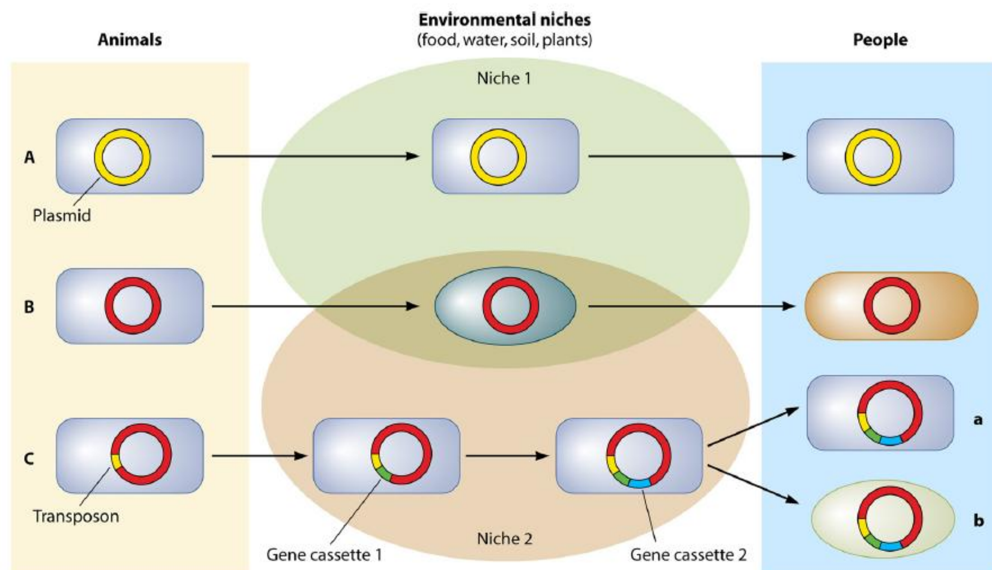


Figure 3.6 Several scenarios represent the genetic transport that occurs as bacteria migrate from animal to human environments. (A) The same host and its indigenous genes in animals are transported unchanged to humans. (B) The genetic structure passes through one or more different hosts, ending in a new host (humans), with a resulting 100% match of DNA. (C) The host and its plasmid-borne genes pass through the environment, picking up gene cassettes en route, with a resulting 100% match for the host only (a) or a low-% match for DNA only (b) (86).

Mechanisms of antimicrobial resistance in bacteria

The discovery for antibacterial agents began around 1910 by Paul Ehrlich, who discovered anti-syphilitic drug named Salvasan, an arsenical arsphenamine. Prontosil was later discovered by Gerhard Domagk. Penicillin and streptomycin were discovered in the late 1930s and early 1940s by Alexander Fleming and Selman Waksman, respectively. Penicillin was a progenitor for many versions of β-lactam antibiotics, e.g., ampicillin, and cephalosporins. Antibacterial agents can be categorized into two main groups, antibacterial obtained from natural sources, e.g., β-lactams and synthetic compounds, e.g., quinolones, according to their sources. The use of antibacterial agents in bacterial infection has saved patients from death, but its use can generate unwanted consequence, i.e., an emergence of antimicrobial resistant bacteria. Antimicrobial agents can be classified according to their modes of actions, which are inhibition of cell wall (β-lactam, cephalosporins), inhibition of protein synthesis (tetracycline, macrolide, lincosamide), inhibition of DNA synthesis (quinolones), inhibition of RNA synthesis (rifampin), competitive inhibition of folic acid synthesis (sulfonamide, trimethoprim), membrane disorganizing (colistin) (87). Although new antimicrobial drugs are still to be developed, it seems useless because bacteria keep developing resistance to any kinds of drug. The major mechanisms of antimicrobial resistance in bacteria are diminished intracellular drug concentration mediated by efflux system, target alteration, drug inactivation, and bypass mechanism.

1. Diminished intracellular drug concentration mediated by influx-efflux system

Bacterial cells have an intrinsic resistance property to toxic substances including antibiotics. In Gram-negative bacteria, outer membrane plays a role in first line barrier, which limits an entry of antibiotics, whereas Gram-positive bacteria are prone to be susceptible to any toxic substances due to their lack of outer membrane. Porins, which locate on outer membrane, are likely a generalized protection mechanism that might not be specific to any class of antibiotics. OprD protein in *Pseudomonas aeruginosa*, unlike the generalized ones, it shows specific activity with its substrate, imipenem. Imipenem can be used for treatment against *P. aeruginosa*

containing intact OprD protein, in contrast to strains carrying mutation in OprD protein, OprD deletion, or low level of OprD expression with intact OprD protein (88). On the other hand, bacteria can develop resistance to antibiotics by activation of efflux pumps. The efflux systems confer antibiotic resistance by pumping antibiotic out of bacterial cells, therefore decrease intracellular drug accumulation. The efflux system comprises three parts, an inner membrane protein, linker protein at periplasmic space, and an outer membrane protein. There are five families of efflux transporters; MFS (major facilitator superfamily), MATE (multidrug and toxic efflux), RND (resistance-nodulation-division), SMR (small multidrug resistance), and ABC (ATP binding cassette) (89). The example of efflux systems are ArcAB-TolC, ArcEF-TolC in *E. coli*, MexAB-OprM, MexCD-OprJ, MexOF-OprN, and MexXY-OprM in *P. aeruginosa*. Quinolone resistance is mediated by activation of efflux systems, although mutations in target region, QRDR of DNA gyrase and DNA topoisomerase IV, and Plasmid-mediated quinolone resistance (PMQR) (90), *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *aac(6')-Ib-cr* are major resistance genes responsible for quinolone resistance. It has been demonstrated that efflux pump mediated responsible for quinolone resistance in many bacterial species, e.g., MexAB-OprM, MexCD-OprJ, and MexEF-OprN in *P. aeruginosa*, AcrAB, AcrEF, and EmrAB in *E. coli*, CmeABC in *C. jejuni*, AdeABC in *Acinetobacter baumannii* (91). The overexpression of efflux system result from mutations in repressor proteins, e.g., mutations in MexR, a repressor protein, leads to derepression of MexAB-OprM in *P. aeruginosa* (92). The efflux system has a broad range of substrate, which can result in cross resistance to other antibiotics. Efflux proteins also play a role in tetracycline resistance. Tetracyline, a drug that inhibits protein synthesis by preventing the attachment of aminoacyl-tRNA to ribosomal protein, has four resistance mechanisms; efflux, ribosomal protection, enzyme inactivation, and unknown mechanisms (93). The efflux proteins responsible for tetracycline resistance belong to MFS, which are divided into 6 groups according to amino acid identity (93). The examples of efflux genes are *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, etc. These efflux proteins are normally associated with mobile genetic elements, e.g., plasmid, which often carry other resistance genes also.

2. Target alteration

After antibiotics entered to bacterial cell through porin channels, it needs to reach and action on target site according to its classes. Therefore, target site alterations caused by mutations are another mechanism conferring resistance in many drugs. Penicillin binding proteins (PBPs) are transpeptidases, which catalyze peptidoglycan crosslinking. PBPs harbor three specific domains: SXXK, (S/Y)XN, and (K/H)(S/T)G (94). The serine of SXXK interacts on carbonyl group of the penultimate D-alanine residue of donor peptide, which releases the last D-alanine to form a covalent acyl-enzyme complex, and then crosslinks with the third L-lysine residue of acceptor peptide (94). β-lactams have a structure similar to D-ala-D-ala, a target for PBPs, which act as competitive inhibitors to bind with PBPs leading to cell wall synthesis inhibition. Many mutations in PBPs have been shown in penicillin resistant bacteria. The β-lactam resistance in methicillin resistant *Staphylococcus aureus* (MRSA) results from production of low affinity PBP, PBP2a, which encoded by *mecA* (95), while mutations in PBP5 decreased affinity for β-lactam was demonstrated in *Enterococcus faecium* (96). PBP1a, PBP2b, PBP2x and sometimes PBP2a were shown to be altered in resistant *Streptococcus pneumonia* strains (94). Another example is target alteration conferring quinolone resistance. Quinolone is a synthetic compound that acts on DNA gyrase and topoisomerase IV. DNA gyrase, encoded by *gyrA* and *gyrB*, functions in catalyzing of negatively supercoilng of DNA, whereas topoisomerase IV, encoded by *parC* and *parE*, functions in decatenating the daughter replicons (91). Mutations in QRDR, position 67 to 106, of *gyrA* decreased susceptibility to quinolones. Moreover, mutation in position 51, which located outside the QRDR was also demonstrated to lower susceptibility to quinolones (97). The most frequent mutation positions in *E. coli* and *Salmonella* are at positions 83 and 87 (98, 99). In *gyrB*, substitutions at position 426 and 447 have been detected in quinolone resistant strains (91). Mutations at positions 78, 80, and 84 of *parC*, and position 445 of *parE* were detected in quinolone resistant *E. coli* (91). Target alterations confer antibiotic resistance also including *rpoB* mutation in rifampicin resistant strain, mutation in 23S rRNA genes in linezolid resistance, and lipid A modifications that interfere binding between colistin and cell membrane in colistin resistance.

3. Production of drug inactivation enzymes

This mode of resistance confers high level resistance to many classes of antibiotics. The prominent example is the production of β-lactamase enzyme, which inactivates β-lactam antibiotics, e.g., penicillin, cephalosporin, by cleaving the β-lactam ring. β-lactamase enzymes can be classified into groups and subgroups according to structure and function (100). The classification based on structure classifies β-lactamase into four groups: class A, B, C, and D, which class A, C, and D are serine active site enzymes, whereas class B is a metallo β-lactamase, which requires one or two zinc ions for their β-lactam hydrolysis. The discovery of β-lactamase began with a restricted spectrum enzymes, e.g., TEM-1, TEM-2, and SHV-1 with an ability to destroy penicillin but not cephalosporins. Mutations in various sites around active site of the original β-lactamase enzymes shift them to expanded activity against higher spectrum drugs. These enzymes are so called extended spectrum β-lactamase (ESBL) with act on newer generations of β-lactam antibiotics. β-lactamase are classify as chromosomal β-lactamase, e.g., chromosomal AmpC β-lactamase in *Citrobacter*, *Enterobacter*, *Serratia*, *Morganella morganii*, and *P. aeruginosa* (101), which normally repressed by repressor protein and plasmid mediated β-lactamase, e.g., Class A β-lactamase. Some of these β-lactamase enzymes are inhibited by inhibitor, e.g., Class A β-lactamase is inhibited by clavulanic acid, tazobactam, and sulbactam, but not for Class C β-lactamase or Class B β-lactamase, which is inhibited by chelating agents, e.g., EDTA or 2-mercaptopropionic acid. Although, inhibitor was used in combination with β-lactam antibiotics, e.g., amoxicillin/clavulanic acid for treatment, but this strategy was overcome by inhibitor resistance β-lactamase production, e.g., in case of inhibitor resistance TEM (IRT) β-lactamase (102). Aminoglycosides exhibit a wide range spectrum against clinically important bacteria, e.g., *E. coli*, *Salmonella*, by binding to 30S ribosomal subunit leading to protein synthesis inhibition. The major mechanism conferring aminoglycosides resistance is a production of aminoglycoside modifying enzymes (AMEs), which are aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside nucleotidyltransferases (ANTs) (103). These enzymes modify the

amino or hydroxyl groups of aminoglycosides in various positions resulting in poor binding to ribosome. The examples of AMEs are: APH(3'), APH(2''), APH(3''), APH(4) for APHs; AAC(1), AAC(3), AAC(2'), and AAC(6') for AACs; ANT(2''), ANT(3''), ANT(4'), ANT(6), and ANT(9) for ANTs (103). Like aminoglycosides, chloramphenicol resistance is often mediated by chloramphenicol acetyltransferase (104). Production of drug inactivating enzymes in macrolide, lincosamide, and streptogramin (MLS) resistance was also demonstrated. Macrolide modifying enzymes are encoded by esterase, and *ereA*, *ereB* for phosphotransferase enzymes, whereas *lnuA* (formerly *linA*) (for lincosamide nucleotidylation) was found among lincosamide resistant Staphylococci (105).

4. Resistance mediated by bypass mechanism

The classical example of this mode of resistance is trimethoprim-sulfamethoxazole (SXT) resistance. SXT inhibits bacterial folic acid synthesis by two sequential steps, i.e., sulfonamide inhibits dihydropteroate synthetase (DHPS), which generates dihydropterate from para-aminobenzoic acid (PABA), trimethoprim subsequently inhibits dihydrofolate reductase (DHFR), which generates tetrahydrofolate from dihydrofolate. SXT resistance is conferred by the overproduction of both DHPS and DHFR enzymes, which caused by promoter mutations (106).

Modes of resistance gene dissemination

Antimicrobial resistance bacteria response to a selective pressure by duplicating themselves or spread the resistance determinants to other bacteria either within species (intraspecies) or between different genera (interspecies). There are two modes of resistance gene spread, which are vertical gene transfer, also known as clonal spread, and horizontal gene transfer, also known as lateral gene transfer. Vertical gene transfer or clonal spread is defined as a transfer of resistant bacteria from parent to offspring. Such bacteria may be preferentially selected under selective pressure and spread by many ways, e.g., poor hygiene, contamination in food products. The emergence of resistant bacteria to a former effective drug has been shown due to clonal expansion (107). In contrast, horizontal gene transfer is a process of transferring a piece of DNA including resistance genes to other bacteria, which comprises conjugation, transformation, and transduction. The conjugation needs a contact between donor and recipient bacteria for transferring genetic material. DNA is transferred by either self-transmissible or mobolizable plasmid (108). Donor cells synthesize multiproteins called mating pair to connect with recipient cells and send a single strand DNA, then completed DNA will be synthesized by recipient. In addition, conjugation also includes chromosomal transfer in form of integrative conjugative elements or ICEs. ICEs have both an integrative and conjugative machine, which offer their integration and excision from host chromosome and transfer by conjugation machinery. Transformation is a process of uptaking free DNA of competence bacteria from environment. Some bacteria, e.g., *Neisseria gonorrhoeae* and *Haemophilus influenzae* have specific sequence recognition, which enhances transformation efficiency (108). Transduction, unlike two former mechanisms, is a process of DNA transferring by help of bacteriophages. The DNA of temperate phage is firstly inserted into donor cell and transferred as host DNA alone (generalized transduction) or transfer its own DNA along with host DNA (specialized transduction). The amount of transferred DNA depends on the size of phage and it can range to 100 kb (108).

Integron element

In a field of antibiotic usage, bacteria have an ability to develop resistance against those antibiotics by acquiring resistance determinants from other bacteria. The classical mechanism in horizontal transfer is conjugation, transformation, and transduction, which all of them have been demonstrated to be important in the movement of resistance genes among clinically important bacteria. Nevertheless, there are other mechanisms conferring resistance genes transfer among such bacteria. Transposition, mediated by transposon element, is a process involving in movement of DNA by requiring no homology between the recombining sequences in contrast to classic recombination, which requires extensive homology between recombining sequences (15). Moreover, bacteria have an efficient tool for capturing resistance genes as a gene cassette, called integron.

Integrons are defined as an assembly platform that incorporate open reading frames (ORFs) of resistance genes in form of gene cassettes, which integrated by site-specific recombination mediated by an integrase (15, 16). Gene cassettes are discrete genetic elements that can be excised as a free circular form in a RecA-independent manner, when moving from one to another site (109), or it can be present as linear sequences that be a part of plasmid or bacterial chromosome (15).

The principle of integrons comprise three major key elements necessary for capturing resistance genes; integrase encoded by *intI*, which belongs to tyrosine recombinase family, a primary recombination site, called *attI*, and a strong promoter, called Pc, which is used for driving an expression of resistance genes inside (Fig 3.8). Gene cassettes, normally present as a single gene, contain resistance gene sequence and additional sequence, called 59 base elements or sometimes called *attC* (15, 110). The gene cassettes carried on integron lack a promoter and expressed from promoter on integron. The *attC* is not highly conserved and varies in length from 57 to 141, which contains boundaried sequences called inverse core site (RYYYAAC) at the 5' end of resistance gene and core site (GTTRRRY) at the opposite end (R = purine, Y = pyrimidine) (16, 111). The cross-over point locates between G and T (16) and mutations in these position reduced cross-over activity. Mutations in AAC of inverse core site also reduced cross-over activity but in lesser extent (111).

The classical feature of integron contains an integrase, *intI*, the integration site, *attI*, which is located beside *intI*, and a promoter, which is located within *intI*. These components are accommodated in the 5' conserved segment (5'CS). The 3' conserved segment (3'CS) contains gene encoded for sulfonamide resistance, *sul1*, a truncated version of the detergent resistance gene, *qacE*Δ*1*, and open reading frame, *orf5* encoding a protein of unknown function (Fig. 3.7). The natural occurring integron designated as In0, which identified in plasmid pVS1, display a structural comprising the 5'CS join directly to 3'CS with no inserted genes (15, 112).

Integron was first thought to be a mobile element (14) based on a fact that the first characterized integron was located in transposon (16). The transposition of integron was then proved that it did not depend on integrase activity, which only mobilized gene cassettes within integron (16). Nonetheless, integron have been shown to be associated with other mobile genetic elements, e.g., plasmid and lead to rapid increase in clinically important bacteria (18). Integron therefore can be divided into two subsets; mobile integrons, which are linked to mobile DNA and play an important role in antibiotic resistance gene spread, and chromosomal superintegron (16). Five classes of mobile integron are classified to date based on sequences of integrase which show 40-58% identity (16, 17). Among all classes of integron, class 1 integron is found extensively in clinically important bacteria and most of known antibiotic resistance cassettes belongs to this class. Class 1 integron is associated with transposon derived from Tn*402* that can be embedded in larger transposon, e.g., Tn*21*, whereas class 2 integron is associated with Tn*7* derivetives (113). Class 3 integron is thought to be located in transposon but less prevalence than class 2 integron (114). Class 4 and Class 5 integron were found in SXT elements in *Vibrio cholerae* and compound transposon in *Vibrio salmonicida*, respectively (16). Chromosomal superintegron was first discovered in chromosome 2 of *V. cholerae*, called *V. cholerae* repeats or VCRs, which have the general characteristics; they are large and carry more than 20 gene cassettes and extensive homology between *attC* sites, and located in chromosome which not associated with other mobile elements (16, 17). It was also identified in closely related bacteria, i.e., xanthomonads and pseudomonads.

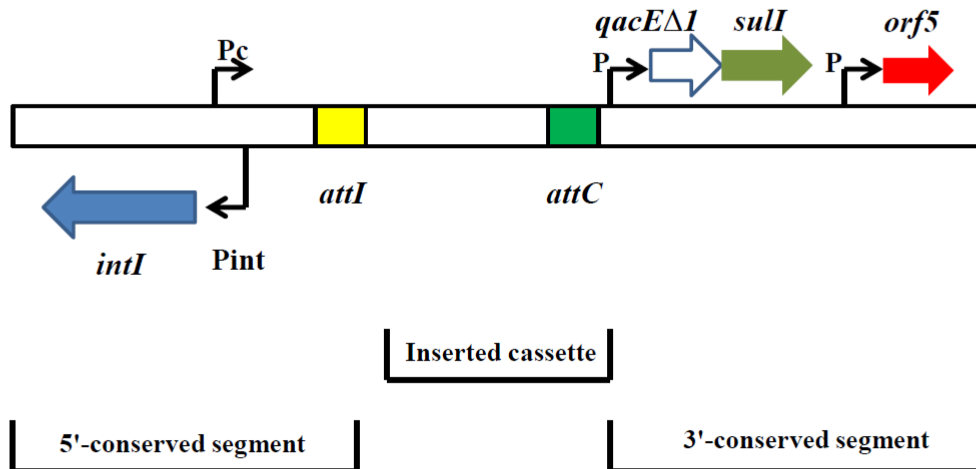


Figure 3.7 General structure of class 1 integron. The 5'CS comprises *intI*, *attI*, and a promoter, whereas the 3'CS comprises *qacE*Δ1, encoding for detergent resistance, *sul1*, encoding for sulfonamide resistance, and *orf5*, encoding protein of unknown function. Arrows indicate transcription direction (modified from ref.115).

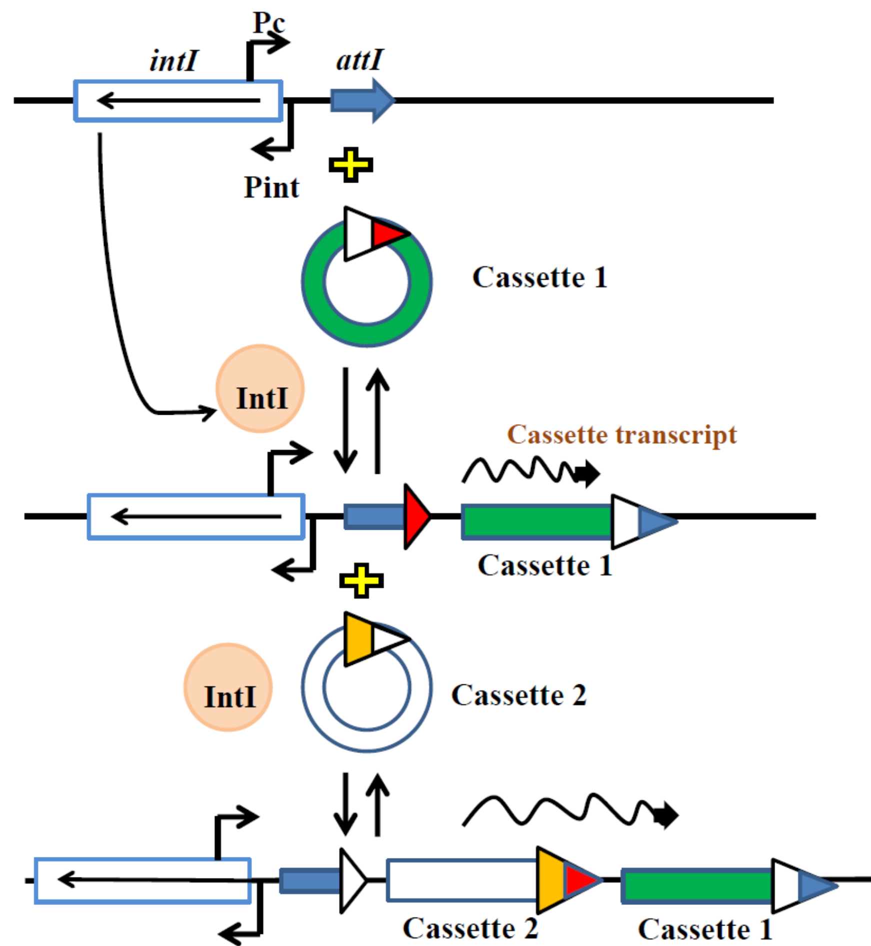


Figure 3.8 Integron-mediated gene capture and model for cassette exchange. Outline of the process by which circular gene cassettes are repeatedly inserted at the specific *attI* site in an integron downstream of the strong promoter Pc. *intI*, integrase encoding gene; IntI, integrase IntI (modified from ref. 17).

Typing methods of *Salmonella* and *E. coli*

The diseases caused by bacterial infection have a great impact on human health with high morbidity and mortality rate. The bacterial contamination in foods is an important factor involving in this situation, which can be transmitted to human via fecal-oral route. In addition, contaminated food can then be spread rapidly via travelers, therefore it is responsible for such strain worldwide. Epidemiological study is important to track the sources of bacteria, investigate an outbreak situation, understand pathogenesis of an infection, and, therefore provide an epidemiological data for control of their spread. The criteria for evaluating typing methods are typeability, an ability to obtain unambiguous results in each time of analysis, reproducibility, an ability to obtain the same result when tested in different times, discriminatory power, ability to differentiate among unrelated strains, ease of performance, and ease of interpretation. Multiple methods can be used for typing bacterial pathogens, which can be classified into phenotypic typing and genotypic typing.

1. Phenotypic methods

Phenotypic methods identify bacteria by observing their phenotypic expression of those bacteria. These methods involve observing in size, shape, biochemical property based on metabolic activity.

1.1 Serotyping

Serotyping is based on the fact that strains of same species can differ in the antigenic determinants expressed on the cell surface. It uses differences in the somatic (O) and flagellar (H) surface antigens to separate strains into distinct serotypes. Several methods can be used for serological testing, e.g., latex agglutination, by mixing bacterial suspension with known panel of O and H antigens.

1.2 Phage typing

Phage typing used an ability of a standard set of bacteriophages to infect certain bacteria. This differential ability of phage infection depends on a specific receptor of phage on bacterial surface, which allow phage to bind to bacterial cell.

1.3 Antibiogram

This typing technique involves a comparison of antibiotic susceptibility pattern among bacterial isolates. This method is ease of performance and a pattern of unusual may indicate an outbreak.

2. Genotypic methods

2.1 Plasmid profile

Plasmids are an extrachromosomal DNA, which are distributed between the daughter cells in cell division. Thus, bacteria from the same clonal groups typically carry the same plasmids, which facilitate comparison among bacterial isolates (116). Plasmid DNA isolation can be done by various methods, e.g., alkaline lysis method, and then separated according to their size by agarose gel electrophoresis. The number and size of plasmid are used to differentiate among bacterial isolates. In addition, extracted plasmids are used in restriction reaction by restriction enzymes and compared in number and size of plasmids. The major problem in plasmid analysis is a conformation changes in plasmids, which may affect the migration property in agarose gel electrophoresis (117).

2.2 Restriction fragment length polymorphism (RFLP)

RFLP patterns are created by digesting chromosomal DNA with restriction enzymes, and separated digested fragments by agarose gel electrophoresis. The digested fragments are difficult to interprete because of many

fragments will be observed. This problem can be solved by using rare cutting enzymes or hybridized DNA to membrane and probed with specific probes. Ribotyping is a form of RFLP, which uses a specific probe to conserved regions of rRNA to probe with hybridized DNA (118), whereas insertion sequences-RFLP (IS-RFLP) use a specific probe to insertion sequences (116). A variant of RFLP is PCR-RFLP, which involve PCR amplification a specific DNA sequences, usually a conserved gene, followed by restriction digestion to generate banding patterns.

2.3 Amplified fragment length polymorphisms (AFLP)

AFLP combines a restriction digestion with PCR amplification. Chromosomal DNA is extracted and digested with enzymes and then linkers, which contain a complimentary sequence with digested fragments, are added in reaction. PCR amplification is carried out by using primers specific to linkers. The number of PCR products are limited by adding one to three additional sequences at the 3' ends of primer. The additional bases will bind randomly to template strand, which will reduce the number of amplicons to a factor of four (119).

2.4 Randomly amplified polymorphic DNA PCR (RAPD-PCR)

RAPD-PCR based on PCR amplification by using an arbitrary primer under low stringency condition. The primers usually contain 6-10 base pairs long (120). The PCR amplicons are then separated by agarose gel electrophoresis. The problem for this method is reproducibility of results due to analysis parameters, e.g., reagents, amplification conditions.

2.5 Repetitive element PCR (Rep-PCR)

Rep-PCR relies on the fact that bacterial species have many repeated DNA sequences throughout their genome. PCR primers are designed to the regions flanking the repeats. When two repeats are close enough, the PCR amplification will occur (121). The differences in banding patterns can result from the number and position of repetitive elements in their genome (122). The examples of repeated sequences used in Rep-PCR are enterobacterial repetitive intergenic

consensus (ERIC), which are 126 base pairs conserved motifs of enteric bacteria (121), and repetitive extragenic palindromic (REP).

2.6 Variable number of tandem repeat (VNTR) analysis and multiple locus VNTR analysis (MLVA)

Bacterial genomes were found to contain repeated sequences throughout their genome from DNA sequencing data. The repeated sequences range from few base to over 100 base pairs in length. PCR primers are designed to anneal an outside of repeated arrays. The size of PCR amplicons depend on the amount of nucleotide base within repeated arrays. MLVA is an alternative approach by combining multiple VNTR loci for typing.

2.7 Multilocus sequence typing (MLST)

MLST determines sequence variability within selected housekeeping genes rather than DNA fragment size determination. PCR amplification of housekeeping genes are performed followed by nucleotide sequencing. The sequencing data is subjected to MLST database, which all sequencing data are assigned as sequence type (ST) according to alleic profile. High quality of sequencing results are needed in case of MLST because it relies on nucleotide base changes (116). If the strains display a unique alleic profile, it will define as a new ST. The advantage of this method is worldwide comparable method which all users can download sequence of primers and upload sequencing results to database to know what STs they have.

CHAPTER IV
MATERIALS AND METHODS

Prevalence of *Salmonella* spp. and *E. coli* in raw chicken meat from supermarkets in Bangkok

1 Sample collection

Two hundred raw chicken meat samples were collected from various supermarkets in Bangkok. The characteristics of samples in this study were sealed-packages, which kept at 4°C. Manufacturing date and expiry date were addressed on each package. Sample collections were performed during July 2010 to May 2011 from 7 supermarkets and 4 major distributors. The collection places were Tops (Central-Pinklao) (68 samples), Lotus (Pinklao) (35 samples), Lotus (Pata-Pinklao) (20 samples), Tang Hua Seng (37 samples), Golden Place (33 samples), Lotus Bangkuntien (6 samples), and Wanglang market (1 sample). Four distributors in this study were distributor 1 (42 samples), distributor 2 (61 samples), distributor 3 (50 samples), and distributor 4 (47 samples). Some supermarkets sold raw chicken meat from more than one distributor, e.g., Tops (Central-Pinklao) sold products from distributor 1 and 3, Golden place sold from distributor 3 and 4. Sample number per time of visit was from 5 to 15 samples. The samples were transferred on ice and brought to laboratory within 30 minutes. Bacterial isolation was performed immediately after arrival at laboratory by using aseptic technique. If the process was delayed, samples would be kept at 4°C until bacterial isolation process was done. Each time of sample collection, expiry date was determined in order to reduce samples variation.

Sample size in this study was calculated from:

$$n = \frac{Z^2 p(1-p)}{d^2} \qquad (123)$$

n = required sample size

Z = confidence level at 95% (standard value of 1.96)

P = estimated prevalence of interested project

d = margin of error at 5% (standard value of 0.05)

Sample size in this study was calculated based on prevalence of *Salmonella*. The average prevalence was 15%, which requires about 200 samples for this study.

2 Bacterial isolation

Salmonella spp. were isolated according to standard method described in ISO 6579 (124) and appropriated method was used for non-pathogenic *E. coli* isolation. All processes were done by using aseptic technique. Twenty five grams of samples were cut, weighed, and aseptically placed in sterile stomacher bag with 225 ml of buffered peptone water (BPW), which is a pre-enrichment media for *Salmonella* spp. and can also be used for *E. coli*. The content in the stomacher bags were then shaken in order to homogenize the content in the stomacher bags by using homogenizer machine for 2 minutes, sealed and then incubated at 37°C for 18 h. BPW helps to recover injured bacterial cells that can be caused by food preservation techniques involving heat, desiccation, preservatives, and high osmotic pressure or pH changes.

For *Salmonella* isolation, 1 ml and 0.1 ml of chicken meat rinse solution were transferred to 2 tubes of 10 ml of tetrathionate broth (TT broth) and Rappaport-Vassiliadis R10 (RV R10 broth), incubated at 37°C for 24 h and 42°C for 24 h, respectively. The broth cultures were then streaked on Salmonella-Shigella Agar (SS Agar) and deoxycholate hydrogen sulfide lactose Agar (DHL Agar), and incubated at 37°C for 24 h. The typical colonies of *Salmonella* spp., which are colorless colony with ferrous sulfide in black color in the center, were confirmed by using biochemical identification test. Biochemical test for *Salmonella* identification were triple sugar iron

(TSI), motile, indole, ornithine decarboxylase (OD), and urea. All *Salmonella* strains were typed further into serogroup and serotype.

For *E. coli* isolation, the content in the stomacher bags were streaked on MacConkey agar and incubated at 37°C for 24 h. The typical colony of *E. coli*, which presented a pink colony with pitting in the center, was confirmed by using biochemical identification test. Biochemical test for *E. coli* identification were TSI, motile, indole, OD, and urea.

Table 4.1 Biochemical test for *Salmonella* spp. and *E. coli* identification used in this study

	TSI	Motile	Indole	OD	Urea
E. coli	A/AG, K/AG	+	+	+	-
Salmonella	K/AGH2S	+	-	+	-

3 *Salmonella* serogrouping and serotyping

For serogrouping, pure colonies of *Salmonella* were reacted with 6 polyvalent *Salmonella* antisera (Serosystem, Clinag, UK). Positive results were observed the agglutination on slides by naked eye.

Salmonella serotyping was performed at the Department of Medical Sciences, Ministry of Public Health. All isolates were serotyped by using slide agglutination. O and H antigens were characterized by agglutination with hyperimmune sera (S & A reagents lab, Ltd, Bangkok, Thailand) and serotype was assigned according to the Kauffmann-White scheme (125).

4 Bacterial storage

Salmonella spp. and *E. coli* were kept frozen at -20°C in Luria-Bertani broth (LB broth) or brain heart infusion broth (BHI broth) plus 20% glycerol with beads inside. Isolates were grown in suitable media and heavy inoculated into storage tubes. After isolates were completely mixed by inverting tubes, supernatants were then removed and stored the tubes at -20°C.

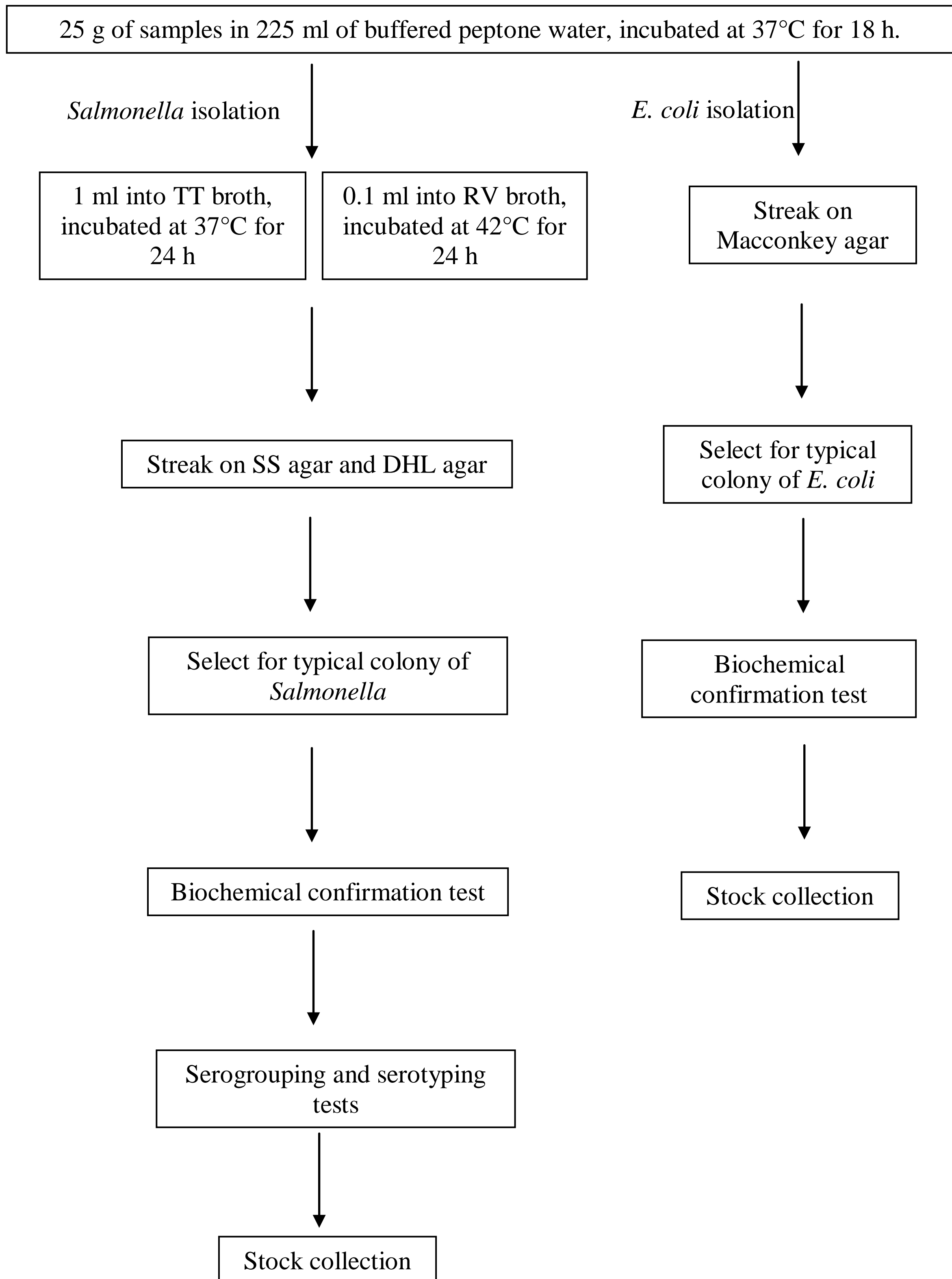


Figure 4.1 Flowchart of *Salmonella* spp. and *E. coli* isolation from chicken meat

Determination of coliform bacteria by most probable number method (MPN method)

Most probable number (MPN) is the multiple tubes technique, which is used to determine the coliform bacteria in food and water specimen. MPN used in this study was performed according to Food microbiology; A laboratory manual (126). There are three levels of testing to determine the presence of coliform bacteria in water and food; presumptive test, confirmatory test and completed test.

Presumptive test:

1. The chicken meat was mixed thoroughly and taken with sterile technique. Eleven grams of food was weighed and transferred into a sterile stomacher bag.

2. Ninety nine ml of buffered peptone water (BPW) was added and then homogenized by homogenizer for 2 minutes. The homogenized sample represented the 10^{-1} dilution.

3. The following additional dilution: 10^{-2}, 10^{-3}, 10^{-4}, was prepared by using the 9-ml BPW tubes.

4. Three tubes of lauryl sulphate tryptose broth (LST broth), each with 9 ml and Durham inside, were inoculated with 1 ml of the three dilutions (10^{-2}, 10^{-3}, 10^{-4} a total of nine tubes).

5. All tubes were incubated at 35°C for 48 hours.

6. Each LST tubes were observed and gently shaked the tube to help release the gas from the medium. If there is a gas bubble trapped in the Durham tube, or effervescence is produced when the tube is gently shaken, score the tube positive. When no gas is produced, the tube is scored negative.

7. The total number of positive tubes at each dilution was determined and interpreted the MPN value in the Table 4.5.

Table 4.2 Most probable number (MPN) estimates for three fermentation tubes per dilution and dilutions representing 10^{-1}, 10^{-2}, 10^{-3} dilution

Number of positive tubes/3 tubes				Number of positive tubes/3 tubes			
10^{-1}	10^{-2}	10^{-3}	MPN/g	10^{-1}	10^{-2}	10^{-3}	MPN/g
0	0	0	<3	3	0	0	23
0	1	0	3	3	0	1	38
1	0	0	3.6	3	1	0	43
1	0	1	7.2	3	1	1	75
1	1	0	7.4	3	2	0	93
1	2	0	11	3	2	1	150
2	0	0	9.2	3	2	2	210
2	0	1	14	3	3	0	240
2	1	0	15	3	3	1	460
2	1	1	20	3	3	2	1100
2	2	0	21	3	3	3	>1100

8. The dilution factor multiplier was determined. Table above is for tubes with dilutions of 10^{-1}, 10^{-2}, and 10^{-3}. The MPN/g value from the table must be converted to the dilution used in this experiment. The lowest dilution used in this study is 10^{-3}, there is a factor of 10^{-2} ($10^{3-}/10^{-1}$) between the table and the sample. Therefore, multiply the table values by $1/10^{-2}$ to convert the dilution.

Confirmatory test:

1. A presumptive-positive LST broth tube was selected for confirmation.
2. Contents of the LST broth tubes were mixed by rapidly rolling each in between hands and using a loop. The contents were then put into the brilliant green lactose bile broth (BGLB broth).
3. The contents of the BGLB broth tubes were mixed as in 2.2.2
4. The BGLB tubes were incubated at 35°C for 24 hr.
5. The BGLB broth tubes were examined for entrapped gas in Durham tubes and recorded tubes with gas as positive.

6. If BGLB broth tubes are positive, this confirms the LST results. If some BGLB broth tubes are negative, MPN/g of coliform in food was calculated based on the proportion of confirmed gassing in BGLB broth tubes.

Completed test using MacConkey agar:

1. The contents of the positive BGLB broth tubes were mixed.
2. The positive BGLB broth was streaked onto MacConkey agar.
3. The plates were incubated at 35°C for 24 hr.
4. MacConkey agar plates were inspected and observed the bright and pink colony with pitting in the center of *E. coli*.

Quality control

Organism	Result
E. coli	Gas bubbles in Durham tube
Proteus vulgaris	No Gas bubbles in Durham tube

Antibiotic susceptibility testing (AST) by disk diffusion method

AST was determined by disk diffusion method according to the standard method of Clinical and Laboratory Standards Institute 2010 guideline (CLSI 2010 guideline) (127). Inoculum was prepared by making a direct saline from overnight cultured bacteria and adjusted to 0.5 McFarland standard. Within 15 minutes after adjusting the turbidity of inoculum, the sterile swab was dipped into the inoculum and rotated several times. The swab was then streaked on Muller-Hinton agar and rotated 60° in order to ensure the distribution of inoculums. The antibiotic disks were placed on the surface of agar by using sterile forceps within 15 minutes, after culture inoculation. Antibiotics used in this study were ampicillin (10 µg), amoxicillin-clavulanic acid (20/10 µg), cephalothin (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefepime (30 µg), ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg) amikacin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25/23.75

μg), chloramphenicol (30 μg), tetracycline (30 μg). The plates were incubated at 35°C for 18 hours. The results were recorded as inhibition zone in diameter and interpreted according to CLSI 2010 guideline (Table 4.6). Quality control strains were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 35218. Quality control was done in parallel with tested strains.

Quality control strains

- *Escherichia coli* ATCC 25922
- *Staphylococcus aureus* ATCC 25923
- *Escherichia coli* ATCC 35218 for β-lactamase/β-lactamase inhibitor testing

Table 4.3 Zone diameter interpretative standard for *Enterobacteriaceae* according to CLSI 2010 (127)

Antibacterial agents	Disk content (μg)	Diameter of inhibition zone (mm)		
		Resistant	Intermediate	Susceptible
Ampicillin	10	≤ 13	14-16	≥ 17
Amoxicillin/clavulanate	20/10	≤ 13	14-17	≥ 18
Cephalothin	30	≤ 14	15-17	≥ 18
Cefuroxime	30	≤ 14	15-22	≥ 23
Cefotaxime	30	≤ 22	23-25	≥ 26
Ceftriaxone	30	≤ 19	20-22	≥ 23
Ceftazidime	30	≤ 17	18-20	≥ 21
Cefepime	30	≤ 14	15-17	≥ 18
Ertapenem	10	≤ 15	16-18	≥ 19

Antibacterial agents	**Disk content (μg)**	**Diameter of inhibition zone (mm)**		
		Resistant	Intermediate	Susceptible
Imipenem	10	≤ 13	14-15	≥ 16
Meropenem	10	≤ 13	14-15	≥ 16
Gentamicin	10	≤ 12	13-14	≥ 15
Amikacin	30	≤ 14	15-16	≥ 17
Ciprofloxacin	5	≤ 15	16-20	≥ 21
Levofloxacin	5	≤ 13	14-16	≥ 17
Chioramphenicol	30	≤ 12	13-17	≥ 18
Tetracycline	30	≤ 11	12-14	≥ 15
Trimethoprim-sulfamethoxazole	1.25/23.75	≤ 10	11-15	≥ 16

Investigation the presence of class 1 integron and their antibiotic resistance genes

1 Total DNA extraction

Total DNA of *Salmonella* (14 isolates) and *E. coli* (106 isolates) were extracted by using PUREGENE™ DNA purification kit with the instruction provided by manufacturer. Briefly, 500 µl of bacterial cell suspension was centrifuged at 15,000 x g for 5 seconds to pellet cell. The supernatant was carefully removed. Total of 300 µl of cell lysis solution was then added to resuspend the pellet and incubated at 80°C for 5 minutes. Cell lysis was added with 1.5 µl of RNase A solution in order to eliminate unwanted RNA, and incubated at 37°C for 60 minutes. After samples were cool to room temperature, 100 µl of protein precipitation solution was added and vortex vigorously to mix this solution with cell lysate. Samples with protein precipitation solution were centrifuged at 15,000 x g for 3 minutes. The precipitated proteins were formed as a tight pellet. Supernatant containing DNA was removed to

1.5 ml centrifuged tube, which contained 300 µl of 100% Isopropanol (2-propanol). 100% Isopropanol was used to precipitate DNA and should be visible as white pellet. Ethanol (70%) was added in order to wash DNA. After 70% ethanol was carefully poured off and air dry for 15 minutes, DNA hydration was added. DNA was rehydrated by incubating samples at 65°C for 1 hour or overnight at room temperature. DNA was stored at -20°C until used.

2 Analysis of DNA by agarose gel electrophoresis

DNA products were analyzed for their sizes and amounts by electrophoresis in 1% agarose gel. Agarose gel was run at 100 volt for 30 minutes and stained with 5µg/ml ethidium bromide for 5 minutes and destained with water for 20 minutes. DNA products were observed on UV transilluminator.

3 Class 1 integron determination

PCR was carried out with specific primers including *intI1* encodes integrase and 5'CS-3'CS for internal segments of integron element (Table 4.7). PCR schematic for class 1 integron detection was shown in Fig. 4.10

For *intI1*, PCR amplification was performed with the following cycling parameters: initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds, with final extension at 72°C for 7 minutes. The PCR products were analyzed by electrophoresis in 1% agarose gel containing 1X TBE buffer. 1 kb plus DNA ladder was used as a marker and positive control, *Pseudomonas aeruginosa* PA67, an integron containing $bla_{\text{IMP-15}}$ strain, was run in parallel with samples. 471 bp in size was interpreted as positive for integrase gene. Negative control was a master mix reagent, which contains all reagents except DNA.

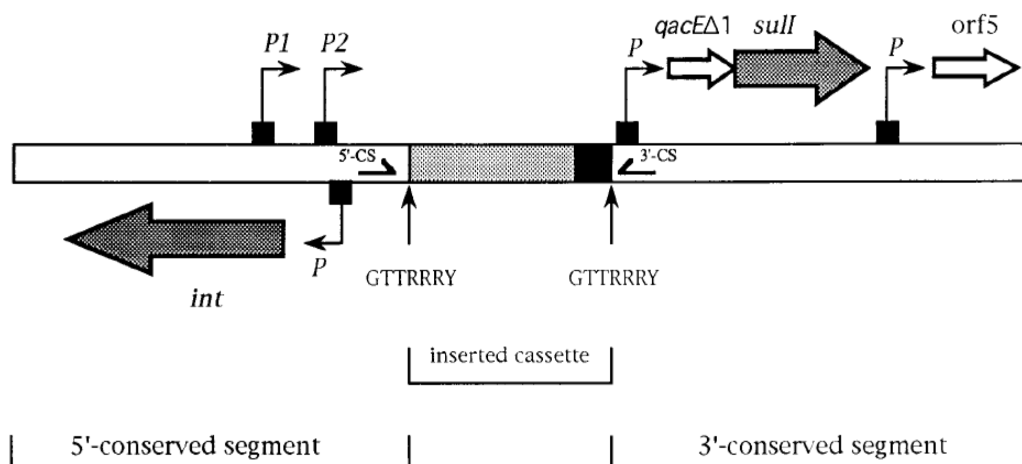


Figure 4.2 PCR schematic for class 1 integron detection in this study (115). PCR with specific primer to *intI1* was used as screening test for the presence of class 1 integron and PCR with specific primers to internal segment of 5'CS and 3'CS was used to detect an inserted resistance genes.

Table 4.4 Primer sequences used to detect class 1 integron, *intI1*, which the expected PCR product size of 471 bp. Primers with specific to 5'CS, 3'CS, and 3'CS 2 were used to identify an inserted resistance gene

Primer name	Sequence	Product size	Reference
intI1 F	5' AAGGATCGGGCCTTGATGTT 3'	471 bp	This study
intI1 R	5' CAGCGCATCAAGCGGTGAGC 3'		
5'CS	5' GGCATCCAAGCAGCAAG 3'	Variable	115
3'CS	5' AAGCAGACTTGACCTGA 3'		
3'CS2	5' GACCTGATAGTTTGGCTGTGAGC 3'		This study

Table 4.5 Recipe of PCR reaction for detecting integrase gene (total volume 50 µl)

Reagent	Volume (µl)
MilliQ water	37.25
10X buffer (with $MgCl_2$)	5
10 mM dNTP	1
Forward primer (10 µM)	2.5
Reverse primer (10 µM)	2.5
DyNAzyme *Taq* polymerase	0.75
Template DNA	1

For 5'CS-3'CS, the binding sites of primers were shown in Fig. 4.11. PCR was performed with the following cycling parameters: initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 1 minute and extension at 72°C for 2 minutes, with final extension at 72°C for 7 minutes. The PCR products were analyzed by electrophoresis in 1% agarose gel containing 1X TBE buffer. One kb plus DNA ladder was used as a marker. *Pseudomonas aeruginosa* PA67, an integron containing $bla_{IMP\text{-}15}$ strain and generate 1,897 bp of PCR product, was run in parallel with samples. Negative control was a master mix reagent, which contains all reagents except DNA.

Table 4.6 Recipe for PCR reaction for 5'CS-3'CS (total volume 25 µl)

Reagent	Volume (µl)
MilliQ water	17.75
10X buffer (with $MgCl_2$)	2.5
25 mM $MgCl_2$	2
10 mM dNTP	0.5
5'CS primer (100 µM)	0.25
3'CS primer (100 µM)	0.25
DyNAzyme *Taq* polymerase	0.75
Template DNA	1

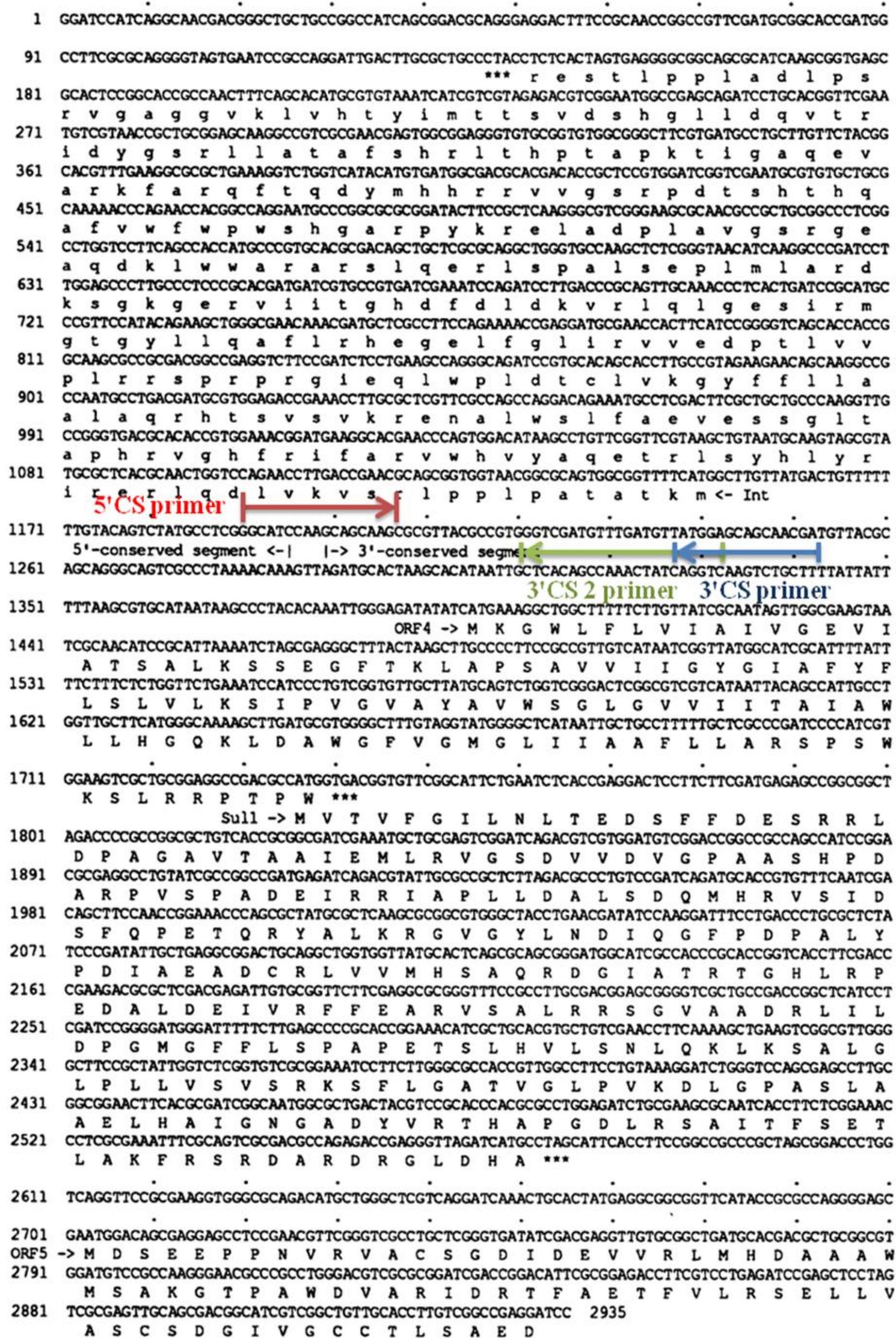


Figure 4.3 The PCR primers for internal segment of 5'CS-3'CS were indicated by red, blue, and green color for 5'CS, 3'CS, and 3'CS2, respectively. The primer sequences were designed from a sequence of plasmid pVS1, an integron with no inserted resistance genes from *P. aeruginosa* (128)

PCR products of 5'CS-3'CS primer were examined a pattern of digestion by using *Afa*I enzyme and recipe for *Afa*I digestion was shown below.

Recipe for *Afa*I analysis

Sterile D.W.	10 µl
*Afa*I	1 µl
10 X T Buffer	2 µl
0.1% BSA	2 µl
PCR product	5 µl
Total	20 µl

The total amount of digested tube was activated at 37 ºC for 1 hr and inactivated at 60 ºC for 15 min. The result of banding patterns was analyzed by electrophoresis in 1% agarose gel containing 1X TBE buffer.

4 Cloning experiment

PCR products amplified from 5'CS-3'CS primers were cloned into a plasmid vector StrataClone™ PCR cloning vector pSC-A-amp/kan according to manufacturer's instruction.

The StrataClone PCR cloning vector mix contains two DNA arms, each charged with topoisomerase I on one end and containing a *loxP* recognition sequence on the other end. The topoisomerase-charged ends have a modified uridine (U*) overhang. *Taq*-amplified PCR products, which contain 3´-adenosine overhangs, are efficiently ligated to these vector arms in a 5 min ligation reaction, through A-U* base-pairing followed by topoisomerase I-mediated strand ligation.

Table 4.7 Recipe for StrataClone™ PCR cloning reaction

Reagents	Volume (µl)
StrataClone™ Cloning buffer	3
PCR product	2
StrataClone™ Vector mix	1

The ligation reaction was prepared by mixing the components shown in Table 4.10. The reaction was mixed gently by repeated pipetting and incubated at room temperature for 5 minutes. When the incubation is complete, the reaction was placed on ice. One tube of StrataClone SoloPack competent cells was thawed on ice for each ligation reaction. After that, the PCR cloning reaction was transformed into 50 µl of competence cell and swirled the tubes gently, then incubated on ice for 20 minutes. The reaction tubes were then heat-pulsed in a 42°C water bath for 45 seconds.The transformation mixture was incubated on ice for 2 minutes. Two hundred and fifty microliters of preheated (42°C) LB medium was added to the reaction tubes. The tubes were then incubated at 37°C for 1 hour with agitation. Finally, 5 µl and 100 µl of transformation mixture was plated on LB-kanamycin with 2% X-Gal plate and incubated at 37°C overnight. The white or light blue colonies were selected for the insertion of PCR products.

5 Transformant clones analysis

5.1 Plasmid extraction

Transformant clones were extracted for recombinant plasmids by using Geneaid High-Speed Plasmid Mini Kit according to manufacturer's instruction. First step, 1.5 ml of an overnight culture was harvested by microcentrifuge (15,000 g) for 1 minute and the supernatant was discarded. The pellet was resuspended in 200 µl of PD 1 buffer by vortex or pipetting. Two hundred µl of PD 2 buffer was added to cells and mixed gently by inverting the tubes 10 times, then were incubated at room temperature for 2 minutes or until the lysate was homologous. Next, 300 µl of PD 3 buffer was added and mixed immediately by gently inverting the tubes 10 times, then microcentrifuge for 3 minutes. Supernatant was loaded onto PD column and microcentrifuge the column for 30 seconds, then the flow-through was discarded and the column was placed back in the tube. Four hundred µl of W 1 buffer was added to the column and microcentrifuge for 30 seconds, then the flow-through was discarded and the column was placed back to the tube. Six hundred µl of Wash buffer was added and microcentrifuge for 30 seconds, then the flow-through was again discarded and the column was placed back to the tube and microcentrifuge again for 3 minutes to dry

the column. The dried column was transferred to a new microcentrifuge tube. Fifty µl of elution buffer or TE was added into the center of the column, let stand for 2 minutes, microcentrifuge for 2 minutes for DNA elution. Finally, purified plasmid was contained in elution tubes.

5.2 Restriction enzyme analysis

In order to find transformant clones, which carried recombinant plasmid, restriction analysis was performed with *Eco*RI digestion. StrataClone^{TM} PCR cloning vector pSC-A-amp/kan contains *Eco*RI flanked PCR product insertion site (Fig. 4.12), which used for screening the clones with specific insert orientation. *Eco*RI digestion was performed with the following reaction (Table 4.11).

Table 4.8 Recipe for digestion reaction for *Eco*RI enzyme

Reagents	Volume (µl)
10x buffer	1
*Eco*RI	1
Recombinant plasmid	4
Sterile water	4

The digestion reaction was incubated at 37°C for 18 hours. The reaction was stopped by heat inactivation at 65 °C for 20 minutes and observed fragment sizes by agarose gel electrophoresis.

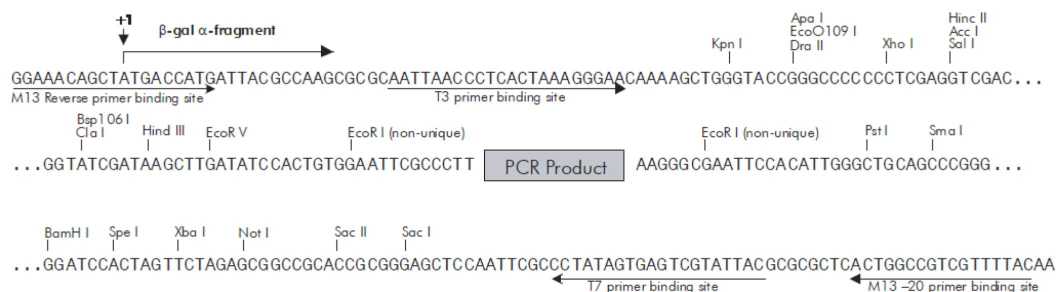


Figure 4.4 pSC-A-amp/kan PCR cloning vector used in cloning experiment

5.3 DNA sequencing and BLAST sequence analysis

The purified plasmid was performed DNA sequencing by using M13 universal sequencing primer. The sequencing data was checked for the quality, i.e., purity, signal before submitting to GenBank database (http://ncbi.nlm.nih.gov).

Plasmid profile study

Plasmids of *Salmonella* spp. and *E. coli* were extracted by using alkaline lysis miniprep (129) and followed by restriction enzyme analysis. A preparation of alkaline lysis solution I, II, and III was described in appendix.

Reagents used in alkaline lysis miniprep

- BHI agar medium containing appropriate antibiotic
- Plasmid-containing bacterial colonies
- Alkaline lysis buffer I
- Alkaline lysis buffer II
- Alkaline lysis buffer III
- 95% and 70% ethanol
- TE buffer

Procedure

1. Single bacterial colony was inoculated into 5 ml BHI broth and grown overnight at 37ºC.

2. One and a half ml of cells were centrifuged, vortex at maximum speed for 20 sec and then discarded supernatant.

3. Pellet was resuspended in 100 µl alkaline lysis buffer I with RnaseA solution and then let sit 5 minutes at room temperature.

4. Two hundred µl of freshly prepared alkaline lysis buffer II was added and mixed by tapping tube with finger and placed on ice for 5 minutes.

5. One hundred and fifty µl of alkaline lysis buffer III was added, vortex 2 sec to mix and returned to ice for 5 minutes.

6. Tubes were centrifuged for 3 minutes and then supernatants were transferred to fresh tubes.

7. Eight hundred µl of 95% ethanol was added and let sit at room temperature for 2 minutes.

8. Tubes were centrifuged for 1 minute at room temperature, supernatant was discarded then pellet was washed with 1 ml of 70% ethanol and let dry under vacuum.

9. Pellet was resuspended in 30 µl of TE buffer and stored at 4ºC for short term or -20ºC or -70ºC for long term storage.

Multilocus sequence typing (MLST) for characterization of *Salmonella* isolates

Salmonella isolates in this study were typed by using multi locus sequencing typing method (MLST). MLST examines alleic profile from selected gene by sequencing of the selected genes. PCR amplification was carried out with specific primers and conditions according to described in MLST database (http://mlst.ucc.ie/mlst/dbs/Senterica).

Seven housekeeping genes used for MLST for *Salmonella*

thrA (aspartokinase+homoserine dehydrogenase)
purE (phosphoribosylaminoimidazole carboxylas)
sucA (alpha ketoglutarate dehydrogenase)
hisD (histidinol dehydrogenase)
aroC (chorismate synthase)
hemD (uroporphyrinogen III cosynthase)
dnaN (DNA polymerase III beta subunit)

PCR amplification

For all seven housekeeping genes, PCR amplification was performed with the following cycling parameters: initial denaturation at 94°C for 5 minutes followed

by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, with final extension at 72°C for 7 minutes. The PCR products were analyzed by electrophoresis in 1% agarose gel containing 1X TBE buffer. 1 kb plus DNA ladder was used as a marker. Recipe for PCR amplication was shown in Table 4.12. The primer sequences and an expected PCR product size in each gene were shown in Table 4.13.

Table 4.9 Recipe of PCR reaction of MLST for *Salmonella* (total volume 50 µl)

Reagent	**Volume (µl)**
MilliQ water	42
10X buffer (with $MgCl_2$)	5
10 mM dNTP	1
Forward primer (100 µM)	0.25
Reverse primer (100 µM)	0.25
DyNAzyme *Taq* polymerase	0.50
Template DNA	1
Total	50

Table 4.10 Primer sequences and an expected PCR product size of 7 housekeeping genes used for perform MLST of *Salmonella*

Primer name	**Primer sequences**	**Product size (bp)**
thrA	F 5'-GTCACGGTGATCGATCCGGT-3' R 5'-CACGATATTGATATTAGCCCG-3'	852
purE	F 5'-ATGTCTTCCCGCAATAATCC-3' R 5'-TCATAGCGTCCCCCGCGGATC-3'	510
sucA	F 5'-AGCACCGAAGAGAAACGCTG-3' R 5'-GGTTGTTGATAACGATACGTAC-3'	643
hisD	F 5'-GAAACGTTCCATTCCGCGCAGAC-3' R 5'-CTGAACGGTCATCCGTTTCTG-3'	894

Primer name	Primer sequences	Product size (bp)
aroC	F 5'-CCTGGCACCTCGCGCTATAC-3' R 5'-CCACACACGGATCGTGGCG-3'	826
hemD	F 5'-ATGAGTATTCTGATCACCCG-3' R 5'-ATCAGCGACCTTAATATCTTGCCA-3'	666
dnaN	F 5'-ATGAAATTTACCGTTGAACGTGA-3' R 5'-AATTTCTCATTCGAGAGGATTGC-3'	833

PCR product clean up

All PCR products were performed a PCR clean up (Gel/PCR DNA Fragments Extraction Kit, Geneaid) in order to remove an excess dNTP, primer, and other unwanted fragments prior submitting for DNA sequencing.

Procedure

1. Up to 100 µl of a reaction product was transferred to a 1.5 ml microcentrifuge tube.

2. Five volume of DF buffer was added to 1 volume of sample and mixed by vortex.

3. DF column was placed in a 2 ml collection tube and the sample mixture from previous step was transferred to the DF column, centrifuged at 15,000 x g for 30 sec.

4. Flow-through was discarded and the DF column was placed and backed in the 2 ml collection Tube.

5. Six hundred µl of Wash Buffer (ethanol added) was added into the center of the DF column and let stand for 1 minute.

6. Tubes were centrifuged at 15,000 x g for 30 seconds, discarded the flow-through. The DF column was placed back in the 2 ml collection tube and centrifuged again for 3 minutes at 15,000 x g to dry the column matrix.

7. Dried column was transferred to a new tube.

8. Twenty to fifty µl of Elution Buffer or TE was added into the center of the column matrix and let stand for at least 2 minutes to ensure the Elution Buffer was completely absorbed by the matrix.

9. Tubes were centrifuged for 2 minutes at 15,000 x g to elute the purified DNA.

Dot blot hybridization

The presence of *intI1* in *Salmonella* and *E. coli* were confirmed by dot blot hybridization. PCR product with specific primer to *intI1* was used for making *intI1* probe and labeled by using High Prime DNA Labeling Kit (Roche Applied Science) according to manufacturer's instruction. DNA was denatured by using alkaline solution and heat and then blotted onto positively charge nylon membrane. Blotted DNA was then hybridized with specific probe and washed excess solution using stringency solution. Detection was done by using CDP-star detection module (Amersham Pharmacia Biotech).

Materials and Reagents

1. Fluorescein-labeled probe DNA
2. Prehybridization buffer: 1/20 dilution of liquid block solution, 5X SSC, 0.1% SDS solution.
3. Hybridization buffer: Prehybridization with labeled probe.
4. 20X SSC: 0.3 M trisodium citrate, 3 M NaCl, pH 7.0.
5. Stringency solution I: 1X SSC, 0.1% (w/v) SDS.
6. Stringency solution II: 0.5X SSC, 0.1% (w/v) SDS.
7. Buffer A: sterile 100 mM Tris-HCl, pH 9.5, 300 mM NaCl.
8. Liquid block solution.
9. Bovine serum albumin (BSA).
10. Tween 20.
11. Antifluorescein antibody/alkaline phosphatase conjugate.
12. CDP-Star detection reagent.
13. Whatman 3MM paper.

14. Saran wrap.

15. Forceps.

16. Autoradiography film.

17. X-ray film cassette: Hypercassette from Amersham Pharmacia Biotech.

Probe labeling

Fluorescein-labeled DNA probe was generated with Fluorescein-High Prime (Roche Applied Science) according to the "random primed" labeling technique. The complementary DNA strand is synthesized by Klenow polymerase using the 3'OH termini of the random oligonucleotides as primers. Fluorescein-12-dUTP is incorporated into the newly synthesized complementary DNA strand. Fluorescein-High Prime kit contains random oligonucleotides, Klenow polymerase, fluorescein-12-dUTP, dATP, dCTP, dGTP, dTTP, and an optimized reaction buffer concentrate in 50% glycerol.

Procedure

1. One µg of template DNA was added to a reaction vial and bring to a final volume of 16 µl with distilled water.

2. DNA was denatured by heating in a boiling water bath for 10 min and chilling quickly in an ice/water bath.

3. Fluorescein-High Prime was mixed thoroughly and 4 µl was added to the denatured DNA. Reaction vial was mixed, centrifuged briefly, and incubated for 1 h or O/N at 37° C.

4. Reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0) and/or by heating to 65° C for 10 min.

DNA blotting

1. A strip of positively charge nylon membrane was cut to the desired size and marked out a grid of 0.5 cm × 0.5 cm squares with a blunt pencil.

2. Membrane was wet in distilled water, allowed to submerge, and left for 10 minutes.

3. 1 M NaOH and 200 mM EDTA, pH 8.2 was added to each sample to give a final concentration of 0.4 M NaOH/10 mM EDTA then heat 100°C for 10 minutes.

4. The wet membrane was placed over the top of an open plastic box so that the bulk of the membrane was freely suspended.

5. Each sample was spun for 5 sec, then 2µl of DNA was blotted onto membrane by using pipette and allow the membrane to dry.

6. Membrane was rinsed briefly in 2X SSC and allowed to dry.

7. Membrane was stored between sheets of Whatman 3MM filter paper for several months at room temperature. For long-term storage, the membrane was placed in a desiccator at room temperature or 4°C.

Hybridization and detection

1. Thirty ml of prehybridization buffer was added and incubated at 60°C (2-4 hours) with rotating.

2. Prehybridization was discarded. Thirty ml of hybridization buffer, which pre-heated at 100°C for 20 minutes, was immediately added and incubated at 60°C overnight.

3. Fifty ml of stringency solution I was added and incubated at 60°C for 15 min.

4. Fifty ml of stringency solution II was added and incubated at 60°C for 15 min.

5. Thirty ml of blocking solution was added and rotated at RT for 1 h.

6. Thirty ml of antifluorescein antibody/alkaline phosphatase conjugate was added and rotated at room temperature for 1 hour.

7. Membrane was washed by using 0.3% Tween 20 for 3 times with 10 min each.

8. Membrane was placed on saran wrap.

9. CDP-Star detection reagent was added and left for 2 minutes.

10. Membrane was placed on new saran wrap.

11. Edge of membrane was sealed and exposed to x-ray film.

Randomly amplified polymorphic DNA (RAPD) PCR

RAPD-PCR is a PCR amplification of genomic DNA by using arbitrary primer, which can bind ramdomly throughout genomic DNA. It is used to determine genetic relatedness among individuals from specific patterns given by using random primer. In this study, primer name R018 (5' GTA TTG CCC T 3') was used to perform PCR with the following cycling parameters: initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 42°C for 30 seconds and extension at 72°C for 1 minute, with final extension at 72°C for 7 minutes. The PCR products were analyzed by electrophoresis in 1% agarose gel containing 1X TBE buffer. 1 kb plus DNA ladder was used as a marker. Recipe for RAPD-PCR is shown in Table 4.14.

Table 4.11 Recipe for RAPD-PCR by using R018 primer

Reagent	Volume (µl)
MilliQ water	18.25
10X buffer (with $MgCl_2$)	2.5
25 mM $MgCl_2$	2
10 mM dNTP	0.5
R018 primer (100 µM)	0.25
DyNAzyme *Taq* polymerase	0.5
Template DNA	1
Total	25

Discriminatory power of RAPD-PCR was calculated according to Hunter and Gaston's formula (130) with the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

Where N is the total number of strains in the sample population, *s* is the total number of types described, and *nj* is the number of strains belonging to the *j*th type. This formula was also used for calculation the discriminatory power of serogrouping and serotyping.

Conjugation experiment

Minimum inhibitory concentration (MIC) of ampicillin and norfloxacin by microdilution broth method

Reagents and materials

1. Cation-Adjusted Muller Hinton Broth (CAMHB)
2. Peptone broth or 0.85% NaCl
3. Antibiotic powders
4. 96 well microtiter plate

Procedure

1. Prepare stock solution of antibiotics and perform two fold dilution to the desired concentrations using CAMHB. Norfloxacin and ampicillin were dissolved in distilled water with drops of 0.1 M NaOH and phosphate buffer saline pH 8.0, respectively. The stock solution can be prepared by using the following formula.

$$W = \frac{1000}{P} \times V \times C$$

P = Potency given by the manufacturer in relation to the base

V = Volume in ml required

C = Final concentration of solution

W = Weight of antimicrobial agent to be dissolved in volume V

2. Inoculum was prepared by suspending overnight cultured bacteria in peptone broth or 0.85% Nacl and adjusted to 0.5 McFarland standard (10^8 CFU/ml). Then suspension was diluted 1:10 to yield 10^7 CFU/ml.

3. In each well, 100 µl of each antibiotics dilutions were mixed with 5 µl of adjusted bacterial suspensions (final concentration 10^5 CFU/ml).

4. Plates were incubate at 35 ºC for 18 hrs and determined MIC value by observing the lowest concentration that can completely inhibit bacterial growth (clear solution in the well). The results were interpreted according to CLSI 2010 (146), and shown in Table 4.15.

5. *E. coli* ATCC 25922 was used as a quality control strain. Negative control was CAMHB containing antibiotics. An acceptable range MIC of *E. coli* ATCC 25922 against ampicillin and norfloxacin are 2-8 µg/ml and 0.03-0.12 µg/ml, respectively.

Table 4.12 MIC interpretation of *Enterobacteriaceae*

Antibacterial agent	**MIC (µg/ml) interpretation**		
	Susceptible	Intermediate	Resistant
Ampicillin	≤ 8	16	≥ 32
Norfloxacin	≤ 4	8	≥ 16

Mating experiment

Donor (integron harboring isolates) and recipient (laboratory strain) were grown separately in 3 ml of BHI broth at 37 °C in shaking incubator. Cell suspensions of donor and recipient were mixed at various donor:recipient ratio, e.g., 1:1, 1:5, 1:10. Then, mixture of suspension was then incubated overnight at 37 °C in shaking incubator. 40 µl of suspensions were spread on MacConkey agar containing appropriate antibiotics. The colonies of transconjugants were observed and investigated further for horizontal transferring of resistance genes, e.g., plasmid profile analysis, PCR for detection the presence of integron, and antibiotic susceptibility pattern.

CHAPTER V
RESULTS

Prevalence of *Salmonella* spp. and *E. coli* in raw chicken meat from supermarkets in Bangkok

From a total of 200 raw chicken meats, *E. coli* was detected in 106 of 200 samples (53%) (Table 5.16). The prevalence of *E. coli*, from higher to lower, were distributor 3, 56.00% (28/50), distributor 2, 54.10% (33/61), distributor 1, 52.39% (22/42), and distributor 4, 48.94% (23/47) (Fig. 5.13). Based on the prevalence of *E. coli* according to collection places, Lotus Bangkuntien exhibited the highest prevalence followed by Lotus Pinklao, Tops Central Pinklao, Golden Place, Tang Hua Seng, Lotus Pata Pinklao, and Wanglang market, at the rate of 66.67% (4/6), 60% (21/35), 55.88% (38/68), 54.55% (18/33), 45.95% (17/37), 40% (8/20), 0% (0/1), respectively (Fig. 5.14). *Salmonella* spp. was detected in 14 of 75 samples (18.67%) (Table 5.16). The prevalence of *Salmonella* spp., from higher to lower, were distributor 2, 30% (3/10), distributor 1, 23.08% (6/26), distributor 3, 14.29% (3/21), and distributor 4, 11.11% (2/18) (Fig. 5.13). Lotus Pinklao exhibited the highest prevalence of *Salmonella* spp. 30% (3/10) among collection places followed by Golden Place 20% (2/10), and Tops Central Pinklao 19.15% (9/47), which Lotus Pata Pinklao, Lotus Bangkuntien, and Wanglang market were not examined (Fig. 5.14).

Table 5.1 Prevalence of *E. coli* and *Salmonella* spp. in sealed packages of chicken meat from supermarkets in Bangkok, Thailand and identification of class 1 integron by PCR

	Prevalence	**Integrase (*intI1*)**
***Salmonella* spp.**	18.67% (14/75)*	42.86% (6/14)
E. coli	53% (106/200)	37.74% (40/106)

* Prevalence of *Salmonella* spp. in this study was calculated from total of 75 samples.

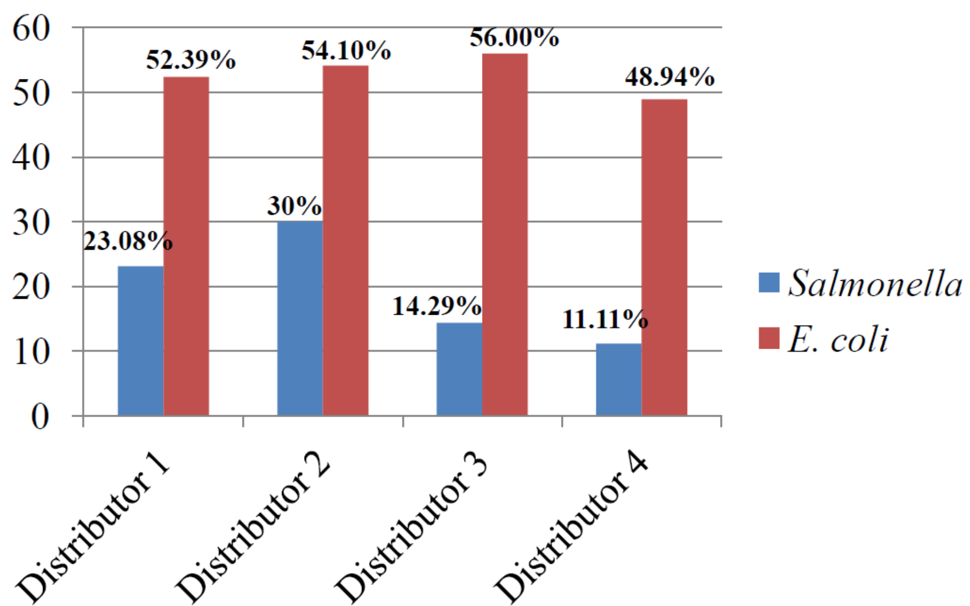


Figure 5.1 Prevalence of *Salmonella* spp. and *E. coli* from raw chicken meats according to distributors

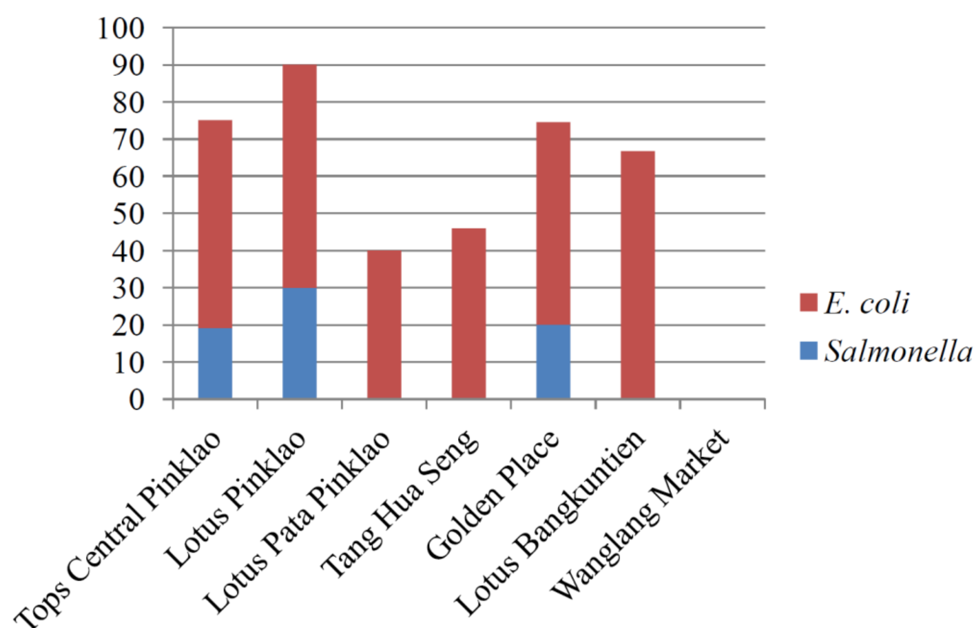


Figure 5.2 Prevalence of *Salmonella* spp. and *E. coli* from raw chicken meats according to collection places*

* Samples from Lotus Pata Pinklao, Lotus Bangkuntien, and Wanglang market were not examined in *Salmonella* prevalence determination.

Serotyping and Serogrouping of *Salmonella* isolates

Salmonella serogroup B was the most frequent serogroup (6 isolates) in this study followed by serogroup C (4 isolates), unidentified serogroup (3 isolates), and serogroup E (1 isolate). Serotype Kedougou (2 isolates), Saintpaul (2 isolates), Schwarzengrund (1 isolate), Agona (1 isolate), Orion (1 isolate), 4,12,27:d:- (1 isolate) Manhattan (1 isolate), Albany (2 isolates), Kiambu (1 isolate), and Altona (2 isolates), were identified in this study.

Determination of coliform bacteria by most probable number method (MPN method)

A total of 60 raw chicken meats were determined for the presence of total coliform bacteria. By using MPN technique, 56.66% of the samples (34/60) were an unacceptable quality according to the criteria of Department of Medical Sciences, Ministry of Public Health (Total coliform < 500/gram of sample). MPN values range from <3 to 46,000 and MPN value of 360 was the most frequent value (7 samples).

Antibiotic resistance profile of *Salmonella* and *E. coli*

Most of *E. coli* isolates showed resistance to ampicillin (72.34%), tetracycline (48.48%), gentamicin (38.30%), and cephalothin (27.66%), with no extended spectrum β-lactamase (ESBL) phenotypes were found (Table 5.17). None of *E. coli* isolates exhibited resistance to cefotaxime, ceftriaxone, ceftazidime, cefepime, imipenem, meropenem, ertapenem, and amikacin. For *Salmonella*, most of isolates exhibited resistance to ampicillin (78.57%), gentamicin (71.43%), and tetracycline (21.43%) (Table 5.18). None of *Salmonella* isolates exhibited resistance to amoxicillin/clavulanic acid, cephalothin, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, cefepime, imipenem, meropenem, ertapenem, ciprofloxacin, levofloxacin, and amikacin. Resistance to fluoroquinolone was found only in *E. coli* at the rate of 10.64%. Multidrug resistant phenotype (resistance to three classes of antibiotics) was also found in both *Salmonella* and *E. coli* isolates. Comparison of antibiotic resistance profile of *Salmonella* and *E. coli* was demonstrated in Fig. 5.15. In *Salmonella* spp., resistance pattern against ampicillin and gentamicin was the most commonly found (10 isolates) followed by ampicillin, gentamicin, and tetracycline (3 isolates). In *E. coli*, resistance pattern against ampicillin, gentamicin, and tetracycline was the most commonly found (8 isolates) followed by ampicillin, cephalothin, and gentamicin (3 isolates), ampicillin, ciprofloxacin, levofloxacin, and gentamicin (2 isolates).

Table 5.2 Antibiotic susceptibility of 14 *Salmonella* spp. isolated from chicken meat from supermarkets in Bangkok by disk diffusion method

Antimicrobial agents	Percentage (%)		
	Susceptible	Intermediate	Resistance
Ampicillin	21.43	-	78.57
Amoxicillin/clavulanic acid	92.86	7.14	-
Cephalothin	100	-	-
Cefuroxime	100	-	-
Cefotaxime	100	-	-
Ceftriaxone	100	-	-
Ceftazidime	100	-	-
Cefepime	100	-	-
Imipenem	100	-	-
Meropenem	100	-	-
Ertapenem	100	-	-
Ciprofloxacin	100	-	-
Levofloxacin	100	-	-
Gentamicin	28.57	-	71.43
Amikacin	100	-	-
Trimethoprim-sulphamethoxazole	82.98	-	17.02
Tetracycline	78.57	-	21.43
Chloramphenicol	ND	ND	ND

ND = not determine

Table 5.3 Antibiotic susceptibility of 46 *E. coli* isolated from chicken meat from supermarkets in Bangkok by disk diffusion method

Antimicrobial agents	Percentage (%)		
	Susceptible	Intermediate	Resistance
Ampicillin	23.4	4.26	72.34
Amoxicillin/clavulanic acid	68.09	25.53	6.38
Cephalothin	38.3	34.04	27.66
Cefuroxime	95.74	2.13	2.13
Cefotaxime	100	-	-
Ceftriaxone	100	-	-
Ceftazidime	100	-	-
Cefepime	100	-	-
Imipenem	100	-	-
Meropenem	100	-	-
Ertapenem	100	-	-
Ciprofloxacin	89.36	-	10.64
Levofloxacin	89.36	-	10.64
Gentamicin	61.7	-	38.30
Amikacin	100	-	-
Trimethoprim-sulphamethoxazole	92.86	-	7.14
Tetracycline*	51.52		48.48
Chloramphenicol**	75.86	6.90	17.24

* A total of 33 samples were used to calculate the percentage of tetracycline susceptibility

** A total of 29 samples were used to calculate the percentage of chloramphenicol susceptibility

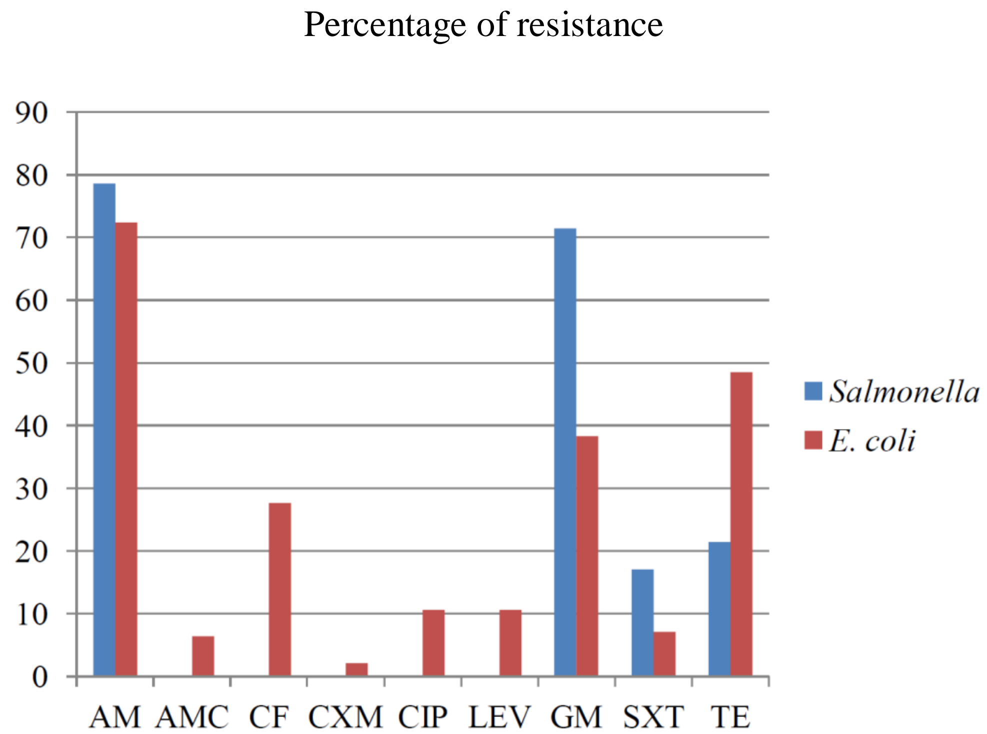


Figure 5.3 A Comparison of percentage of antibiotic resistance between *Salmonella* spp. and *E. coli*

AM = ampicillin, AMC = amoxicillin/clavulanate, CF = cephalothin, CXM = cefuroxime, CIP = ciprofloxacin, LEV = levofloxacin, GM = gentamicin, SXT = trimethoprim/sulfamethoxazole, TE = tetracycline

Class 1 integron determination

Prevalence of *intI1* by using PCR amplification

Salmonella and *E. coli* isolates were screened for the presence of class 1 integron by using PCR with specific primers to *intI1*. An expected PCR products size is 471 bp (Fig. 5.16). Integron harboring *Salmonella* and *E. coli* were found 42.86% (6/14) and 37.74% (40/106), respectively (Table 5.16).

Identification of resistance cassettes inserted in class 1 integron

Integron carrying isolates, characterized by showing positive result for PCR amplification using *intI1* primer, were identified for resistance cassettes by using PCR with specific primer to 5'CS and 3'CS followed by DNA sequencing. The isolates were selected based on antibiotic resistance pattern by disk diffusion, which isolates that exhibited large scale of resistance were selected. Firstly, two *E. coli* isolates, E9 and E12, which exhibited resistance to a variety class of antibiotics (E9 resisted to β-lactam, fluoroquinolones, and aminoglycosides, E12 resisted to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole), were selected. Both E9 and E12 showed multiple bands in PCR amplification (Fig. 5.17). PCR products were then cloned to plasmid vector StrataClone™ PCR cloning vector pSC-A-amp/kan. Plasmids containing PCR products were then verified and determined an insert orientation by using *Eco*RI restriction analysis (Fig. 5.21). Resistance cassettes of E9, 1 kb and 700 bp PCR products, were *aadA23* (accession number FN252409) and *dfrA5* (accession number GU562437), respectively. E12 was found to carry *aadA4* (accession number FQ482074) from 850 bp of PCR product. *Salmonella* Kedougou, Sal162 which showed resistance ampicillin, gentamicin, and tetracycline, was also selected. One kb PCR product of Sal162 matched well with *aadA22* (accession number AB434537). Integrase negative isolates were also characterized and some of integrase negative isolates (E17 and Sal130) were also found to carry resistance genes. Interestingly, E17 was found to carry *lnuF* from 500 bp of PCR product while Sal130 carried Δ*aadA22* and *lnuF* from 650 bp of PCR product (accession number EU118119) (Fig. 5.18). Surprisingly, *lnuF*, which conferred lincosamide resistance, was found among Gram-negative bacteria. Features of characterized *E. coli* and *Salmonella* were summarized

in Table 5.19. The PCR products of other integron carrying isolates were shown in Fig. 5.19 and Fig. 5.20. The PCR products were analyzed further by restriction enzyme analysis. The digested PCR products with *Afa*I enzyme was shown in Fig. 5.19. Finally, the nucleotide sequence analysis of 3 completed resistance cassettes, which indicated primer binding site, core site, open reading frame of resistance cassettes, and inverse core site, were shown in Fig. 5.22-5.24.

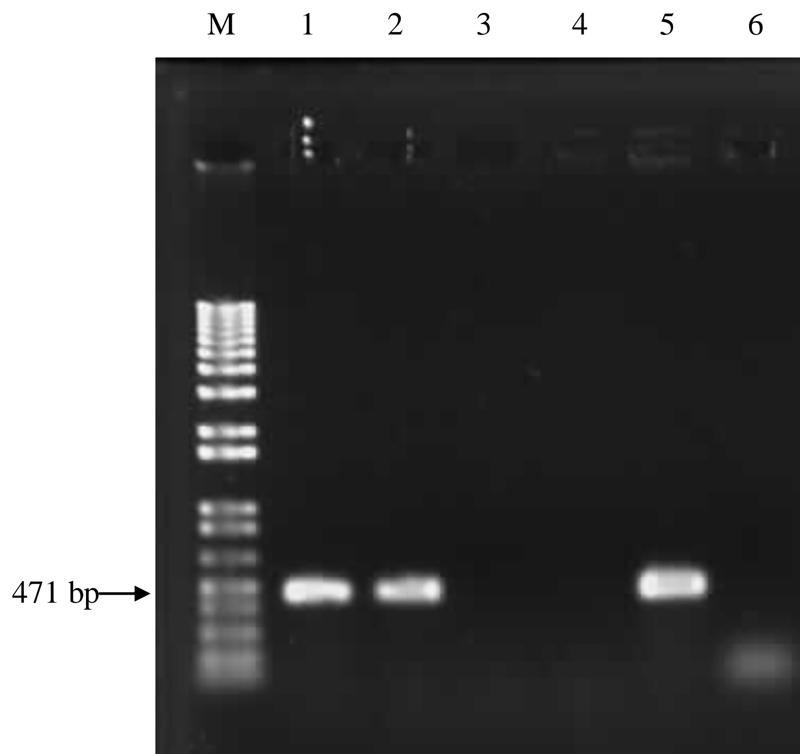


Figure 5.4 PCR with specific primers to *intI1* generate 471 bp of PCR product. Lane M, 1 kb plus DNA ladder; Lane 1-4, samples; Lane 5, positive control, PA 67 *Pseudomonas aeruginosa* carrying *bla*$_{IMP-15}$; Lane 6, negative control

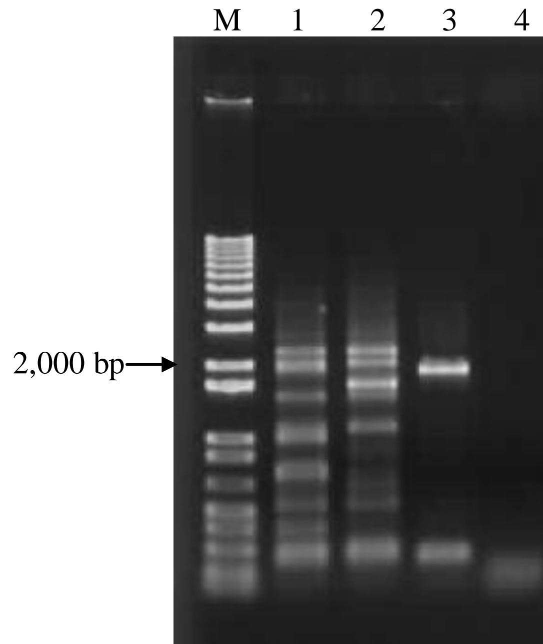


Figure 5.5 PCR using 5' CS and 3' CS primers of two *E. coli* isolates, E9 and E12. Lane M, 1 kb plus DNA ladder; Lane 1 *E. coli* E9; Lane 2, *E. coli* E12; Lane 3, PA 67 *Pseudomonas aeruginosa* possessed integron carrying 1,897 bp PCR product of bla_{IMP-15} was used as positive control; Lane 4, negative control

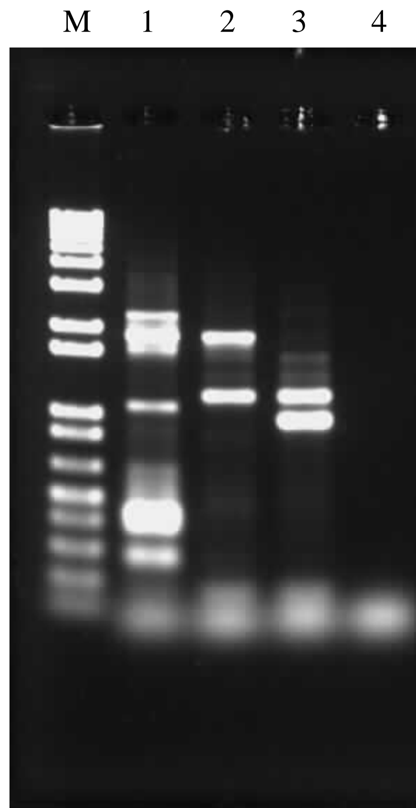


Figure 5.6 PCR using 5' CS and 3' CS primers of *E. coli* and *Salmonella*. Lane M, 1 kb plus DNA ladder; Lane 1 *E. coli* E17; Lane 2, *Salmonella* Sal130; Lane 3, *Salmonella* Sal162; Lane 4, negative control

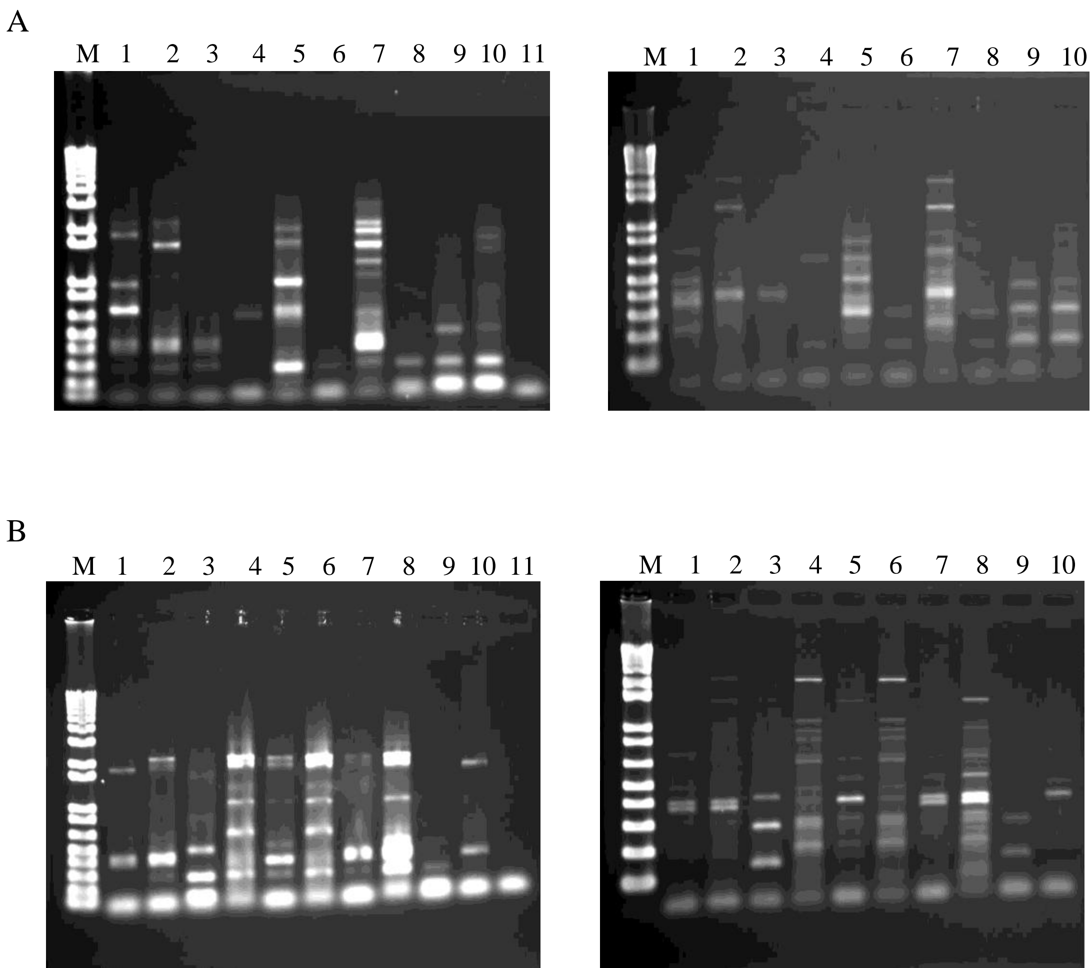


Figure 5.7 (A and B) PCR with specific primer to 5'CS-3'CS (left) and digested PCR products pattern with *Afa*I enzyme. For the left side of Fig. A, Lane M, 1 kb plus DNA ladder; Lane 1, E9; Lane 2, E12; Lane 3, E29; Lane 4, E30; Lane 5, E38; Lane 6, E46; Lane 7, E60; Lane 8, E66; Lane 9, E72; Lane 10, E74; Lane 11, negative control. For the left side of Fig. B, Lane M, 1 kb plus DNA ladder; Lane 1, E97; Lane 2, E104; Lane 3, E106; Lane 4, E110; Lane 5, E112; Lane 6, E114; Lane 7, E119; Lane 8, E123; Lane 9, E134; Lane 10, E135; Lane 11, negative control. For the right side, Lane M, 1 kb plus DNA ladder; Lane 1-10, digested PCR products with *Afa*I enzyme of Lane 1-10 of the left picture.

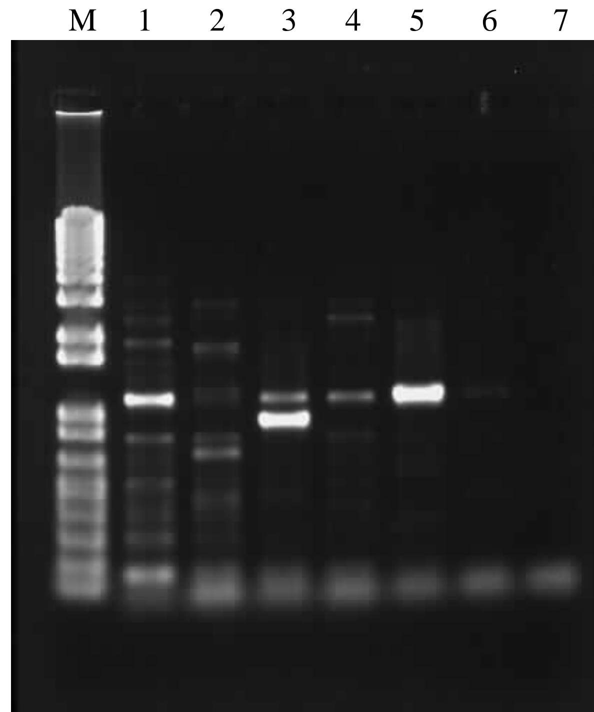


Figure 5.8 PCR with specific primers to 5' CS-3' CS of 6 integron carrying *Salmonella* isolates. Lane M, 1 kb plus DNA ladder; Lane 1, Sal 143; Lane 2, Sal 145; Lane 3, Sal 162; Lane 4, Sal 165; Lane 5, Sal 178; Lane 6, Sal 180; Lane 7, negative control

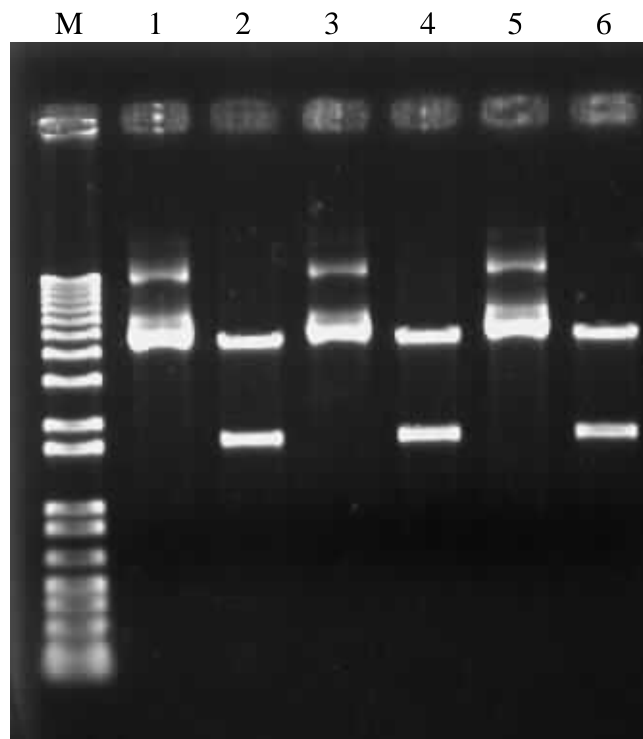


Figure 5.9 Identification of tranformants, which carried recombinant plasmids by *Eco*RI digestion analysis. Lane M; 1 kb plus DNA ladder, Lane 1, 3, 5 Undigested plasmid; Lane 2, 4, 6 *Eco*RI digested plasmids

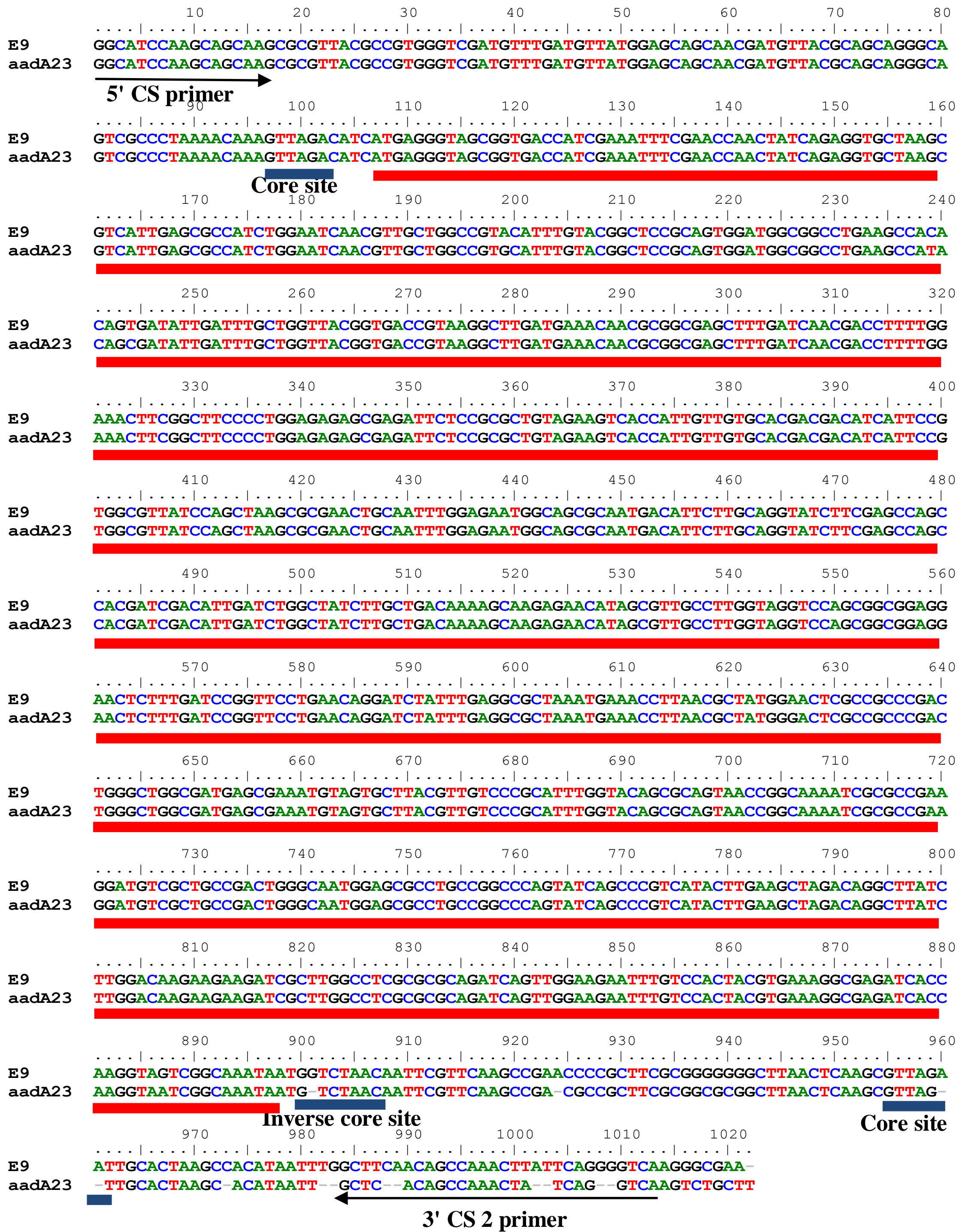


Figure 5.10 Nucleotide sequence analysis of class 1 integron containing *aadA23* in E9. Primer binding site, core site, and inverse core site were indicated. An open reading frame of *aadA23* was shown in red bar

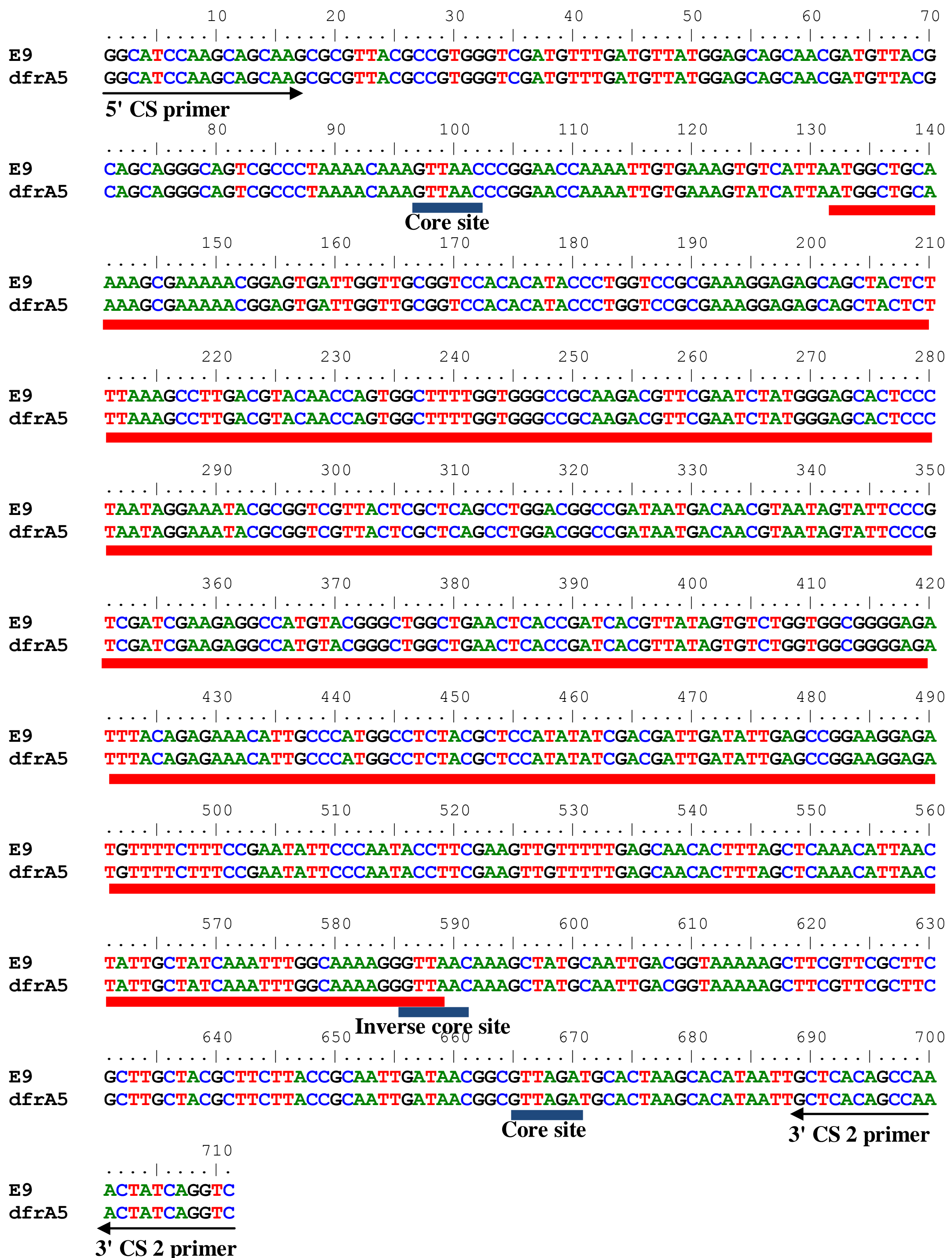


Figure 5.11 Nucleotide sequence analysis of class 1 integron containing *dfrA5* in E9. Primer binding site, core site, and inverse core site were indicated. An open reading frame of *dfrA5* was shown in red bar

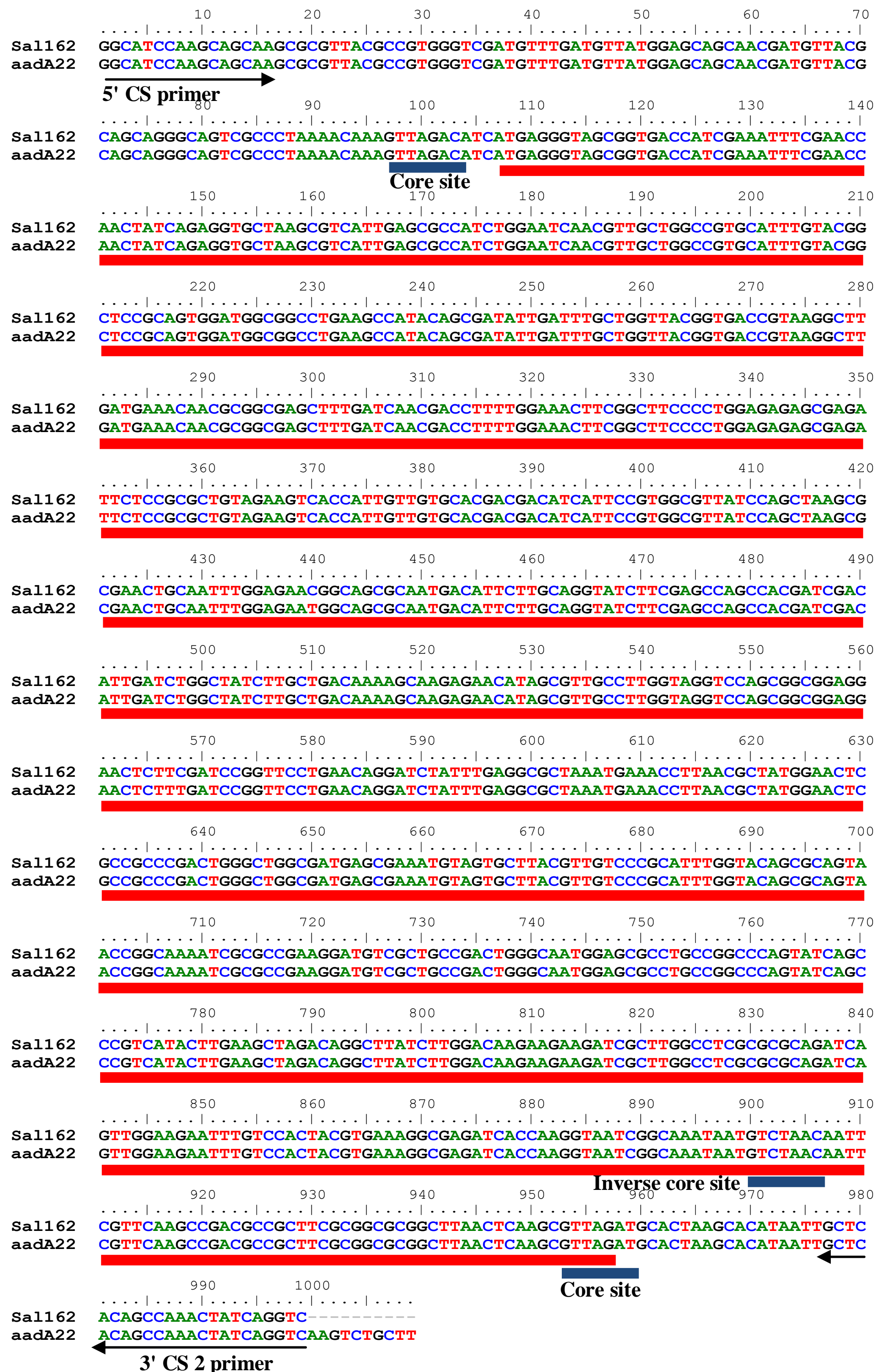


Figure 5.12 Nucleotide sequence analysis of class 1 integron containing *aadA22* in Sal162. Primer binding site, core site, and inverse core site were indicated. An open reading frame of *aadA22* was shown in red bar.

Table 5.4 Summary features of characterized *Salmonella* and *E. coli* carrying class 1 integron in this study

	intI1	**5'CS-3'CS**	**Resistance cassette (s)**	**Resistance phenotype**	**Accession number**	**Source**[1)]
E9 (*E. coli*)	+	1 kb	*aadA23*	AM, CIP, LEV, GM	FN252409	Swine holding pen from Brazil
E9 (*E. coli*)	+	700 bp	*dfrA5*	AM, CIP, LEV, GM	GU562437	Animal from Australia
E12 (*E. coli*)	+	850 bp	*aadA4*	CIP, LEV, GM, SXT	FQ482074	-
E17 (*E. coli*)	-	500 bp	*lnuF*	AM, CF, GM	EU118119	-
Sal130 (*S.* Kedougou)	-	650 bp	Δ*aadA2*, *lnuF*	AM, GM	EU118119	-
Sal162 (*S.* Kedougou)	+	1 kb	*aadA22*	AM, GM, TE	AB434537	Cheese from Egypt

[1)] The integron database at http://integrall.bio.ua.pt/

AM = ampicillin, CF = cephalothin, GM = gentamicin, CIP = ciprofloxacin, LEV = levofloxacin, SXT = trimethoprim-sulfamethoxazole, TE = tetracycline

Prevalence of *intI1* by using Dot blot hybridization technique

Considering the results of resistance cassettes identified in integrase negative bacteria (E 17 and Sal 130), indicated either low sensitivity of detection by using PCR or a diverse sequences of *intI1* among species. Therefore, dot blot hybridization by using *intI1* specific probe was used to confirm the PCR results. Dot blot hybridization, which could detects 61 from 106 of *E. coli* isolates (57.55%), yielded higher detection compared with PCR test (37.74%). The equality of detection was found in *Salmonella*, which could detects 6 from 14 *Salmonella* isolates (42.86%). In characterized integrase negative bacteria (E 17 and Sal 130), dot blot hybridization can detect one of these isolate (E 17), but another (Sal 130) cannot be detected (Fig. 5.25). Comparison of *intI1* detection between *intI1* and dot blot hybridization was shown in Table 5.20. Even though dot blot hybridization emphasizes a low sensitivity of PCR method, the failure of detection by dot blot hybridization was found in 5 PCR positive isolates, i.e., E183, E186.2, Sal145, Sal178, and Sal180.

E2	E2	E3	E3	E4	E4	E5	E5	E6	E6	E8	E8	E9	E9	E11	E11	E12	E12	E13.1
E13.1	E13.2	E13.2	E14.1	E14.1	E14.2	E14.2	E14.3	E14.3	E14.4	E14.4	E15.1	E15.1	E15.2	E15.2	E16	E16	E17	E17
E29	E29	E30	E30	E31	E31	E32	E32	E38	E38	E42	E42	E43	E43	E44	E44	E46	E46	E47
E47	E48	E48	E53	E53	E54	E54	E55	E55	E57	E57	E59	E59	E60	E60	E61	E61	E62	E62
E66	E66	E69	E69	E70	E70	E71	E71	E72	E72	E73	E73	E74	E74	E78	E78	E79	E79	E89
E89	E93	E93	E97	E97	E99	E99	E104	E104	E106	E106	E107	E107	E108	E108	E109	E109	E110	E110
E111	E111	E112	E112	E113	E113	E114	E114	E116	E116	E119	E119	E122	E122	E123	E123	E124	E124	E126
E126	E127	E127	E129	E129	E131	E131	E132	E132	E134	E134	E135	E135	E136	E136	E137	E137	E138.1	E138.1
E138.2	E138.2	E140	E140	E141	E141	E142	E142	E144	E144	E146	E146	E148	E148	E149	E149	E150	E150	E151.1
E151.1	E151.2	E151.2	E153	E153	E154	E154	E156	E156	E157	E157	E158	E158	E159	E159	E162	E162	E166	E166
E167	E167	E169	E169	E171	E171	E172	E172	E173	E173	E181	E181	E182	E182	E183	E183	E186.1	E186.1	E186.2
E186.2	E187	E187	E190	E190	E191	E191	E192	E192	E193	E193	E196	E196	E197	E197	E198	E198	E200	E200
S130	S130	S138	S138	S143	S143	S145	S145	S154	S154	S160	S160	S162	S162	S164	S164	S165	S165	S178
S178	S180	S180	S186	S186	S190	S190	S193	S193	+	+	-	-						

Positive control Negative control

Figure 5.13 Dot blot hybridization of *Salmonella* and *E. coli* by using *intI1* specific probe. Solid-lined box represents E17 and dashed box represents Sal130. Positive control and negative control are shown as indicated by arrow heads

Table 5.5 Comparison of *intI1* detection between PCR and dot blot hybridization

Strain	PCR for *intI*	Dot blot hybridization	Strain	PCR for *intI*	Dot blot hybridization
E2	-	+	**E69**	-	-
E3	-	-	**E70**	-	+
E4	-	-	**E71**	-	+
E5	-	-	**E72**	+	+
E6	-	-	**E73**	-	-
E8	-	-	**E74**	+	+
E9	+	+	**E78**	-	+
E11	-	-	**E79**	-	-
E12	+	+	**E89**	-	-
E13.1	-	-	**E93**	-	-
E13.2	-	-	**E97**	+	+
E14.1	-	-	**E99**	-	+
E14.2	-	-	**E104**	+	+
E14.3	-	-	**E106**	+	+
E14.4	-	-	**E107**	-	+
E15.1	-	+	**E108**	-	+
E15.2	-	+	**E109**	-	-
E16	-	-	**E110**	+	+
E17	-	+	**E111**	-	-
E29	+	+	**E112**	+	+
E30	+	+	**E113**	-	-
E31	-	-	**E114**	+	+
E32	-	+	**E116**	-	-
E38	+	+	**E119**	+	+
E42	-	+	**E122**	-	+
E43	-	-	**E123**	+	+
E44	-	+	**E124**	-	+
E46	+	+	**E126**	-	-
E47	-	-	**E127**	-	-
E48	-	-	**E129**	-	+
E53	-	+	**E131**	-	-
E54	-	-	**E132**	-	+
E55	-	-	**E134**	+	+
E57	-	+	**E135**	+	+
E59	-	-	**E136**	+	+
E60	+	+	**E137**	+	+
E61	-	-	**E138.1**	-	-
E62	-	-	**E138.2**	-	-
E66	+	+	**E140**	-	-

Strain	**PCR for *intI***	**Dot blot hybridization**	**Strain**	**PCR for *intI***	**Dot blot hybridization**
E141	+	+	**S160**	-	+
E142	-	-	**S162**	+	+
E144	-	-	**S164**	-	-
E146	-	+	**S165**	+	+
E148	-	-	**S178**	+	-
E149	+	+	**S180**	+	-
E150	-	-	**S186**	-	-
E151.1	-	-	**S190**	-	-
E151.2	-	-	**S193**	-	-
E153	+	+			
E154	+	+			
E156	-	+			
E157	+	+			
E158	-	+			
E159	+	+			
E162	+	+			
E166	+	+			
E167	-	-			
E169	+	+			
E171	-	+			
E172	-	+			
E173	+	+			
E181	+	+			
E182	-	-			
E183	+	-			
E186.1	-	-			
E186.2	+	-			
E187	-	-			
E190	-	-			
E191	+	+			
E192	+	+			
E193	-	-			
E196	+	+			
E197	+	+			
E198	-	-			
E200	+	+			
S130	-	-			
S138	-	+			
S143	+	+			
S145	+	-			
S154	-	+			

Plasmid profile study

Although integron was not a mobilizable platform, It was shown to be associated with other mobile genetic elements, e.g., plasmid and transposon. This study investigated the role of plasmid in integron carrying bacteria by performing plasmid profile study. From a total of 33 integron carrying *E. coli*, 25 plasmid patterns were found (Fig. 5.26 and Fig. 5.27). The same plasmid patterns were found in both *E. coli* isolates collecting from the same place and different places (Lane 1 and Lane 9 were *E. coli* that collected from different places, Lane 7 and Lane 8 were *E. coli* that collected from the same place) (Fig. 5.26). For *Salmonella*, The unique plasmid pattern was demonstrated among 6 integron carrying *Salmonella* (Fig. 5.28).

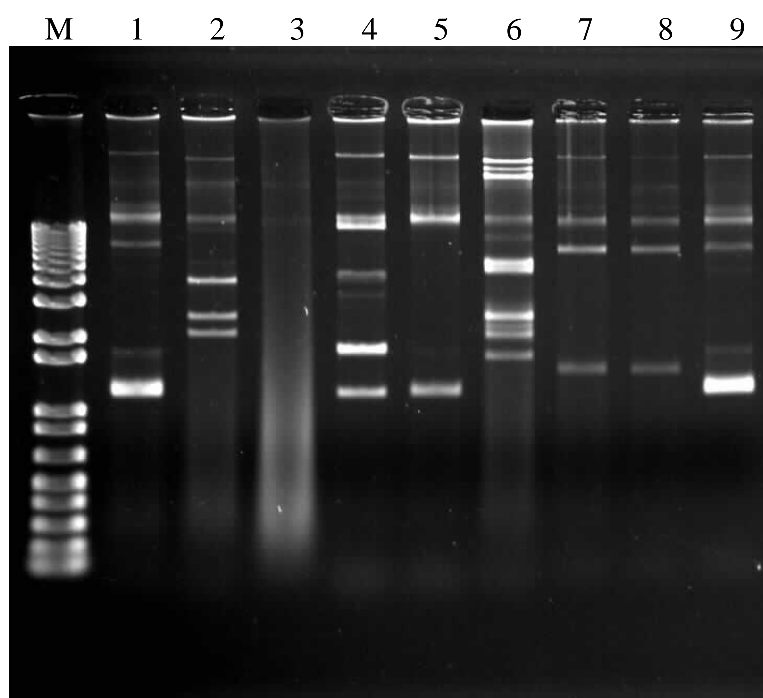
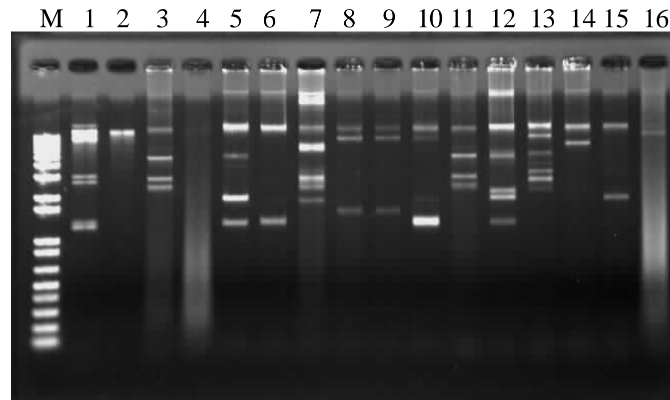


Figure 5.14 Plasmid profile of *E. coli* carrying integron isolated from chicken meats. Lane M, 1 kb plus DNA ladder; Lane 1, E60; Lane 2, E123; Lane 3, E134; Lane 4, E166; Lane 5, E169; Lane 6, E173; Lane 7, E181; Lane 8, E183; Lane 9, E186.2

A



B

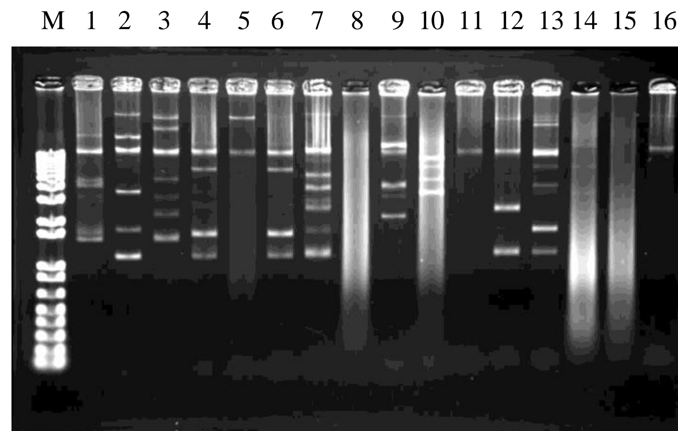


Figure 5.15 Plasmid profiles of other *E. coli* carrying integron isolates. In Fig. A, Lane M, 1 kb plus DNA ladder; Lane 1, E9; Lane 2, E12; Lane 3, E123; Lane 4, E134; Lane 5, E166; Lane 6, E169; Lane 7, E173; Lane 8, E181; Lane 9, E183; Lane 10, E186.2; Lane 11, E119; Lane 12, E191; Lane 13, E192; Lane 14, E196; Lane 15, E197; Lane 16, E200. In Fig. B, Lane M, 1 kb plus DNA ladder; Lane 1, E29; Lane 2, E97; Lane 3, E104; Lane 4, E110; Lane 5, E112; Lane 6, E114; Lane 7, E135; Lane 8, E136; Lane 9, E137; Lane 10, E141; Lane 11, E149; Lane 12, E153; Lane 13, E154; Lane 14, E157; Lane 15, E159; Lane 16, E162.

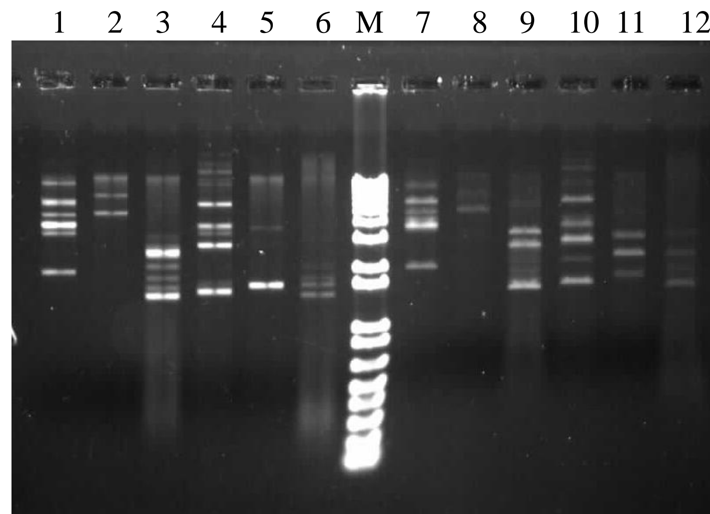


Figure 5.16 Plasmid profiles of 6 *Salmonella* carrying integron isolates and their restriction enzyme analysis. Lane M, 1 kb plus DNA ladder; Lane 1, Sal143; Lane 2, Sal145; Lane 3, Sal162; Lane 4, Sal165; Lane 5, Sal178; Lane 6, Sal 180; Lane 7-12, *Eco*RI digested products plasmid of Sal143, Sal145, Sal162, Sal165, Sal178, and Sal180, respectively

RAPD-PCR of *Salmonella* isolates

Salmonella isolates in this study were typed by using antibiotic resistance profiles, serotype, serogroup, and plasmid profiles with an additional molecular typing method, RAPD-PCR. RAPD-PCR is a simple PCR-based technique that can be used to discriminate between strains. We used RAPD-PCR to determine genetic relatedness among *Salmonella* isolates in combination with other methods. A total of 9 RAPD patterns were found among 14 *Salmonella* isolates by using R018 primer (Fig. 5.29). The same RAPD patterns were pattern I, II, III, and IX. We investigated further by gathering these typing results together. Three out of four repetitive RAPD patterns (pattern I, II, and IX) showed well correlation with serogrouping and serotyping results (Table 5.21). Moreover, the well correlated strains was collected at different times. The discriminatory index of RAPD-PCR, serotyping, and serogrouping were calculated according to Hunter and Gaston's formula in which the higher discriminatory power, the closer to discriminatory power of 1. The RAPD-PCR identified 9 distinct groups consisting of 3 strains in group 1, 2 strains in group 2, 3, and 9, and 1 strain in the remaining groups. Serogrouping identified 4 distinct groups consisting of 3 strains in group 1, 6 strains in group 2, 1 strain in group 3, and 4 strains in group 4. Serotyping identified 10 distinct groups consisting of 2 strains each in 4 groups and 1 strain each in 6 groups. The discriminatory index according to Hunter and Gaston's formula of RAPD-PCR, serotyping, and serogrouping was 0.934, 0.956, and 0.736, respectively.

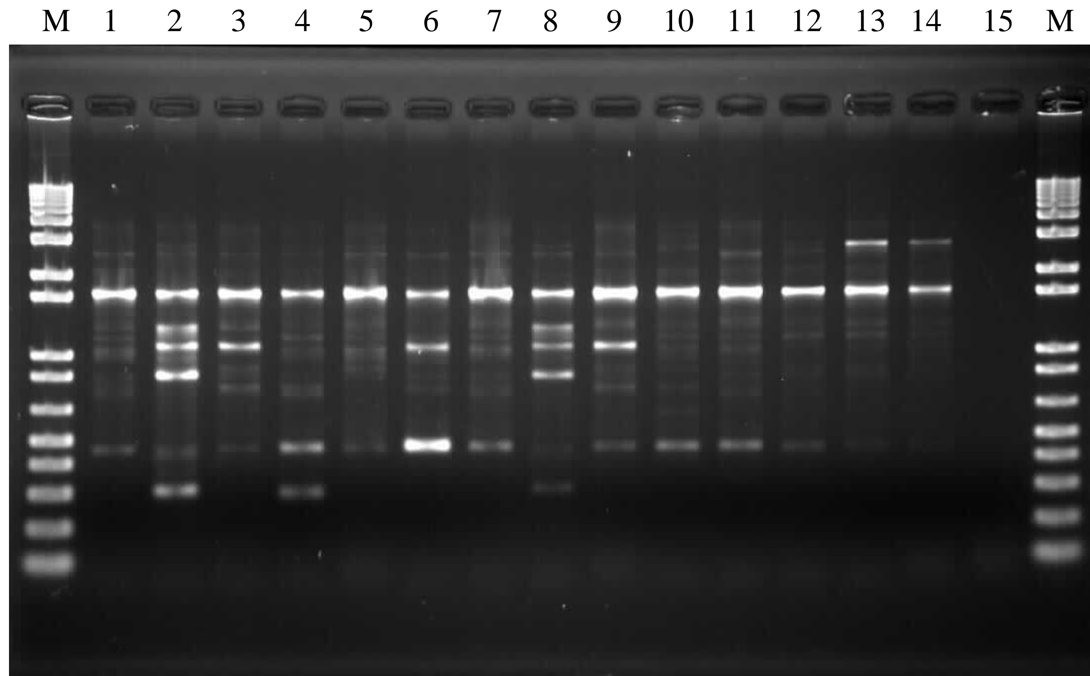


Figure 5.17 RAPD-PCR of *Salmonella* isolates. Lane M, 1 kb plus DNA ladder; Lane 1, Sal130; Lane 2, Sal138; Lane 3, Sal143; Lane 4, Sal145; Lane 5, Sal154; Lane 6, Sal160; Lane 7, Sal162; Lane 8, Sal164; Lane 9, Sal165; Lane 10, Sal178; Lane 11, Sal180; Lane 12, Sal186; Lane 13, Sal190; Lane 14, Sal193; Lane 15, negative control.

Table 5.6 Relationship of *Salmonella* isolates demonstrated by various typing methods

Isolate	Serogrouping	Serotyping	RAPD PCR	Resistance phenotype	Place of collection[1)]	Collection date
Sal 130	Unidentified serogroup	Kedougou	I	AM, GM	A1	Jan 2, 2011
Sal 138	B	Saintpaul	II	AM, GM	B2	Mar 14, 2011
Sal 143	B	Schwarzengrund	III	AM, GM	B2	Mar 14, 2011
Sal 145	B	Agona	IV	-	B2	Mar 14, 2011
Sal 154	E	Orion	V	AM, GM	A3	Apr 4, 2011
Sal 160	C	Manhattan	VI	AM, GM	A3	Apr 4, 2011
Sal 162	Unidentified serogroup	Kedougou	I	AM, GM, TE	A1	Apr 4, 2011
Sal 164	B	Saintpaul	II	AM, GM	A1	Apr 4, 2011
Sal 165	B	4,12,27:d:-	III	AM, GM	A1	Apr 4, 2011
Sal 178	C	Albany	VII	AM, SXT, TE	A1	Apr 19, 2011
Sal 180	Unidentified serogroup	Albany	I	AM, GM, TE	A1	Apr 19, 2011
Sal 186	B	Kiambu	VIII	-	C4	Apr 25, 2011
Sal 190	C	Altona	IX	-	C4	Apr 25, 2011
Sal 193	C	Altona	IX	AM, GM	A3	May 3, 2011

[1)] A, B, and C represent supermarkets, whereas 1, 2, 3, and 4 represent major distributor companies

MLST of *Salmonella* isolates

Salmonella had been shown to be spread globally either through travelers or foods. It is important to perform global epidemiology using molecular typing methods, which generate worldwide comparable data, e.g., MLST. MLST was chosen for global epidemiology purpose. PCR products of seven housekeeping genes were shown in Fig. 5.30. Four *Salmonella* isolates (Sal138, Sal162, Sal165, and Sal193), which represents RAPD pattern II, I, III, and IX, respectively, were submitted to MLST analysis. The MLST results showed 4 sequence type (ST), i.e., ST 50, ST 96, ST 1543, and ST 1549 found in this study (Table 5.22). From MLST database, ST 1543 and ST 1549 were collected from human stools in Vietnam, while ST 50 and ST 96 were collected from many countries, e.g., Denmark, Taiwan, Chile, and Tunisia (Table 5.23).

Table 5.7 Allelic profile of 4 *Salmonella* isolates in this study

Samples	**Allelic profile** ***thrA-purE-sucA-aroC-hisD-hemD-dnaN***	**ST**	**Countries**
Sal138	17-6-12-5-9-18-21	50	Chile, Denmark, U.S.A., Germany, Australia
Sal162	141-95-9-417-262-15-4	1543	Vietnam
Sal165	3-41-15-43-49-49-47	96	Tunisia, U.S.A., Taiwan, Denmark, Scotland
Sal193	102-426-9-347-496-78-394	1549	Vietnam

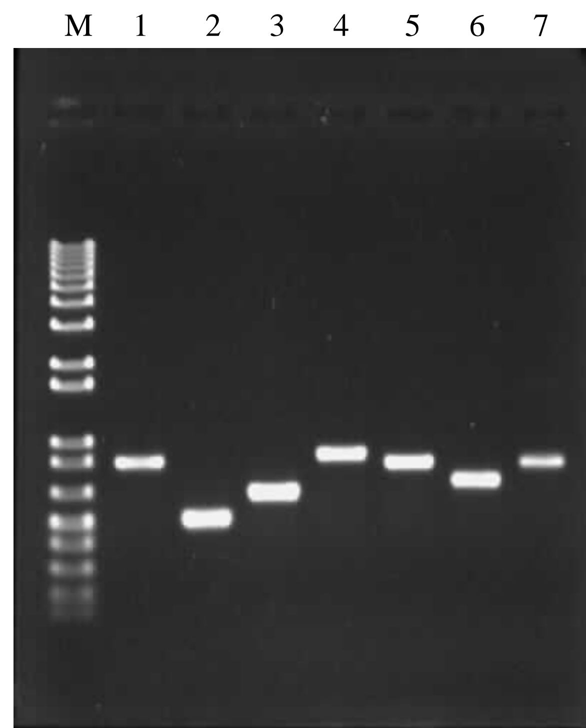


Fig. 5.18 PCR products of seven housekeeping genes used for MLST for *Salmonella*. Lane M, 1 kb plus DNA ladder; Lane 1, *thrA*, 852 bp; Lane 2, *purE*, 510 bp; Lane 3, *sucA*, 643 bp; Lane 4, *hisD*, 894 bp; Lane 5, *aroC*, 826 bp; Lane 6, *hemD*, 666 bp; Lane 7, *dnaN*, 833 bp.

Table 5.8 Strain information of four *Salmonella* sequence types in this study

ST	Strain	Serotype	Host	Country	Year
50	1115/72	Kingston	Animal Feed	Chile	1972
	0410W75689	Saintpaul	Human	Denmark	2004
	0307T28755	Saintpaul	Human stool	Denmark	2003
	0306T23309	Saintpaul	Human	Denmark	2003
	0702W56738	Saintpaul	Human stool	Denmark	2007
	0208W18237	Saintpaul	Human stool	Denmark	2002
	ma873	Saintpaul	*Onychogalea fraenata*	Australia	2002
	M299	Saintpaul	-	USA	2003
	M203	Saintpaul	-	Philippines	2001
	M109	Saintpaul	-	Australia	2001
	SGSC4920	Saintpaul	-	-	-
	05-02659	Saintpaul	Crocodile	Germany	2005
	SARA22	Saintpaul	Human	U.S.A.	-
	SARB55	Saintpaul	Human	U.S.A.	-
	01W15422	Saintpaul	Human	Denmark	-

ST	Strain	Serotype	Host	Country	Year
96	9836/08	Schwarzengrund	Human	Tunisia	2008
	M81	Schwarzengrund	-	South Africa	2001
	M69	Schwarzengrund	-	South Africa	2001
	M217	Schwarzengrund	-	USA	2001
	M2	Schwarzengrund	-	USA	2001
	S/20050414	Schwarzengrund	Human	Scotland	2005
	H2535	Schwarzengrund	-	Taiwan	2001
	H2500	Schwarzengrund	-	Denmark	2001
	3222	Schwarzengrund	Human carrier	Germany	1985
	S/20042666	Schwarzengrund	Human	Scotland	2004
	SARB57	Schwarzengrund	-	Scotland	1988

ST	Strain	Serotype	Host	Country	Year
1543	20062	-	Human	Vietnam	2009

ST	Strain	Serotype	Host	Country	Year
1549	20114	-	Human	Vietnam	2009

Conjugation experiment

In conjugation experiment, integron containing bacteria were used as a donor. *E. coli* JM 109, *E. coli* TOP10, and *E. coli* DH5α were used as a recipient strains. *E. coli* TOP10 is a streptomycin resistant strain while *E. coli* JM 109 and *E. coli* DH5α are nalidixic resistant strains. *E. coli* E9 (resistance to AM, CIP, LEV, GM) and *E. coli* E12 (resistance to CIP, LEV, GM, SXT) were selected to be a donor strains and *E. coli* TOP10 for recipient strain. Transconjugants were selected for non-lactose fermenter that can grow on MacConkey agar containing ampicillin (100 μg/ml) and spectinomycin (100 μg/ml). No transconjugants were observed in all experiments. E9 and E12 were checked for resistance background to spectinomycin by plating on MacConkey agar containing ampicillin (100 μg/ml) and spectinomycin (100 μg/ml). Both E9 and E12 grew on selective plate and cannot be used in experiment. *E. coli* E60 (resistance to AM, SXT, TE) *E. coli* DH5α, and *E. coli* TOP10 were determined MIC value by microdilution broth before performing conjugation experiment. MIC of norfloxacin and ampicillin was shown in Table 5.24. Norfloxacin failed to use for selection of transconjugants because both donor and recipient were sensitive to norfloxacin.

Table 5.9 MIC determination by microbroth dilution method of E60, *E. coli* JM109, and *E. coli* DH5α to norfloxacin and ampicillin by microdilution broth.

	Norfloxacin (μg/ml)	Ampicillin (μg/ml)
E60 (donor)	0.5	256
E. coli JM109 (recipient)	0.5	4
E. coli DH5α (recipient)	< 0.25	8
E. coli ATCC 25922 (quality control)	0.0625	8

Acceptable range for *E. coli* ATCC 25922.

Norfloxacin = 0.03-0.12 μg/ml, Ampicillin = 2-8 μg/ml

CHAPTER VI
DISCUSSION

The contamination of antibiotic resistant bacteria in food producing animals has been reported, which antibiotic usage in animals was related to an emergence of such strains (4, 5, 78-80). In addition, these bacteria not only protect themselves from an effect of antibiotics, but they also transfer resistant genes horizontally or vertically to other bacteria. Therefore, the number of antibiotic resistant bacteria was increased.

This study monitored the prevalence of *Salmonella* spp. and *E. coli* in chicken meats from supermarkets in Bangkok, Thailand. These two bacterial species were selected based on hypothesis that horizontal gene transfer might occur between pathogenic bacteria, *Salmonella* spp. and normal flora *E. coli*. *E. coli* in this study was not tested for any virulent factors and designated as normal flora. The reason why *Campylobacter* spp., the main pathogenic bacteria found in chicken, was excluded in this study was an inconvenience for culture condition. *Campylobacter* spp. requires a microaerophilic condition for their growth and various detection methods can be used. PCR method can give a rapid diagnosis, but it is expensive, labor intensive, and cannot provide a certain strains for further typing or AST. The culture methods including selective culture showed better efficiency than membrane filtration technique, but it takes 2-3 days for incubation and cannot identify some less common species (131). Another aspect for *E. coli* as a microflora was that it can act as a reservoir for resistance genes and accessible to transfer to pathogenic bacteria. The transferability of resistance genes was evidenced by metagenomic study (132).

Salmonella spp. isolation was performed according to standard method ISO 6579 with modification on enrichment media. TT broth was used to enrich *Salmonella* spp. instead of RV broth because of its ease of preparation, and incubation temperature is 35°C, which was proper for our laboratory. Unfortunately, TT broth cannot detect any of 125 chicken samples, therefore an RV broth was used instead for

the last 75 samples. RV broth was demonstrated to give a higher sensitivity than TT broth (133), and it was shown to be suitable for high microbial load foods (134) as oppose to TT broth that is suitable for low microbial load foods (135). The MPN analysis reflected a high microbial load in chicken meats, 56.66% of which were unacceptable quality with the highest value of 46,000, and it absolutely explained why TT broth cannot be used in this study. The prevalence of *Salmonella* spp. in this study may present an underestimate of true prevalence of *Salmonella* spp. in chicken due to a low sample size. However, the prevalence of *Salmonella* spp. was calculated from the total of 75 samples and showed at the rate of 18.67% (14/75). The contamination of *Salmonella* did not meet the criteria of department of livestock development, which *Salmonella* spp. must not be found in 25 grams of samples.

The result from this study differed from the previous study in Thailand, which the prevalence of *Salmonella* spp. was 57 % (12). The sample type from that study did not specify, whether it was a sealed packages and it might lead to a great differ from this study. This study also showed a higher contamination rate than that reported from Washington, D.C., USA (4.2%) (11), and Morocco (0%) (136), but lower than that reported from England (33%) (71) and U.S.A. (35%) (137). *Salmonella* spp. were found in all distributors, which distributor 1 gave the highest isolates (6 isolates) followed by distributor 2 (3 isolates), distributor 3 (3 isolates), and distributor 4 (2 isolates). There were 3 isolates from distributor 3 obtained from different collection times, which were Sal 154 and Sal 160 collected in April 4, 2011, and Sal 193 collected in May 3, 2011. There is an announcement from distributor that their products were controlled to guarantee to be antibiotic-free production and delivered a *Salmonella*-free products to consumers (58). However, the fact that *Salmonella* were found in all distributors warrants a sterilization and proper cook of chicken meat for consumer.

Serogrouping and serotyping were used for typing *Salmonella* isolates based on O and H antigen and also used for confirmation *Salmonella* strains obtained from this study. *Salmonella* group B was the most commonly found (6 isolates) followed by group C (4 isolates). Serotypes presented in this study were diverse, which *S.* Kedougou, *S.* Albany, and *S.* Altona were the most common serotypes. *S.* Albany was shown to be the top serotypes isolated from animals in Thailand during

2003-2006 (44-46, 74). Moreover, *S.* Agona and *S.* Schwarzengrund found in this study were also often isolated in animals. Noteworthily, the top serotypes isolated from animals, i.e., *S.* Enteritidis, *S.* Weltevreden, *S.* Stanley, *S.* Anatum, *S.* Corvallis was similar to that isolated from human (44-46, 74). The consumption of contaminated foods might be a key factor in conferring to this situation. In addition, *S.* Schwarzengrund was increased in Thailand (138) and was shown to be spread globally via food products and cause more illnesses in Denmark and United States (139). Our results were discorded from the previous study in Thailand, which *S.* Mbandaka was the most frequently isolated (12). *Salmonella* serotypes trend to be differ in each countries, e.g., *S.* Emek, *S.* London, and *S.* Agona were the most frequent serotypes reported in Vietnam (69), *S.* Istanbul, and *S.* Agona in U.S.A. (137), *S.* Hadar and *S.* Brancaster in Senegal (140), *S.* Enteritidis, *S.* Hadar, and *S.* serotype 4,12:b:-(II) in Spain (68).

The prevalence of *E. coli* in this study (53%) showed a high contamination rate, which may be described by the frequent presence of *E. coli* in animal production process, environments, and workers. *E. coli* in this study was from two isolation methods, which are standard isolation method and MPN analysis. MPN method had a higher sensitivity of detection compared with standard method, which MPN identified 12 of *E. coli* isolates more than standard method. The reasonable explanation was MPN method has two enrichment steps, which BGLB enhances growth of coliform bacteria and inhibit growth of Gram-positive bacteria and many Gram-negative bacteria. However, *E. coli* from both methods was count once. The prevalence of non-type *E. coli* in chicken meat varied from 21.7% in USA (11), to 39% in Thailand (73). In addition, MPN analysis was used to determine total coliform bacteria in chicken. Coliform bacteria are commonly used as an indicator for sanitary of foods and water, which comprise *E. coli*, *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia*. The MPN value ranged from <3 to 46,000 with 56.66% (34/60) violated allowable total coliform bacteria (MPN <500/g) present in chicken meat according to criteria of Department of Medical Sciences, Ministry of Public Health. These indicated a poor sanitary of chicken, which can cause severe illnesses even in the absence of pathogenic bacteria, *Salmonella*, and warrant the proper cook for the consumers.

Salmonella spp. and *E. coli* in this study showed high percentage of resistance against ampicillin, gentamicin, and tetracycline by disk diffusion method. Multi-drug resistant (MDR) phenotype, referred as a resistance against at least three classes of antibiotics, was found in both *Salmonella* spp. and *E. coli*. Sal 162 and Sal 180 were resistance to β-lactam, aminoglycosides, and trimethoprim-sulfamethoxazole, whereas Sal 178 was resistance to β-lactam, trimethoprim-sulfamethoxazole, and tetracycline. MDR in *E. coli* comprised resistance to β-lactam, fluoroquinolones, and aminoglycosides (2 isolates), resistance to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole (1 isolate), resistance to β-lactam, aminoglycosides, and tetracycline (8 isolates), and resistance to chloramphenicol, aminoglycosides, and tetracycline (2 isolates). The resistance against these drugs in this study might be consequently of the usage of these antibiotics in animals. In Thailand, an antibiotic usage data in animals could not be shown clearly and it cannot be used to clarify the correlation between antibiotic usage in animals and resistance pattern found in this study. Nevertheless, the resistance pattern found in this study is likely the result of such event as supported by previous study (4-6). In some countries where the antibiotic usage data is available, e.g., Norway and Denmark, it was demonstrated that an emergence of fluoroquinolones resistant strains were linked to fluoroquinolones usage in animals (4, 5). This study reflected a continuously use of antibiotics in food producing animals and might lead to an emergence of untreatable strains in the near future. Resistance to fluoroquinolones was found in *E. coli* at the rate of 10.64% whereas it was not found in *Salmonella* spp. Fortunately, ESBLs, which exhibit resistance to extended spectrum cephalosporins and limit option for treatment, were not found in this study. The similar resistance pattern was reported in Vietnam, which resistance to tetracycline and ampicillin were the highest and MDR was observed (70), the high percentage of resistance against tetracycline, sulfamethoxazole, streptomycin, ampicillin was reported in Korea (141), high percentage of resistance to sulfisoxazole, tetracycline and streptomycin was observed in Thailand (12). However, the ESBL-producing strains isolated from chicken were reported in Portugal (142), Ireland (143), Spain (144). The difference in resistance pattern indicated a difference antibiotic usage in animals in each country. Although

most strains in this study were susceptible to most of antibiotics tested, but they will absolutely develop themselves more resistance if antibiotic usage still existing.

Generally, antibiotic resistant bacteria can spread resistance determinants to other bacteria via horizontal gene transfer and vertical gene transfer. This study investigated a role of horizontal gene transfer by focusing on integron element. Integron has a special character in gathering of resistance genes as gene cassettes and usually associated with mobile genetic elements (18). Therefore, integron has an important role contributing in horizontal gene transfer. Class 1 integron was detected by PCR with specific primers to *intI1*, and dot blot hybridization. The prevalence of class 1 integron was 42.86% (6/14) in *Salmonella* spp. and 37.74% (40/106) in *E. coli*. For *E. coli*, the result showed similar to that reported in Korea (42%) (141). The different rates were reported in Japan (11.6%) (19), in Greece (49.2%) (20). The prevalence of class 1 integron in *Salmonella* in this study was lower than reported in Japan (90%) (19). Resistance genes identified in this study were *aadA2*, *aadA4*, *aadA22*, *aadA23*, *dfrA5*, and *lnuF*. Most characterized strains contained cassettes for aminoglycoside resistance and these cassettes were common in class 1 integron (145, 146). Interestingly, *lnuF* conferring lincosamide resistance containing in class 1 integron was rarely found in Gram-negative bacteria, which indicated an occurrence of resistance genes transfer between Gram-positive bacteria to Gram-negative bacteria or vice versa. *lnuF* had been reported in class 2 integron from Ruiz E *et al* (146). Additionally, integrase negative bacteria also carry resistance gene cassettes. The reasonable explanation was the low sensitivity of PCR method or the mutations or deletion in *intI1* itself. Dot blot hybridization indicated a low sensitivity of PCR method, which it gave a higher rate of detection in *E. coli*. Although dot blot hybridization can restore the result of PCR in E17, it showed the same result with PCR in Sal130 (Fig. 4). In case of Sal130, the loss of *intI1* might occur during an integration-excision event. Nevertheless, the failure of detection by dot blot hybridization was found in 5 PCR positive isolates, i.e., Sal145, Sal178, Sal180, E183, and E186.2. The result could be explained by technical error, which may occur during blotting step, hybridization step, blocking step, and detection step. These 5 isolates should be repeated with separated membrane in order to finalize the presence of class 1 integron. In addition, conjugation experiment was used to determine the horizontal

gene transfer among integron carrying bacteria. No transconjugants were observed in any experiments due to a limited recipient strains and drugs. Streptomycin was used instead of spectinomycin for selecting transconjugants between E9 and E12 (donors) and *E. coli* TOP10 (recipient) because streptomycin would expect to kill E9 and E12. Unfortunately, both E9 and E12 can grow in MacConkey agar containing ampicillin (100 µg/ml) and streptomycin (100 µg/ml) and cannot be used in this experiment. The other recipient strains, i.e., *E. coli* JM109 and *E. coli* DH5α were used, which both of them were resistant to nalidixic acid due to a mutation in DNA gyrase, *gyrA*. Norfloxacin was used instead of nalidixic acid due to nalidixic acid powder was not available. Norfloxacin is an antibiotic belongs to fluoroquinolones and its structure is similar to nalidixic acid. Therefore, it would expect to use in this experiment. The MIC result of donor and recipient strains, which showed sensitive in all tested strains, indicated norfloxacin cannot be used. Moreover, direct transformation of plasmid from integron carrying bacteria (E9, E12, and Sal162) was failed because their plasmid were too large (2.5 kb to >12 kb). Electroporation which give a high efficiency should be performed in order to prove this role.

A genetic relatedness among these bacteria was assessed by using typing methods based on phenotypic, and genotypic background. The same plasmid pattern found in *E. coli*, collected from either same place or different places, indicates that the clonal spread also plays a role in the bacterial spread. *Salmonella* typing scheme was divided into the phenotypic method, including serogrouping, serotyping, and resistance pattern incorporated with demographic data, and the genotypic method, including RAPD-PCR, plasmid profile study, and MLST. Serogrouping is a typing method based on agglutination with specific sera to identify the variation in O antigens, whereas serotyping determines an antigenic variation in O and H antigens. The O antigens are one part of lipopolysaccharide (LPS) and major targets for adaptive immune response. The O antigens are synthesized by a set of sugar synthases and transferases, which are encoded by *rfb* (147). The *rfb* showed different sequences among different serogroups and it suggested that this gene was acquired horizontally and might occur multiple times. This gene shuffling might explain why the same *S.* Albany in this study (Sal178 and Sal180) belongs to different serogroups. A comparison of discriminatory power among three typing method, RAPD-PCR,

serogrouping, and serotyping was calculated according to Hunter and Gaston's formula (130). An acceptable level of discrimination depends on many factors, but an index of greater than 0.90 would be more desirable. Serotyping showed the highest discriminatory index of 0.956 followed by RAPD-PCR with an index of 0.934. Serogrouping showed the lowest index of 0.736. The result of various typing methods incorporated with demographic data strongly supports the role of clonal spread among these bacteria. Sal 130 and Sal 162 were RAPD type I, unidentified serogroup, serotype Kedougou that collected from the same place and distributor but in different times. Sal 138, Sal 164 and Sal 190, Sal 193, which were collected from different companies, distributors, and times, showed well correlated in serogrouping, serotyping, and RAPD-PCR results. Nevertheless, these typing methods have some limitations, e.g., O antigens variation, reproducibility and inter-lab comparison of RAPD-PCR. MLST, a sequencing based typing was performed in order to finalize all results and it can be used to compare results with other studies. All of 14 *Salmonella* isolates should be analyzed but due to the cost of the test and limited funding, 4 *Salmonella* isolates were selected based on RAPD-PCR pattern. The result showed ST 50, ST 96, ST 1543, and ST 1549, which matched well with strains from many countries, e.g., Vietnam, Denmark, Chile, USA, and Tunisia. The results of MLST indicated a standardized method, which provides unambiguous and comparable data and is also suited for epidemiological and evolution pathway study. The correlation of MLST and serotyping or serogrouping could not be done in this study. Nevertheless, the different *Salmonella* serotypes with the same ST were demonstrated, e.g., S. Typhimurium and S. Typhimurium var. Copenhegen with the same MLST type (148), and this data also supports that the *Salmonella* typing scheme should be changed from traditional typing to MLST.

CHAPTER VII
CONCLUSION

A contamination of antibiotic resistant bacteria in food was demonstrated worldwide. The main factor enhancing an emergence of such bacteria is an overuse of antibiotics in food producing animals. In Thailand, there were many studies demonstrated the contamination of antibiotic resistant bacteria in food producing animals, which indicated the antibiotic usage in animals even though the antibiotic usage data in Thailand could not be shown clearly. Nevertheless, little is known about genetic mechanism conferring antibiotic resistance and their molecular characteristics. Therefore, the study on prevalence and molecular characterization of antibiotic resistant bacteria in chicken meat in Thailand was conducted.

In this study, 200 of sealed packages chicken meat were performed to determine the prevalence of *Salmonella* spp. and *E. coli*. The prevalence of *Salmonella* spp. and *E. coli* were 18.67% (14/75) and 53% (106/200), respectively. The prevalence of *Salmonella* spp. was calculated from a total of 75 samples due to an ineffectiveness of TT broth, an enrichment media for *Salmonella* spp., which had been modified from ISO 6579. RV broth was demonstrated to give a higher sensitivity than TT broth and suitable for high microbial load samples. This study supported the effectiveness of RV broth in enrichment of *Salmonella* spp. and should be used for the future study. MPN analysis showed that 56.66% (34/60) violated the limit of allowable coliform bacteria in chicken meat, which has the highest value of 46,000. Serogrouping and serotyping were used to type all 14 *Salmonella* isolates, whereas *E. coli* in this study was not characterized by both tests. *Salmonella* group B was the most commonly found (6 isolates) followed by group C (4 isolates), unidentified serogroup (3 isolates), and group E (1 isolates). *Salmonella* serotypes in this study was similar to the top of *Salmonella* serotypes in animals in Thailand, of which *S.* Kedougou, *S.* Albany, and *S.* Altona were the most common serotypes. *S.* Agona and *S.*

Schwarzengrund were found in this study, the latter has been shown to be an international spread. Antibiotic resistance profiles of *Salmonella* spp. and *E. coli* were determined by disk diffusion method according to CLSI 2010. Most of *Salmonella* spp. and *E. coli* exhibited high percentage of resistance against ampicillin, gentamicin, and tetracycline, which the percentage of resistance against ampicillin, gentamicin, and tetracycline in *Salmonella* spp. and *E. coli* were 78.57%, 71.43%, 21.43%, and 72.34%, 38.30%, 48.48%, respectively. Resistance to fluoroquinolones, chloramphenicol, trimethoprim-sulfamethoxazole, and amoxicillin-clavulanic acid was observed. Eventhough ESBLs phenotype, as screened by double disk and combination disk method, were not found in this study, but MDR phenotypes were found in both *Salmonella* spp. and *E. coli*. A presence of class 1 integron was demonstrated by PCR and dot blot hybridization. The PCR showed that class 1 integron was found in 42.86% (6/14) and 37.74% (40/106) in *Salmonella* spp. and *E. coli*, respectively. Resistance genes identified in this study were *aadA2*, *aadA4*, *aadA22*, and *aadA23* (for aminoglycoside resistance); *dfrA5* (for trimethoprim resistance); and *lnuF* (for lincosamide resistance). Moreover, integrase negative bacteria also carried resistance cassettes (E17 and Sal130). Therefore, dot blot hybridization was used to confirm the PCR result. Dot bot hybridization can restore the result of PCR in E17, whereas it showed the same result with PCR in Sal130. This result indicated a low sensitivity of PCR method and dot blot should be used instead. However, the failure of detection was found in 5 PCR positive isolates. Finally, a genetic relatedness among these bacteria was determined by using phenotypic methods and genotypic methods. The same plasmid patterns found in *E. coli*, either collected from the same place or different places, indicates that clonal spread might play a role in conferring resistance genes spread. The results of typing methods incorporated with demographic data of *Salmonella* supported the role of clonal spread. Nevertheless, O antigen variations that might occur via gene shuffling may lead to misinterpretation of the results. The same *S*. Albany (Sal178 and Sal 180) but different serogroups were found in this study. MLST, which provides unambiguous and comparable data, was selected in order to finalize all results. All 14 *Salmonella* isolates should be analyzed to compare with serotyping result, but due to its cost and limited funding, 4 *Salmonella* isolates was

submitted to MLST analysis. MLST results were ST 50, ST 96, ST 1543, and ST 1546.

In conclusion, this study demonstrated that antibiotic resistant bacteria and integron elements now have been spread among food producing animals and can be spread to human through consumption of undercooked food. Therefore, molecular characterization of these bacteria from other sources is important in order to track down sources of such strains and provides insight information to control and prevent the spread of these bacteria. An effective molecular typing methods, e.g., MLST should be replaced the traditional typing methods, which have a variation, e.g., serotyping or provide ambiguous data. Antibiotic usage in humans and animals should be tightly monitored in order to limit the emergence of antibiotic resistant bacteria.

REFERENCES

1. World Health Organization. Available at http://www.who.int/mediacentre/factsheets/fs237/en/.
2. World Health Organization. The Global Burden of Disease. 2004 updates. Geneva, 2008. Available at http://www.who.int/healthinfo/global_burden_disease/GBD_report_2004update_full.pdf.
3. Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, et al. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J Antimicrob Chemother 2004; 53: 28-52.
4. Endtz HP, Ruijs GJ, van Klingeren B, Jansen WH, van der Reyden T, Mouton RP.Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J Antimicrob Chemother 1991; 27: 199-208.
5. Norström M, Hofshagen M, Stavnes T, Schau J, Lassen J, Kruse H. Antimicrobial resistance in *Campylobacter* jejuni from humans and broilers in Norway. Epidemiol Infect 2006; 134: 127-30.
6. Angulo FJ, Johnson KR, Tauxe RV, Cohen ML. Origins and consequences of antimicrobial-resistant nontyphoidal *Salmonella*: implications for the use of fluoroquinolones in food animals. Microb Drug Resist 2000; 6: 77-83.
7. Van TT, Moutafis G, Tran LT, Coloe PJ. Antibiotic resistance in food-borne bacterial contaminants in Vietnam. Appl Environ Microbiol 2007; 73: 7906-11.
8. Nógrády N, Tóth A, Kostyák A, Pászti J, Nagy B. Emergence of multidrug-resistant clones of *Salmonella* Infantis in broiler chickens and humans in Hungary. J Antimicrob Chemother 2007; 60: 645-48.
9. Boyle F, Morris D, O'Connor J, Delappe N, Ward J, Cormican M. First report of extended-spectrum-beta-lactamase producing *Salmonella*

enterica serovar Kentucky isolated from poultry in Ireland. Antimicrob Agents Chemother 2010; 54: 551-53.

10. Angkititrakul S, Chomvarin C, Chaita T, Kanistanon K, Waethewutajarn S. Epidemiology of antimicrobial resistance in *Salmonella* isolated from pork , chicken meat and humans in Thailand. Southeast Asian J Trop Med Public Health 2005; 36: 1510-15.
11. Zhao C, Ge B, De Villena J, Sudler R, Yeh E, Zhao S, White DG, Wagner D, Meng J. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., area. Appl Environ Microbiol 2001; 67: 5431-36.
12. Minami A, Chaicumpa W, Chongsa-Nguan M, Samosornsuk S, Monden S, Takeshi K, et al. Prevalence of foodborne pathogens in open markets and supermarkets in Thailand. Food Control 2010; 21: 221–26.
13. Stokes HW, Gillings MR. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. FEMS Microbiol Rev 2011; 35: 790-819.
14. Stokes HW, Hall RM. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol Microbiol 1989; 3: 1669-83.
15. Bennett PM. Integrons and gene cassettes: a genetic construction kit for bacteria. J Antimicrob Chemother 1999; 43: 1-4.
16. Mazel D. Integrons: agents of bacterial evolution. Nat Rev Microbiol 2006; 4: 608-20.
17. Cambray G, Guerout AM, Mazel D. Integrons. Annu Rev Genet 2010; 44: 141-66.
18. Guerra B, Soto SM, Argüelles JM, Mendoza MC. Multidrug resistance is mediated by large plasmids carrying a class 1 integron in the emergent *Salmonella enterica* serotype [4,5,12:i:-]. Antimicrob Agents Chemother 2001; 45:1305-08.
19. Ahmed AM, Shimabukuro H, Shimamoto T. Isolation and molecular characterization of multidrug-resistant strains of *Escherichia coli* and

Salmonella from retail chicken meat in Japan. J Food Sci 2009; 74: M405-10.

20. Vasilakopoulou A, Psichogiou M, Tzouvelekis L, Tassios PT, Kosmidis C, Petrikkos G et al. Prevalence and characterization of class 1 integron in *Escherichia coli* of poultry and human origin. Foodborne Pathog Dis 2009; 6: 1211-18.
21. Threlfall EJ. Epidemic *Salmonella* typhimurium DT 104--a truly international multiresistant clone. J Antimicrob Chemother 2000; 46: 7-10.
22. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, et al. Food-related illness and death in the United States. Emerg Infect Dis 1999; 5: 607-25.
23. Roberts T. WTP estimates of the societal costs of U.S. food-borne illness. American journal of agricultural of economics 2007; 89: 1183-1188.
24. Schmidt RH, Goodrich RM, Archer DL, Schneide KR. General overview of the causative agents of foodborne illness. Available at http://edis.ifas.ufl.edu.
25. Centers for Disease Control and Prevention (CDC). Preliminary FoodNet Data on the incidence of infection with pathogens transmitted commonly through food--10 States, 2008. MMWR Morb Mortal Wkly Rep 2009; 58: 333-37.
26. Centers for Disease Control and Prevention (CDC). Preliminary FoodNet Data on the incidence of infection with pathogens transmitted commonly through food--10 States, 2009. MMWR Morb Mortal Wkly Rep 2010; 59: 418-22.
27. Rushton J., Viscarra R., Guerne Bleich E. and McLeod A. Impact of avian influenza outbreaks in the poultry sectors of five South East Asian countries (Cambodia, Indonesia, Lao PDR, Thailand, VietNam) outbreak costs, responses and potential long term control. World Poultry Sci. J 2005; 61: 491–513.
28. Schultz M. Theobald Smith. Emerg Infect Dis 2008; 14: 1940-42.
29. Bäumler AJ, Tsolis RM, Ficht TA, Adams LG. Evolution of host adaptation in *Salmonella enterica*. Infect Immun 1998; 66: 4579-87.

30. Pui CF, Wong WC, Chai LC, Tunung R, Jeyaletchumi P, Noor Hidayah MS. *Salmonella*: A foodborne pathogen. Int Food Res J 2011; 18: 465-473.

31. Crosa JH, Brenner DJ, Ewing WH, Falkow S. Molecular relationships among the *Salmonelleae*. J Bacteriol 1973; 115: 307-15.

32. Euzéby JP. Revised *Salmonella* nomenclature: designation of *Salmonella enterica* (ex Kauffmann and Edwards 1952) Le Minor and Popoff 1987 sp. nov., nom. rev. as the neotype species of the genus *Salmonella* Lignieres 1900 (approved lists 1980), rejection of the name *Salmonella choleraesuis* (Smith 1894) Weldin 1927 (approved lists 1980), and conservation of the name *Salmonella typhi* (Schroeter 1886) Warren and Scott 1930 (approved lists 1980). Request for an opinion. Int J Syst Bacteriol 1999; 49: 927-30.

33. Reeves MW, Evins GM, Heiba AA, Plikaytis BD, Farmer JJ 3rd. Clonal nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of *Salmonella bongori* comb. nov. J Clin Microbiol 1989; 27: 313-20.

34. Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. *Salmonella* nomenclature. J Clin Microbiol 2000; 38: 2465-67.

35. Garcia-del Portillo F, Foster JW, Finlay BB. Role of acid tolerance response genes in *Salmonella* typhimurium virulence. Infect Immun 1993; 61: 4489-92.

36. Ohl ME, Miller SI. *Salmonella*: a model for bacterial pathogenesis. Annu Rev Med 2001; 52: 259-74.

37. Francis CL, Starnbach MN, Falkow S. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella* typhimurium grown under low-oxygen conditions. Mol Microbiol 1992; 6: 3077-87.

38. Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. Bull World Health Organ 2004; 82: 346-53.

39. Mayer CA, Neilson AA. Typhoid and paratyphoid fever –prevention in travellers. Aust Fam Physician 2010; 39: 847-51.

40. Raffatellu M, Wilson RP, Winter SE, Bäumler AJ. Clinical pathogenesis of typhoid fever. J Infect Dev Ctries 2008; 2: 260-66.
41. Cleary P, Browning L, Coia J, Cowden J, Fox A, Kearney J, et al. A foodborne outbreak of *Salmonella* Bareilly in the United Kingdom. Euro Surveill 2010; 15: 2.
42. Barton Behravesh C, Mody RK, Jungk J, Gaul L, Redd JT, Chen S, et al. 2008 outbreak of *Salmonella* Saintpaul infections associated with raw produce. N Engl J Med 2011; 364: 918-27.
43. Hohmann EL. Nontyphoidal salmonellosis. Clin Infect Dis 2001; 32: 263-69.
44. Bangtrakulnonth A, Tishyadhigama P, Bunyaraksyotin G, Nantanawoot P. Annual Report of Confirmed *Salmonella* and *Shigella* in Thailand 2003. The National *Salmonella* and *Shigella* Center, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. 2003.
45. Bangtrakulnonth A, Bunyaraksyotin G, Nantanawoot P. Annual Report of Confirmed *Salmonella* and *Shigella* in Thailand 2004. The National *Salmonella* and *Shigella* Center, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. 2004.
46. Bangtrakulnonth A, Pornruangwong S, Pulsrikarn C. Annual Report of Confirmed *Salmonella* and *Shigella* in Thailand 2005. The National *Salmonella* and *Shigella* Center, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. 2005.
47. Gordon MA, Banda HT, Gondwe M, Gordon SB, Boeree MJ, Walsh AL. Non-typhoidal *salmonella* bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence. AIDS 2002; 16: 1633-41.
48. Bronzan RN, Taylor TE, Mwenechanya J, Tembo M, Kayira K, Bwanaisa L. Bacteremia in Malawian children with severe malaria: prevalence, etiology , HIV coinfection, and outcome. J Infect Dis 2007; 195: 895-904.
49. Diniz-Santos DR, Silva LR, Silva N. Antibiotics for the empirical treatment of acute infectious diarrhea in children. Braz J Infect Dis 2006; 10: 217- 27.

50. Jacoby GA. Mechanisms of Resistance to Quinolones. Clin Infect Dis 2005; 41: S120-26.

51. de Souza RB, Magnani M, Ferrari RG, Kottwitz LBM, Sartori D, Tognim MCB, et al. Detection of quinolone-resistance mutations in *Salmonella* spp. strains of epidemic and poultry origin. Braz J Microbiol 2011; 42: 211-215.

52. Reyna F, Huesca M, González V, Fuchs LY. *Salmonella typhimurium gyrA* mutations associated with fluoroquinolone resistance. Antimicrob Agents Chemother 1995; 39: 1621-23.

53. Turner AK, Nair S, Wain J. The acquisition of full fluoroquinolone resistance in *Salmonella* Typhi by accumulation of point mutations in the topoisomerase targets. J Antimicrob Chemother 2006; 58: 733-40.

54. Chiu CH, Wu TL, Su LH, Chu C, Chia JH, Kuo AJ, et al. The emergence in Taiwan of fluoroquinolone resistance in *Salmonella enterica* serotype Choleraesuis. N Engl J Med 2002; 346: 413-19.

55. Ferrari R, Galiana A, Cremades R, Rodriguez JC, Magnani M, Tognim MC, et al. Plasmid-mediated quinolone resistance by genes *qnrA1* and *qnrB19* in *Salmonella* strains isolated in Brazil. J Infect Dev Ctries 2011; 5: 496-98.

56. Sjölund-Karlsson M, Howie R, Rickert R, Krueger A, Tran TT, Zhao S, et al. Plasmid-mediated quinolone resistance among non-typhi *Salmonella enterica* isolates, USA. Emerg Infect Dis 2010; 16: 1789-91.

57. Taguchi M, Kawahara R, Seto K, Inoue K, Hayashi A, Yamagata N, et al. Plasmid-mediated quinolone resistance in *Salmonella* isolated from patients with overseas travelers' diarrhea in Japan. Jpn J Infect Dis 2009; 62: 312-14.

58. http://www.betagro.com/products_en.php?g_id=2&s_id=1&c_id=6

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

60. Jay MT, Garrett V, Mohle-Boetani JC, Barros M, Farrar JA, Rios R, et al. A multistate outbreak of *Escherichia coli* O157:H7 infection linked to

consumption of beef tacos at a fast-food restaurant chain. Clin Infect Dis 2004; 39: 1-7.

61. Wendel AM, Johnson DH, Sharapov U, Grant J, Archer JR, Monson T, et al. Multistate outbreak of *Escherichia coli* O157:H7 infection associated with consumption of packaged spinach, August-September 2006: the Wisconsin investigation. Clin Infect Dis 2009; 48: 1079-86.

62. Grant J, Wendelboe AM, Wendel A, Jepson B, Torres P, Smelser C, et al. Spinach-associated *Escherichia coli* O157:H7 outbreak, Utah and New Mexico, 2006. Emerg Infect Dis 2008; 14: 1633-36.

63. Vogt RL, Dippold L. *Escherichia coli* O157:H7 outbreak associated with consumption of ground beef, June-July 2002. Public Health Rep 2005; 120: 174-78.

64. Thompson JS, Hodge DS, Borczyk AA. Rapid biochemical test to identify verocytotoxin-positive strains of *Escherichia coli* serotype O157. J Clin Microbiol 1990; 28: 2165-68.

65. Kilonzo-Nthenge A, Nahashon SN, Chen F, Adefope N. Prevalence and antimicrobial resistance of pathogenic bacteria in chicken and guinea fowl. Poult Sci 2008; 87: 1841-48.

66. White DG, Zhao S, Sudler R, Ayers S, Friedman S, Chen S, et al. The isolation of antibiotic-resistant *Salmonella* from retail ground meats. N Engl J Med 2001; 345: 1147-54.

67. Limawongpranee S, Hayashidani H, Okatani AT, Ono K, Hirota C, Kaneko K, et al. Prevalence and persistence of *Salmonella* in broiler chicken flocks. J Vet Med Sci 1999; 61: 255-59.

68. Domínguez C, Gómez I, Zumalacárregui J. Prevalence of *Salmonella* and *Campylobacter* in retail chicken meat in Spain. Int J Food Microbiol; 72: 165-68.

69. Luu QH, Fries R, Padungtod P, Tran TH, Kyule MN, Baumann MP. Prevalence of *Salmonella* in retail chicken meat in Hanoi, Vietnam. Ann N Y Acad Sci 2006; 1081: 257-61.

70. Van TT, Moutafis G, Istivan T, Tran LT, Coloe PJ. Detection of *Salmonella* spp. in retail raw food samples from Vietnam and characterization of their antibiotic resistance. Appl Environ Microbiol 2007; 73: 6885-90.

71. Harrison WA, Griffith CJ, Tennant D, Peters AC. Incidence of *Campylobacter* and *Salmonella* isolated from retail chicken and associated packaging in South Wales. Lett Appl Microbiol 2001; 33: 450-54.

72. Nzouankeu A, Ngandjio A, Ejenguele G, Njine T, Ndayo Wouafo M. Multiple contaminations of chickens with *Campylobacter*, *Escherichia coli* and *Salmonella* in Yaounde (Cameroon). J Infect Dev Ctries 2010; 4: 583-586.

73. Hanson R, Kaneene JB, Padungtod P, Hirokawa K, Zeno C. Prevalence of *Salmonella* and *E. coli*, and their resistance to antimicrobial agents, in farming communities in northern Thailand. Southeast Asian J Trop Med Public Health 2002; 33 Suppl 3: 120-26.

74. Bangtrakulnonth A, Tishyadhigama P. Annual Report of Confirmed *Salmonella* and *Shigella* in Thailand 2006. The National *Salmonella* and *Shigella* Center, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. 2006.

75. Overdevest I, Willemsen I, Rijnsburger M, Eustace A, Xu L, Hawkey P et al. Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. Emerg Infect Dis 2011; 17: 1216-22.

76. McEwen SA, Fedorka-Cray PJ. Antimicrobial use and resistance in animals. Clin Infect Dis 2002; 34: S93–S106.

77. Apata DF. Antibiotic resistance in poultry. Int. J. Poult. Sci 2009; 8: 404-408.

78. Smith KE, Besser JM, Hedberg CW, Leano FT, Bender JB, Wicklund JH, et al. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992-1998. Investigation Team. N Engl J Med 1999; 340: 1525-32.

79. Kruse H, Johansen BK, Rørvik LM, Schaller G. The use of avoparcin as a growth promoter and the occurrence of vancomycin-resistant *Enterococcus* species in Norwegian poultry and swine production. Microb Drug Resist 1999; 5: 135-39.

80. Pantosti A, Del Grosso M, Tagliabue S, Macrì A, Caprioli A. Decrease of vancomycin resistant-enterococci in poultry meat after avoparcin ban. Lancet 1999; 354: 741-42.

81. 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: 365-71.

82. Zhang XY, Ding LJ, Fan MZ. Resistance patterns and detection of *aac(3)-IV* gene in apramycin-resistant *Escherichia coli* isolated from farm animals and farm workers in northeastern of China. Res Vet Sci 2009; 87: 449-54.

83. Winokur PL, Vonstein DL, Hoffman LJ, Uhlenhopp EK, Doern GV. Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob Agents Chemother 2001; 45: 2716-22.

84. Salyers AA, Gupta A, Wang Y. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol 2004; 12: 412-16.

85. Lester SC, del Pilar Pla M, Wang F, Perez Schael I, Jiang H, O'Brien TF. The carriage of *Escherichia coli* resistant to antimicrobial agents by healthy children in Boston, in Caracas, Venezuela, and in Qin Pu, China. N Engl J Med 1990; 323: 285-89.

86. Marshall BM, Levy SB. Food animals and antimicrobials: impacts on human health. Clin Microbiol Rev 2011; 24: 718-33.

87. Alanis AJ. Resistance to antibiotics: are we in the post-antibiotic era?. Arch Med Res 2005; 36 :697-705.

88. Naenna P, Noisumdaeng P, Pongpech P, Tribuddharat C. Detection of outer membrane porin protein, an imipenem influx channel, in *Pseudomonas aeruginosa* clinical isolates. Southeast Asian J Trop Med Public Health 2010; 41: 614-24.

89. Webber MA, Piddock LJ. The importance of efflux pumps in bacterial antibiotic resistance. J Antimicrob Chemother 2003; 51: 9-11.

90. Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev 2009; 22: 664-89.

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

92. Adewoye L, Sutherland A, Srikumar R, Poole K. The mexR repressor of the mexAB-oprM multidrug efflux operon in *Pseudomonas aeruginosa* characterization of mutations compromising activity. J Bacteriol 2002; 184: 4308-12.

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

94. Zapun A, Contreras-Martel C, Vernet T. Penicillin-binding proteins and beta-lactam resistance. FEMS Microbiol Rev 2008; 32: 361-85.

95. Hartman BJ, Tomasz A. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J Bacteriol 1984; 158: 513-16.

96. Fontana R, Aldegheri M, Ligozzi M, Lopez H, Sucari A, Satta G. Overproduction of a low-affinity penicillin-binding protein and high-level ampicillin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother 1994; 38: 1980-83.

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

98. Eaves DJ, Randall L, Gray DT, Buckley A, Woodward MJ, White AP, et al. Prevalence of mutations within the quinolone resistance-determining region of *gyrA*, *gyrB*, *parC*, and *parE* and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*. Antimicrob Agents Chemother 2004; 48: 4012-15.

99. Yoshida H, Bogaki M, Nakamura M, Nakamura S. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob Agents Chemother 1990; 34: 1271-72.

100. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 1995; 39: 1211-33.

101. Carattoli A. Plasmid-mediated antimicrobial resistance in *Salmonella enterica*. Curr Issues Mol Biol 2003; 5: 113-22.

102. Chaïbi EB, Sirot D, Paul G, Labia R. Inhibitor-resistant TEM beta- lactamases: phenotypic, genetic and biochemical characteristics. J Antimicrob Chemother 1999; 43: 447-58.

103. Vakulenko SB, Mobashery S. Versatility of aminoglycosides and prospects for their future. Clin Microbiol Rev 2003; 16: 430-50.

104. Murray IA, Shaw WV. O-Acetyltransferases for chloramphenicol and other natural products. Antimicrob Agents Chemother 1997; 41: 1-6.

105. Leclercq R, Courvalin P. Intrinsic and unusual resistance to macrolide, lincosamide, and streptogramin antibiotics in bacteria. Antimicrob Agents Chemother 1991; 35: 1273-76.

106. Huovinen P. Resistance to Trimethoprim-Sulfamethoxazole. Clin Infect Dis 2001; 32: 1608–14.

107. Klugman KP. The role of clonality in the global spread of fluoroquinolone-resistant bacteria. Clin Infect Dis 2003 Mar; 36: 783-85.

108. Ochman H, Lawrence JG, Groisman EA. Lateral gene transfer and the nature of bacterial innovation. Nature 2000; 405: 299-304.

109. Collis CM, Hall RM. Gene cassettes from the insert region of integrons are excised as covalently closed circles. Mol Microbiol 1992; 6: 2875-85.

110. Hall RM, Stokes HW. Integrons: novel DNA elements which capture genes by site-specific recombination. Genetica 1993; 90: 115-32.

111. Stokes HW, O'Gorman DB, Recchia GD, Parsekhian M, Hall RM. Structure and function of 59-base element recombination sites associated with mobile gene cassettes. Mol Microbiol 1997; 26: 731-45.

112. Hall RM, Collis CM. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol Microbiol 1995; 15: 593-600.

113. Radström P, Sköld O, Swedberg G, Flensburg J, Roy PH, Sundström L. et al. Transposon Tn*5090* of plasmid R751, which carries an integron, is related to Tn*7*, Mu, and the retroelements. J Bacteriol 1994; 176: 3257-68.

114. Collis CM, Kim MJ, Partridge SR, Stokes HW, Hall RM. Characterization of the class 3 integron and the site-specific recombination system it determines. J Bacteriol 2002; 184: 3017-26.

115. Lévesque C, Piché L, Larose C, Roy PH. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob Agents Chemother 1995; 39: 185-91.

116. Foley SL, Lynne AM, Nayak R. Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. Infect Genet Evol 2009; 9: 430-40.

117. Olsen JE, Brown DJ, Skov MN, Christensen JP. Bacterial typing methods suitable for epidemiological analysis. Applications in investigations of salmonellosis among livestock. Vet Q 1993; 15: 125-35.

118. Chisholm SA, Crichton PB, Knight HI, Old DC. Molecular typing of *Salmonella* serotype Thompson strains isolated from human and animal sources. Epidemiol Infect 1999; 122: 33-39.

119. Savelkoul PH, Aarts HJ, de Haas J, Dijkshoorn L, Duim B, Otsen M, et al. Amplified-fragment length polymorphism analysis: the state of an art. J Clin Microbiol 1999; 37: 3083-91.

120. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 1990; 18: 6531-35.

121. 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: 6823-31.

122. Georghiou PR, Doggett AM, Kielhofner MA, Stout JE, Watson DA, Lupski JR, et al. Molecular fingerprinting of *Legionella* species by repetitive element PCR. J Clin Microbiol 1994; 32: 2989-94.

123. Naing L, Winn T, and Rusli BN. Practical issues in calculating the sample size for prevalence studies. Arch Orofac Sci 2006; 1: 9-14.

124. International Organization for Standardization (ISO) 6579. (1998): Microbiology of animal feeding stuff-horizontal method for the detection of *Salmonella*, ISO, Geneva.

125. Grimont, P. A. D. (2007): Antigenic formulae of the *Salmonella* serovars. F.X.Weil. [9th ed.]. Paris, France., WHO Collaborating Center for Reference and Research on *Salmonella*. Institut Pasteur. 166 p.

126. Yousef, A.E. and Carlstrom, C. (2003): Food Microbiology. A laboratory manual. John Wiley and Sons, Inc., Hoboken, NJ.

127. Clinical and Laboratory Standard Institute (2010): Performance standards for antimicrobial susceptibility testing; 20th information supplement (M100- S20). Clinical Laboratory Standard Institute, Wayne, Pa.

128. Bissonnette L, Roy PH. Characterization of In0 of *Pseudomonas aeruginosa* plasmid pVS1, an ancestor of integrons of multiresistance plasmids and transposons of gram-negative bacteria. J Bacteriol 1992; 174: 1248-57.

129. 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 (Volume 1). 5th ed. United States of America: Wiley; 2002.

130. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 1988; 26: 2465-66.

131. Kulkarni SP, Lever S, Logan JM, Lawson AJ, Stanley J, Shafi MS. Detection of *Campylobacter* species: a comparison of culture and polymerase chain reaction based methods. J Clin Pathol 2002; 55: 749-53.

132. Sommer MO, Church GM, Dantas G. The human microbiome harbors a diverse reservoir of antibiotic resistance genes. Virulence 2010; 1: 299-303.

133. Pal A, Marshall DL. Comparison of culture media for enrichment and isolation of *Salmonella* spp. from frozen Channel catfish and Vietnamese basa fillets. Food Microbiol 2009; 26: 317-19.

134. June GA, Sherrod PS, Hammack TS, Amaguana RM, Andrews WH. Relative effectiveness of selenite cystine broth, tetrathionate broth, and rappaport-vassiliadis medium for recovery of *Salmonella* spp. from raw flesh, highly contaminated foods, and poultry feed: collaborative study. J AOAC Int 1996; 79: 1307-23.

135. Hammack TS, Amaguana RM, June GA, Sherrod PS, Andrews WH. Relative effectiveness of selenite cystine broth, tetrathionate broth, and Rappaport-Vassiliadis medium for the recovery of *Salmonella* spp. from foods with a low microbial load. J Food Prot 1999; 62: 16-21.

136. Abdellah C, Fouzia FR, Abdelkader C, Rachida BS and Mouloud Z. Prevalence and anti-microbial susceptibility of *Salmonella* isolates from chicken carcasses and giblets in Meknès, Morocco. Afr J Microbiol Res 2009; 3: 215-19.

137. White DG, Zhao S, Sudler R, Ayers S, Friedman S, Chen S et al. The isolation of antibiotic-resistant *Salmonella* from retail ground meats. N Engl J Med 2001; 345: 1147-54.

138. Bangtrakulnonth A, Pornreongwong S, Pulsrikarn C, Sawanpanyalert P, Hendriksen RS, Lo Fo Wong DM et al. *Salmonella* serovars from humans and other sources in Thailand, 1993-2002. Emerg Infect Dis 2004; 10: 131-36.

139. 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: 726-31.

140. Cardinale E, Perrier-Gros-Claude JD, Tall F, Cisse M, Gueye EF, Salvat G. Prevalence of *Salmonella* and *Campylobacter* in retail chicken carcasses in Senegal. Rev Elev Med Vet. Pays. Trop 2003; 56: 13–16.

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

142. Machado E, Coque TM, Cantón R, Sousa JC, Peixe L. Antibiotic resistance integrons and extended-spectrum β-lactamases among Enterobacteriaceae isolates recovered from chickens and swine in Portugal. J Antimicrob Chemother 2008; 62: 296-302.

143. Boyle F, Morris D, O'Connor J, Delappe N, Ward J, Cormican M. First report of extended-spectrum-β-lactamase-producing *Salmonella enterica* serovar Kentucky isolated from poultry in Ireland. Antimicrob Agents Chemother 2010; 54: 551-53.

144. Brinas L, Moreno MA, Zarazaga M, Porrero C, Saenz Y, Garcia M et al. Detection of CMY-2, CTX-M-14, and SHV-12 β-lactamases in *Escherichia coli* fecal-sample isolates from healthy chickens. Antimicrob Agents Chemother 2003; 47: 2056-58.

145. Firoozeh F, Zahraei-Salehi T, Shahcheraghi F, Karimi V, Aslani MM. Characterization of class I integrons among *Salmonella enterica* serovar Enteritidis isolated from humans and poultry. FEMS Immunol Med Microbiol 2012; 64: 237-43.

146. Ruiz E, Sáenz Y, Zarazaga M, Rocha-Gracia R, Martínez-Martínez L, Arlet G et al. *qnr*, *aac(6')-Ib-cr* and *qepA* genes in *Escherichia coli* and *Klebsiella* spp.: genetic environments and plasmid and chromosomal location. J Antimicrob Chemother 2012; Jan 4 [Epub ahead of print].

147. Kingsley RA, Baumler AJ. Host adaptation and the emergence of infectious disease: the *Salmonella* paradigm. Mol Microbiol 2000; 36: 1006-14.

148. Sukhnanand S, Alcaine S, Warnick LD, Su WL, Hof J, Craver MP et al. DNA sequence-based subtyping and evolutionary analysis of selected *Salmonella enterica* serotypes. J Clin Microbiol 2005; 43: 3688-98.

APPENDIX

REAGENTS FOR MOLECULAR PARTS

1) 10X TBE buffer

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

All ingredients were mixed until Tris base and EDTA was completely dissolved. Forty ml of 0.5M EDTA, pH 8.0 was added and brought volume to 1 L with distilled water. The solution was mixed and stored at room temperature.

2) 6X loading buffer

Bromphenol blue	0.025	g
Glucose	4	g
Distilled water	10	ml

All ingredients were mixed and stored at room temperature.

3) 20X SSC solution

NaCl	175.3	g
Sodium citrate	88.2	g
Distilled water	800	ml

All ingredients were mixed thoroughly. Solution was adjusted to pH 7.0 by using HCl and brought volume to 1 L with distilled water. The solution was sterilized by autoclaving at 121°C for 15 minutes, and stored at room temperature.

4) Stringency solution I

A total volume of 50 ml of solution containing 1X SSC, and 0.1% SDS was prepared by mixing 0.5 ml of 10% SDS, and 2.5 ml of 20X SSC in 47 ml of distilled water.

5) Stringency solution II

A total volume of 50 ml of solution containing 0.5X SSC, and 0.1% SDS was prepared by mixing 0.5 ml of 10% SDS, and 1.25 ml of 20X SSC in 48.25 ml of distilled water.

6) Buffer A

Buffer A was prepared by mixing 50 ml of 1M Tris-Cl, pH 9.5, and 50 ml of 3M NaCl in 400 ml of distilled water (100 mM Tris-Cl, pH 9.5, 300 mM NaCl). The solution was sterilized by autoclaving at 121°C for 15 minutes, and stored at room temperature. A fresh preparation is important for this buffer.

7) Blocking solution

Blocking solution was prepared by dissolving 1.5 g of skim milk in 30 ml of buffer A (5% skim milk in buffer A).

8) Antifluorescein antibody/alkaline phosphatase conjugate

Antifluorescein antibody/alkaline phosphatase conjugate was prepared by mixing 0.15 g of bovine serum albumin, and 6 µl of antifluorescein antibody/alkaline phosphatase conjugate in 30 ml of buffer A.

9) Prehybridization buffer

A total volume of 30 ml solution containing 0.05% liquid block solution, 5X SSC, and 0.1% SDS was prepared by mixing 1.5 ml of liquid block solution, 7.5 ml of 20X SSC, and 0.3 ml of 10% SDS in 20.7 ml of distilled water.

10) Hybridization buffer

Hybridization buffer was prepared by adding labeled probe into prehybridization buffer. The hybridization buffer was denatured by boiling at 100°C for 20 minutes and immediately placed on ice before use.

11) Alkaline lysis buffer I

Glucose	1.8	g
1 M Tris-cl, pH 8.0	5	ml
0.5 M EDTA, pH 8.0	4	ml
Distilled water to make	200	ml

All ingredients were mixed, autoclaved at 121°C for 15 minutes, and stored at 4°C.

12) 1M Tris-cl pH 8.0

Tris base	121.1	g
Distilled water	700	ml

All ingredients were mixed thoroughly by stirring. Solution was adjusted to pH 8.0 by using HCl and brought volume to 1 L with distilled water. The solution was sterilized by autoclaving at 121°C for 15 minutes, and stored at room temperature.

13) 0.5M EDTA pH 8.0

Na2 EDTA.2H2O	37.22	g
Distilled water	140	ml

All ingredients were mixed, adjusted to pH 8.0 by using 10M NaoH, and brought volume to 200 ml with distilled water.

14) 10M NaOH

Eighty grams of NaOH was dissolved in 200 ml of distilled water.

15) Alkaline lysis buffer II

A desired volume of the final concentration of 0.2M NaOH, and 1% SDS solution was freshly prepared from 10M NaOH and 10% SDS.

16) 10% SDS solution

Ten grams of SDS powder was dissolved in 90 ml of distilled water and brought volume to 100 ml with distilled water.

17) Alkaline lysis solution III

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

.

BIOGRAPHY

NAME	Mr. Chaiyaporn Chaisatit
DATE OF BIRTH	11 July 1984
PLACE OF BIRTH	Nakhonsawan, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 2002-2005: Bachelor of Science (Medical Technology) Mahidol University, 2009-2012: Master of Science (Microbiology)
RESEARCH GRANTS	The Siriraj Graduate Thesis Scholarships
HOME ADDRESS	12/125 Samakki Road, Tumbol Tha-Sai, Ampur muang Nonthaburi, Nonthaburi, Thailand 11000 Tel. 090-7679402 E-mail: cchaisatit_28@hotmail.com