RESISTANCE PLASMID METAGENOMICS IN HOSPITALIZED PATIENTS' GUT MICROBIOME

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RESISTANCE PLASMID METAGENOMICS IN HOSPITALIZED PATIENTS' GUT MICROBIOMES

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ABSTRACT

Nosocomial infections with multidrug-resistant pathogens cause colossal problems for a healthcare system. In theory, pathogens, environmental bacteria, and normal flora can regularly exchange their resistance genes among themselves. Resistance genes spread among these bacteria are the result of antibiotic usage in hospitals, gene transfer by plasmids and mobile genetic elements, and the presence of reservoirs of resistance genes, such as gut microbiome in patients. This study collected stool specimens from 7 patients from two different wards that had been hospitalized for longer than 7 days with more than two different classes of antibiotic use within 1 month. Extracted gut microbiomes' plasmids from patients and one healthy volunteer, with no history of hospitalization and no antimicrobial use for three years, were used for the study. Plasmids were extracted by alkaline lysis method, and were purified by CsCl-EtBr gradient centrifugation or enzyme treatment with lambda exonuclease, exonuclease I and Phi29 DNA polymerase. The *454* high-throughput sequencing (GS Junior) revealed DNA outputs from approximately 9 to 81 Mega base-pairs. By an integration of NCBI BLAST and Pfam, most predicted resistance proteins conferred the resistance to most antimicrobial drug groups, and some belonged to unknown DNA. In addition, by using ResFinder and BioEdit for analysis, highly similar *aph(3')*-III gene variants together with flanking regions were found in both patients and a healthy control indicating that these resistance genes have been transferred between a community and hospitals. Several nosocomial resistance genes, namely *ant(6)-Ia*, *ermB*, *lnuB*, *tetL* and *tetU*, conferring resistance to aminoglycosides, lincosamide, macrolides, streptogramin B and tetracycline, spread among different patients within the same ward and between different wards. Several other resistance genes responsible for resistance to beta-lactam, rifampin, chloramphenicol, trimethoprim, sulfonamide, and fosfomycin were also detected in one patient. However, discovered resistance genes from both Pfam and Resfinder have not been related to antimicrobial drug treatment. It may be the result of linkage disequilibrium of genes and some resistance mechanisms on chromosome. Moreover, some present plasmid types were reported that linked with the discovered resistance genes, though resistance genes and plasmid replicons were not on the same sequences. From our findings, there were limitations on resistance gene sequencing coverage, because the tremendous size of DNA from gut microbiomes is about 10^8 times of sequencing output for each individual patient. Future study needs better plasmid extraction methods and higher outputs of DNA for sequencing to disclose enormous gut microbiome plasmids.

KEY WORDS: RESISTANCE GENE/ GUT MICROBIOME/ PLASMID/ HIGH-THROUGHPUT SEQUENCING/ METAGENOMICS

143 pages

การศึกษาเมตาจีโนมิกส์ของพลาสมิดดื้อยาในเชื้อประจำถิ่นในลำไส้ของผู้ป่วยที่นอนโรงพยาบาล
RESISTANCE PLASMID METAGENOMICS IN HOSPITALIZED PATIENTS' GUT MICROBIOMES

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บทคัดย่อ

การติดเชื้อดื้อยาในโรงพยาบาลเป็นปัญหาที่สำคัญมากในระบบสาธารณสุข เชื้อก่อโรค, เชื้อในสิ่งแวดล้อม และเชื้อประจำถิ่นสามารถแลกเปลี่ยนยีนดื้อยาซึ่งกันและกันและเกิดการแพร่กระจายยีนดื้อยาได้ การแพร่กระจายยีนดื้อยามาจากปัจจัยต่างๆ ได้แก่ การใช้ยาต้านจุลชีพ, กลไกการส่งต่อยีนดื้อยาโดยพลาสมิดและ mobile genetic elements, และเชื้อที่เป็นแหล่งกักเก็บยีนดื้อยา การศึกษาครั้งนี้ได้เก็บอุจจาระจากผู้ป่วย 7 คนจากหอผู้ป่วยอายุรกรรมและหอผู้ป่วยอายุรกรรมวิกฤติ โดยผู้ป่วยอยู่โรงพยาบาลมากกว่า 7 วัน และได้รับยาต้านจุลชีพมากกว่า 2 กลุ่มภายใน 1 เดือน อุจจาระควบคุมเก็บมากจากคนสุขภาพดี 1 คนที่ไม่ได้นอนโรงพยาบาลและไม่ได้รับประทานยาต้านจุลชีพมาเป็นเวลา 3 ปี พลาสมิดถูกนำมาสกัดจากอุจจาระด้วยวิธี plasmid alkaline lysis และถูกนำไปทำให้บริสุทธิ์ด้วย CsCl-EtBr gradient centrifugation หรือ enzyme treatment with lambda exonuclease, exonuclease I และ Phi29 DNA polymerase พลาสมิดที่ได้ถูกนำมาอ่านลำดับเบสด้วย *454* High-throughput sequencing (GS Junior) และถูกอ่านความยาวได้ 9 ถึง 81 ความยาวเบส การใช้เครื่องมือทางชีวสารสนเทศ NCBI BLAST และ Pfam, แสดงถึงโปรตีนที่ถูกสร้างให้มีความสามารถในการดื้อต่อยาต้านจุลชีพเกือบทุกชนิดซึ่งเคยถูกค้นพบบนโครโมโซม พลาสมิด และบนตำแหน่งซึ่งไม่สามารถระบุได้ นอกจากนี้การใช้เครื่องมือทางชีวสารสนเทศ ResFinder และ BioEdit ยังชี้ให้เห็นลำดับเบสของยีน *aph(3′)*-III และลำดับเบสข้างเคียงว่ามีความใกล้เคียงกันทั้งเชื้อในลำไส้ผู้ป่วยและและในลำไส้กลุ่มควบคุม แสดงว่ายีนดื้อยานี้มีการส่งต่อกันระหว่างเชื้อที่พบในชุมชนและโรงพยาบาล นอกจากนี้ยังพบยีนดื้อยาที่เคยพบระบาดในโรงพยาบาล ได้แก่ *ant(6)-Ia*, *ermB*, *lnuB*, *tetL* และ *tetU* ที่ดื้อต่อยาในกลุ่ม aminoglycosides, lincosamide, macrolides, streptogramin B และ tetracycline แพร่กระจายระหว่างผู้ป่วยในหอผู้ป่วยเดียวกันและหอผู้ป่วยที่ต่างกัน อีกทั้งในผู้ป่วย 1 คนจากหอผู้ป่วยอายุรกรรมยังมียีนดื้อยาต่อยาในกลุ่ม beta-lactam, rifampin, chloramphenicol, trimethoprim, sulfonamide และ fosfomycin อย่างไรก็ตามยีนดื้อยาที่พบไม่สัมพันธ์กับยาต้านจุลชีพที่ผู้ป่วยได้รับ ปรากฏการณ์ที่เกิดขึ้นอาจมาจาก linkage disequilibrium ของยีน และ กลไกการดื้อยาอาจเกิดขึ้นบนโครโมโซมที่ปะปนมาได้ นอกจากนี้ไม่มีดีเอ็นเอเส้นใดที่บรรจุทั้งยีนดื้อยาและลำดับเบสที่บอกชนิดของพลาสมิด แต่พลาสมิดบางชนิดที่พบเคยมีรายงานว่าสัมพันธ์กับยีนดื้อยา ในการศึกษาครั้งนี้มีข้อจำกัดบางประการคือ ดีเอ็นเอจากเชื้อในลำไส้มีความหลากหลายและมีปริมาณมากว่าประสิทธิภาพของเครื่องมือ high-throughput sequencing ที่จะเผยให้เห็นลำดับเบสของดีเอ็นเอ ได้ถึง 10^8 เท่า รวมทั้งงบประมาณที่จำกัด การศึกษาในอนาคตจึงต้องการวิธีสกัดพลาสมิดและเครื่อง high-throughput sequencing ที่ดีขึ้นในการศึกษาเมตาจีโนมิกส์ของพลาสมิดดื้อยาจากเชื้อในลำไส้ต่อไป

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LIST OF ABBREVIATIONS

Abbreviation	Term
aac	aminoglycoside acetyltransferase gene
ant	aminoglycoside nucleotidltransferase gene
aph	aminoglycoside phosphotransferase gene
arr	Rifampin ADP-ribosyltransferase gene
ATP	Adenosine triphosphate
bla	β-lactamase gene
bp	Base pair
BLAST	Basic Local Alignment Search Tool
cat	Chloramphenicol acetyltransferase
CAUTI	Catheter-associated urinary tract infection
CCD	Charge coupled device
CDC	Centers for Disease Control and Prevention
CLABSI	Central line-associated blood stream infection
CsCl	Cesium Chloride
D-ala-D-ala	D-alanyl-D-alanine
DHFR	Dihydrofolate reductase
dfr	Trimethoprin-resistance dihydrofolate reductase gene
DNA	Deoxyribonuclease
dNTP	Deoxynucleoside 5’-triphosphate
dsDNA	double-strand DNA

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
erm	Erythromycin resistance methylase
ESBL	Extended spectrum β-lactamase
EtBr	Ethidium Bromide
gyrA	Gyrase A gene
HGT	Horizontal Gene transfer
ICU	Intensive Care Units
Inc	Incompatibility group
KDa	Kilodalton
lnu	Lincosamide nucleotidyltransferase
MDR	Multidrug Resistance
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
NNIS	National Nosocomial Infection Surveillance
OprD	Outer membrane porin protein D
PDR	Pan-drug resistant
Pfam	Protein families database
PPi	Pyrophosphate
QRDR	Quinolone resistance-determining regions
ResFinder	Acquired antimicrobial resistance gene finder
SDS	Sodium Dodecyl Sulfate
ssDNA	Single-strand DNA

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
sul1	dihydropteroate synthase 1
TE	Tris-EDTA
VAP	Ventilator-associated pneumonia
XDR	Extremely drug resistant

CHAPTER I
INTRODUCTION

Antimicrobial agents play an important role in the selection of resistant bacteria. Some bacteria are resistant to some agents in nature without antimicrobial drug pressure. For example, the infection of Gram-negative bacteria is not treated with vancomycin (1). The drug binds to D-ala-D-ala of the pentapeptide of peptidoglycan, so it hardly penetrates through lipid bilayer of the outer membrane to peptidoglycan in the periplasmic space of Gram-negative bacteria. Anaerobic bacteria intrinsically resist to aminoglycoside because the drug is transported into the cytoplasm by oxygen-dependent process (2). In addition, acquired drug resistance is happened under the antimicrobial drugs usage. The antimicrobial agents are more used for treatment in patients, food animals and waste water. Drug resistant bacteria are selected with the agents, and their resistance genes can be spread to the environment by gene transfer mechanisms.

The acquired resistance is composed of several mechanisms. They include the target mutation, drug-modifying enzyme, drug titration, and bypass mechanism. Firstly, mutations for drug resistance occurred on drug-binding target or other processes involving drug influx and efflux. Before drugs bind to their target, their passing through cell wall and cell membrane is the important step. The permeability change is influent on the uptake of the antimicrobial drugs. Loss of OprD that is responsible for imipenem uptake in *Pseudomonas aeruginosa* causes its resistance to carbapenem (3). In addition, the change of drug-target binding affinity leads to drug resistance. DNA gyrase that unwind DNA for replication is the target of fluoroquinolone. The agent causes accumulative mutation at *gryA* that decrease drug-target binding affinity (4). In drug efflux process, increase removal of antibiotics is important to decrease drug activity. Mutations in MexR repressor cause the derepression of MexAB-oprM multidrug efflux operon in *P. aeruginosa* (5). Secondly, the other common mechanism is target modification by drug-inactivating

enzymes. β-lactamase hydrolyzes the β-lactam ring of β-lactam drugs, such as penicillins and cephalosporins, in order to inactivate them (6). Moreover, target titration is a crucial mechanism that increases such more targets that drug cannot destroy all. Not only trimethoprim resistance occurred by DHFR-binding affinity change, but also overproduction of DHFR was observed in Enterobacteria (7). Futhermore, bypass mechanism that bacteria use as the optional metabolism is a resistance pathway. Plasmid-born sulfonamide resistance gene, *sul1*, expresses the high affinity dihydropteroate synthase with PABA, but low binding to sulfonamide (8).

These acquired resistance mechanisms establish resistance gene candidates that are ready to be transferred to descendants and neighbors. Gene transfer is composed of two types including vertical and horizontal gene transfer. Vertical gene transfer is the genes transmission from parent to descendants. Resistance genes usually locate on chromosome, and they spread among intraspecies bacteria. In the other hand, horizontal gene transfer (HGT) spreads genes from one bacterial cell to neighbors. This process more rapidly expands resistance genes among bacteria than the other because the genes are able to translocate across different bacterial species. Resistance genes are not transferred by chromosomal replication. HGT is composed of three main mechanisms including transformation, transduction and conjugation. Transformation is the uptake of foreign DNA fragments into bacterial cells, and is integrated into chromosome or plasmid by recombination. Transduction is the DNA transfer into bacterial host by bacteriophage, and then integration is occurred as transformation. Conjugation is the most common mechanism among drug-resistant bacteria. DNA fragments are transferred from one bacterial cell to other by plasmid.

Plasmid is the powerful tools for gene transmission within the same and among different bacterial species. This property occurs by plasmid modules such as replication and conjugation modules. Replication module involves the host range and incompatibility group of plasmid. ColE1 plasmids restrict the *Enterobacteriaceae*, whereas IncP plasmid can replicate in almost Gram negative bacteria (9). Different plasmids that have the same replication modules cannot stay in the same host, and are classified in the same incompatibility group (Inc). Conjugation module is composed of mobilization and mating-pair formation parts. Both are important for the gene transfer

from host to neighbor. Conjugation module is used to classify plasmid into three main groups including conjugative, mobilizable, and non-mobilizable plasmid. Significantly, both replication and conjugation modules are also traced back the epidemiological pathway of plasmid. In the investigation of resistance gene spread among *E.coli* and *Salmonella* spp., Eliza Bielak *et al*. found that IncI1 plasmid was responsible for $bla_{\text{TEM-52C}}$, whereas IncX1A spread $bla_{\text{TEM-52B}}$ (10).

When the resistant bacteria encounter bacterial community that is called microbiome, resistance genes are hypothetically transferred to them with horizontal gene transfer mechanism. They could become reservoir of resistance genes, and disseminate resistance genes to other bacteria including pathogens. Microbiome is the bacterial community in the same habitat such as soil microbiome, wastewater microbiome, oral microbiome, skin microbiome, and gut microbiome. A few studies revealed the resistance gene pool in soil, wastewater, food animals, and healthy humans. However, resistance genes metagenomic has never studied in hospitalized patients which are under the strong antibiotic use. In addition, the most closet and large microbiome to human is gut microbiome. The microbiome is bigger, so the resistance gene reservoir is more powerful. Moreover, plasmid is probably the main tools for gene spread among microbiome. Recently, one study showed that plasmids transfer carbapenem resistance gene, bla_{KPC}, among *Enterobacteriaceae* bacteria in same and different patients' gut (11). However, this study did not include all gut bacteria. They combine the culture method and high-throughput sequencing to track the epidemiology of resistance gene spread. Therefore, the study that reveals resistance plasmid transfer in gut microbiome should be studied further.

There are many studies that explored the association among antibiotic use, resistance gene spread, gut bacteria, or microbiome. Human oral microbiome from amoxicillin-used children was higher resistant than those from subjects who did not use any drug, and antibiotic resistome are shared between soil and gut microbiome and human pathogen (12-14). Also, antibacterial agents cause persistent resistant bacteria after they have been ceased for a long time such as *ermG* persistence in *Bacteroides thetaiotaomicron* after clindamycin usage at least 18 weeks, and various resistant genes found in healthy human which were not treated with antibiotics at least 1 year (12, 14). Moreover, plasmids carried several resistance genes that were found among

pathogens in wastewater treatment plant microbiome (15). Therefore, the evidence determines that antibiotics probably force drug resistance to stay in microbiomes; although, the drug application is ceased for a long time, and plasmids mostly play a crucial role for resistant gene spread between microbiome and human pathogen. However, resistant plasmids have never been studied among gut microbiome in hospitalized patient treated with antibiotics. Therefore, this study will find out how plasmids are responsible for resistance genes transfer in hospitalized patients' colonic microbiome to human pathogens in order to plan some approach to control antibiotic resistance gene spread in the hospital in the future.

In the study of plasmid pool in gut microbiome, traditional method can cause the bias about unculturable bacteria. Metagenomics is the answer for solving this problem. There are two approaches including functional selection plus Sanger sequencing and DNA selection without cloning plus using next-generation sequencing and bioinformatics (16). The functional approach can receive DNA sequence and prove function; however, it may lose the original DNA, require more labour, and have low DNA output. In contrast, the latter give enormous sequence data, and use less time for study, but data analysis is quite complicate. Several bioinformatics tools can be developed and mixed together to help analyze the resistance plasmid including NCBI blast, ResFinder, Pfam, and Python programming (17-19). Therefore, the latter approach may suitable for the study of resistance plasmids in gut microbiome that contains high abundant DNA.

Moreover, plasmid extraction and purification should produce good qualities including chromosomal free and high-yield of plasmid before processing in high-throughput sequencing. There are three interesting plasmid purification methods. They are alkaline lysis plus anion exchange chromatography, CsCl-EtBr gradient centrifugation, and enzyme treatment. Alkaline lysis separate plasmid from DNA, but have the contamination of some chromosomal DNA and other component such as carbohydrate and protein. Anion exchange chromatography uses the affinity of DNA to traps DNA with anion column, and gets rid of others; however, contaminated chromosome can be bound (20). CsCl-EtBr gradient centrifugation tends to be better because it can separate closed-circular plasmid from linear chromosomal DNA; however, this method has ever purified plasmid among single clone, and not used for

DNA from microbiome (21). The last method is lambda exonuclease and exonuclease I treatment that confers linear DNA digestion, yet may have low yield of DNA for high-throughput sequencing (22, 23). Phi29 DNA polymerase is the high processive enzyme to amplify DNA (24). It can amplify DNA with rolling circle DNA synthesis. It is possible that the combination of enzymes produce more purified plasmid. Therefore, all plasmid extraction and purification method should be compared for plasmid preparation for high-throughput sequencing.

CHAPTER II
OBJECTIVES

The objectives of this study are:

1. To purify the plasmids from gut microbiome for high-throughput DNA sequencing
2. To identify drug resistome collected in human gut microbiome by *454* high-throughput DNA sequencing
3. To determine the molecular epidemiology of resistance plasmids among gut microbiomes in a hospital
4. To identify type of plasmids carrying drug resistance genes
5. To elucidate the spread of drug resistance genes associated with plasmids

CHAPTER III
LITERATURE REVIEW

3.1 Clinical significance of nosocomial infection with multidrug resistance bacteria

Nosocomial infections cause a colossal problem for healthcare system. They lead to the prolonged duration of hospitalization, higher morbidity and mortality, and more medical cost. In 2002, the National Nosocomial Infection Surveillance (NNIS) system of the Centers for Disease Control and Prevention (CDC) of the United States showed that approximately 98,000 patients died from the infection with hospitalized pathogens (25). Recently, NNIS has concluded the direct hospital cost for nosocomial infection that overall payment is at least $28 billion (25). Not only in the United States has been suffered, but also other parts of the world have faced on this problem. The data from the intensive care units (ICU) of 36 countries in Latin America, Asia, Europe, and Africa were determined that average length of stay of patients with nosocomial infections was 17.9 days, which was longer than that of patients without nosocomial infections (6.2 days). Moreover, the mortality rate from these types of infections was 23.5% which was more than that from patients without infections (10%) (26). In Thailand, there were 13.8% of patients in the hospital were infected with nosocomial pathogens, and 6.7% of these group died with nosocomial infections. Also, the medical cost of antimicrobial drugs for treatment of nosocomial infection was high to 5919.50 baht per one hospitalization (27).

The most common factor making nosocomial infections difficult to solve is the infection with multidrug resistance (MDR) bacteria. For instance, patients infected with MRSA, and MDR *P. aeruginosa* have more mortality rate than those infected with drug-susceptible bacteria, and have more significance when they are compared with patients without infection (28, 29). Multidrug resistance bacteria can be spread within patients easily with many reasons including medical device, healthcare environment, and the usage of antimicrobial drugs. Central line-associated blood

stream infection (CLABSI), catheter-associated urinary tract infection (CAUTI), and ventilator-associated pneumonia (VAP) are linked with nosocomial infection (25, 26). Healthcare environment, such as surface of bed and bedside table, can be contaminated with MDR bacteria by healthcare clothing, wrong disinfectant method, and incorrect hand washing (25, 30). Moreover, the most important factor is the usage of antimicrobial drugs, which are the selective pressure for the emergence of multidrug resistance bacteria. Any use of antibiotics, either appropriate or inappropriate use, can lead to drug resistance bacteria emerging and increase mortality rate with these bacteria in the end (25, 29, 31). In 1990, Aswapokee et al. revealed about the antibiotic usage by doctors from a big hospital in Thailand that there were the high inappropriate use of antimicrobial drugs, and this problem resulted from the lacking of the evidence of infection. It probably results from the lack of understanding of drug resistance mechanisms and the slow identification of pathogens and their resistance determinants (29, 31).

3.2 Epidemiology of multidrug resistance bacteria in other countries and Thailand

Organizations of both developing and developed countries underlined "ESKAPE" superbugs containing *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species as the most powerful global MDR pathogens in hospitals (32-34). There are three defined words about multidrug resistance organisms including multidrug resistant (MDR), extremely drug resistant (XDR), and pan-drug resistant (PDR) bacteria. MDR bacteria are resistant to more than two groups of antimicrobial drugs, and XDR bacteria mean bacteria resistant to almost all of drugs except the last choice of all available drugs, such as colistin or vancomycin, and PDR bacteria are determined that they can counter against all antimicrobial drugs. E. D'Agata showed that there are the increases of infected rated of ESKAPE superbugs and other bacteria from 1994 to 2002 including 1% to 16% for MDR *P. aeruginosa,* 4% to13% for MDR *Enterobacter* spp., 0.5% to 17% for MDR *Klebsiella* spp., 0.2% to 4% for MDR *E. coli*. (34). In addition to ESKAPE superbugs, other MDR bacteria were reported, such

as *Streptococcus pneumoniae, Proteus* spp., and *Serratia marcescens* (34-37). More seriously than normal MDR infection, in China, 84.4% of 77 XDR *Enterobacteriaceae* isolates were resistant to carbapenem by producing KPC-2 carbapenemase (38). Also, for Gram-positive, vancomycin-resistant MRSA were reported, and *vanA* operon causing the change of the cell wall composition transferred from *Enterococcus faecalis* by plasmid (39). Terribly, one study concluded the data from many research and reported that 1 to 61 patients were infected with colistin-resistant *Acinetobacter baumannii* from many countries in several continents (40). It should be concerned because colistin is the last drug of choice for the treatment of infection with Gram-negative bacteria. Moreover, MDR pathogen can cause more serious problems if they spread rapidly in many areas; for instance, the gene for NDM-1, which produces a metallo-β-lactamase conferring carbapenem and all β-lactam antibiotic resistance, together with other resistant genes that were spread among *E. coli* and *K. pneumoniae.* This caused multidrug-resistance emerging in India, Pakistan, and the United Kingdom by both clonal spread and especially horizontal gene transfer with plasmids (41, 42).

In Thailand, the problems from the infection with MDR bacteria both Gram-positive and Gram-negative are severe too. Lately, Asian Network for Surveillance of Resistant Pathogens determined that, in Asian countries including Thailand, *Acinetobacter* spp., *P. aeruginosa*, *S. aureus*, and *K. pneumoniae* are the most common pathogens in the hospitals. Multidrug-resistance rates of *Acinetobacter* spp. and *P. aeruginosa* were high to 82% and 42.8%, and their extensively drug-resistant rates were 51.1% and 4.9%. Also, mortality rate reached to 38.9%, and was related with improper empirical antimicrobial therapy (43).

Gram-negative bacterial infection is resistant to many antimicrobial drugs very rapidly and widely. For *A. baumannii* and *P. aeruginosa*, they are the first and second MDR pathogens which infect patients in hospitals. In 2002, 208 clinical samples from *A. baumannii* infections and colonization were performed the epidemiological analysis. 98% of the infections were nosocomial infection when patients stay in hospital with an average of 26 days. The most common factors were antimicrobial drug treatment therapy before *A. baumannii* infection and medical devices. Fifty seven percent of *A. baumannii* isolates were resistant to most

antimicrobial drugs including 3rd generation cephalosporins, 4th generation cephalosporins, beta-lactam/beta-lactamase inhibitors, aminoglycosides, fluoroquinolones, co-trimoxazole, and carbapenem (not reported with colistin), and patients infected with *A. baumannii* had 54.7% mortality (44). Recently, 27 of 52 clinical *A. baumannii* isolated from tracheal aspirates of VAP patients and 19 of 44 environmental *A. baumannii* isolates were integron-positive; also, 91% and 72% of integron-positive isolates were resistant to more than 5 antibiotics and resistant to imipenem, respectively. Moreover, there was a sharing of some patterns of antibiotic resistance, and integron class between patients and environmental isolates. It determined that integron, which can integrate into both chromosome and plasmid, was a tools for resistance gene spread among bacteria (45). For other pathogens, during 2007-2009, more than 70% of MDR *P. aeruginosa* from university hospital and regional hospital are resistant to carbapenem, and there are four clones spread between different hospitals. Although this study did not focus on carbapenem resistance among different clones within the same hospital and between hospitals, carbapenem resistant genes may spread by horizontal gene transfer (46).

In addition, enteric bacteria were often resistant to many antimicrobial drugs in the hospital. Methee Chayakulkeeree *et al.* showed that the infections with Gram-negative bacilli reached to 30.1%; and the prevalence rates of ESBL-producing pathogens were 56.5% *K. pneumoniae*, 33.3% *E. coli*, 2.6% *P. aeruginosa*, and 6.8% *A. baumannii* (47). Also, one study showed that 37 ESBL enteric bacteria from patients in Siriraj Hospital were resistant to ceftazidime, an extended spectrum beta-lactam drug, and other non-beta-lactam drugs. When they were studied for their resistance genes, it was shown that $bla_{\text{VEB-1}}$ was found in the different clones of bacterial species including *E. coli*, *E. cloacae*, *E. sakazakii*, and *K. pneumoniae* and in the same clone of *E. cloacae*. Also, $bla_{\text{VEB-1}}$ together with $bla_{\text{OXA-10-like}}$ and *arr*-2-like gene was contained in the same integron, and this integron was located on mostly conjugative plasmids. It meant that multidrug resistance genes were occurred in enteric bacteria, and could be spread by horizontal gene transfer, and sometimes the integron could jump to chromosome and spread by vertical transfer (48). Recently, some rare resistance genes can be found; for example, a presence of 8 *qnrA* positive isolates out of 100 extended-spectrum beta-lactamase (ESBL) producing *Escherichia*

coli (*E. coli*) isolated from university-hospitalized patients. Double mutations in the QRDR region (Ser83Leu and Asp87Asn in GyrA) were found in 4 out of 8 *qnrA* positive isolates. These 4 isolates were resistant to ciprofloxacin, while the isolates without double mutations were still susceptible to this drug (49).

On the other hand, the infection with Gram-positive bacteria is difficult to cure as well. For example, heterogeneous subpopulations of three MRSA strains of patients were resistant to vancomycin in Thailand. They grew at 4 mg/ml of vancomycin and had MICs at more than 8 mg/ml. When this study performed molecular typing with *Sma*I restriction enzyme, two in three strains were the same clone. As the report in the past, vancomycin resistance in MRSA probably occur horizontal gene transfer of vanA operon from *Enterococcus faecalis* to MRSA, so it is possible that MRSA received this resistance operon from *E. faecalis* and spread within its clone (39, 50).

3.3 Evolution of resistance gene emerging

The emerging of drug resistance genes in pathogens evolves from several factors including antibiotic usage, gene transfer mechanism and reservoirs of resistance genes

3.3.1 Antimicrobial agent as selective pressure for resistance gene

It is acceptable that antibacterial agents being from antibiotic producer in natural environment and man-made circumstances select resistant genes to make microbes survive in the antibacterial-rich hostile environment (51, 52). For billions years ago, bacteria producing antibiotics have gradually derived genetic tools to tolerate the biochemical agents, which are antibiotic resistant-genes, in order to protect themselves. Glycopeptide-producing bacteria such as *Streptomyces toyocaensis* and *Amycolatopsis orientalis* belong to glycopeptide-resistance gene like *vanA*, *vanH*, and *vanX* in enterococci, and their antibiotics have influence on neighbor cells to develop resistant genes (51-55).

On the other hand, antimicrobial resistances have been rapidly emerging since antibacterial drug discovery as seen in figure 3.1 (56). This study showed the

timeline between antimicrobial agent development and drug-resistance bacteria emerging. Antimicrobial agents discovery are always followed by drug-resistance bacteria. For example, penicillin and penicillinase-producing *S. aureus*, methicilin and methicillinase-producing *S. aureus*, vancomycin and vancomycin resistance *Enterococci*, cephalosporin and ESBL-producing G- bacteria, quinolone and quinolone-resistant *E.coli* and *N. gonorrhoeae*. Human have used the agents in several way such as prophylaxis and therapy in human, domestics, agricultural and etc. These affect the drug resistance emerging in these population; for instance, the decreasing fluoroquinolones susceptibilities among *P. aeruginosa*, *P. mirabilis*, and *E. coli* is associated with the increasing fluoroquinolone uses in patients (57). The more several drug are used, the more various resistant gene types are collected, so multidrug resistance is originated among these hosts. It is possible that hospital having the highest and most various antimicrobial drug usage is good place for living of multidrug-resistant bacteria.

Antibacterial agents force the resistance on bacteria by the overexpression of natural resistant genes, or acquired the resistant genes by mutation or acquisition from other bacterial cells with horizontal gene transfer, and the selection of resistant clones to survive in the final (51-53, 57, 58). There are several examples. Firstly, the mutation of Ser-83 to Leu of *gyrA* gene of unrelated clinical *A. baumannii* isolates lead to quinolone resistance (59). Secondly, *ampC*, which is a constitutively resistance gene on the chromosome of many bacteria in *Enterobacteriaceae*, such as *Enterobacter* spp., *Citrobacter freundii*, and etc., is overexpressed in *Salmonella* spp. The *ampC* is not located on the chromosome of *Salmonella* spp., but the *ampC* is overexpressed when it is located on plasmid under IS*Ecp* with the *ampC* promotor. Sequence analysis found that $bla_{\text{CMY-7}}$ on a plasmid was derived from chromosomal *ampC* of *C. freundii* (60). In the onother example, there were the spreading of $bla_{\text{OXA-72}}$ carbapenemase gene on a plasmid among *A. baumannii* within the same clone and between different clones in Taiwan (diversity carbapenem, oxa72, Taiwan), and recently, one famous carbapenem resistance gene; $bla_{\text{NDM-1}}$, carried on multidrug resistance plasmid spread to many countries including India, Pakistan, and the United Kingdom with the same and different clones of *Klebsiella pneumonia* (41, 61).

3.3.2 Horizontal gene transfer of resistance gene

In addition to selective pressure, gene transfer is a crucial machine to spread resistant genes. There are two pathways that confer resistant-gene transfer including vertical and horizontal gene transfer. Vertical gene transfer (VGT) means the inheritance of gene from parent to descendant while horizontal gene transfers (HGT) serve as the movement of gene from one bacterial cell to others including intra- and interspecies transmission (62). Both vertical and horizontal transfer can cause resistance gene spread within same and between different host species. For example, fluoroquinolone-resistant *E. coli* in human was closely linked with the clones isolated from chicken and porcine (63, 64); whereas, IncX1A and IncI1 were the major plasmids carrying $bla_{\text{TEM-52}}$ gene among *S. enterica* and *E. coli* growing from animal and human, and Tn3 was the mobile genetic element transferring $bla_{\text{TEM-52}}$ between different plasmids(10). However, with the mechanism, HGT spread genes more rapidly than VGT. HGT is divided into 3 types including transformation, transduction and conjugation.

3.3.2.1 Transformation

Transformation is the transfer mechanism of naked DNA with competent bacterial cells. This mechanism follows since the competent cell induction, DNA fragmentation, DNA uptake and DNA integration into chromosome or plasmid (65, 66). In natural environment, more than 90 bacterial species are identified to cause transformation such as *N. gonorrhoeae*, *H. influenzae*, *T. thermophilus*, *H. pyrori*, *S. pneumonia*, *B. subtilis*, and etc. Some require recognition sequence for DNA uptake whereas other is recognition sequence independence (65, 66). For example, *B. subtilis* and *S. pneumoniae* do not recognize the specific sequence, whereas *N. gonorrhoeae* and *H. influenzae* accept 10bp of 5′-GCCGTCTGAA-3′ and 9bp of 5′-AAGTGCGGT-3′ recognition sequence before DNA uptake (65, 67, 68). Transformation is subdivided into 2 types including active DNA releasing and passive DNA releasing transformation. Whereas active process remains unclear, passive mechanism from the lysis of dead cells faces on many barriers for gene transfer. For instances, DNases in human serum and environment which degrade 90% of DNA fragment within a few minutes, and large chromosome and plasmid were destroyed after 4 hours exposure with serum (68). Also, recipients still have restriction systems to destroy DNA

fragment, if DNA is transferred as double strand. When DNA can be transferred to recipient, it should have enough length of homologous sequence or specific-similar sequence in order to integrate itself into host chromosome through homologous or illegitimate recombination, respectively (68). Transfer frequency ranges from 3.6×10^{-6} to 1.13×10^{-9} per recipient (69).

3.3.2.2 Transduction by bacteriophage

Transduction is the HGT mechanism by bacteriophage. Bacteriophage replicates itself within bacterial donors and then the descendants pack their DNA and host DNA into their particles. The descendants can infect other bacterial cells, and can transfer of donor DNA to the recipients. The size of transferred DNA is approximately not more than 100 kb (65, 66, 70). Depending on the capture mechanisms of donor DNA and the DNA donor insertion into recipient DNA, transduction is divided into 2 types including generalized and specialized transduction. Generalized transduction catch DNA fragment randomly and cause insertion by recombination whereas specialized transduction capture and insert DNA by the recombination between phage attachment site (*att*P) and bacterial attachment site (*att*B). Transfer frequency is approximately 10^{-7} per recipient (69). Even if phage is the most abundant organisms, transduction is the least studied mechanism.

3.3.2.3 Conjugation by conjugative transposon and plasmid

Conjugation requires cell-to-cell contact between donor and recipient to transfer DNA. It can occur between bacteria, genera, phyla and domains. Conjugation can transfer both chromosomal and extrachromosomal DNA (plasmid). Its transfer frequency by plasmid ranged from 2×10^{-6} to 1.4×10^{-4} per recipient, and increased when it was induced by antibiotic (69). Some studies pointed the link between antibiotics and HGT that antibiotic as selective pressure activated SOS response and led to activate the transfer modules of MGEs on plasmid, and conjugative transposons to chromosome (71-73). It implied that this mechanism probably the most common resistance gene transfer under antibiotic force. DNA can be transferred in conjugation mechanism by both conjugative transposon and plasmid (65, 66, 68, 74). The mechanism starts from nicking dsDNA to ssDNA at *oriT* located on conjugative transposon or plasmid sequence, form relaxome with mobilizable proteins, and move to the recipient through the transfer apparatus. However, plasmids

require the replication maintenance to stay as extrachromosomal DNA by themselves, whereas conjugative transposon does not have replication module, and have to integrate into the chromosome of recipients by a recombinase.

For conjugative transposon, there were several studies about the spreading of antibiotic resistance genes among bacteria. For examples, Tn*916* and Tn*1545* families of conjugative transposons were found to carry *tetM* (tetracycline and minocycline resistance gene) in Gram-positive bacteria such as *E. faecalis* and *S. pneumoniae*, whereas CTnDOT spread *tetQ*, and *erm* (erythromycin resistance gene) among *Bacteroides* spp., but these conjugative transposons can be found in other bacteria (75, 76). The inserted positions onto chromosome were various that caused the transposition within the chromosomal host. Some positions may be highly specific in one species, but low specific in other species; for instance, Tn916 integrates in several positions in *E. faecalis*, but in only *att*916 site in *C. difficile*. However, the evidence of conjugative transposon in spreading multidrug resistance genes among bacteria is hardly found.

About the other conjugal element, plasmids can carry more than one resistance genes, and cause both intercellular and intergenus transmission. Also, plasmid can carry other MGEs such as integron, which can help resistance genes translocation between chromosome and plasmid, or in the same genome (77). For example, class 1 integron containing $bla_{\text{VEB-1}}$, bla_{OXA}-like, and *arr-2*-like gene cassette is spread among enteric bacteria by its location on conjugative plasmid (48). Moreover, some plasmid can carry both virulence gene and resistance such resistance virulence plasmid of *Salmonella enterica* serovar Choleraesuis carrying virulent operon *spv* and resistance gene including *sulI* and $bla_{\text{TEM-1}}$ (78). Furthermore, plasmid spread drug resistance genes among both Gram-negative bacteria by conjugation such as IncXIA transferring $bla_{\text{TEM-52}}$ among *Enterobacteriaceae* bacteria in both human and poultry, and Gram-positive bacteria by non-pheromone dependent conjugative plasmid such as board-host-range pAM830 spreading *vanA* among *E. faecalis and S. aureus*; also, pheromone responsive plasmid can spread gene in Gram-positive bacteria, but it is unclear (10, 79). It is more interesting for trans-gram conjugation. In nature, resistance gene can be transferred from Gram-positive bacteria to Gram-negative bacteria, and vice versa. Some studies suggest that hybrid bireplicon plasmid

is responsible to transfer from Gram-positive to Gram-negative bacteria such as *aphA-3* from *Enterococcus* spp. to *Campylobacter coli*, and pRSF1010 IncQ broad host range plasmid can mobilize from *E. coli* to *Streptomyces lividans*, since this plasmid do not specify host replication modules (80).

3.3.3 Reservoir of resistance gene

In nature, resistance genes originate from pathogen and antibiotic producer to resist antibiotics followed figure 3.1(52). Also, some organisms have resistance genes for other function in their metabolism such as $bla_{\text{CTX-M}}$ is possibly originated in *Kluyvera* spp. which is environmental bacteria. If these bacteria live in the same area, this community is called microbiome, resistance genes are collected in them named "resistome". In the world, there are several microbiome such as marine microbiome, soil microbiome, plant microbiome, food animal microbiome and human microbiome, and they are linked together follow figure 3.2 (51). There were several studies that implied enormous resistomes in these microbiomes, for examples, soil microbiome can resist to almost drugs, and approximately 25% isolates were resistant to 7 numbers of antibiotics, and healthy human microbiome 95 unique resistance genes from metagenomic analysis including known and unrevealed genes (13, 14). It is pointed that these microbiomes play crucial roles as intermediate host for not only resistance gene transfer to pathogens, but also, resistance gene requirement from pathogens (51). The more antibiotic are used, the more resistance genes are transferred. Importantly, hospitalized human microflora is the nearest resistance gene reservoir with clinical pathogens.

3.3.3.1 Human microbiome and human colonic microbiome

Human microbiome means the entire set of microbial species that live in the human body (81). They are composed of four dominated phyla of bacteria including *Fermicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*. They are all Gram-positive, Gram-negative and anaerobic bacteria. Also, archaea are found in human body espescially *Methanobrevibacter* spp. (81, 82). Each site has different proportion and number of microbiome followed by figure 3.3. There are some reports about resistance genes pool in each human microbiome; for examples, *tet* and *erm* genes found in oral microbiome are related to gut microbiome even located in

different mobile genetic elements (83). It is pointed that resistance genes are transferred between different sites of microbiome by the evolution of MGEs, and later these reservoirs are ready to give their resistance genes to new coming bacteria including pathogen. Interestingly, human colonic microbiome are the biggest population and have the most anaerobe which is difficult to reveal their resistance gene pool (81).

Human colon is composed of $10^{11\text{-}12}$ microbial cells per grams which contain at least 100 times genes of human (81, 84). It means that there are great opportunities to transfer genes in the big community, and the major population is *Bacteroides* spp. which is anaerobe bacteria. Human colonic microbiome has several important roles (81, 85). First, they give some benefits for host such as interfering with pathogen colonization. Second, they become opportunistic pathogen especially *B. fragillis* and *B. thetaiotaomicron* when immune system of host is compromised. The last one, they may turn the efficient reservoir of antibiotic resistance genes such as the more opportunity of cell-to-cell contact for conjugation. Some studies found that some resistance genes were transferred in colon; for example, *Bactoroides* spp. became to interestingly resistant to antibiotics e.g. tetracycline, and erythromycin (76). Some studies indicate some resistance genes which transferred between colonic microbiome including intragenus and intergenus bacteria without the barrier of different cell walls. For example, more than 99% identity of *ermB* was spread among Gram-positive bacteria including *S. pneumoniae*, *C. perfringens*, *Enterococcus* spp., and *Staphylococcus* spp. and Gram-negative bacteria including *Bacteroides* spp.; also, seriously, *vanB,* which is contributing to glycopeptide resistance, was found among *Enterococci* spp. and *Clostridium innocuum* (85). Recently, 95 antibiotic resistance genes by metagenomics and 115 those genes by culturable method were revealed in healthy human gut microbiome (14). Therefore, colonic microbiome has the good opportunity to be a reservoir of the resistance gene pool.

3.4 Resistance gene colonization in hospitalized patient's gut microbiome

Resistance genes among hospitalized patient's microbiome can be originated from 2 sources. Firstly, when patient get the long-term of antibiotic treatment, human microbiome are forced with selective pressure and have the mutation by themselves whereas, the other way which is more common, they acquired resistance genes from bacteria in hospital environment including pathogens and environmental bacteria when these bacteria colonize in human body. There are several risk factors for colonization or infection in the hospital. One study collected clinical data from several researches, and found that common risk factors including age, underlying disease, illness severity, prolonged hospitalization, and exposure of invasive device increased the colonization or infection of multidrug resistance bacteria namely MRSA, VRE, *C. difficile*, and ESBL Gram negative bacilli (86). For the most abundant bacterial collection area, there are also some reports about pathogen colonization in patients. Imipenem (IMP)-resistant *P. aeruginosa* colonizing among patients' gut after hospitalization were detected after 11 days admission (87). Also, patients' gut were colonized by multidrug resistance *A. baumannii* after hospitalization within the first week (88). It means that pathogens probably transfer resistance genes to flora under high selective pressure in hospital. When multidrug resistance bacteria colonize or infect each part of human bodies, this is influent on resistance genes transfer between those bacteria and normal flora. Some studies showed that several resistance genes can be collected among human microbiome in some parts of human bodies. Seville et al showed that *ermB*, *ermV*, *ermE* (erythromycin resistance gene), and *tetQ* (tetracycline resistance gene) were abundant among oral and gut microbiome with cultural method and selected with specific antibiotic, whereas *tetM* was found mostly in oral environment and *tetW* had the most prevalent among gut microbiota (83). It means that resistance genes can be spread among different microbiome by themselves or can be sent to other bacteria including pathogens and environmental bacteria which are carrier in sending resistance genes to other areas. Moreover, the same resistance genes can be found between difference areas with the same or different proportions. Since these resistance genes have ever been found in pathogen. It probably implied that these resistance genes may be originated from pathogens or these microbiomes are source of resistance genes for resistance pathogens. However, there is no study about the pool of resistance genes in hospitalized patient's gut

microbiome without the limit of anaerobic condition. Also, the mechanism of resistance gene transfer between microbiome is the interesting point for finding route of resistance genes in order to plan to prevent the resistance genes spread.

3.5 Plasmids as the common tools for resistance gene transfer

Plasmid is the most common reported tool for resistance gene transmission. One review collected the information of resistance plasmid among several host including environment, animal, and human (89). For example, *repU* and *repQ* spread qnrS2 among bacteria in environment, *repA/C* carried $bla_{\text{VEB-1}}$ among *Enterobacteriaceae* in human, and *repN* transferred *qnrB19* cassette among *S.* Typhimurium in poultry. Some plasmid types expanded some resistance genes or resistance gene cassettes among the narrow host such as $bla_{\text{CTX-M-3}}$ spread among pigs with *repN* in Spain. However, several plasmid types were shown that were able to perform resistance gene transmission among several host genera. Interestingly, repI1 carrying $bla_{\text{CMY-2}}$ among *E. coli* and *S. enterica* in human, cattle, pet, and poultry in many countries. These evidences point that plasmid is crucial for resistance gene spread, and associated with the epidemiological transmission.

3.6 Plasmid characteristic and organization

Architecture of plasmid is composed of five modules namely replication and stability, mobilization, mating-pair formation, establishment, and adaptive modules (62). Firstly, the replication and stability module controls true copy number of plasmids in different hosts and makes sure that plasmids have opportunities to be transferred to every descendant. Processes of replication initiation and replication control are different among different plasmid types; however, these control mechanisms always occur at the origin of replication (90). Secondly, the mobilization module (MOB) involves the cleavage of plasmid DNA at the *oriT* site and the cooperation of DNA with relaxase protein for transferring to the new host. Thirdly, the mating-pair formation module (MPF) generates type 4 secretion systems (T4SS), which are the channel for nucleoprotein complex transfer to the recipient. From

properties of both MOB and MPF, plasmids are divided into three types including conjugative, mobilizable, and non-transmissible plasmids. Conjugative plasmids can be transferred by themselves having both MOB and MPF modules, whereas mobilizable plasmids, which do not have the MPF, cannot be transmitted by themselves and need to coexist with conjugative plasmids in the same host. Non-transmissible plasmids contain neither MOB nor MPF, so they cannot be transferred to neighbors. Fourthly, the establishment module is located on the conjugal leading region of conjugative plasmid, which is the first part of DNA for conjugation, and contains many genes shared by various plasmids such as anti-restriction systems. This region is crucial for plasmid living in the new genetic status. Last, adaptive module is the most various and carries beneficial genes for fitness such as antibiotic-resistant genes. In addition, there are various hotspots on this module for the captures of mobile genetic elements such as transposon (62, 91).

3.6.1 Plasmid typing

Plasmid modules are applied for plasmid typing in order to find the pathways of resistant gene spread (10, 92-95). In the first time, plasmids were classified by incompatibility groups (Inc). In the test, one plasmid from donor is transferred to recipient carrying other plasmid. If both plasmids cannot be compatible with each other, they are in the same incompatibility group. There are 37 incompatibility groups that were found in enterobacteria and *S. aureus*. However, plasmid typing with incompatibility groups has some problems. In incompatibility testing, donor plasmid may be inhibited with surface exclusion of recipients, so this gives the false positive for the trial. Also, the marker gene fails to be transfer with donor plasmid, and use more labor.

Then, the other typing, replicon (rep) typing, was discovered (62, 92, 93). The principle depends on the replication module. The control of replication (replicon) directly reflects the incompatibility groups. Replication inhibitors are divided into two main types including countertranscript RNA and Rep protein dimer (90, 93). The countertranscript RNA binds to an RNA primer for a Rep protein transcription, and this binding leads to cessation of the expression of Rep that is required for the initiation of replication. The iterons are direct repeats close to the origin of replication

and the rep gene. These repeats will be bound with Rep protein for the initiation of replication. When two plasmids have the same replicon type in the same host, they can separately replicate in the initiation. However, the replication is gradually inhibited, when plasmid copy number increases. One plasmid will be eliminated with the preprimer degradation or trans-acting of Rep proteins as protein dimers. Countertranscript RNA, which have base complimentary with preprimer, binds to these preprimer, and cause the inhibition. Rep monomer can initiate the replication; in contrast, dimer inhibits this mechanism. Rep dimer is formed when the amount of Rep increased. In the same way of countertranscripts RNA, the replication is inhibited by Rep dimer when replication increase. Two plasmids having the same replicon compete for the replication until countertranscript RNA or Rep dimers inhibit one's replication. From these control mechanisms, two plasmids that are competing will be incompatible and will be assigned into the same incompatibility group. There are 22 different basic replicons, which are based on autonomous replication and its control region involving incompatibility group, including repFIA, repFIB, repFIC, repFIIA, $repFII_S$, repA/C, FrepB, rep9, repI1, repB/O, repK, repHI1, repHI2, repL/M, repN, repP, repQ, repT, repU, repW, repX and repY. However, Rep typing has one pitfall namely multiple replicons in one plasmid; for example, plasmid P307 had repFIB, repFIC, repFIIA and repI1. Up to now, rep typing has been performed by hybridization with replicon probes or PCR-based replicon typing. In high-throughput sequencing era, sequence-based replicon typing should be developed for plasmid typing (92, 93, 96).

In addition, conjugative tools including mobilization and mating-pair formation modules are the new interesting ones for plasmid typing (62, 94, 95). Mobilization module is directly important for plasmid spread, so this module relates to the epidemiological resistance plasmid. Smillie *et al.* bring 1730 complete plasmid in GenBank into the alignment of relaxase. Mob typing can classified plasmid into 6 families including MOB_V, MOB_Q, MOB_P, MOB_H, MOB_F, and MOB_C. However, this typing cannot use for the typing of non-mobilizable plasmid, and has been less studied. In addition, mating-pair formation (MPF) module is essential for plasmid transfer as mobilization modules, and encodes T4SS. Plasmids that have this module can be transferred by themselves. VirB4 protein present in all T4SS is used for the target of T4SS typing. There are 4 families based on MPF module including MPF_G,

MPF_T, MPF_I, and MPF_F. In conclusion, the combination of MOB typing and MPF typing represent the typing scheme of the conjugative transfer system.

Recently, plasmid multi-locus sequence typing (pMLST) is sequence-based plasmid typing (62, 97, 98). This typing used several core genes including replication, conjugation, anti-restriction, and surface exclusion associated genes. This method looks promising and as good as the conventional MLST scheme for the chromosomes, but there are only a few studies on a discovery of alleles of the plasmids, only some incompatibility groups were identified, namely IncI1, IncN, IncH1, and IncH2.

3.7 Identification of resistance genes in gut bacteria

It is really important to study the transfer of resistance genes between gut microbiomes as reservoirs and pathogens. In the past, whole genome of gut microbiome was studied by cultured-based or classical method since it is convenient to be performed; however, this way has some limitations because gut microbiome is composed of both aerobic and anaerobic bacteria which this method is suitable for only aerobic microorganism, and causes the bias and incomplete study. On the other hand, there is a new approach method. Metagenomics is the study of genomes from bacterial community without the limitation of aerobic and anaerobic condition by direct DNA extraction from specimen.

3.7.1 Culture-based method

Culture-based method is the standard way to grow microbes. Many studies evidenced that resistance genes occurred in the mammalian colon by this method. They found virtually identical copies of the resistance genes among pathogen and gut bacteria. Several culture-based studies showed some resistance genes which were found in pathogen in gut microbiome with high similarlity such as *ermG, ermB, ermF, tetM, tetQ*, and *vanB* (85). It is indicated that these resistance genes were transferred between colonic microbiome and pathogen. However, these resistance genes were probably recruited from only aerobic bacteria. Although culture-based method is not good for anaerobic bacteria with some reasons such as difficulty and cost, some study used high-efficient culturable methods under anaerobic condition and high-throughput

sequencing to show high uncovered species; however, the abundance of uncultured species was still lower than metagenomic study (99).

3.7.2 Metagenomic approach

Metagenomics is the study of genomes from bacterial community without the limitation of aerobic and anaerobic condition by direct DNA extraction from specimen, so this method is suitable for the study of gut microbiome. For example, one study comparing both methods in human gut microbiota revealed that metagenomics could identify unknown resistance genes including ESBL genes which culture-based method cannot detect (14). Also, when I compare between traditional and metagenomics approach in taxonomy. I found that under cultivation-based method, colonic microflora belonged to many gram negative bacteria in the Order Enterobacteriales, but a few gram positive bacteria. In contrast, under metagenomics, Fermicutes and Bacteriodes are the most organisms (81). It pointed that metagenomics show previous unreveal bacteria in gut microbiome. Also, if researches pay attention in drug resistance, metagenomics are still divided into 2 types including cloning with drug selection and sequencing from DNA library with Sanger sequencing, and direct sequencing from extracted DNA with next generation sequencing. Both have different strong and weak point. The first one can construct longer reads, but it is biased because of cloning, and total output is lesser. On the other hand, the other give enormous sequences, but a little more errors (16).

3.7.2.1 Next generation sequencing

Since the target of metagenomics is enormous DNA from bacterial community, sequencing technology should be high-throughput sequencing for high-throughput genomic analysis. Previously, Sanger sequencing is the popular for sequencing of short DNA sequence and is the gold standard method for gene identification. The output of Sanger sequencing is about 800 bp read lengths, and 800 bp per run. If this method was used for metagenomics, enormous runs would be necessary, and it would make high cost. Nowadays, next generation sequencing is developed for high-throughput sequencing and applied for metagenomics. There are several platforms of next generation sequencing which were used for DNA from metagenomics in several studies.

Firstly, 454-sequencing platform are mixed with the principle of emPCR and pyrosequencing (100-103). In emPCR step, single DNA molecule linked with oligonucleotide adaptor is attached with complementary on microscale bead, and then amplification constructs clonal amplified DNA per one bead. Next step, each clonal amplified bead will be contained into each tiny well, and pyrosequencing will be started. Pyrosequencing is the reaction originated from the releasing pyrophosphate (PPi) in nucleotide-incorporation process which perform light signal each time of sequencing.454 pyrosequencing platform is divided into 2 types including GS FLX (bigger platform) and GS Junior (minor platform). The result from GS FLX about 400 bp read length and 500 Mb per run whereas GS Junior gives the same read length, and 35 Mb per run. The error is less than 0.1%.

Secondly, Illumina platform is composed of bridge amplification and sequencing by DNA polymerase with reversible terminators (100-104). In bridge amplification, adaptor-ligated DNA template is bound with complementary adaptor randomly on surface of flow cell, and cluster amplified DNA is produced with amplification. Different sequencing from 454 sequencing, Illumina sequences cluster-amplified DNA with polymerase reaction. Nucleotides used for DNA synthesis are labeled with fluorescein and blocked with terminator at 3′-OH group of ribose sugar. When DNA are synthesize each time, fluorescent signal is reported, and then, fluorescent labels and terminators are cleaved in order to prepare DNA for new sequencing reaction. Illumina gives approximately 150 bp read length, 2 Gb per run of Illumina Miseq, 30 Gb per run of Illumina GAIIx, and 600 Gb per run of Illumina HiSeq2000. Its error rate is less than 1%.

Thirdly, SOLiD platform is integrated with emPCR and ligase-mediated sequencing (100-103). This method gives about 50 bp read length and 100 Gb per runs. This emPCR likes the one in 454 sequencing platform. Ligase-mediated sequencing is divided into 2 step including primer-incorporated round and complementary specific dibase octamer round. Beginning by annealing a first-round primer (N) with adaptor sequencs (N) on clonal amplified DNA, and then DNA ligase extends complementary specific dibase octamer from primer. The octamer have 2 specific dibase and concatenated two types of non-specific 3 bases such as 3′-TTnnnzzz-5′-label. After octamer extension, zzz oligomers together with label are

cleaved, and new round of complementary specific dibase octamer round is begun. When ligation reaches final cycles, the old primer (N) is cleavage, and the new primer (N-1) is incorporated, and the new extension of complementary specific dibase octamer is started again. The result of sequencing is from specific dibase combination.

Fourthly, single-molecule realtime sequencing platform or PacBio platform, which is the only one among next generation sequencing platform, does not perform amplification (100-104). Single DNA molecules are added with poly A and hybridized with poly T attached on surface, and then they are sequenced by DNA polymerase with fluorescent-cleaved nucleotide. Its output is approximately shorter than 1500 base pairs read lengths and 100 Mb per run. Its error rate is about 12.86%

The new one, Ion Torrent Personal Genome Platform, has been the last high-throughput sequencing machine (104, 105). DNA amplification likes emPCR in 454 sequencing platform. However, this machine detects nucleotide incorporation with ion-sensitive semiconductor. The output of this machine is approximately 200 base pair read length. Total base is 20-50 Mb on 314 chip, 100-200 Mb on 316 chip, and 1 Gb on 318 chip. This machine gives the error rate about 1.71%

Among total next-generation sequencing platform, 454 sequencing give the lowest error rate (0.1%), and its longest reads help assembly process which is beneficial for corrected metagenome construction.

3.7.2.2 Bioinformatic tools

It is difficult to analyze large molecules of DNA sequences in metagenomic analyses. Both the differences of DNA mix and their enormous amount are important factors that affect the accuracy of analyses. Bioinformatic tools are used to solve this problem. Bioinformatics is the usage of computer to manage biological data including DNA and amino acid sequences. One web-based program cannot solve everything in metagenomics, so the integration of several web-based programs and computer program writing and compilation are very crucial. If the study requires resistance plasmid metagenomics, the desired tools should include all plasmid, resistance gene, and protein databases. NCBI has the biggest databases of nucleotide and protein sequences that contain several types of DNA, such as human DNA, bacterial chromosome, bacterial plasmid and etc., and NCBI BLAST is the basic local alignment search tool (17). There are five types of NCBI BLAST including nucleotide

BLAST, protein BLAST, blastx (query is translated nucleotide and database is protein), tblastn (query is protein and database is translated nucleotide) and tblastx (both query and database are translated nucleotide). In addition, Pfam is the protein family database (18). It supports the multiple alignment tool for protein family search, and use profile Hidden Markov Models (HMMs) for finding protein domain. This method is suitable for the prediction of both reported and unreported proteins that have the same protein domain by sequences. Besides, ResFinder is the database that specifies the acquired drug resistance genes (19). This tool is crucial for the identification of resistance genes that have ever been carried on plasmids.

3.8 Resistance plasmid metagenomics among microbiome

In metagenomics era, plasmids from microbiome are the interesting target for the studying of virulence or resistance gene pool; however, few data was shown in this area. Metagenomics is divided into two types including functional and sequence-based screening. Sequence-based studies have to use the enough competent plasmid extraction without contaminated chromosome and high-throughput sequencing together (106, 107); in contrast, functional-based studies used metagenomic library by vector combined with Sanger sequencing(15, 108, 109). Previously, for resistance plasmid studies, plasmid purification, plasmid extraction kit with an exonuclease that destroys sheared chromosomal DNA was used for plasmid inclusion in sequence-based method, and next-generation sequencing together with some bioinformatics tools showed some known antibiotic resistance genes and some plasmid sequences. No investigation indicated the association of resistance gene and plasmid type. It was possible that known resistance genes relate with contaminated chromosome or plasmid. On the other hand, functional-based studies used plasmid extraction, made and transformed DNA library into *E. coli*, and select *E.coli* that resist to the indicated antibiotics. These resistance genes were disclosed by Sanger sequencing. However, this method showed only the resistance genes, and did not find plasmid sequence.

CHAPTER IV
MATERIALS AND METHODS

4.1 Studied population

Studied population is divided into 2 groups namely inpatient general medicine ward and I.C.U. medicine ward, and each group has 4 patients. There are several inclusion criteria including adult patients' age within 18-80 years, hospitalized for longer than 7 days, receiving antibiotics more than three different classes within the past 1 month and currently on at least one antibiotic. Stools from patients can be obtained and shows the presence of multidrug resistance pathogens, such as Methicillin-Resistant *Staphylococcus aureus*, Extended-Spectrum β-lactamase-producing Gram-negative bacteria, Pandrug resistance *Acinetobacter baumannii*, or *Pseudomonas aeruginosa,* and resigned informed consent. For exclusion criteria, if patients refuse to be in the study, or have illness status too poor with the score of 8 – 9 in the Modified Mary E. Charlson Classification (110) (Figure 4.1), which is difficult to be collected stool. Clinical information from patients including admission date, stool collection date, surgery and medical device data, admission history in 6 months, previous multidrug resistance infection and antibiotic treatment within 3 months, current multidrug resistance infection and antibiotic treatment, and Modified Mary E. Charlson Classification.

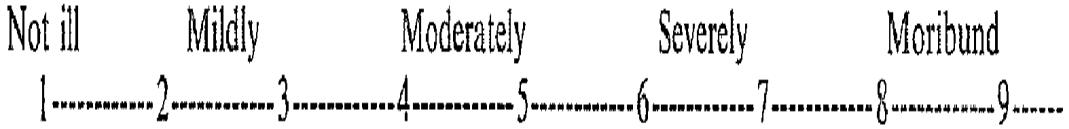

Figure 4.1 Illness status in the Modified Mary E. Charlson Classification (110)

4.2 Control

The plasmids used as controls for plasmid purification with CsCl-EtBr gradient centrifugation are plasmids in various sizes from five clinical drug resistance isolates including ESBL *Escherichia coli* no. 58, 108, 215 and ESBL *Klebsiella pneumonia* no. 197, 232. Cloning vector pUCP24, which have 4,036 base pairs, is the control of plasmid purification with enzyme digestion and phi29 DNA polymerase amplification. Stool from Thai healthy human who does not receive antimicrobial drugs at least 3 year was negative control for this project.

4.3 Stool collection

To collect stool with least contamination, lockable stool collecting boxes were sterilized with autoclave at 121 °C for 20 minutes before stool collection. Stool was released from patients to clean bed pan, and divided about 100 – 200 grams of stool to sterile stool collecting boxes. After that, stools were divided into 50 ml centrifuged tubes and kept frozen at minus 20 °C.

4.4 Plasmid extraction

Before plasmid extraction, bacterial cells were separated from human cells and food sediment. In a 50 ml centrifuge tube, stool was suspended with 15 ml of H_2O at 100 g 4˚C for 3 minutes and supernatant was collected (repeated 3 times) into a new 50 ml centrifuge tube. After that, supernatant was centrifuged to pull bacterial cells down to the bottom of the centrifuge tube, and supernatant was discarded. To eliminate pigment in stool, which might inhibit PCR reaction, bacterial cells were washed with 20 ml of PBS pH 7.4 at 7000 g 4 ˚C for 10 minutes, and this step was repeated until supernatant was clear. Bacterial cells reached 5-6 grams, before they subjected for plasmid extraction with Geneaid Plasmid Maxi Kit. Bacterial cells were resuspended into 10 ml of PM1 buffer containing GTE and RNase. Then, 200 U of lysostaphin (10 U/µl lysostaphin in PBS) and 1 ml of 150 mg/ml lysozyme were added to digest cell wall of both Gram positive and Gram negative bacteria. The DNA

solution was inverted 10 times, and incubated at 37 ˚C for 60 minutes. Ten ml of PM2 buffer having NaOH/SDS was added into DNA solution to lyse both Gram positive and Gram negative cell walls, inverted 10 times, and stood at room temperature for 2 minutes. After that, 10 ml of PM3 buffer as potassium acetate was mixed into solution in order to renature plasmid DNA by inverting the tube 10 times. Proteins and denatured chromosome, which cannot be renatured with PM3 buffer, were precipitated at 10000 g, 4 ˚C for 30 minutes, and this step is repeated until supernatant was clear. Supernatant was passed through Plasmid Maxi Column (anion chromatography column) soaked with 10 ml of PEQ buffer by gravity flow, DNA binds to column, and filtrate was discarded. After that, DNA-binding column was washed with PW by gravity flow, and then DNA was eluted with PEL buffer into a new 50 ml centrifuge tube by gravity flow. Next, plasmid DNA were precipitated with 9 ml of isopropanol at -20 ˚C overnight, centrifuged at 10,000 g 4 ˚C for 30 minutes, and supernatant was removed. For DNA purity, DNA pellet was washed with 5 ml of 75% ethanol at 10,000 g 4 ˚C for 15 minutes, supernatant was removed, and DNA pellet was air-dried for 10 minutes. Finally, DNA was dissolved in 1 ml of TE buffer. DNA was electrophoresed in 1% agarose gel in order to check whether DNA was extracted.

4.5 Plasmid purification

4.5.1 Circular plasmid purification by CsCl-EtBr gradient centrifugation

When circular plasmid was extracted by plasmid maxi preparation, it was probably contaminated with chromosomal DNA which were sheared and shown as linear DNA. Therefore, CsCl-EtBr gradient centrifugation was used to separate them with the different density. Chromosome, which was linear DNA, was apart to be lower buoyant density, whereas plasmid, which is circular DNA, is higher buoyant densityas in Figure 4.2. One g of DNA solution is mixed with 1 g of CsCl and 20 µl of EtBr; then, the density of solution was adjusted to 1.55 g/ml. After that, the mixture was transferred into thick wall polycarbonate (Beckman), which resist to 120,000 RPM maximum speed, and was sealed with mineral oil. Then, 1.55 g/ml of DNA solution

was centrifuged with TLA120.2 rotor of tabletop ultracentrifuge at 80,000 rpm 20 ˚C for 20 hours in order to separate linear DNA from circular DNA.

When this process was finished, the lower band was collected into 1.7 ml microfuge tube under UV light. EtBr was eliminated with 1 volume of isoamyl alcohol at 450 g for 3 minutes, the pink solution in upper phase was discarded, and this step was repeated until the upper phase was clear. After that, the lower phase was collected, and was added with 3 ml of TE. This solution was passed through 10 kDa Amicon tube in order to desalted CsCl at 3000 rpm 20 ˚C for 20 minutes. This step was repeated 3 times. Last time, DNA solution on filter was collected to a new 1.7 ml microfuge tube. To control of this method, several sizes of clinical plasmid were used as positive control. These plasmids were extracted from *Escherichia coli* no. 58, 108, 215 and *Klebsiella pneumoniae* numbers 197 and 232 by the same plasmid extraction method as in samples.

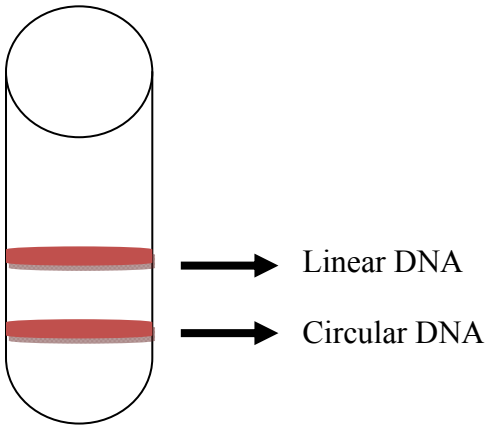

Figure 4.2 Diagram shows linear and circular DNA bands separated with CsCl-EtBr ultracentrifugation

4.5.2 Circular plasmid purification by enzyme digestion and plasmid amplification by phi29 DNA polymerase

This method used lambda exonuclease which was able to digest linear double strand DNA (ds DNA), but could not digest circular DNA despite nick site (New England Biolab). However, dsDNA digestion with lambda exonuclease brought

about contaminated single strand DNA (ssDNA). Exonuclease I was used for ssDNA digestion. Therefore, circular plasmid was more purified by both enzymes. In the first step, 50 µl of reaction was composed of MQ water, 5 µl of 10X lambda exonuclease buffer, 30 µl of 30-50 ng/µl of DNA solution, and 5 µl of 5 U/µl of lambda exonuclease. After that, the reaction was incubated at 37 ˚C for 45 minutes, and was inactivated at 75 ˚C for 30 minutes. To purify DNA product, the reaction was added with PD3 buffer of Geneaid high-speed plasmid mini kit, inverted 10 times, and centrifuged at 15,000 g for 3 minutes. Then, DNA supernatant was passed through PD column at 15,000 g for 1 minute. DNA was bound to PD column, and was washed with 400 µl of W1 buffer at 15,000 g for 1 minute together with 600 µl of Wash buffer respectively. DNA-binding column was dried at 15,000 g for 3 minutes, and then it was absorbed with 30 µl of elution buffer for 2 minutes before DNA was eluted at 15,000 g for 2 minutes. The quality of reaction was checked by electrophoresis in 1% agarose gel.

To eliminate contaminated ssDNA, DNA product from digestion process with lambda exonuclease was performed with exonuclease I. Thirty µl of reaction was consisted of MQ water, 3 µl of 10X exonuclease I buffer, 20 µl of DNA product, and 3 µl of 20 U/µl of exonuclease I. The reaction tube was incubated at 37 ˚C for 45 minutes, and was inactivated at 75 ˚C for 30 minutes. After that, DNA product was purify like the process after lambda exonuclease digestion, but was eluted in 20 µl of elution buffer. DNA product was electrophoresed into 1% agarose gel to indicate the decontamination of chromosome as linear DNA.

After chromosomal DNA decontamination, it probably reduced DNA yield which had residual circular DNA, and might not be enough for sequencing. So, circular plasmid DNA was amplified with phi29 DNA polymerase. This enzyme amplifies circular DNA template to multimeric strand DNA by rolling circle replication, required neither heating nor cooling for its function, and had high processivity (more than 70 kb) (111, 112). The 25 µl of reaction contained MQ water, 2.5 µl of 10X phi29 buffer, 0.5 µl of 10 mg/ml BSA, 0.5 µl of 10 mM dNTP, 10 µl of 10 uM exo-resistant random hexamer, 1 µl of DNA template, 0.75 µl of 10U/µl phi29 DNA polymerase. DNA templates dissolved in water were denatured at 95˚C for 5 minutes, and then random primer was added. After that temperature was reduced to

30˚C for 5 minutes, and cooled to 4 ˚C for 10 minutes. Next step, other reagents was mixed into the reaction, and templates were amplified at 30˚C for 16 hours (New England Biolab) (24).

For these purification and amplification methods, uncut and cut pUCP24 was used to evaluate the functions of these enzymes.

4.6 High-throughput DNA sequencing by Gs Junior 454 pyrosequencing

For high-throughput DNA sequencing by GS Junior 454 pyrosequencing (Roche) (100, 102), there are three main processes including rapid library preparation, emPCR amplification, and sequencing as shown in Figure 4.3.

$$\mu l \text{ of library per tube} = \frac{\text{desired molecules per bead} \times 2.4 \times 10^6 \text{ beads per tube}}{\text{library concentration (in molecules/}\mu l)}$$

Figure 4.3 The equation for the amount of DNA following desired molecules per bead

4.6.1 Rapid Library Preparation

600-900 base pair length of DNA libraries were recommended for emPCR amplification and sequencing, this process followed the Rapid Library Preparation Method Manuals. Five hundreds to one thousand ng of DNA templates were diluted with TE buffer to final volume of 100 µl in the Nebulizer cup, added with 500 µl of Nebulization buffer, and fragmented by nebulization at 30 psi of nitrogen for 1 minute. After that, nebulized DNA was mixed with 2.5 ml of PBI Buffer, and DNA solution was filtered through a column from the Qiagen MiniElute PCR Purification Kit at 13,000 rpm for 30 seconds. DNA-binding column was washed with 750 µl of PE buffer at 13,000 rpm for 1 minute, and dried by spin-rotate spin at 13,000 rpm for 2 minutes. Then, DNA was eluted to PCR tube with 16 µl of TE buffer, and repaired the end of fragment in order to prepare DNA for adaptor ligation. The fragment end repair

mixture was composed of 2.5 µl of RL 10X buffer, 2.5 µl of RL ATP, 1 µl of RL dNTP, 1 µl of RL T4 Polymerase, 1 µl of RL PNK, 1 µl of RL *Taq* polymerase, and DNA sample. This mixture was run on thermocycler with 25 ˚C for 20 minutes, 72 ˚C for 20 minutes, and 4 ˚C on hold. When fragment end repair program was finished, 1 µl of RL adaptor and 1 µl of RL ligase were added into the reaction tube to attach adapter, and incubated at 25ºC for 10 minutes.

On other hand, AMPure beads were prepared for the selection of appropriated DNA fragment by mixing with the proportional amount of Sizing solution. One twenty five µl of AMPure beads having adaptor sequence and being magnetic particles were aliquot, and were precipitated with the Magnetic Particle Concentrator (MPC). Then, AMPure beads were diluted into 73 µl of TE buffer, mixed with 500 µl of Sizing solution, and kept on ice until adaptor ligation at DNA sample has finished. After that, Adaptor-ligated DNA was added into AMPure beads, and was incubated at room temperature for 5 minutes. The adaptors on DNA having appropriated sizes were attached with the adaptors on AMPure beads within sizing solution. After that, DNA-binding AMPure beads were pelleted on the wall of tube with MPC, and supernatant was discarded. For other two times of sizing, the pellet was added with 100 µl of TE buffer and 500 µl of Sizing solution, incubated at room temperature for 5 minutes, and pelleted on MPC. After supernatant was discarded, selected DNA-captured AMPure beads were washed with 1 ml of 70% ethanol on MPC, and were dried at room temperature for 2 minutes.

DNA library was assessed with both quality and quantitation before emPCR amplification process. Firstly, DNA on AMPure beads were dissolved with 53 µl of TE buffer, AMPure beads were separated on the wall of the tube with MPC, and 51 µl of supernatant containing DNA library was transfer to a new 1.5 ml microcentrifuge tube. One µl of DNA library was aliquot and run on an Agilent Bioanalyzer High Sensitivity DNA chip to evaluate the size of the library. Average fragment length should be between 600-900 base pairs, and lower size cut-off was less than 10% below 350 base pairs. Secondly, the quantity of DNA library was measured by using a 96-well plate fluorometer. Before the measurement of DNA library, standard curve was prepared by serial dilution of RL Standard. Ninety µl of 2.5×10^9 molecule/µl of RL standard was mixed with 90 µl of TE buffer. One hundred and

twenty µl of solution was transferred to 60 µl of TE buffer in second tube, and process the same serial dilution in other tubes containing 60 µl of TE buffer until 7th tube. Fifty µl of each standard and of DNA library was measured the relative fluorescence units (RFU) for the sample. The serial dilution and concentration of standard was shown in Table 4.1. Standard curve was plot with RFU of standard, and construct the linear equation as below.

$$Y = mX + c$$

X means the RFU of sample
Y means the concentration of sample
m means the constant as the slope of the line
c means the constant as the point crossing the *y*-axis

The correlation coefficient for the linear regression (R^2) must be more than or equal to 0.9, so this standard equation can be used for the calculation of sample concentration by replacing RFU of the sample in X value.

Finally, DNA libraries are diluted to concentration at 10^7 molecules, and then were kept in -20ºC until use, but not more than 2 months.

Table 4.1 The serial dilution and the concentrations of the RL standard (Roche)

Dilution tube number (dilution factor)	RL Stardard (mol/ µl)
1 (no dilution)	2.50×10^9
2 (2/3rd dilution of tube 1)	1.67×10^9
3 (2/3rd dilution of tube 2)	1.11×10^9
4 (2/3rd dilution of tube 3)	7.41×10^8
5 (2/3rd dilution of tube 4)	4.94×10^8
6 (2/3rd dilution of tube 5)	3.29×10^8
7 (2/3rd dilution of tube 6)	2.19×10^8
8 (2/3rd dilution of tube 7)	1.46×10^8

4.6.2 emPCR amplification and clonal DNA-amplified bead enrichment

The principle of this step is the amplification of single-DNA molecule to clonal template in water-in-oil emulsion compartment before sequencing with GS Junior 454 high-througput sequencing. After finishing DNA library preparation, DNA solution of each sample was diluted to 4×10^6 in order to calculate the amount DNA aliquot to use for required DNA molecules per bead in emPCR amplification. The amount of DNA for emPCR amplification was calculated as Figure 4.4. Before choosing the accurate amount of DNA molecules per bead in emPCR amplification in order to perform 454 pyrosequencing, it was titrated into 2 values including 2 and 6 DNA molecules per bead in emPCR amplification to analyze the number of amplified beads. For the reason, the amount of amplified bead reflected to the amount of DNA template per bead which affects the efficiency of sequencing later. These below steps were explained about 1×SVE (1 small emulsion tube) for titration, but libraries prepared for sequencing became 4×SVE (4 small emulsion tube per one library).

Firstly, emPCR condition was prepared by 3 parts including emulsion oil, Pre-emulsion, and Live Amplification Mix. Emulsion oil was shaked with TissueLyser tube rack in the outer row at 25 Hz for 2 minutes. After that, 290 µl of 1X Mock Amplification Mix was added into emulsion oil, tube rack was inverted 2 – 3 times to mix, and shaked with the TissueLyser in the outer row at 25 Hz for 5 minutes to become Pre-emulsion. Live Amplification Mix was composed of reagent for amplification as Table 4.2.

Table 4.2 Master mix of Amplification mix for 1×SVE and 4×SVE (Roche)

Reagent	**Volume (µl)**	
	1×SVE	**4×SVE**
Mol. Bio. Grade Water	70	280
emPCR Additive	90	360
5× Amplification Mix	47.5	190
Amplification Primer	13.75	55
emPCR Enzyme Mix	12.5	50
PPiase	0.5	2
Total	246.75	987

Secondly, this step was the attachment of DNA library aliquot to capture beads. Eighty µl of capture beads (per reaction) were spun down with bench top minifuge for 10 seconds, the tube was rotated 180°, and beads were spun down again for 10 seconds. Supernatant was removed and beads were washed twice with 1 ml of 1× Capture Bead Wash Buffer TW, and supernatant was discarded after each wash. Capture beads were resuspended in 40 µl of 1× Capture Bead Wash Buffer TW and kept in room temperature. In the other hand, dsDNA in the library aliquot was denatured to ssDNA at 95 °C for 2 minutes and 4°C on hold (at least 5 minutes). Then, the amount of DNA, which followed the calculation of the required amount of DNA molecules per bead, was added to the washed capture beads in order to perform DNA capturing with beads, and later, they were mixed with 215 µl of Live Amplification Mix harmoniously to become capture library.

In next process including emulsification and amplification, capture library was pipetted into Pre-emulsion tube, and then, this tube was inverted 3 times and shaken with TissueLyser in the outer row at 15 Hz for 5 minutes. The mixture was dispensed into PCR tube in the proportion of 100 µl of mixture per 1 well (1 tube of emulsified mixture per 10 wells). After that, the reaction was performed as shown in Table 4.3

Table 4.3 Condition of emPCR amplification (Roche)

Number of cycles	temperature	Time
1	94°C	4 minutes
50	94°C	30 seconds
	58°C	4.5 minutes
	68°C	30 seconds
1	10°C	On hold

After amplification, the beads were recovered from emulsion compartment, clonal DNA-carrying beads were enriched, and they were annealed with sequencing annealing for GS Junior sequencing. Firstly, PCR emulsion was collected with a 16 gauge blunt, flat tip needle connected with a 10 ml syringe from PCR tube. If emulsions were from 4 SVE, they can be pooled in only one syringe. Then, 100 µl of isopropanol was added to each tube, and drawn to collect the remaining beads in the tube with the same syringe. The syringe was inverted and drawn in 3 ml of air, and next, the blunt needle onto the syringe was changed to a blue filter from a GS FLX Titanium emPCR Filters. The syringe was vortex at maximum speed for 5 minutes, and then the contents of emulsion were discarded through the filter into a waste bottle. In this step, clonal DNA-amplified beads were retained into syringe. Then, the beads were washed and recovered from all emulsion compartments by isopropanol and Enhancing Fluid XT. Air was drawn about 3 ml, and later 5 ml of isopropanol were drawn into the syringe. The contents in the syringe were vortex at maximum speed for 5 seconds, and emulsion components were expelled through the filter. The washing step with isopropanol was repeated 3 times. Like a washing with isopropanol, after 3 ml of air was drawn into syringe, 3 ml of Enhancing Fluid XT was sucked into the syringe, and it was shaken at maximum speed for 5 seconds, and emulsion components were discarded through the filter. This step was repeated 3 times too. Then, beads were collected from the syringe to perform DNA library bead enrichment. 500 µl of Enhancing Fluid XT was drawn into the syringe, and it was vortex at maximum speeds for 5 seconds. The syringe was drawn in 3 ml of air again, the filter from the syringe was removed and the bead suspension was dispensed in a new 1.7 ml microfuge tube. The new collection tube was spin for 10 seconds, rotated the tube

180°, and spin again for 5 seconds (this process was called the spin-rotate-spin chain). The supernatant was discarded. To collect all beads in the syringe, the beads were collected with 500 µl of Enhancing Fluid XT in the same process for 3 times.

Secondly, the adaptors on clonal DNA-amplified beads were used to link with enrichment primer in order to select only clonal DNA-amplified beads from unamplified bead with enrichment beads. For DNA bead preparation for enrichment, twice, the collected beads from the before step were incubated with 1 ml of Melt solution, which helped to separated dsDNA of clonal-amplified DNA onto beads to ssDNA so as to bind to enrichment primer, at room temperature for 2 minutes, and then the beads were spin down with the spin-rotate-spin chain, and supernatant was discarded. Then, 1 ml of Annealing Buffer XT, which prepared beads for attaching of the adaptor to enrichment primer, was mixed to the beads in the tube with vortex, the beads were spin down with the spin-rotate-spin chain, and the supernatant was discarded. This step was repeated once. After that, 30 µl of Annealing Buffer XT and 6 µl of Enrichment primer (24 µl for 4×SVE) were mixed into the tube with vortex, and incubated in a heat block at 65°C for 5 minutes, and then immediately cooled on ice for 2 minutes. Then, 500 µl of Enhancing Fluid XT was mixed into the bead suspension in tube with vortex, and the bead was spin down by spin-rotate-spin chain, and the supernatant was discarded. This step was repeated again. Then, 800 µl of Enhancing Fluid XT was added into the tube and vortex.

For Enrichment beads, these beads were magnetic particles and had the primer to bind to the enrichment primer onto DNA-amplified beads. Twenty µl of Enrichment beads (80 µl for 4×SVE) were placed on MPC until they were pulled on the wall of tube, and supernatant was discarded. Two times, 500 µl of Enhancing Fluid XT was added on the tube, the mixture was shaken with vortex, the Enrichment Beads were pelleted on MPC until supernatant was clear, and the supernatant was discarded. Then, the tube was removed from the MPC, and 20 µl of Enhancing Fluid XT was added into the tube (80 µl for 4×SVE).

For the process of the selection of only DNA-amplified beads, all prepared magnetic Enrichment beads were mixed with the prepared DNA-amplified beads in a tube with vortex, and then, the tube was rotated on the Labquake at room temperature for 5 minutes. The rotated tube was placed on MPC for 4 minutes, inverted several

times and waited for 4 minutes to pellet the DNA-amplified beads attached with Enrichment Beads. Next, the supernatant was discarded. Six to ten times of washing until no visible white DNA beads in supernatant, the beads were mixed with 500 µl of Enhancing Fluid XT with vortex, and the DNA-amplified beads attached with Enrichment Beads were pelleted on the MPC for 4 minutes, and the supernatant was discarded. After that, the tube of the DNA-amplified beads attached with Enrichment Beads was added with 700 µl of Melt solution, which destroyed the binding between the enrichment primers onto DNA-amplified beads and the primer onto the magnetic Enrichment beads, and mixed with vortex for 5 seconds. The tube was placed in the MPC until the magnetic Enrichment beads were pelleted, and the supernatant containing only enriched DNA-amplified beads was transferred into a 1.7 ml collection tube. The step since adding Melt solution was repeated once, but in the repeated step, supernatant was transferred into the same 1.7 ml collection tube. Then, the collection tube containing DNA-amplified beads was spun down with spin-rotate-spin chain, and the supernatant was discarded. To clear the contaminated magnetic Enrichment beads, Annealing buffer was used because the magnetic beads had lower density than DNA-amplified beads when they were in Annealing buffer XT. Three times, 500 µl of Annealing Buffer XT was added into the collection tube, and the component was vortex, and the DNA amplified beads was spun down by spin-rotate-spin chain, and the supernatant is discarded.

Finally, DNA-amplified beads were bound to Sequencing primer for preparation the bead before sequencing, they were resuspended in 30 µl of Annealing Buffer XT (120 µl for 4×SVE), and mixed with 6 µl of Sequencing primer (24 µl for 4×SVE). The mixture was incubated at 65°C for 5 min, and then promptly on ice for 2 minutes. After that, three times, the reaction was mixed with 500 µl of Annealing Buffer XT, and the DNA-amplified beads bound with sequencing primers were spun down by spin-rotate-spin chain, and the supernatant was discarded. In the end, 100 µl of Annealing Buffer XT was added to the bead pellet and shaken with vortex. To evaluate the number of beads from emPCR amplification and DNA amplified bead enrichment, the beads were between 500,000 to 2,000,000 beads, which were measured with the GS Junior Bead Counter (Appendix A). The tube of beads was stored at +2°C to +8°C, and sequenced within two weeks.

4.6.3 Sequencing

After clonal DNA-carrying beads were annealed with sequencing primer, their sequencing was performed by GS Junior genome sequencer (Roche). DNA beads were filled into each microwell in a picotiter plate by one bead per one well (total 250,000 wells), and DNA was performed the pyrosequencing reaction with three enzymes on beads (enzyme bead) including DNA polymerase, ATP sulfurylase, and luciferase. The reaction was shown in Appendix A. The clonal DNA on beads were incorporated with nucleotide by DNA polymerase, and after that, pyrophosphates were released. In the next step, pyrophosphate (PPi) was reacted to ATP, and this product were cofactor of luciferase in changing of luciferin to oxyluciferin and light which was detected by a charge coupled device (CCD) camera. So, the light emitted was proportional to the amount of a particular nucleotide incorporated.

In the method, some reagents, Packing beads, Enzyme beads, and DNA beads had to be prepared before loading them into picotiter plate device by layer as seen in Table 4.3. For reagent preparation, CB buffer in the bottle was mixed with 6.6 ml of Supplement CB. 40 ml of this mixture was transferred to a new 50 ml conical tube, and was added with 6.5 µl of apyrase, which degraded unincorporated nucleotide before another nucleotide was added in sequencing process, to become BB2 reagent. In the preparation on Enzyme beads and PPiase beads, each tube of both were mixed with 1 ml of BB2 at medium speed, and the beads in each tube were pelleted into MPC, and supernatant was removed. Three times, both bead types were washed with 1 ml of BB2, and supernatant was discarded. After the third washing, Enzyme beads were resuspended with 400 µl of BB2, and PPiase beads was resuspended with 410 µl of BB2 at medium speed. PPiase beads were kept on ice whereas Enzyme beads were brought to prepared Enzyme pre-layer and Enzyme post-layer with BB2 as Table 4.4. In the preparation of Packing and DNA beads in layer 2, 3 times, Packing beads were washed with 1 µl of BB2, and they were centrifuged at 10,000 rpm 5 minutes, and supernatant was discarded. After the third washing, Packing beads were resuspended in 200 µl of BB2 by using a vortex at high speed, and kept on ice. On the other hand, 6 µl of Control beads, which were used in the control of sequencing, were added to the DNA beads, and all beads were spun down, and the volume in the tube was adjusted to 100 µl by removing supernatant. 500 µl of BB2 was added to the mixed beads, and

they were incubated on a lab rotator at room temperature for 20 minutes. Then, DNA beads were spun down, and supernatant was removed to leave 50 µl in the tube. DNA beads was mixed three reagents including 65 µl of BB2, 40 µl of polymerase, and 20 µl of polymerase cofactor at low speed for 5 seconds, and incubated on the lab rotator at room temperature for 10 minutes. After that, 175 µl of prepared Packing beads was mixed into the DNA mixture at low speed and incubated on the lab rotator at room temperature for 5 minutes to finish the preparation of layer 2.

Before loading layer as in Table 4.4, the PicoTiterPlate (PTP) and Bead deposition device (BDD) was constructed. This device was the tool for deposition of beads in each layer. Before the deposition, the device had to be soaked with 350 µl of BB2 by loading in the larger holes of BBD device without air bubbles as in Figure 4.5, and spun at 1620 RCF for 5 minutes. After that, BB2 was pipetted out and discarded through the larger hole. Then, 350 µl of pre-layer Enzyme beads in layer1 was deposited with the same process of BB2 soaking. After centrifugation, Enzyme beads were deposited into PTP. For the other layer, they were deposited with the same process; however, there were some different times of the centrifugation; 15 minutes for the DNA and Packing bead loading, 10 minutes for Post-layer Enzyme bead loading, and 5 minutes for PPiase bead loading. When these processes were finished, PTP was removed and placed into the cartridge frame of GS Junior sequencer which was pre-washed and primed. The mode of sequencing was Shotgun sequencing. After about 10 hours of sequencing, the images of light were translated into a sequence data with the GS Junior algorithm about 2 hours. Low quality of reads including dot fail, mix fail, too short quality, and too short primer read were excluded.

Table 4.4 Bead layers in the PicoTiterPlate deposition (Roche)

Bead Layer	**Bead Type**
Layer 1	Enzyme Beads Pre-layer
Layer 2	DNA and Packing Beads
Layer 3	Enzyme Beads Post-layer
Layer 4	PPiase beads

4.7 Bioinformatics and statistical analysis

The main difficulty of resistance plasmid metagenomic analysis was how to relate resistance gene with plasmid carrying them. The bioinformatics processes of this study included De Novo DNA assembly, nucleotide blast, identification of known and unknown DNA, gene annotation, identification of resistance gene, resistance genes and flanking regions alignment, and sequence-based plasmid typing. Work flow of bioinformatics analysis is shown in Appendix C.

4.7.1 De Novo DNA assembly

The aim of assembly was that assemble sequences should be long as much as possible. Total reads was assemble to contigs with the criteria including at least 40 bp overlapping length and minimum 90% overlapping identity by Newbler Assembler which was associated with 454 pyrosequencing (Roche) (113). After assembly, sequence data was divided into 5 types including contigs (read-concatenated assemble sequences), singlets (non-overlapping sequence), repeat sequences (repetitive sequences to at least 70 reads), outlier sequences (chimeric sequence or assembler artifacts) and too short sequences (shorter than 50 bp sequences). All types except too short sequences were performed data analysis.

4.7.2 Nucleotide blast and the identification of known and unknown sequences

Selected sequences were blasted with nucleotide (nt) dataset in NCBI by BIOPIECE program. The aligned hits were generated with an e-value of 10^{-7}, and top hits having the best bit score were retrieved. After that, the alignment length of selected hits were plotted on histogram, and arranged with cumulative curve to observe the frequency of alignment length. Equal to and more than 80% of alignment length was used to be cut-off for known and unknown sequence characterization because the highest peak of frequency was near 100% of alignment length, and the protection against error from next-generation sequencing. For known sequences, their DNA types were categorized into 6 groups including gDNA, plasmid, mobile genetic elements, phage, human, and other such as mouse and virus.

4.7.3 Gene annotation, identification of resistance genes, and multiple alignments of resistance genes together with flanking regions

Resistance gene were identified with two method including alignment with nucleotide sequences of resistance gene by ResFinder, and resistance gene annotation by Pfam. Firstly, ResFinder provided drug-resistance nucleotide dataset, and resistance hits were selected with equal to and more than 30% of identity. Also, resistance hits were confirmed with NCBI blastx (protein annotation with non-redundant protein dataset from nucleotide sequences). Next step, resistance genes identified by ResFinder were brought to find the relation of resistance gene transfer among hospitalized patients and the healthy subject. Nucleotide sequence containing resistance genes and translated resistance proteins were performed the multiple alignments with Bioedit.

Secondly, Pfam was the protein family alignments. All nucleotide sequences were translated to genes by BIOPIECE before, and translated proteins were included when they were composed with more than 33 amino acids. After that, the proteins were performed the protein alignment with Pfam. Predicted resistance proteins were selected with the resistance index that is in the table 4.5. However, for Pfam alignment, e-values from 10^{-10} to 1 were evaluated with specificity by comparing with ResFinder-matched genes. The highest specific and lowest error e-value was indicated to be cut-off for Pfam alignment. After that, resistance hits by Pfam were linked with known and unknown sequences from NCBI nucleotide blast, and were categorized into known and unknown resistance genes.

4.7.4 Plasmid typing

Twenty-two different basic replicons, with each sequence of autonomous replication and its control region involving in incompatibility group, were used for plasmid typing. These replicons were generated into in-house replicon database. Although no sequences contained both resistance genes and replicon, all query sequences were aligned with replicon dataset in order to find plasmid types among gut microbiome. Indirectly, plasmid types together with their previous reported resistance genes were linked with the present resistance genes to estimate the route of resistance gene spread.

Table 4.5 Resistance index from Pfam

PFAM_ID	PFAM_NAME	PFAM_DESCRIPTION	RESISTED_DRUG
PF00873	ACR_tran	AcrB/AcrD/AcrF_family	MDR
PF04439	Adenyl_transf	Streptomycin_adenylyltransferase	Aminoglycosides
PF03756	AfsA	A-factor_biosynthesis_hotdog_domain	Aminoglycosides
PF10706	Aminoglyc_resit	Aminoglycoside-2"-adenylyltransferase	Aminoglycosides
PF02522	Antibiotic_NAT	Aminoglycoside_3-N-acetyltransferase	Aminoglycosides
PF01636	APH	Phosphotransferase_enzyme_family	Aminoglycosides
PF04655	APH_6_hur	Aminoglycoside/hydroxyurea_antibiotic_resistance_kinase	Aminoglycosides
PF12120	Arr-ms	Rifampin_ADP-ribosyl_transferase	Rifamycin
PF02673	BacA	Bacitracin_resistance_protein_BacA	Bacitracins
PF00144	Beta-lactamase	Beta-lactamase	Beta-lactams
PF13354	Beta-lactamase2	Beta-lactamase_enzyme_family	Beta-lactams
PF00302	CAT	Chloramphenicol_acetyltransferase	Chloramphenicol
PF08077	Cm_res_leader	Chloramphenicol_resistance_gene_leader_peptide	Chloramphenicol
PF07931	CPT	Chloramphenicol_phosphotransferase-like_protein	Chloramphenicol
PF00186	DHFR_1	Dihydrofolate_reductase	Trimethoprim
PF06442	DHFR_2	R67_dihydrofolate_reductase	Trimethoprim
PF07564	DUF1542	Domain_of_Unknown_Function_(DUF1542)	Beta-lactams
PF02698	DUF218	DUF218_domain	Glycopeptides
PF03764	EFG_IV	Elongation_factor_G,_domain_IV	Tetracyclins
PF13536	EmrE	Multidrug_resistance_efflux_transporter	MDR
PF06308	ErmC	23S_rRNA_methylase_leader_peptide_(ErmC)	MLS
PF08051	Ery_res_leader1	Erythromycin_resistance_leader_peptide	Macrolides
PF08057	Ery_res_leader2	Erythromycin_resistance_leader_peptide	Macrolides
PF05139	Erythro_esteras	Erythromycin_esterase	Macrolides
PF02388	FemAB	FemAB_family	Beta-lactams
PF07554	FIVAR	Uncharacterised_Sugar-binding_Domain	Beta-lactams
PF07091	FmrO	Ribosomal_RNA_methyltransferase_(FmrO)	Aminoglycosides
PF04632	FUSC	Fusaric_acid_resistance_protein_family	fusaric acid
PF13515	FUSC_2	Fusaric_acid_resistance_protein-like	fusaric acid
PF12805	FUSC-like	FUSC-like_inner_membrane_protein_yccS	fusaric acid
PF00903	Glyoxalase	Glyoxalase/Bleomycin_resistance_protein/Dioxygenase_superfamily	Glycopeptides
PF13669	Glyoxalase_4	Glyoxalase/Bleomycin_resistance_protein/Dioxygenase_superfamily	Glycopeptides
PF05168	HEPN	HEPN_domain	Aminoglycosides
PF07827	KNTase_C	KNTase_C-terminal_domain	Aminoglycosides
PF00753	Lactamase_B	Metallo-beta-lactamase_superfamily	Beta-lactams
PF12706	Lactamase_B_2	Beta-lactamase_superfamily_domain	Beta-lactams
PF08253	Leader_Erm	Erm_Leader_peptide_	MLS
PF12750	Maff2	Maff2_family	Tetracyclins
PF13999	MarB	MarB_protein	MDR
PF01914	MarC	MarC_family_integral_membrane_protein	MDR

Table 4.5 Resistance index from Pfam (cont.)

PFAM_ID	PFAM_NAME	PFAM_DESCRIPTION	RESISTED_DRUG
PF01047	MarR	MarR_family	MDR
PF12802	MarR_2	MarR_family	MDR
PF01554	MatE	MatE	MDR
PF05223	MecA_N	NTF2-like_N-terminal_transpeptidase_domain	Beta-lactams
PF00893	Multi_Drug_Res	Small_Multidrug_Resistance_protein	MDR
PF01909	NTP_transf_2	Nucleotidyltransferase_domain	Aminoglycosides
PF08352	oligo_HPY	Oligopeptide/dipeptide_transporter,_C-terminal_region	MDR
PF13376	OmdA	Bacteriocin-protection,_YdeI_or_OmpD-Associated	Polymyxin
PF05569	Peptidase_M56	BlaR1_peptidase_M56	Beta-lactams
PF11183	PmrD	Polymyxin_resistance_protein_PmrD	Polymyxin
PF03340	Pox_Rif	Poxvirus_rifampicin_resistance_protein	Rifampicin
PF00398	RrnaAD	Ribosomal_RNA_adenine_dimethylase	MLS
PF09357	RteC	RteC_protein	Tetracyclins
PF08050	Tet_res_leader	Tetracycline_resistance_leader_peptide	Tetracyclins
PF08076	TetM_leader	Tetracycline_resistance_determinant_leader_peptide	Tetracyclins
PF02909	TetR_C	Tetracyclin_repressor,_C-terminal_all-alpha_domain	Tetracyclins
PF08361	TetR_C_2	MAATS-type_transcriptional_repressor,_C-terminal_region	Tetracyclins
PF08362	TetR_C_3	YcdC-like_protein,_C-terminal_region	Tetracyclins
PF08359	TetR_C_4	YsiA-like_protein,_C-terminal_region	Tetracyclins
PF08360	TetR_C_5	QacR-like_protein,_C-terminal_region	Tetracyclins
PF00440	TetR_N	Bacterial_regulatory_proteins,_tetR_family	Tetracyclins
PF07739	TipAS	TipAS_antibiotic-recognition_domain	MDR
PF04294	VanW	VanW_like_protein	Glycopeptides
PF02557	VanY	D-alanyl-D-alanine_carboxypeptidase	Glycopeptides
PF04892	VanZ	VanZ_like_family_	Glycopeptides
PF03734	YkuD	L,D-transpeptidase_catalytic_domain	Beta-lactams

CHAPTER V
RESULTS

5.1 Personal information from hospitalized patients and healthy human volunteer

Sixteen patients who stayed at inpatient general medicine ward and I.C.U. medicine ward (n = 8, inpatient general medicine ward; n = 8, I.C.U. medicine ward) were examined in this study. The data are given in the Table 5.1. All patients were involved with medical device or surgery and were treated with at least 3 antibiotics within one month except p12 that obtained two antimicrobial drugs. Their stools were collected after they were admitted more than 7 days. The MDR bacteria-infected data from record were not written by collectors. About the healthy human, she had stayed in Nakhon Pathom and Nakhon Sawan province for 3 years, and had not been received any antimicrobial drug and any hospitalization for 3 years.

Table 5.1 Patient information

ID	Sex	Age	Admission Date	Ward	Stool-collected Date	medical device or surgery	Antibiotic treatment
p1	Female	51	28-12-2009	Gen. Med.	09-03-2010	Yes	colistin, meropenem, vancomycin
p2	Female	76	18-02-2010	Gen. Med.	10-03-2010	Yes	metronidazole, meropenem, doripenem
p3	Female	69	02-03-2010	Gen. Med.	12-03-1010	Yes	meropenem, cefazolin, levofloxacin
p4	Female	38	25-02-2010	Gen. Med.	25-03-2010	Yes	meropenem, amikacin, piperacillin/tazobactam
p5	Female	63	16-03-2010	Gen. Med.	09-04-2010	Yes	piperacillin/tazobactam, imipenem, colistin metronidazole,
p6	Female	63	15-03-2010	Gen. Med.	09-04-2010	Yes	ceftriaxone, cefotaxime, fosfomycin
p7	Female	77	25-04-2010	Gen. Med.	29-04-2010	Yes	ceftriaxone, tazocin, meropenem, colistin
p8	Female	19	09-04-2010	Gen. Med.	07-04-2010	Yes	colistin, vancomycin, vericonazole meropenem, azithromycin,
p9	Male	53	29-12-2010	ICU Med.	06-01-2011	Yes	vancomycin, metronidazole
p10	Female	72	22-12-2010	ICU Med	14-01-2011	Yes	vancomycin, meropenem, ceftriaxone
p11	Male	50	24-11-2010	ICU Med	15-01-2011	Yes	vancomycin, colistin, meropenem, gancyclovir
p12	Male	69	30-10-2010	ICU Med	17-01-2011	Yes	meropenem, colistin
p13	Male	43	22-02-2011	ICU Med	28-02-2011	Yes	vancomycin, meropenem, colistin
p14	Female	64	16-06-2011	ICU Med	25-06-2011	Yes	vancomycin, imipenem, colistin
p15	Female	69	23-03-2011	ICU Med.	28-06-2011	Yes	vancomycin, meropenem, colistin
p16	Female	80	10-02-2011	ICU Med	01-07-2011	Yes	meropenem,vancomycin, levofloxacin

Gen. Med. = General Internal Medicine ward

ICU Med. = Internal Medicine Intensive Care Unit

5.2 Plasmid extraction and purification test

5.2.1 Alkaline lysis and chromotography

On these 17 stool samples, bacterial cells were pelleted before plasmid extraction by human cell lysis and bacterial cell pellet collection. When plasmid was extracted with lysozyme and lyzostaphin treatment and alkaline lysis method in plasmid extraction kit, the results are shown in figure 5.1. Plasmid from gut bacteria was compared with reference plasmid pUCP24. Plasmid from gut bacteria had the smear bands of several plasmid types and contaminated chromosome whereas the reference one had sharp bands of three plasmid forms, supercoiled, nicked-circular and homopolymer forms, contaminated with smear band of chromosome.

5.2.2 Cesium chloride – ethidium bromide (CsCl-EtBr) gradient centrifugation

Based on the standard plasmid purification method, gut plasmids were purified by CsCl-EtBr gradient centrifugation after they were extracted with plasmid extraction kit. Mixed plasmids in various sizes from five clinical drug resistance isolates (ESBL *E. coli* no. 58, 108, 215 and ESBL *K. pneumoniae* no. 197, 232) were used as reference plasmids. About mixed reference plasmids, mixed plasmids and contaminated chromosome were separated into two bands (Left in Figure 5.2). The upper was chromosome, and the lower was plasmid. On the other hand, gut plasmid was massive with chromosome in one band (Right in Figure 5.3). Therefore, CsCl-EtBr gradient centrifugation was not advantageous for plasmid purification.

5.2.3 Plasmid enrichment by enzyme treatment

The other method for plasmid purification test was enzyme treatment. Lambda exonuclease and exonuclease I were used to digest chromosome which is linear DNA form. Undigested and digested pUCP24 were served to evaluate this method for chromosome decontamination. Before plasmids were treated with lambda exonuclease, digested pUCP24 showed the band of linear plasmid form (lane 4) while undigested pUCP24 displayed several plasmid patterns except linear form (lane 1). With lambda exonuclease treatment, almost linear pUCP24 (lane 5) were disappeared

while other forms were affected a little bit (lane 2). To further remove contaminated single-stranded DNA from lambda exonuclease treatment, the exonuclease I was applied. Smeared DNA from undigested (lane 3) and digested (lane 6) pUCP24 disappeared by this enzyme. Both enzymes were beneficial for chromosomal elimination, gut plasmid was done with both. Almost smear bands of gut plasmid were disappeared (lane 8 and 9). It was speculated that almost contaminated chromosome in gut plasmid was removed.

After chromosome decontamination, linear DNA was removed until the remaining amount of circular plasmid would be too low to be performed high-throughput sequencing. Circular plasmid DNA was multiplied with phi29 DNA polymerase in order to have enough amount of plasmid DNA for high-throughput sequencing. This enzyme had high accuracy and processitivity. Both reference plasmid, pUCP24, and gut plasmid were raised with this enzyme (Figure 5.4). The bands in agarose gel were shown as smear bands with thick light shade in the upper. This appearance meant concatenated DNA from amplification by phi29 DNA polymerase.

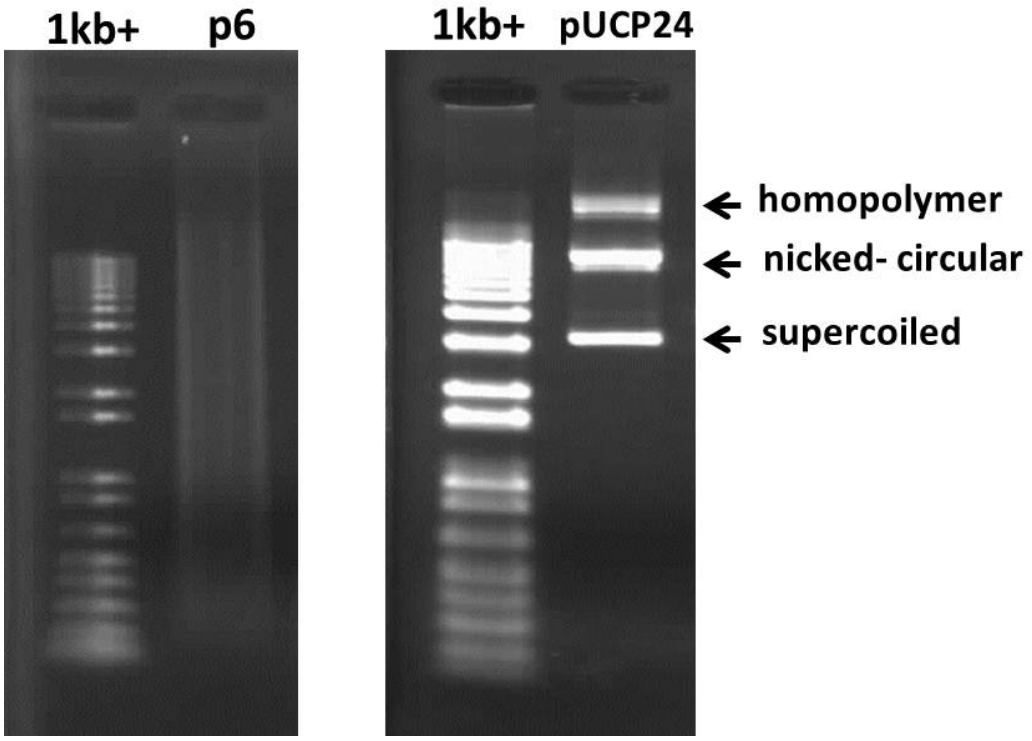

Figure 5.1 Extracted plasmid DNA from one patient (p6) and pUCP24 as reference plasmid

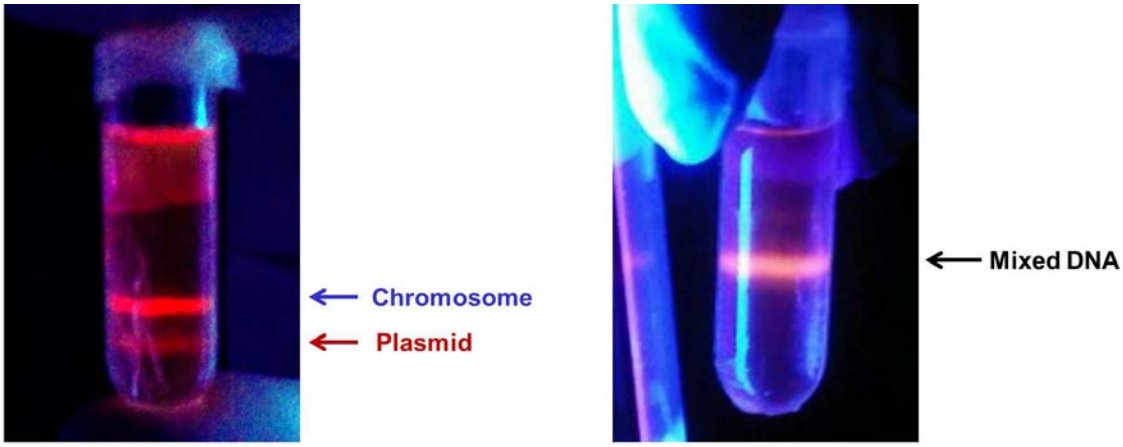

Figure 5.2 Plasmid purification by CsCl-EtBr gradient centrifucation; left was mixed reference plasmids; right was gut plasmid.

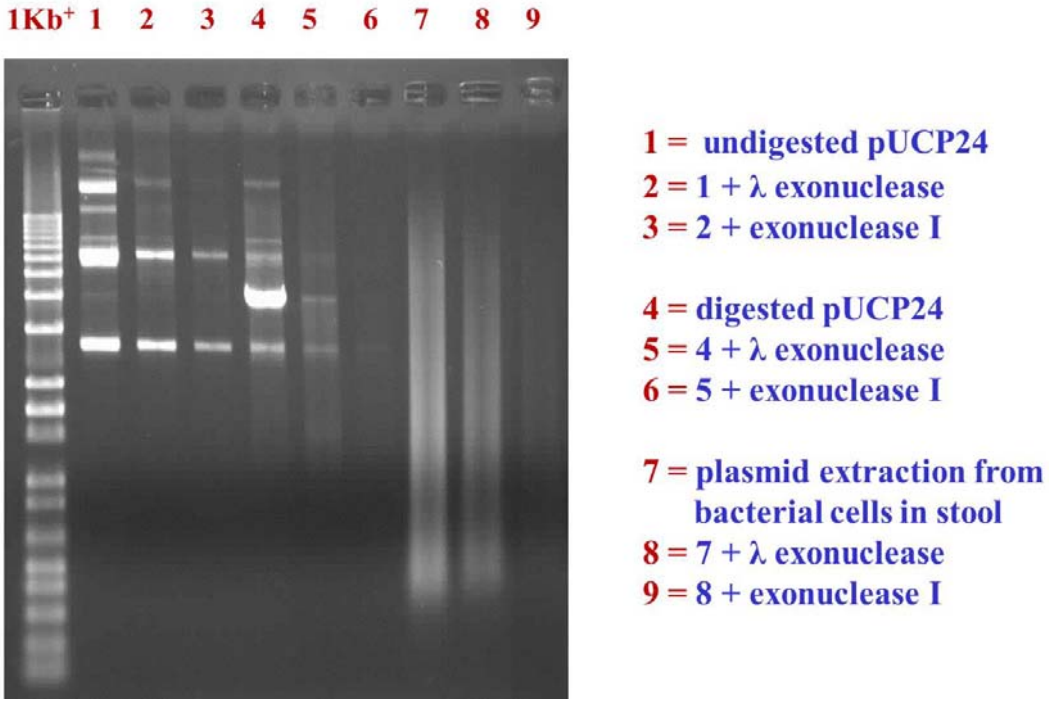

Figure 5.3 Plasmid DNA of reference plasmid (pUCP24) and gut plasmid treated with lambda (λ) exonuclease and exonuclease I. Lane 1 was undigested pUCP24, lane 2 was undigested pUCP24 treated with λ exonuclease, lane 3 was undigested pUCP24 treated with λ exonuclease and exonuclease I, Lane 4 was digested pUCP24; lane 5 was digested pUCP24 treated with λ exonuclease, lane 6 was digested pUCP24 treated with λ exonuclease and exonuclease I, Lane 7 was undigested gut plasmid, lane 8 was undigested gut plasmid treated with λ exonuclease, and lane 9 was undigested pUCP24 treated with λ exonuclease and exonuclease I.

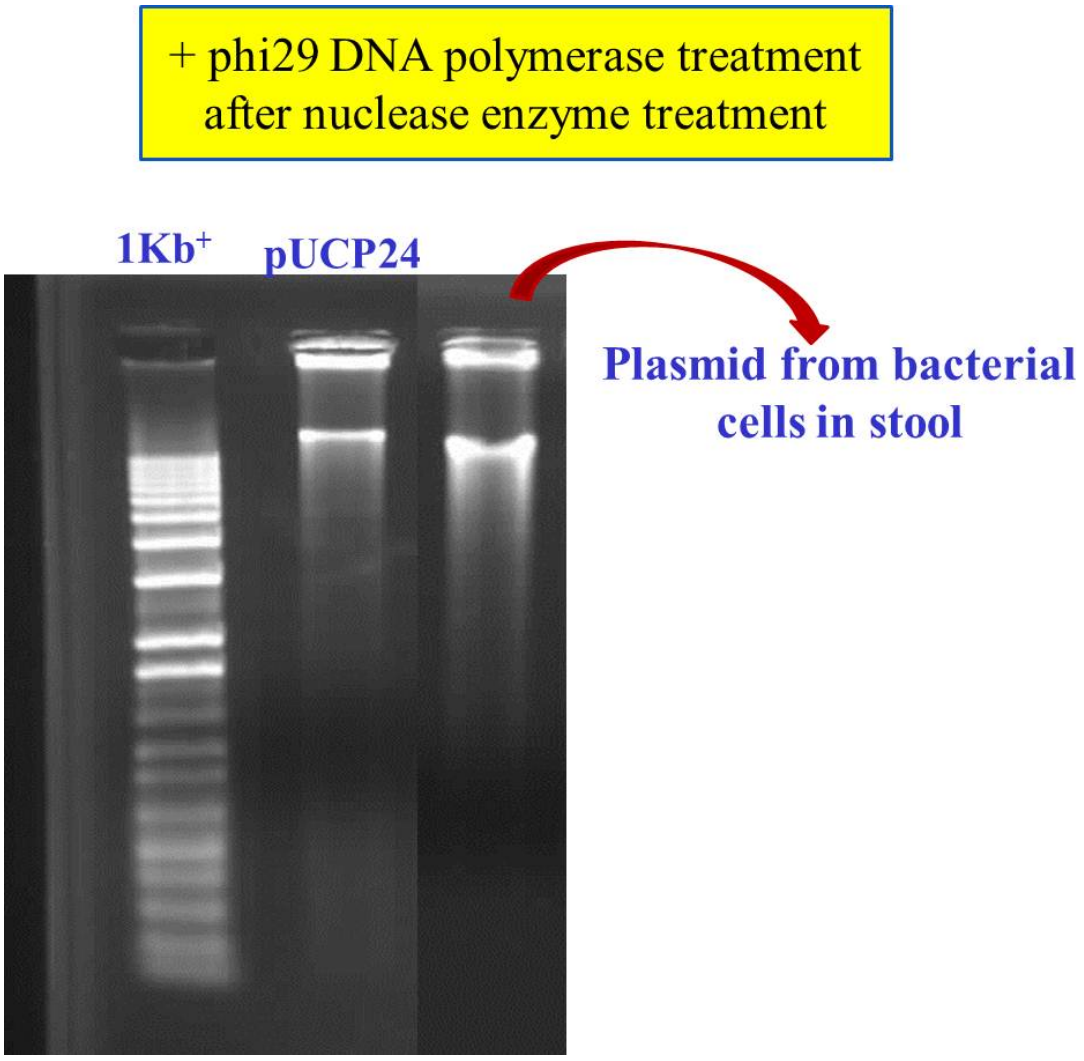

Figure 5.4 DNA multiplication with phi29 DNA polymerase. After the treatment with λ exonuclease and exonuclease I, undigested pUCP24 and undigested gut plasmid were amplified with phi29 DNA polymerase, and showed the concatenated multiplied DNA.

Table 5.2. Output data by GS Junior high-throughput sequencing

Sample	DNA molecules /bead for titration	total bases (bp)	average read length (bp)	total sample raw well in 500,000 wells	% of used well	% of total pass filtered well	% of dot well	% of mix well	% of tooshort seq_well
Control	3	81,489,471	458.33	256,353	51.3	69.4	4.1	5.7	19.9
P1	6	8,830,210	257.09	67,118	13.4	53.5	1.0	21.4	23.7
P4	2	16,433,616	355.68	149,029	29.8	33.5	11.8	35.4	19.3
P5	4	25,795,590	402.94	191,298	38.3	33.5	5.0	25.5	35.0
P6	3	51,342,190	449.24	149,486	29.9	76.5	3.5	3.7	15.9
P6enz	2w	17,941,338	243.87	120,374	24.1	64.2	5.9	7.0	21.7
P10	3	76,359,547	475.81	238,994	47.8	67.1	2.1	5.5	24.9
P12	6	11,028,363	319.7	145,104	29.0	23.8	23.2	28.8	22.3
P16	6	9,695,920	265.52	153,379	30.7	24.0	24.4	27.9	22.2

5.3 High-throughput sequencing

High-throughput sequencing platform used in this study was GS Junior 454 pyrosequencing. The DNA amount for performing highthrouput sequencing must be more than 1,000 ng. Plasmid DNA was sheared to 600-900 base pair length, and single-DNA molecule was amplified to clonal template, and DNA was subjected to the pyrosequencing reaction as shown in material and method. The output should be up to 35 mega base pairs, but only 3 samples which were control, p6, and p10 reached that expectation (Table 5.2). The amount of output were concordant with average read length and read numbers. For example, DNA output of p6 and p6enz, which were plasmid with and without enzyme treatment respectively, were different. Total output of p6 was more than that of p6enz; likewise, the average read length of p6 was longer than that of p6enz.

5.4 Categorization of DNA sequences

After that, DNA reads were performed De Novo assembly with Newbler. Data were divided into 6 types namely assembled, partially assembled, singleton, repeat, outlier and too short sequences, which were described in material and method. MostDNA types except too short sequence were used in bioinformatic analysis. DNA sequences were used in blastn search, and were categorized into 2 types including known and uncharacterized sequences as described in material and method. Of every samples, the percent of aligned length with non-redundant nucleotide database in NCBI were closed to 100% (Figure 5.5 and 5.6). However, some samples had small peaks at different points. The small peaks were at 10%, and 50% of aligned length in control and p10, respectively. Therefore, this study used 80% of aligned length to be digested-off for DNA classification, because the highest peaks were near 100% of aligned length, and the errors from sequencing at the end of reads should not be more than 20% of read length. The unknown sequences were lesser than 80% aligned length, whereas known sequence were equal to or more that 80% aligned length.

When total reads of each samples were categorized, most samples have more unknown sequences than known ones except p10 as shown in Table 5.3. Known sequence of p10 was 52.83%, while unknown sequence of that was 47.17%. In

contrast, unknown reads of others were more than 50%. Whether read length affected the identification of DNA read with non-redundant DNA sequence in NCBI database, average read length was related to the percentage of known and unknown reads. It showed that number of known and unknown sequences were irrelevant with the average read length (Figure 5.7). For instance, average read length of p1, p6enz and p16 were similar, but the percentage of unknown sequence was different with 54.36%, 75.93%, and 87.25%, respectively. On the other hand, average read lengths of control, p6 and p10 were similar, but the percentage of known reads differed a bit with 17.84%, 10.66%, and 52.83%, respectively.

Known sequences of each sample were subdivided into 6 groups namely genomic DNA (gDNA), plasmid, mobile genetic element (MGE), phage, human, and other (other animal or plant DNA) (Table 5.4). By the plasmid purification method without enzyme treatment, gDNA and plasmid were the first and second proportions of known DNA, respectively, while less MGE were found. Hardly any phage, human, and other were appeared. However, they still were less than unknown part.

When gut microbiome's DNA hits by plasmid purification with and without enzyme treatment were compared, their proportions had some differences. From figure 5.8 and Table 5.4, ratio of plasmids in p6 were only 0.5% of total reads; on the other side, plasmids DNA hits in p6 with enzyme treatment (p6enz) were higher (21.68%).

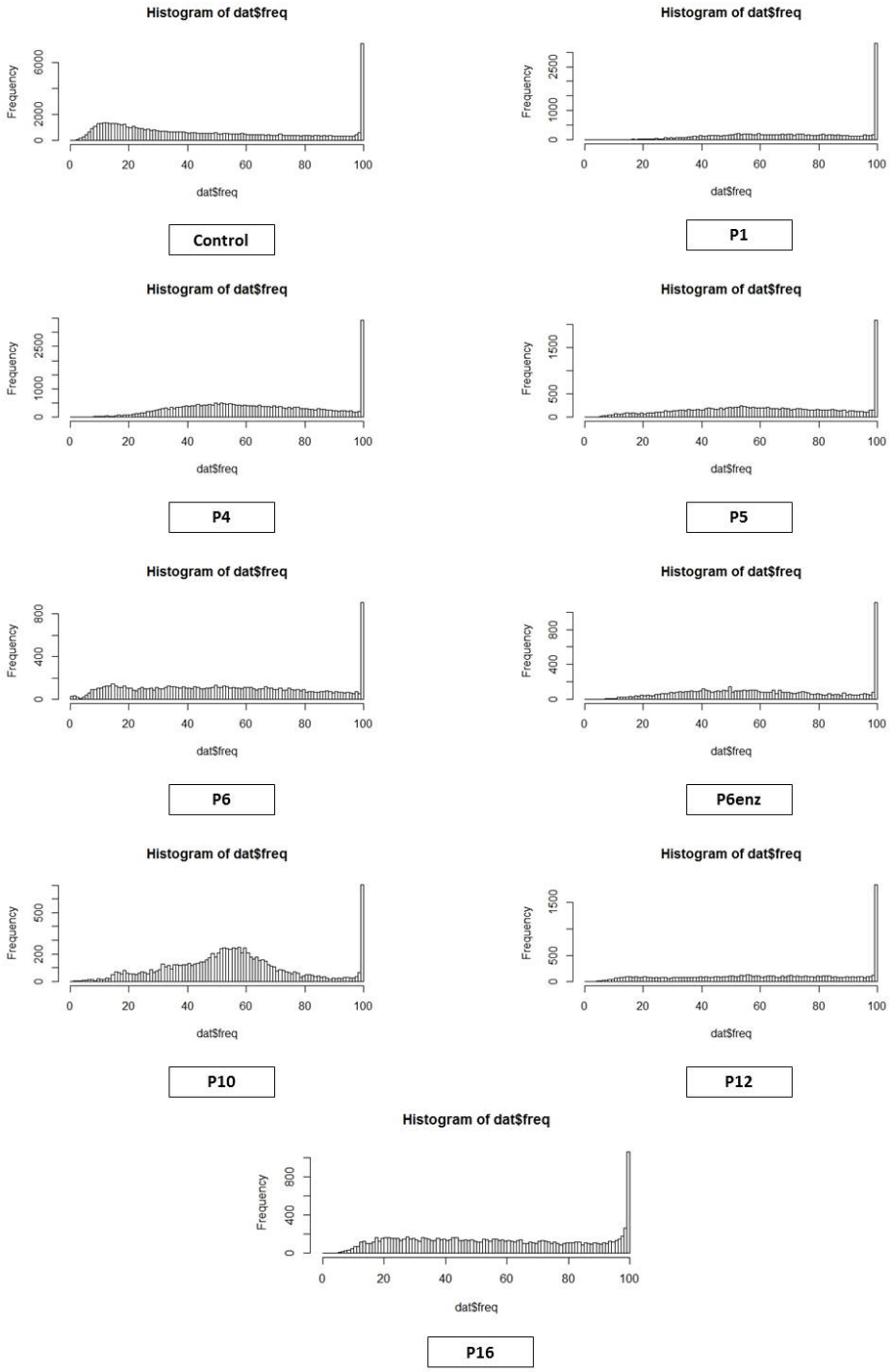

Figure 5.5 Histogram of the percentage of aligned length with nr NCBI database by blastn

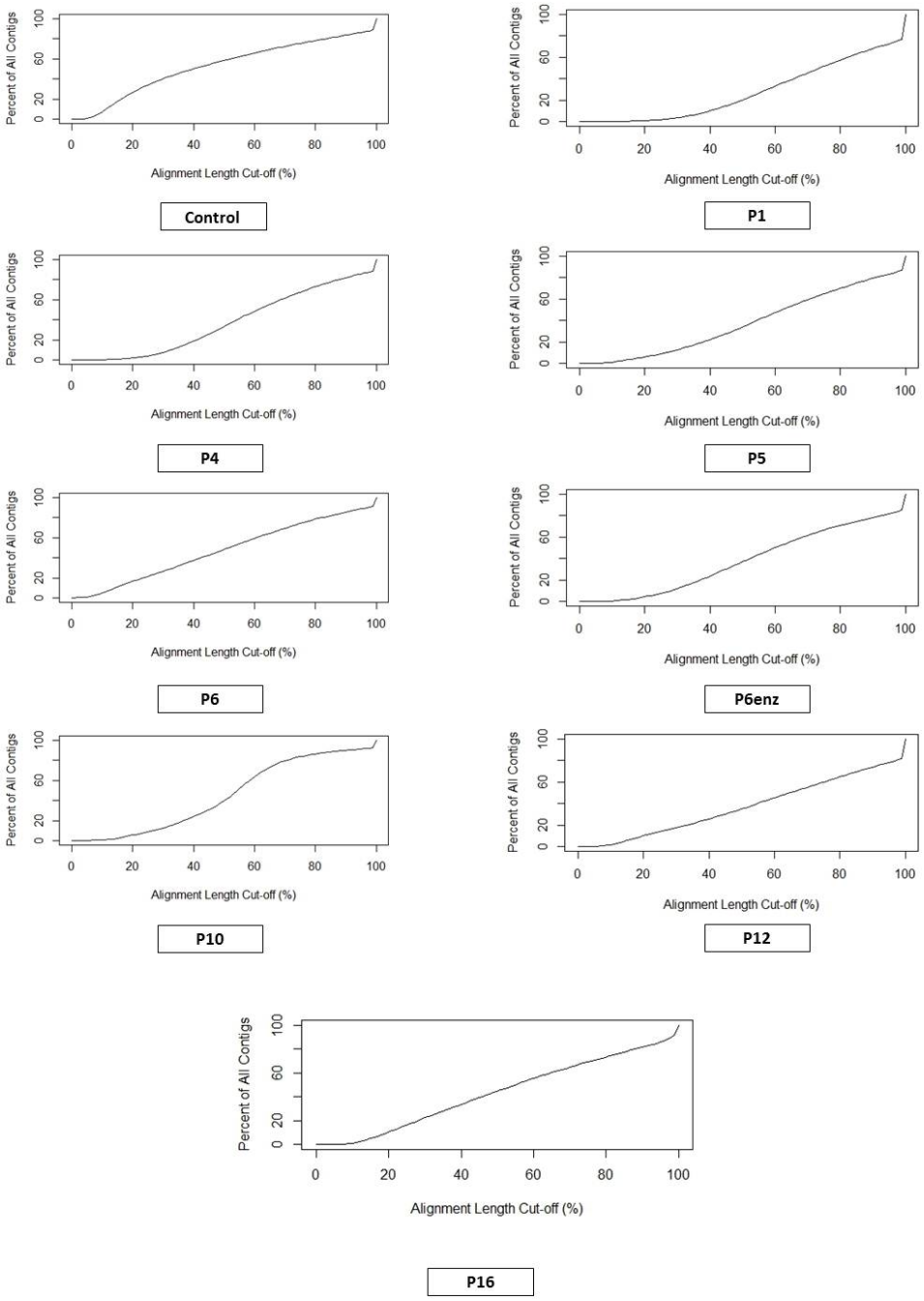

Figure 5.6 Cumulative curves of the percentage of aligned length with non-redundant nucleotide database by blastn

Table 5.3 Categorization of DNA reads

Sample	total_reads % (number)	Average_read_length (bp)	Known_reads % (number)	Unknown_reads % (number)
Control	100 (170,680)	458.33	17.84 (30,457)	82.16 (140,223)
P1	100 (37,612)	257.09	45.54 (17,130)	54.46 (20,482)
P4	100 (56,667)	355.68	31.43 (17,812)	68.57 (38,855)
P5	100 (72,862)	402.94	34.36 (25,038)	65.64 (47,824)
P6	100 (126,552)	449.24	10.66 (13,495)	89.34 (113,057)
P6enz	100 (93,974)	243.87	24.07 (22,621)	75.93 (71,353)
P10	100 (200,673)	475.81	52.83 (106,024)	47.17 (94,649)
P12	100 (37,241)	319.70	36.11 (13,449)	63.89 (23,792)
P16	100 (36,954)	265.52	12.75 (4,713)	87.25 (32,241)

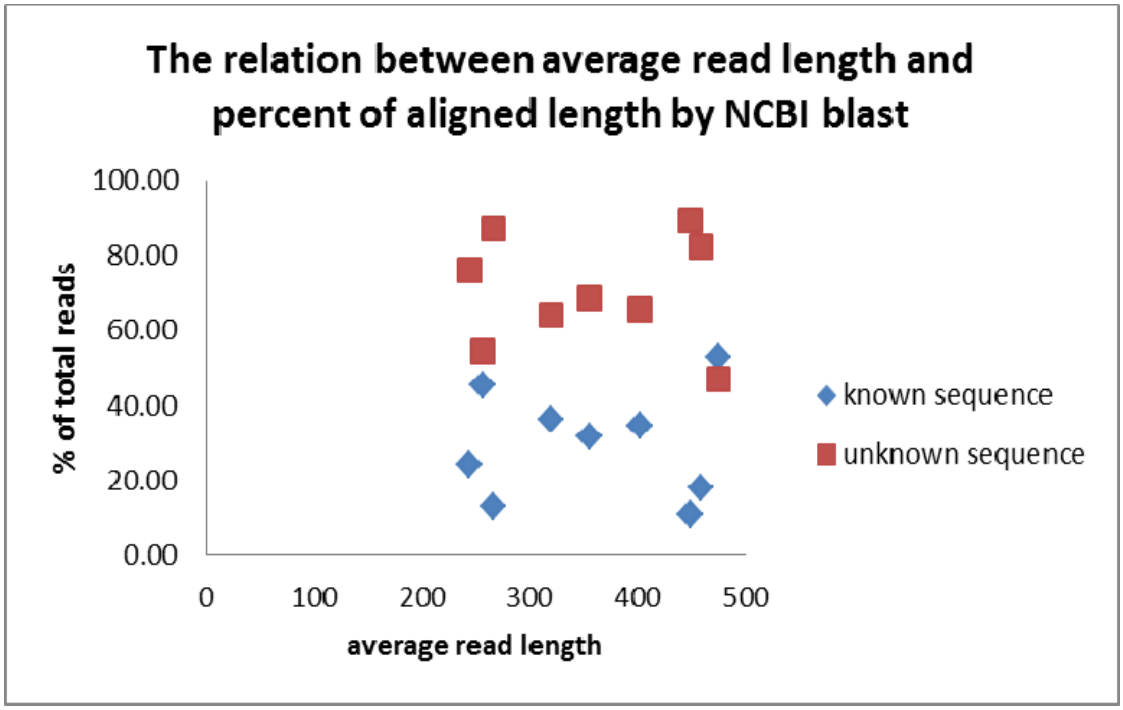

Figure 5.7 The relation between the average read length and the percent of known and unknown sequence

Table 5.4 The ratio of each DNA type

	% type reads of total reads (number of reads)						
	Chromosome or plasmid	**Plasmid**	**MGE**	**Phage**	**Human**	**Other**	**Unknown**
Control	17.51 (29885)	0.26 (448)	0.05 (85)	0.01 (18)	0.01 (12)	0.01 (9)	82.16 (140223)
p1	39.58 (14888)	5.67 (2133)	0.29 (108)	0.003 (1)	0.00 (0)	0.00 (0)	54.46 (20482)
p4	28.95 (16404)	2.39 (1355)	0.01 (7)	0.03 (16)	0.05 (30)	0.00 (0)	68.57 (38855)
p5	29.64 (21597)	4.36 (3175)	0.35 (252)	0.01 (6)	0.01 (4)	0.01 (4)	65.64 (47824)
p6	10.16 (12861)	0.50 (628)	0.003 (4)	0.00 (0)	0.00 (2)	0.00 (0)	89.34 (113057)
p6enz	2.38 (2244)	21.68 (20376)	0.00 (0)	0.00 (1)	0.00 (0)	0.00 (0)	75.93 (71353)
p10	44.24 (88785)	7.76 (15579)	0.83 (1660)	0.00 (0)	0.00 (0)	0.00 (0)	47.17 (94649)
p12	36.05 (13426)	0.03 (13)	0.01 (3)	0.01 (3)	0.01 (4)	0.00 (0)	63.89 (23792)
p16	11.82 (4367)	0.13 (48)	0.80 (294)	0.01 (4)	0.00 (0)	0.00 (0)	87.25 (32241)

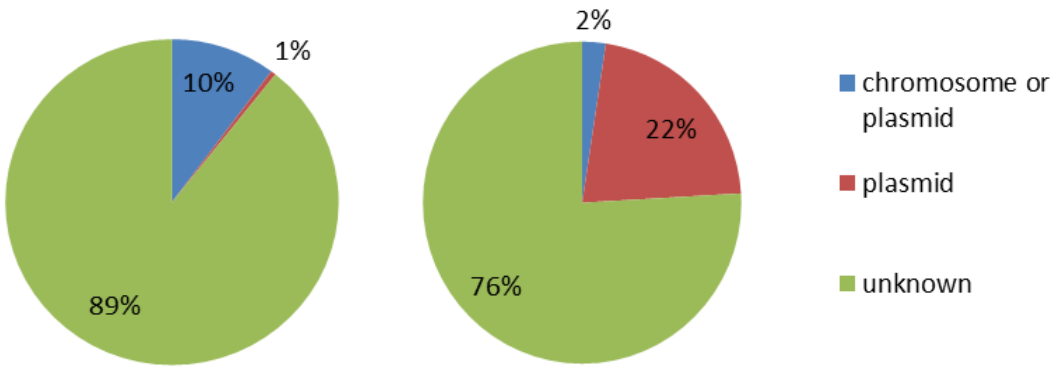

Figure 5.8 Proportion of gut microbiome's DNA hits by DNA purification without (left) or with enzyme treatment (right)

5.5 Finding acquired resistance genes

In order to find resistance genes that were transferred by plasmids, all nucleotide reads were aligned with resistance genes database in ResFinder that collected acquired resistance genes. From table 5.5, several nosocomial resistance genes, namely *ant(6)-Ia*, *erm(B)*, *lnu(B)*, *tet(L)* and *tet(U)*, which confer resistance to aminoglycoside, lincosamide, macrolide, streptogramin B and tetracycline, spread among p1 and p5, which stayed in general medicine ward, and p10, which admitted in ICU medicine ward. Also, several resistance genes being responsible for beta-lactam, rifampin, chloramphenicol, trimethoprim, sulphonamide, and fosfomycin were detected in one patient, p4. Surprisingly, there were some resistance genes, *tetO*, *tet40*, *aph(3')-III* and *catS*, conferring resistance to tetracycline, aminoglycoside and chloramphenicol in control. Although resistance genes of each sample were not corresponded to antibiotic treatment, these were acquired resistance genes by plasmid.

Table 5.5 Acquired resistance genes found among patients and the healthy human

Sample	Used antimicrobial drugs	Resistance gene	% Identity	HSP/Query length	Contig	Predicted phenotype	References	Reference species	Reference country
Control	No	*tetO*	99.76	1647 / 1920	contig00387	Tetracycline resistance	M20925	*Streptococcus mutans*	USA
		tet40	100	807 / 1221	contig03077	Tetracycline resistance	FJ158002	*Uncultured bacterium*	UK
		aph(3')-III	100	644 / 795	contig03732	Aminoglycoside resistance	M26832	*Campylobacter coli*	France
		catS	83.16	374 / 492	IH7MQL401CEH3Q	Phenicol resistance	X74948	*Streptococcus pyogenes*	France
P1	colistin, meropenem, vancomycin	*aph(3')-III*	99.87	796 / 795	contig00005	Aminoglycoside resistance	M26832	*Campylobacter coli*	France
		ermB	99.86	738 / 738	contig00005	Macrolide resistance	X82819	*Enterococcus* sp.	Switzerland
		ant(6)-Ia	100	909 / 909	contig00007	Aminoglycoside resistance	AF330699	*Enterococcus faecium*	Germany
		tetL	100	1377 / 1377	contig00009	Tetracycline resistance	M29725	*Streptococcus agalactiae*	N/A
		msrC	99.1	1108 / 1479	contig00099	Macrolide, Lincosamide and Streptogramin B resistance	AY004350	*Enterococcus faecium*	N/A
		tetU	99.37	318 / 318	contig00136	Tetracycline resistance	U01917	*Enterococcus faecium*	USA
		lnuB	99.72	707 / 804	contig00236	Lincosamide resistance	AJ238249	*Enterococcus faecium*	France

Table 5.5 Acquired resistance genes found among patients and the healthy human (continued)

Sample	Used antimicrobial drugs	Resistance gene	%Identity	HSP/Query length	Contig	Predicted phenotype	References	Reference species	Reference country
P4	meropenem, tazocin, amikin	*bla*$_{TEM-124}$	99.03	723 / 858	contig00176	Beta-lactam resistance	AY327540	*Morganella morganii*	Italy
		sul1	100	840 / 840	contig00223	Sulphonemide resistance	AY224185	*Escherichia coli*	France
		catB3	99.75	399 / 633	contig01173	Phenicol resistance	AJ009818	*Salmonella Typhimurium*	Italy
		bla$_{OXA-1}$	99.82	560 / 831	contig01547	Beta-lactam resistance	J02967	*Escherichia coli*	Canada
		aac(6')-Ib	99.79	466 / 606	contig01569	Aminoglycoside resistance	M21682	*Klebsiella pneumoniae*	USA
		aac(6')Ib-cr	100	398 / 519	contig01569	Fluoroquinolone and aminoglycoside resistance	EF636461	*Klebsiella pneumoniae*	Argentina
		arr-3	100	328 / 453	contig02506	Rifampicin resistance	JF806499	*Escherichia coli*	China
		arr-6	100	328 / 453	contig02506	Rifampicin resistance	JF922883	*Pseudomonas putida*	Brazil
		dfrA5	100	288 / 474	contig03100	Trimethoprim resistance	X12868	*Enterobacteriaceae*	Sweden
		fosA	99.73	376 / 420	GX6IIXK01B05U1	Fosfomycin resistance	NZ_ACWO01000079	*Klebsiella* sp.	USA
		fosA	100	376 / 420	GX6IIXK01BBAE1	Fosfomycin resistance	NZ_ACWO01000079	*Klebsiella* sp.	USA
		fosA	96.13	362 / 420	GX6IIXK01AXWIV	Fosfomycin resistance	NZ_ACZD01000244	*Klebsiella* sp.	USA
		fosA	98.25	343 / 420	GX6IIXK01A4KYP	Fosfomycin resistance	NZ_ACWO01000079	*Klebsiella* sp.	USA

Table 5.5 Acquired resistance genes found among patients and the healthy human (continued)

Sample	Used antimicrobial drugs	Resistance gene	% Identity	HSP/Query length	Contig	Predicted phenotype	References	Reference species	Reference country
P5	tazocin,	*ant(6)-Ia*	100	909 / 909	contig00029	Aminoglycoside resistance	AF330699	*Enterococcus faecium*	Germany
	tienam,	*aph(3')-III*	100	795 / 795	contig00094	Aminoglycoside resistance	M26832	*Campylobacter coli*	France
	colistin	*ermB*	99.86	738 / 738	contig00094	Macrolide resistance	X82819	*Enterococcus* sp.	Switzerland
		tetL	100	1067 / 1377	contig00162	Tetracycline resistance	M29725	*Streptococcus agalactiae*	N/A
		tetM	99.7	1327 / 1920	contig00162	Tetracycline resistance	X92947	*Enterococcus faecalis*	Switzerland
		tetU	99.37	318 / 318	contig00349	Tetracycline resistance	U01917	*Enterococcus faecium*	USA
		lnuB	99.86	706 / 804	contig01046	Lincosamide resistance	AJ238249	*Enterococcus faecium*	France
		aac(6')-Ii	99.64	549 / 549	contig01341	Aminoglycoside resistance	L12710	*Enterococcus faecium*	France
		tetU	97.92	240 / 318	H28AZ0S01DO4TQ	Tetracycline resistance	U01917	*Enterococcus faecium*	USA
		tetU	97.55	286 / 318	H28AZ0S01DFOHQ	Tetracycline resistance	U01917	*Enterococcus faecium*	USA
		tetU	98.99	198 / 318	H28AZ0S01CHSRQ	Tetracycline resistance	U01917	*Enterococcus faecium*	USA
		tetU	98.94	284 / 318	H28AZ0S01CP9YX	Tetracycline resistance	U01917	*Enterococcus faecium*	USA

Table 5.5 Acquired resistance genes found among patients and the healthy human (continued)

Sample	Used antimicrobial drugs	Resistance gene	% Identity	HSP/Query length	Contig	Predicted phenotype	References	Reference species	Reference country
P6	metronidazole, ceftriaxone,	*tetM*	99.53	1926 / 1920	contig00024	Tetracycline resistance	AM990992	*Staphylococcus aureus*	Netherlands
	cefotaxime,	*catA2*	95.86	532 / 642	HY0BG1R01DKIM0	Phenicol resistance	X53796	*Escherichia coli*	UK
	fosfomycin	*dfrA12*	98.71	388 / 498	HY0BG1R01B85I8	Trimethoprim resistance	AB571791	*Salmonella Typhimurium*	Japan
		dfrA12	100	395 / 498	HY0BG1R01C85WQ	Trimethoprim resistance	AB571791	*Salmonella Typhimurium*	Japan
P6enz		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
P10	vancomycin, meropenem,	*aac(6')-Ii*	99.64	549 / 549	contig00010	Aminoglycoside resistance	L12710	*Enterococcus faecium*	France
	ceftriaxone	*dfrG*	100	498 / 498	contig00119	Trimethoprim resistance	AB205645	*Staphylococcus aureus*	Japan
		tetL	99.93	1377 / 1377	contig00235	Tetracycline resistance	AY081910	*Enterococcus faecium*	Germany
		aph(3')-III	100	795 / 795	contig00387	Aminoglycoside resistance	M26832	*Campylobacter coli*	France
		aac(6')-aph(2'')	100	1236 / 1440	contig00393	Aminoglycoside resistance	M13771	*Enterococcus faecalis*	France
		ant(6)-Ia	100	909 / 909	contig00410	Aminoglycoside resistance	AF330699	*Enterococcus faecium*	Germany
		ermB	99.86	738 / 738	contig00426	Macrolide resistance	X82819	*Enterococcus* sp.	Switzerland
		lnuB	99.86	706 / 804	contig00475	Lincosamide resistance	AJ238249	*Enterococcus faecium*	France
		tetU	80.63	284 / 318	H3CCAFC01CZ6EV	Tetracycline resistance	U01917	*Enterococcus faecium*	USA

Table 5.5 Acquired resistance genes found among patients and the healthy human (continued)

Sample	Used antimicrobial drugs	Resistance gene	% Identity	HSP/Query length	Contig	Predicted phenotype	References	Reference species	Reference country
P12	meropenem, colistin	NA	NA	NA	NA	NA	NA	N/A	N/A
P16	meropenem, vancomycin, levofloxacin	*tetW*	99.31	1166 / 1920	contig00027	Tetracycline resistance	FN396364	*Streptococcus suis*	Italy
		ermB	99.78	458 / 738	contig00053	Macrolide resistance	U18931	*Clostridium perfringens*	Australia
		ermB	98.78	490 / 738	H2N0LBF01BJ92S	Macrolide resistance	U18931	*Clostridium perfringens*	Australia

5.6 Predicted resistance protein family in gut microbiome

Pfam was used for finding novel resistance gene sequences that have not been reported before. Both known and unknown DNA sequences were translated to protein by Biopiece program. More than or equal to 100 nucleotides, which were translated to be amino acid, were selected. Protein families were predicted with Pfam, which was protein family database at e-value 1.0. If e-value is higher, the error is more. From figure 5.9, the histograms of all results were shown that Pfam-predicted protein hits had high prevalence at 1e-03 to 1. Pfam-predicted translated proteins were tested for the specificity of resistance protein detection. This study chose sequences that were matched with acquired resistance genes in ResFinder as goal standard. In table 5.6, each translated protein probably had only one or more than one matching with one or several e-value. The digested-off of e-value was change from 1 to 1e-10, and the correct and wrong results were counted and calculated the specificity in Table 5.7. When e-value was decreased by digestedting false results out, the specitivity was increased until e-value at 1e-06, which was quite stable point. If e-value was lesser than 1e-06, several true results were removed. Therefore, e-value at 1e-06 was selected to be digested-off for the alignment of translated proteins with Pfam.

From table 5.8, all samples had resistance translated hits about 1% of total translated proteins, and almost except p1 had the proportion of resistance translated protein from unknown hits more than that of known hits. Also, by different plasmid purification methods, total resistance protein family hits of p6 were 0.73% while that of p6enz were 0.14% (Figure 5.10). It showed that plasmid purification method with enzyme treatment probably lost of resistance protein detection.

For all samples by plasmid purification method without enzyme treatment, almost resistance proteins from known hits belonged to gDNA, other matched with plasmid and MGE respectively (table 5.9). For example, p6, almost known hits matched with genomic DNA were 27 predicted protein; also, other corresponded with plasmid were 1 predicted protein. In contrast, translated resistance proteins from p6 with enzyme treatment balanced between gDNA and plasmid, which were two predicted proteins.

From table 5.10, all translated proteins (both known and unknown sequences) matched with resistance protein families were chosen by Pfam resistance

index as in materials and methods, and were related to antimicrobial drug usage. For all patients, drug resistance protein families were corresponded with drug treatment (gray boxes) except that some resistance protein families, fosfomycin and quinolone resistance protein, were not in Pfam resistance index. However, other resistance protein familes that were not correlated with antimicrobial drug treatment were found. In addition, all samples had resistance protein families to beta-lactams, glycopeptides, aminoglycosides, tetracyclines, trimethoprim, bacitracins, macrolide-lincosamide-streptogramin B, multidrugs and fusaric acid, whereas chloramphenicol, rifamycins, and polymyxin resistance proteins were found in some samples. Interestingly, several drug resistance proteins except rifamycins and polymyxin were found in control. When resistance translated proteins of between p6 and p6 with enzyme treatment were compared, resistance translated proteins of p6 with enzyme treatment were quite less than p6.

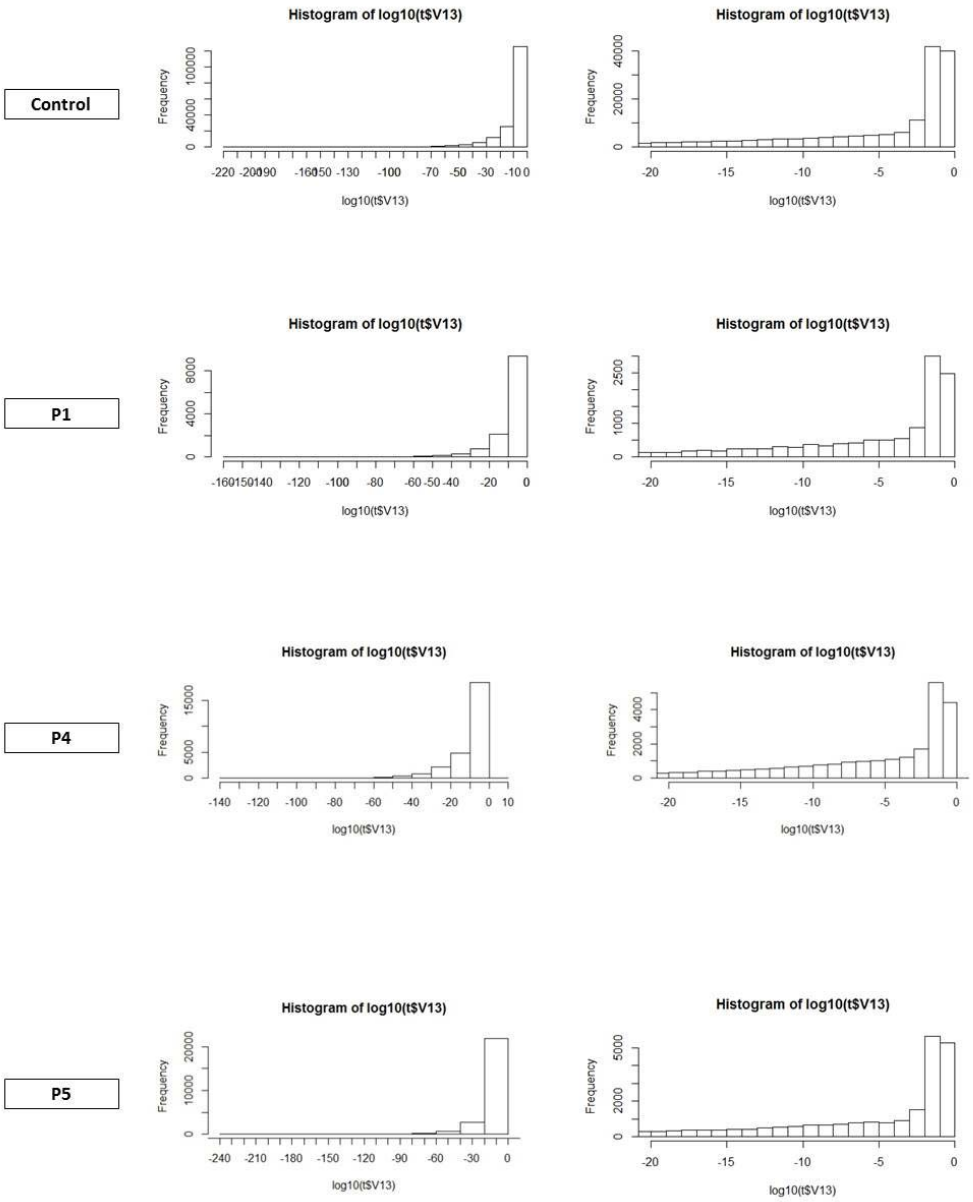

Figure 5.9. Histogram of e-value of protein family identification by pfam

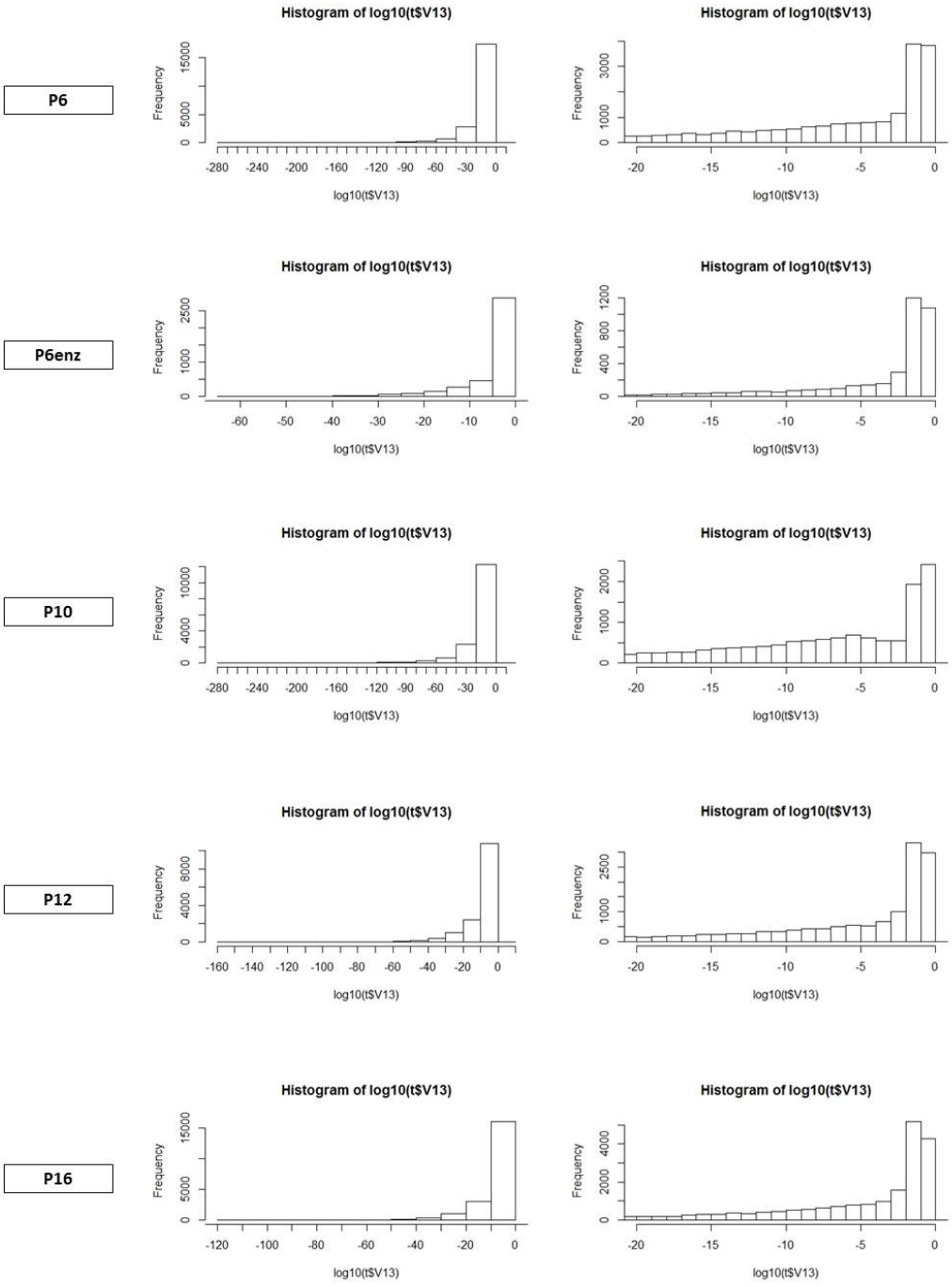

Figure 5.9 Histogram of e-value of protein family identification by pfam (continued)

Table 5.6 The example of pfam results

Translated protein	Pfam_name	e-values
con_contig03077__3077_1:0:818:+	Asp-Al_Ex	0.053
con_contig03732__3732_1:93:734:+	APH	2.3e-22
con_IH7MQL401CEH3Q_21755_1:0:317:-	CAT	3.1e-42
p1_contig00005__5_4:1775:2512:-	RrnaAD	1.8e-72
p1_contig00005__5_4:1775:2512:-	DUF4610	0.1

Table 5.7 Specificity of Pfam test

	Pfam_digested-off										
	≤1	≤1e-01	≤1e-02	≤1e-03	≤1e-04	≤1e-05	≤1e-06	≤1e-07	≤1e-08	≤1e-09	≤1e-10
Specificity for predicted resistance protein family	37.68	41.27	4364	42.59	43.40	45.10	53.33	51.16	53.66	53.66	57.89
No. of true predicted protein family hits	26	26	24	23	23	23	23	21	21	21	21

Table 5.8 Proportion of resistance translated protein hits

	No. of hits (% of total hits)								
	Control	**p1**	**p4**	**p5**	**p6**	**p6enz**	**p10**	**p12**	**p16**
Resistance translated proteins from known hits	270 (0.17)	34 (0.25)	78 (0.23)	50 (0.21)	28 (0.14)	4 (0.05)	36 (0.23)	28 (0.14)	67 (0.24)
Resistance translated proteins from unknown hits	1256 (0.81)	32 (0.23)	154 (0.46)	112 (0.46)	113 (0.58)	6 (0.08)	104 (0.66)	151 (0.26)	129 (0.47)
total resistance translated proteins hits	1526 (0.99)	66 (0.48)	232 (0.70)	294 (1.21)	141 (0.73)	10 (0.14)	140 (0.89)	79 (0.40)	131 (0.71)
total translated proteins	154,364 (100)	13,701 (100)	33,323 (100)	24,326 (100)	19,431 (100)	7,280 (100)	15,643 (100)	19,606 (100)	27,532 (100)

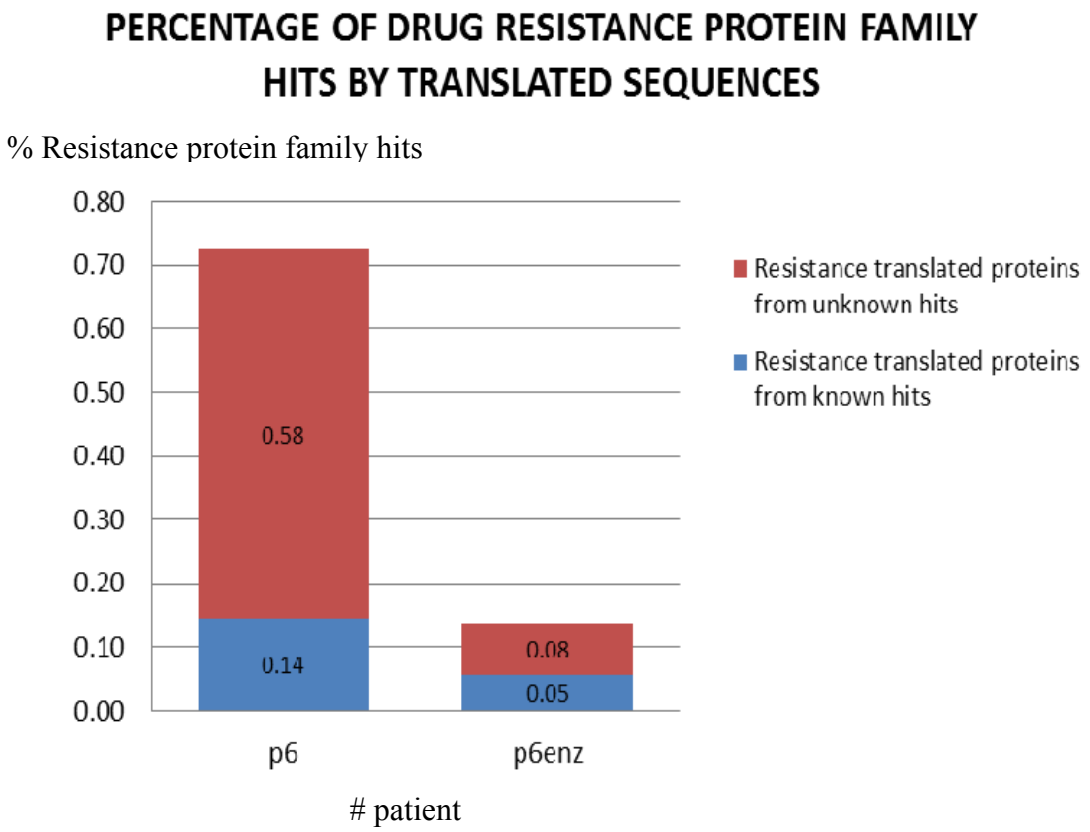

Figure 5.10 Graph for comparing the percentage of resistance protein family hits between p6 and p6 with enzyme treatment

Table 5.9 Number of resistance protein hits matched with each DNA type

	Control	p1	p4	p5	p6	p6enz	P10	p12	p16
gDNA	254	33	72	47	27	2	27	28	67
Plasmid	14	1	8	3	1	2	5	0	0
MGE	2	0	0	0	0	0	4	0	0
Phage	0	0	0	0	0	0	0	0	0
Human	0	0	0	0	0	0	0	0	0
Other	0	0	0	0	0	0	0	0	0
Unknown	1256	32	154	112	113	6	104	51	129
Total	1526	66	234	162	141	10	140	79	196

Table 5.10. Classification of each resistance protein hits

	Number of resistance protein hits (% of total protein hits)								
Resistance to	**Control**	**p1**	**p4**	**p5**	**p6**	**p6-enz**	**p10**	**p12**	**p16**
Beta-lactams	249 (0.16)	15 (0.11)	35 (0.11)	48 (0.20)	24 (0.12)	2 (0.03)	32 (0.20)	12 (0.06)	21 (0.08)
Glycopeptides	103 (0.07)	9 (0.07)	6 (0.02)	15 (0.06)	6 (0.03)	0 (0.00)	18 (0.12)	5 (0.03)	3 (0.01)
Aminoglycosides	63 (0.04)	4 (0.03)	3 (0.01)	14 (0.06)	6 (0.03)	0 (0.00)	10 (0.06)	5 (0.03)	12 (0.04)
Tetracyclins	167 (0.11)	9 (0.07)	28 (0.08)	22 (0.09)	16 (0.08)	5 (0.07)	23 (0.15)	23 (0.12)	20 (0.07)
Macrolides	2 (<0.01)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Chloramphenicol	4 (<0.01)	0 (0.00)	3 (0.01)	0 (0.00)	5 (0.03)	0 (0.00)	0 (0.00)	0 (0.00)	7 (0.03)
Trimethoprim	27 (0.02)	5 (0.04)	4 (0.01)	8 (0.03)	8 (0.04)	0 (0.00)	12 (0.08)	1 (0.01)	2 (0.01)
Bacitracin	47 (0.03)	4 (0.03)	1 (<0.01)	5 (0.02)	7 (0.04)	2 (0.03)	1 (0.01)	3 (0.02)	12 (0.04)
MLS	25 (0.02)	5 (0.04)	6 (0.02)	5 (0.02)	14 (0.07)	0 (0.00)	14 (0.09)	2 (0.01)	5 (0.02)
MDR	822 (0.53)	13 (0.09)	120 (0.36)	42 (0.17)	52 (0.27)	1 (0.01)	28 (0.18)	28 (0.14)	113 (0.41)
fusaric acid	17 (0.01)	2 (0.01)	22 (0.07)	3 (0.01)	3 (0.02)	0 (0.00)	2 (0.01)	0 (0.00)	0 (0.00)
Rifamycins	0 (0.00)	0 (0.00)	1 (< 0.01)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Polymyxins	0 (0.00)	0 (0.00)	3 (0.01)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (<0.01)
Fosfomycin	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Quinolones	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

*Gray box means patients received antimicrobial drug in each corresponding row.

5.7 Alignment of resistance genes among hospitalized patients and control

DNA sequences, which had the same genes were aligned with Bioedit. Resistance genes together with flanking regions were aligned to find the homology among samples (figure 5.11-5.18). In the first group, resistance genes spread among p1, p5, and p10 were ant6-Ia, *lnuB*, *tetL*, and *tetU*. The *ant6-Ia,* which is aminoglycoside O-nucleotidyltransferase, modifides aminoglycoside by adenylation. This gene had the same pattern among these patients, but was different from the reference gene of *Enterococcus feacium* found in animal and human in Germany. There were many substitution mutations and amino acid changing in coding sequences. Also, the flanking regions of *ant6-Ia* among them were identical, but different from reference sequence.

lnuB is lincosamide nuclotidyltransferase, which inactivate lincosamide antibiotics. The *lnuB* of p1, p5 and p10 had the same substitution with G at C145 position in the sequence of *E. faecium* HM1025 that were collected in France. This replacement caused amino acid change from histidine to glutamic acid at position 49. Also, p1 had guanine insertion which differed from reference and other resulted in amino acid change from asparagine to lysine at position 146 and stop codon later. In addition, there were six nucleotide changes from reference in p10, and caused amino acid substitutions at postion 236 (lysine to arginine), 237 (lysine to phenylalanie) and 238 (valine to cysteine). Whereas the coding sequences among the patients were different a little bit, the upstream regions among them were identical.

tetL is the efflux protein that are responsible for tetracycline resistance. *tetL* of p1 and p5 were identical with the reference genes of *Streptococcus agalactiae* plasmid, but that of p10 had two positions of substitutions, C140T and G280T. These caused amino acid change from phenylalanine and valine to lysine at the position 47 and 94 respectively. For flanking regions, the downstream regions of p1 and p10 were identical while the upstream regions of p1and p5 had high homology, which were different from p10.

tetU is responsible for tetracycline resistance with unknown mechanism. From figure 5.14, assembled sequence of p1 and p5 were identical and had the same two substitutions when they were compared with reference sequence of *Enterococcus*

faecium. However, the other unassembled sequence of p5 and p10 were different. Moreover, the pattern of flanking regions among them had the same trend with the coding sequences. p1 and p5 had the same upstream and downstream regions of contigs whereas the others were dissimilar.

aac-Ii which is 6′-N-aminoglycoside acetyltransferase performed aminoglycoside resistance by acetylation. p5 and10 had two substituted positions, when they were compared with reference gene of *Enterococcus faecium* from France. Adenine replaced thymine at position 380 and guanine at position that caused amino acid changes (valine to glutamic acid and aspatic acid to asparagine. Moreover, both upstream and downstream regions were identical and similar with reference.

ermB is rRNA adenine N-6-methyltransferase which change rRNA target of erythromycin antibiotics. The coding sequences of p1, p5 and p10 were identical and had the same substitutions from adenine to guanine which caused amino acid change from isoleucine to valine at position 226. For p16, there was one deletion in coding sequence, so amino acid had frameshift mutation. Besides, the flanking regions among p1, p5 and p10 were identical, but they differed from p16 a bit.

tetM express ribosomal protecting proteins from the inhibition by tetracycline. The genes found in p5 and p6 had several different position including insertion and substitution each other. However, tetM of p5 was more closed to that of reference *Enterococcus faecalis* than p6. *tetM* of p6 like *tetM* of *Staphylococcus aureus* from Netherlands. In the same way, their flanking regions were dislike.

aph(3′)-III is 3′5″-aminoglycoside phosphotransferase being responsible for kanamycin, neomycin, paromomycin, ribostamycin, lividomycin, butirosin, and gentamycin in aminoglycosides. It was found in both patients within the same (p1 and p5) and different wards (p10), and the healthy human (control). Their genes were very high homologous to one another and like the reference of *Campylobacter coli* from France; although, p1 had adenine insertion in only one position which caused amino acid frameshift. Moreover, the flanking regions among them were quite similar.

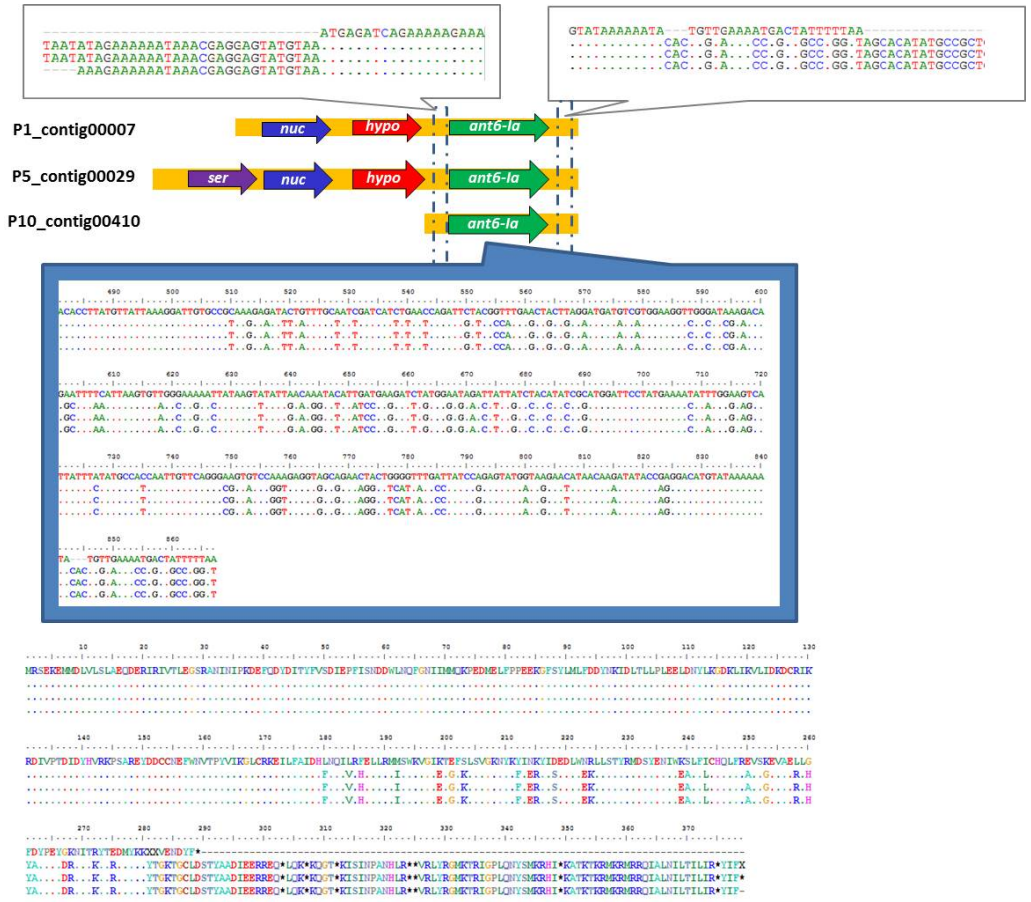

Figure 5.11 The alignment of ant6-Ia among p1, p5 and p10 with Bioedit

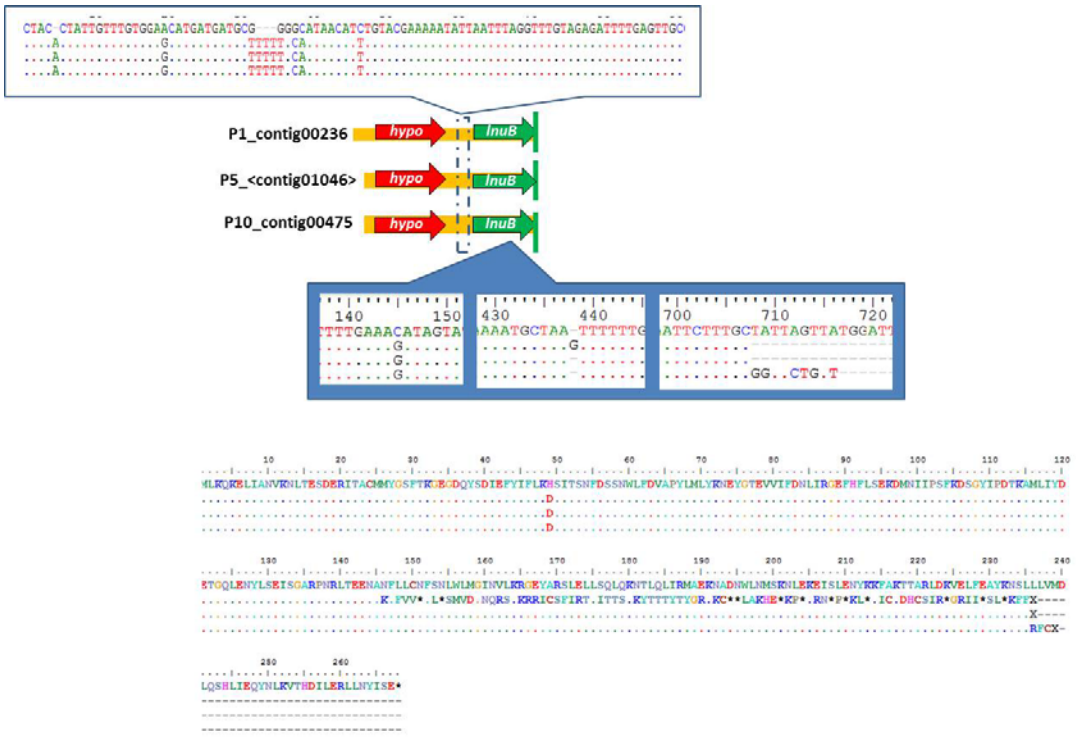

Figure 5.12 The alignment of *lnuB* among p1, p5 and p10 with Bioedit

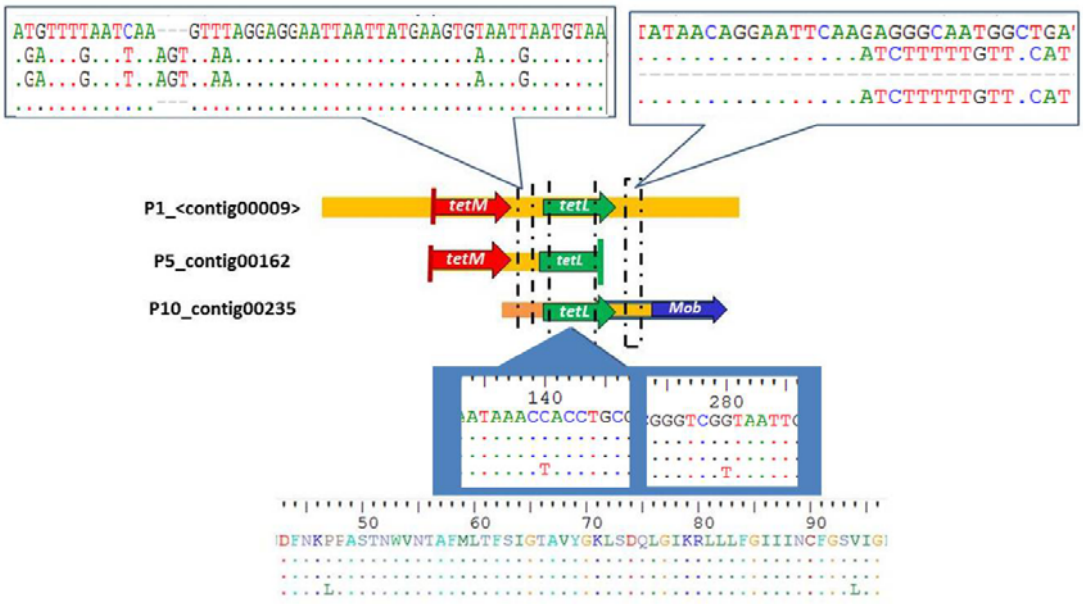

Figure 5.13 The alignment of *tetL* among p1, p5 and p10 with Bioedit

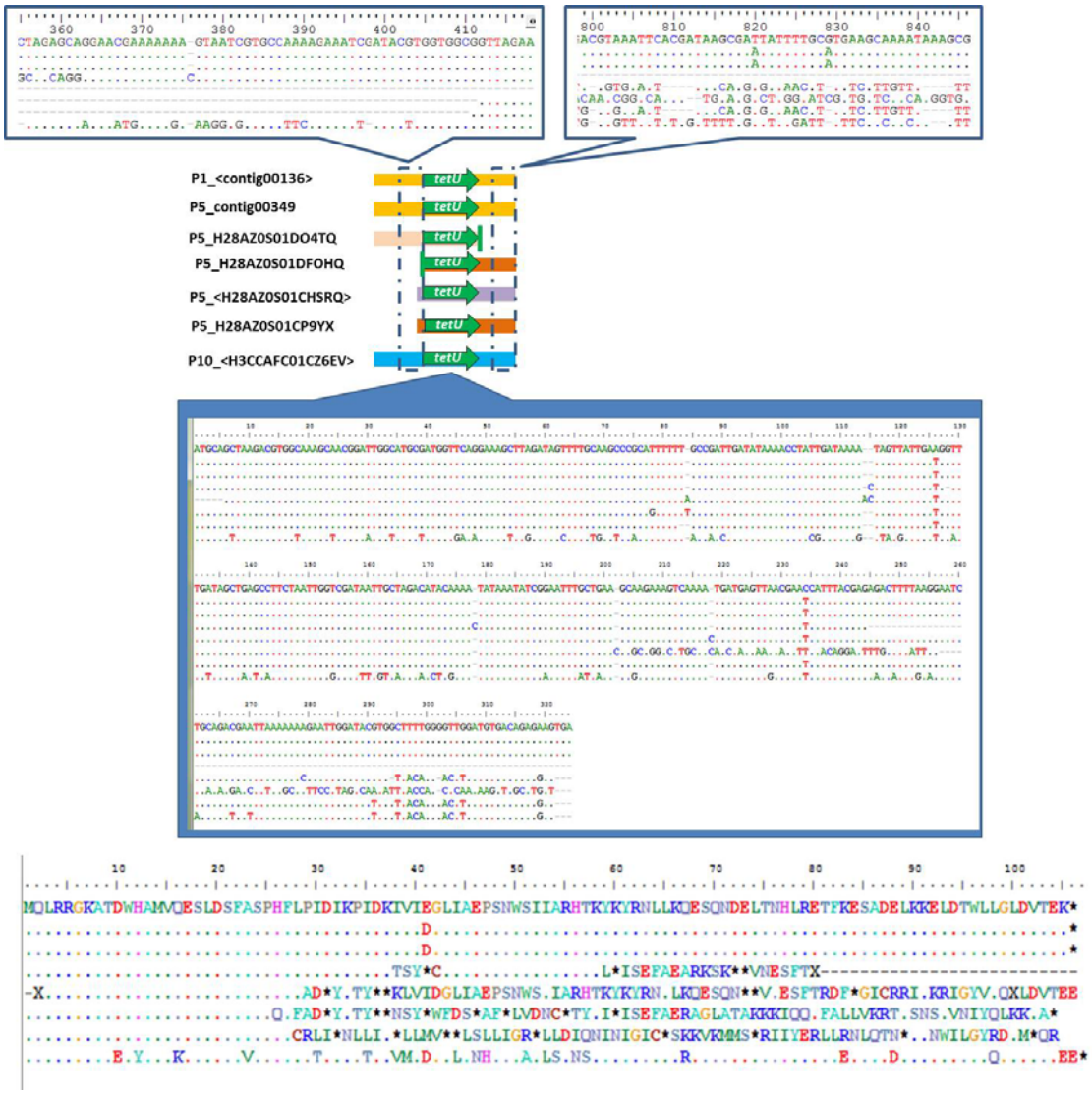

Figure 5.14 The alignment of *tetU* among p1, p5, and p10 with Bioedit

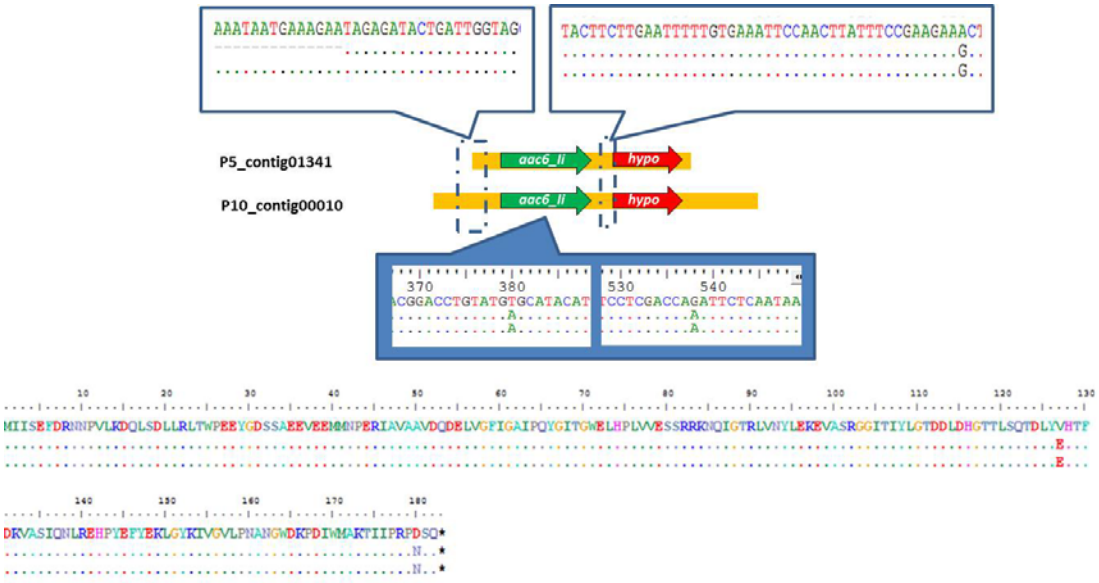

Figure 5.15 The alignment of *aac6-Ii* among p5 and p10 with Bioedit

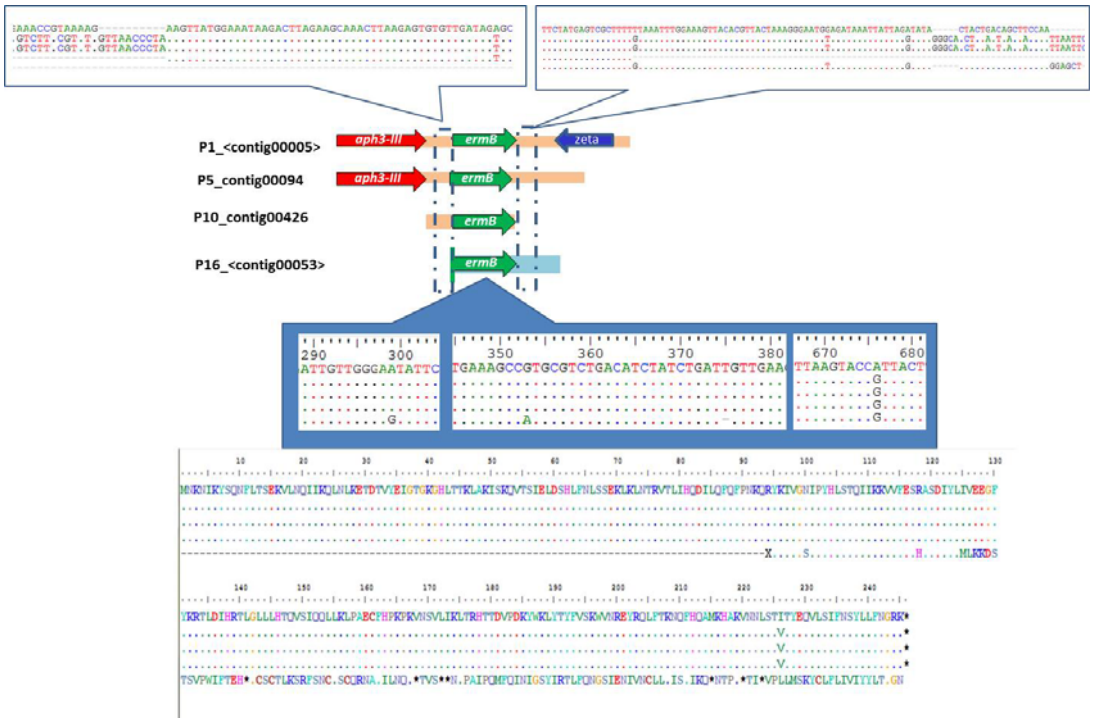

Figure 5.16 The alignment of *ermB* among p1, p5, p10, and p16 with Bioedit

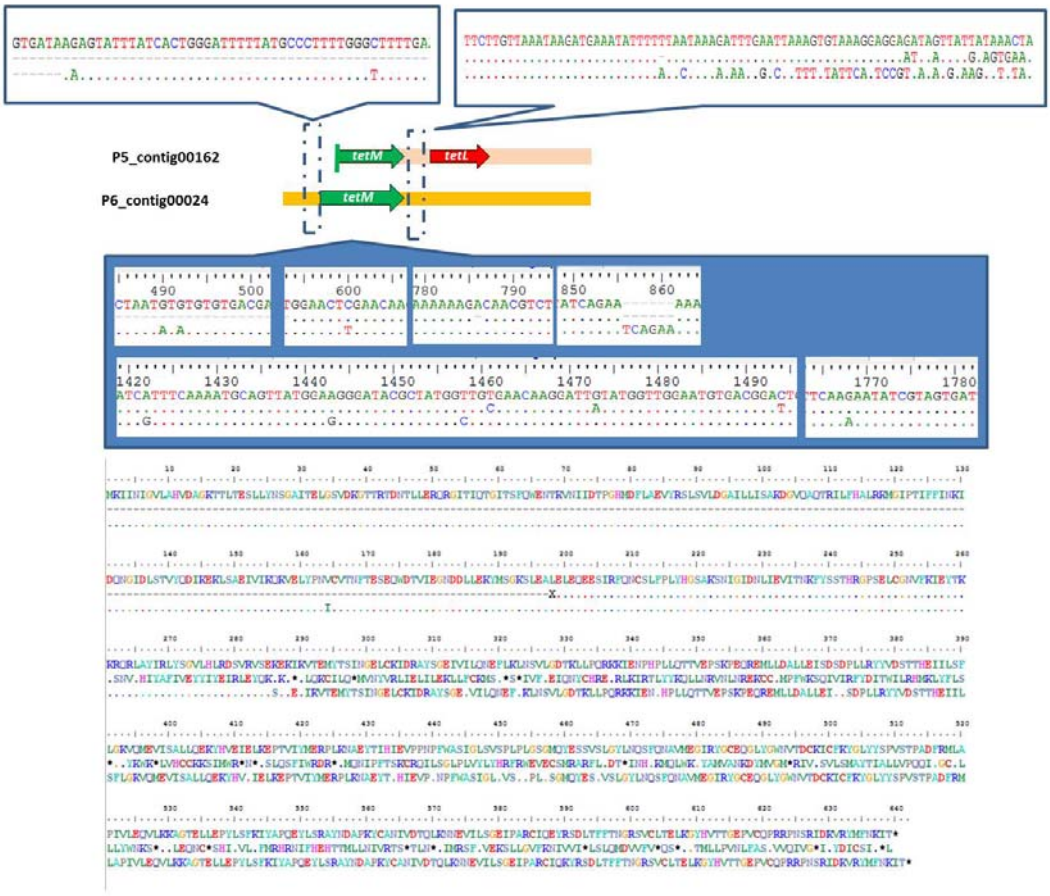

Figure 5.17 The alignment of *tetM* among p5 and p6 with Bioedit

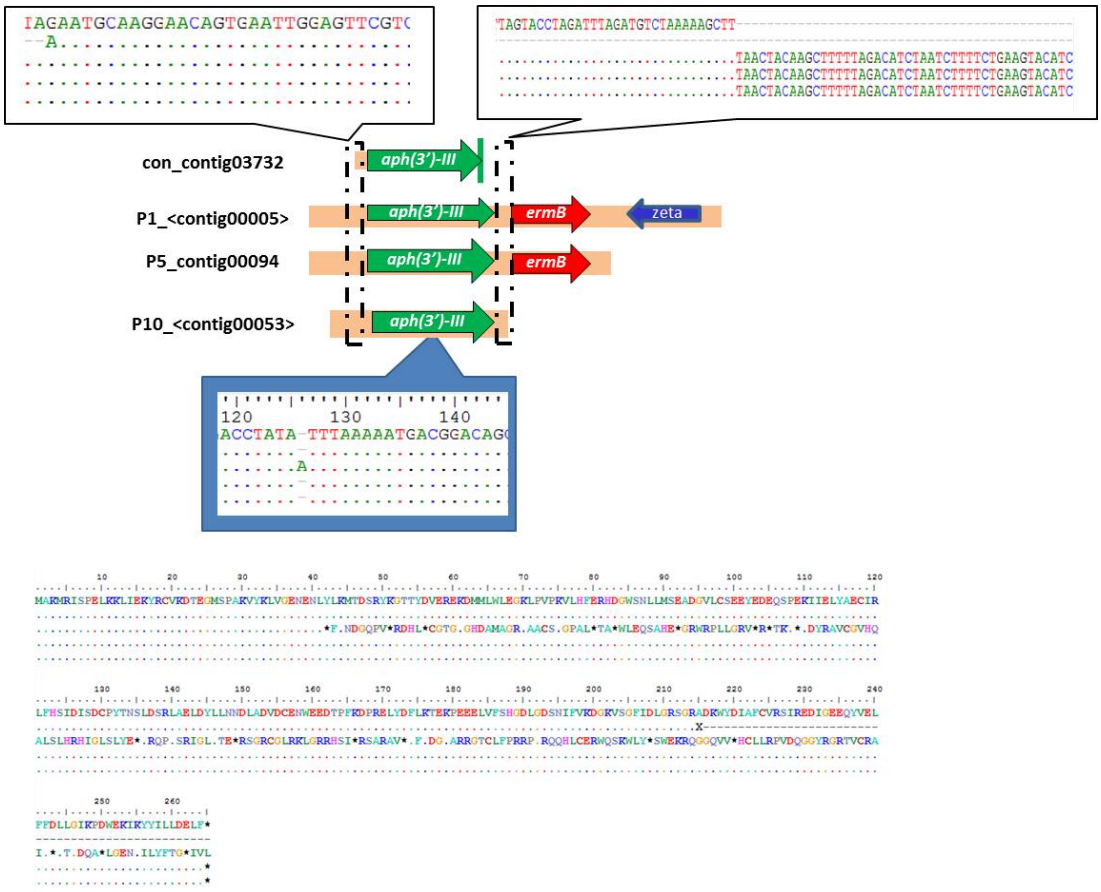

Figure 5.18 The alignment of *aph(3')-III* among control, p1, p5, and p10 with Bioedit

5.8 The relation of resistance genes among hospitalize patients' gut microbiome

The similarity of resistance genes among all patients and control were related into the diagram in the Figure 5.19. Thick line means the sharing resistance from the same origin, dashed line means the sharing resistance genes from the different origins, and the yellow balloon means the individual resistance genes. About the sharing resistance genes from the same origin, *ant(6)-Ia*, *ermB*, *lnuB* and *tetL* conferring resistance to aminoglycosides, lincosamide, macrolides, streptogramin B and tetracycline, spread among p1 and p5 from the general medicine ward and p10 from the ICU medicine ward. *aac(6)-Ii*, which is responsible for aminoglycosides resistance, was shared between p5 and p10. *tetU* conferring tetracycline resistance was identical between p1 and p5. Interestingly, *aph(3')-III* was shared among p1, p5, p10 and control.

About the sharing resistance genes from the different origins, *ermB* variant can be found in p16 from the ICU medicine ward that differed from *ermB* of p1, p5 and p10. *tetM* of p5 was different from that of p6 that stayed in the same ward. Also, several characters of *tetU* were found in the same patient, p5, and between different patients in the same ward, p1 and p5, and between different patient in the different wards, p1, p5, and p10.

About the individual genes, *catS*, *tetO*, and *tet40* conferring chloramphenicol and tetracycline were owned by control. p1 belonged to *msrC* being responsible for macrolide, lincosamide and streptograminB resistance. There were several resistance genes in p4 including *aac(6')-Ib* or *aac(6')-Ib-cr*, *arr-3* or *arr-6*, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-124}}$, *catB3*, *dfrA5*, *fosA* and *sul1* conferring resistance to aminoglycoside, rifampicin, beta-lactam, chloramphenicol, trimethoprim, fosfomycin and sulphonamide. *catA2* and *dfrA12*, which conferred resistance to chloramphenicol and trimethroprim, were found in only p6. P10 owned *aac(6')-aph(2'')* and *dfrG*, which were responsible for aminoglycoside and trimethroprim resistance. P16 had the individual *tetW* conferring tetracycline resistance.

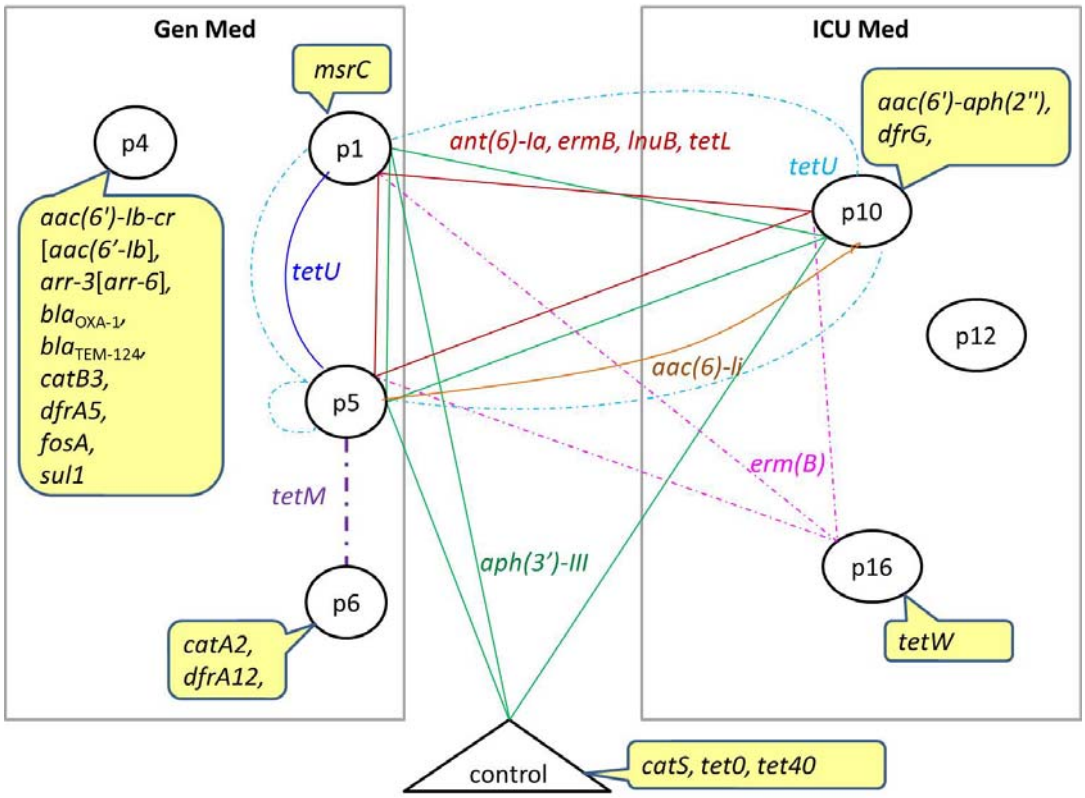

Figure 5.19 The linkage of resistance genes among patients and the healthy human. There were 3 categories. Firstly, solid line represented the spread of the similar resistance genes with similar flanking regions. Secondly, dash line represented the spread of the similar resistance genes with different flanking regions. Last, the genes in yellow boxes meant the individual resistance genes.

5.8 Association of plasmid types and resistance genes

To find correlation between plasmid types and their carried resistance genes, this study searched the sequences that carried both resistance genes and plasmid-typing sequences. Rep typing corresponding incompatibility groups of plasmids were used for this objective. In this study, there were 22 rep types including A/C, HI1, HI2, I1, FIA, FIB, FIC, FIIA, FIIS, FrepB, K, K/B, L/M, N, B/O, P, Q, T, U, W, X, and Y. However, none containing both rep types target and resistance gene on the same sequences. Therefore, this study could show only plasmid types and resistance sequences in the DNA pools, and previously reported resistance genes of each plasmid types as shown in table 5.10. It was shown that almost present resistance genes had never been reported in each plasmid types, except for *aac(6')-Ib-cr* of p4 which have been identified in rep FIA, FIB, FIIA, HI2, and N plasmids.

Table 5.10 Resistance genes and plasmid type with rep typing

ID	rep_type	No_read	Previous-report resistance gene association	Present resistance_gene
Control	Q	2	*bla*$_{CTX-M-53}$, *qnrS2, aac(6')-Ib, bla*$_{OXA}$	*aph(3')-III, catS, tetO, tet40*
	HI2	2	*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *armA*, *qnrA1*[*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6')-Ib-cr*], *bla*$_{CMY8}$, *cat, tetDCAR, aphA*	
	FIIS	1	N/A	
p1	HI1	1	*bla*$_{TEM-1}$, *tetARCD*	*ant(6)-Ia, aph(3')-III, ermB, lnuB, msrC, tetL, tetU*
p4	HI1	148	*bla*$_{TEM-1}$, *tetARCD*	*aac(6')-Ib* or *aac(6')-Ib-cr*, *arr-3* or *arr-6*, *bla*$_{OXA-1}$, *bla*$_{TEM-124}$, *catB3, dfrA5*, *fosA, sul1*
	HI2	637	*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *armA*, *qnrA1*[*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6')-Ib-cr*], *bla*$_{CMY8}$, *cat, tetDCAR, aphA*	
	FIIA	29	*armA*, *qnrB4* [*armA*, *bla*$_{CTX-M-14}$, *bla*$_{DHA-1}$, *bla*$_{SHV-12}$, *aac(6')-Ib-cr*	
	N	278	*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$[*bla*$_{TEM-1}$, *aac(6')-Ib-cr*], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA, qnrA3, qnrB2* [*bla*$_{CTX-M-3}$, *aac(6')-Ib-cr*], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1*[*lap-2*, *aac(6')-Ib-cr*], *bla*$_{OXA}$, *aadA1, sul1, tetAR*	
	FIB	1	*bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6')-Ib-cr*], *bla*$_{SHV-2}$, *bla*$_{SHV-12}$, *bla*$_{TEM-1}$	
	FIC	2	*aac(3')-IV*	
	FIA	37	*bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6')-Ib-cr*], *bla*$_{DHA-1}$, *bla*$_{TEM-1}$, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6')-Ib-cr*], *qnrB4* [*armA, bla*$_{CTX-M-14}$, *bla*$_{DHA-1}$, *bla*$_{SHV-12}$, *aac(6')-Ib-cr*	
	U	200	*qnrS2, sul1, aadA2, tetAR*	
	W	108	*bla*$_{VIM-1}$, *sul1*, *dfrB2*	
	FrepB	31	*bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-3}$, *cat, aad, sul, tet*	
	FIIS	131	N/A	
p5	HI1	12	*bla*$_{TEM-1}$, , *tetARCD*	*aac(6')-Ii, ant(6)-Ia, aph(3')-III, ermB, lnuB, tetL, tetM, tetU*
	HI2	15	*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *armA*, *qnrA1*[*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6')-Ib-cr*], *bla*$_{CMY8}$, *cat, tetDCAR, aphA*	
	N	8	*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$[*bla*$_{TEM-1}$, *aac(6')-Ib-cr*], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA*, *qnrA3*, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6')-Ib-cr*], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1*[*lap-2, aac(6')-Ib-cr*], , *bla*$_{OXA}$, *aadA1, sul1, tetAR*	
	U	1	*qnrS2, sul1, aadA2, tetAR*	
	W	1	*bla*$_{VIM-1}$, *sul1*, *dfrB2*	
	FrepB	1	*bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-3}$, *cat, aad, sul, tet*	
	FIIS	4	N/A	

Table 5.10 Resistance genes and plasmid type with rep typing (continued)

ID	rep_type	No_read	Previous-report resistance gene association (62, 89)	Present resistance_gene
p6	HI1	12	*bla*$_{TEM-1}$, *tetARCD*	*catA2, dfrA12, tetM*
	HI2	37	*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *armA*, *qnrA1*[*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6')-Ib-cr*], *bla*$_{CMY8}$, *cat, tetDCAR, aphA*	
	I1	1	*dfrA1, aadA1, aadB, aadA2, bla*$_{CMY2}$, *bla*$_{CMY7}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, bla$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, bla$_{CTX-M-15}$[bla$_{TEM-1}$, aac(6')-Ib-cr], *bla*$_{CTX-M-24}$, *bla*$_{SHV-12}$, *bla*$_{TEM-1}$, *bla*$_{TEM-52}$, *bla*$_{VIM-1}$, *mphA*, *qnrA1*[*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6')-Ib-cr*], *aph*	
	N	26	*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$[*bla*$_{TEM-1}$, *aac(6')-Ib-cr*], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA*, *qnrA3*, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6')-Ib-cr*], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1*[*lap-2, aac(6')-Ib-cr*], *bla*$_{OXA}$, *aadA1, sul1, tetAR*	
	FIB	1	*bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6')-Ib-cr*], *bla*$_{SHV-2}$, *bla*$_{SHV-12}$, *bla*$_{TEM-1}$,	
	FIC	3	*aac(3')-IV*	
	FIA	4	*bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6')-Ib-cr*], *bla*$_{DHA-1}$, *bla*$_{TEM-1}$, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6')-Ib-cr*], *qnrB4* [armA, *bla*$_{CTX-M-14}$, *bla*$_{DHA-1}$, *bla*$_{SHV-12}$, *aac(6')-Ib-cr*	
	U	7	*qnrS2, sul1, aadA2, tetAR*	
	W	2	*bla*$_{VIM-1}$, *sul1*, *dfrB2*	
	FrepB	2	*bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-3}$, *cat, aad, sul, tet*	
	FIIS	11	N/A	
p6enz	HI1	5	*bla*$_{TEM-1}$, *tetARCD*	N/A
	HI2	33	*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *armA*, *qnrA1*[*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6')-Ib-cr*], *bla*$_{CMY8}$, *cat, tetDCAR, aphA*	
	N	24	*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$[*bla*$_{TEM-1}$, *aac(6')-Ib-cr*], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA*, *qnrA3*, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6')-Ib-cr*], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1*[*lap-2, aac(6')-Ib-cr*], *bla*$_{OXA}$, *aadA1, sul1, tetAR*	
	FIA	1	*bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6')-Ib-cr*], *bla*$_{DHA-1}$, *bla*$_{TEM-1}$, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6')-Ib-cr*], *qnrB4* [armA, *bla*$_{CTX-M-14}$, *bla*$_{DHA-1}$, *bla*$_{SHV-12}$, *aac(6')-Ib-cr*	
	U	1	*qnrS2, sul1, aadA2, tetAR*	

Table 5.10 Resistance genes and plasmid type with rep typing (continued)

ID	rep_type	No_read	Previous-report resistance gene association	Present resistance_gene
p10	N	2	*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$[*bla*$_{TEM-1,}$ *aac(6')-Ib-cr*], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA*, *qnrA3*, *qnrB2* [*bla*$_{CTX-M-3,}$ *aac(6')-Ib-cr*], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1*[*lap-2, aac(6')-Ib-cr*], *bla*$_{OXA}$, *aadA1, sul1, tetAR*	*aac(6')-aph(2"), aac(6')-Ii, ant(6)-Ia, aph(3')-III, dfrG, ermB, lnuB, tetL, tetU*
p12	N	1	*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$[*bla*$_{TEM-1,}$ *aac(6')-Ib-cr*], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA*, *qnrA3*, *qnrB2* [*bla*$_{CTX-M-3,}$ *aac(6')-Ib-cr*], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1*[*lap-2, aac(6')-Ib-cr*], *bla*$_{OXA}$, *aadA1, sul1, tetAR*	N/A
p16	n/a		n/a	*ermB, tetW*

CHAPTER VI
DISCUSSION

Nowadays, infections with MDR pathogens bring the terrible problems into a health care system. Theoretically, resistance genes can be commonly exchanged among pathogens, environmental bacteria and normal flora. Crucial factors for resistance gene transfer among them are antimicrobial drug usage, gene transfer mechanism, and reservoir of resistance genes. According to natural selection theory, antimicrobial drugs are probably the selective pressure for resistance genes. The more antimicrobial drugs are used, the more resistance genes are forced to be occurred by mutation, and especially transfer. Of three resistance gene transfer mechanisms, transformation, transduction and conjugation, conjugation by plasmid is the most common tool for resistance gene transfer. If multidrug resistance pathogens carrying resistance plasmids confront with microbiome, resistance genes should be easily transferred to the bacterial community. In the past, several studies indicated the evidence of resistance gene emerging after antimicrobial drug treatment, the carrying of resistance genes or resistance mobile genetic elements by plasmid, and the collection of drug resistance genes in some bacterial community such as oral and gut microbiome of healthy humans (10, 12, 14, 76). Interestingly, gut microbiome should become the abysmal depth of resistance gene pools that most closed to human. However, resistance plasmids have never been studied among gut microbiome in hospitalized patient under antibiotics treatment condition; also, the linkage of plasmid types that is responsible for resistance gene spread have never been revealed. The outcomes of these perspectives can determine the basic knowledge of resistance plasmid study and can assist heath care workers to plan some approach to control antibiotic resistance gene spread in the hospital. This study has been designed to the pilot scale, and has initiated with sixteen patients from General Internal Medicine ward (8 patients) and Internal Medicine Intensive Care Unit (8 patients), Siriraj Hospital. They passed inclusion criteria including admission more than 7 days,

treatment with more than 2 groups of antimicrobial drugs, and infection with at least one multidrug-resistance bacteria. These criteria reflect the real situation under the selective pressure of drugs and the source of resistance genes in hospital. Also, this study has required to test the importance of antibiotics for resistance genes selection, so one healthy subject chosen as control did not treated with antimicrobial drugs and did not admitted in hospital for three year. Although no study indicated the persistent time of drug-resistant bacteria, one report showed that resistance clone survived at least 18 months (114). 5-10 grams of stools from all samples have been collected. The study has taken bacterial cells from stools as the representative of gut microbiome. The previous studies showed that approximately 45.1% of dry weight and 10% of wet weight of stools was bacterial cells, and its density is about 10^{12} cells/ml (82, 115). If we use 5 grams of wet stool, approximately 0.5 grams or 10^{11} cells of bacteria will be collected. Then, bacterial cells have been separated from food sediments with low-speed centrifugation and human cell lysis with purified water. This method is good enough to purify bacterial cells because the result has showed few contaminated human chromosome.

After the complete sampling, the experimental design that proves the aims of this study is divided into five parts. They are to extract and purify plasmid, to identify DNA types of output from plasmid extraction, to reveal drug resistome collected into gut microbiome, to determine the molecular epidemiology of resistance gene spread, and to establish the association of drug resistance genes spread and plasmid types.

Firstly, plasmid should be most purified in order to prove the aim of study. Commonly, plasmid extraction is always contaminated with chromosomal DNA, and some methods help plasmid to more refine such as CsCl-EtBr gradient centrifugation. This study used three logical pathways to extract and purify plasmid namely alkaline lysis and chromatography purification method (Maxi-plasmid preparation kit) plus CsCl-EtBr gradient ultracentrifugation, Maxi-plasmid preparation set plus linear DNA digestion with lambda exonuclease and exonuclease I plus DNA amplification with phi29 DNA polymerase, and only alkaline lysis plus chromatography purification method. Therefore, all methods have been tested in order to find the best method to purify plasmid for this study.

Commonly, plasmid extraction confers plasmid DNA and contaminated chromosome. Contaminated chromosome is linearized DNA whereas plasmid is closed-circular DNA. With alkaline lysis method, strong base destroys base pairing of both chromosomal and plasmid DNA. DNA strand of chromosome can be separated, but that of closed circular plasmid cannot be sequestered because of the structural mesh. When pH is neutralized, plasmid DNA can be renatured, but linear chromosome cannot. However, some chromosomal DNA can be done. The output from alkaline lysis can be contaminated with other impurity such as salt, SDS and protein. Anion exchange chromatography was fused with alkaline lysis in this study in order to purify DNA from other. This chromatography exchanges interesting molecules that had negative charge such as DNA with its negative charge molecules, and the required molecules are eluted with solution (20). In the result, reference plasmid (pUCP24) was presented three sharp bands of plasmid forms and smear band of contaminated chromosome as expected. In contrast, gut plasmid showed only smear bands. It is probable that plasmids of gut bacteria had the variation of size and copy number, and their smear band was mixed with contaminated chromosome.

CsCl-EtBr gradient centrifugation is the traditional method to separate closed circular DNA from linear or nicked circular DNA. With high gravity force, CsCl is parted, and heavy Cs^+ diffuses towards to the bottom, and constructs gradient condition. EtBr intercalates into DNA molecules and twists DNA to have more superhelix density. In this study, this method has been used to combine with alkaline lysis and chromatography to purify closed-circular plasmid. The result has been shown that mixed plasmid (resistance plasmid from five bacterial isolates) from plasmid maxi-preparation can be separated into two thick bands. The upper is chromosomal and linearized DNA, and the lower is closed circular plasmid. However, gut plasmid did not act as reference plasmid. It displays only one thick band, and faint smear bands. These evidences have been reported in other studies; for examples, G. D. Clark Walker revealed that two closed-circular bands from mitochondrial fractions were separated from linear DNA band from nucleus of yeast. The first closed-circular DNA having a density of 1.684 g/cm^3 was characterized as mitochondrial DNA whereas the other having a density of 1.701 g/cm3 was supposed that belonged to other type of mitochondria or peroxisome in yeast (21). In congruent with the other study, the

closed-circular DNA were constructed to have four different initial superhelix densities, and each was separated one another by CsCl-EtBr gradient centrifugation because the initial superhelix density affected the saturation of intercalated EtBr into DNA strand, and this made the DNA density different at the separated point (116). For our gut plasmids, it is possibly that they had the different superhelix densities, but they are not visualized because gut plasmids have much several types and each has too low amount to be seen. It is concordant with the appearance of gut plasmids from the step of plasmid purification by anion exchange chromatography.

Last method used to combine with plasmid maxi preparation was enzymes treatment. The required enzyme should digest only chromosomal DNA that is linear form. Lambda exonuclease can perform the elimination of linear DNA, but did not digest nicked and closed circular DNAs that are plasmid (22). The result has shown that lambda exonuclease can destroy digested pUCP24 (the band in 4,036 bps position), and does not manipulate other forms of undigested pUCP24. It has been indicated that lambda exonuclease can eradicate linear DNA. However, the digested pUCP24 remain because of uncompleted reaction of too low amount of lambda exonuclease. In the same manner, gut plasmid after lambda exonuclease intervention was decreased as the smear band was faint. Since by-product from lambda exonuclease treatment is single-stranded DNA from linear chromosomal DNA (22), exonuclease I was used for ssDNA digestion in order to clear chromosomal DNA completely. Exonuclease I attacks at 3′-hydroxyl group and splits each mononucleotide, but it has a little endonuclease activity (23). After the exonuclease I treatment, the result has shown that most bands of digested pUCP24 were disappeared, but two bands of undigested DNA that were supercoiled and homopolymer DNA were still present. It has been determined that exonuclease I degrades single-stranded DNA. It is possible that they have been lost by exonuclease I because of remaining endonuclease activity of the enzyme (23).

Plasmid products from DNA purification methods have been brought into high-throughput sequencing steps. However, plasmids from chromatography plus CsCl-EtBr gradient centrifugation method have been discarded, because this method did not help eliminate chromosomal DNA. In addition, plasmid from chromatography plus enzyme treatment should be increased because the amount of plasmid is too low

to be performed high-throughput sequencing. Phi29 DNA polymerase is beneficial for DNA amplification. The enzyme uses rolling-circle amplification like the natural replication of circular plasmid (24). The result showed that both amplified pUCP24 and gut plasmid were concatenated DNA. This is the characteristic of amplification with phi29 DNA polymerase because of its multiply-primed rolling circle amplification. It is pointed that gut plasmids have been raised by this enzyme. Although phi29 DNA polymerase is selective for the amplification of circular DNA template, linear chromosomal DNA were still amplified by this enzymes (24). After sequencing, the data has shown that this enzyme amplified more redundant positions that biased to represent the gut plasmid population. It is concordant with the previous study that phi29 DNA polymerase tended to amplify small circular DNA more than large circular DNA (107, 111). However, this study did not evaluate the size of plasmids.

There were three plasmid-purification methods, which were plasmid extraction by an anion-exchange chromatography alone, an anion-exchange chromatography followed by enzymatic treatments, and chromatography plus CsCl-EtBr gradient ultra-centrifugation. The chromatography method alone has been selected for further study step, as the plasmid purification by chromatography plus CsCl-EtBr gradient did not make better purified plasmids. Plasmid DNA have been sequenced with GS Junior pyrosequencing platform, because *454* sequencing has been shown to give the lowest error rate (0.1%), and its longer reads help assembly process better, which is beneficial for metagenome construction (100-103). In this study, after sequencing, the amounts of sequencing output have been very different. It could be resulted from several reasons, for examples, the length of clonally amplified templates, the titration in emPCR step, and number of raw wells used for analyses. The average read lengths of p1, p4, p6 with enzyme treatment (p6enz), p12, and p16 were lower than 400 bps in concordant with the output of these samples that were lower than 20 Mb. In an emPCR step, DNA was calculated that how many DNA molecules would yield the single clonally amplified DNA per one bead. If number of DNA molecules is too high or too low, mixed or dot wells types of errors will be resulted, respectively. The table 5.2 shows that samples using calculated and selected copy numbers of 2, 4, and 6 DNA copies from titration had more than 10% of mixed or dot well whereas

sample using 3 copies from titration had less than 10% of mixed and dot wells. About the number of raw wells in sequencing step, control and p10 samples having the high outputs had approximately 50% of raw in total wells, while the other samples giving the low outputs had less than 40% of raw in total wells.

In data analysis, they have firstly been classified into known and unknown sequences by percent of alignment length as cut-off. The total sequences have been performed by blastn with non-redundant nucleotide database in NCBI. Blastn has been chosen to search the homologous sequences, because it covers for both intra and inter species comparison (17). The results from blastn have been chosen, if it has the highest bit-score. The bit score is important, because it reflects the length of the hits (117, 118). After that, the percent of alignment length has been calculated from the aligned length divided by the total length of query. The percent of alignment length has been plotted with histogram and cumulative distribution curve as shown in figure 5.5. The highest data was at more than 90% of alignment length. The sequences should have the long coverage, if they have been reported in the database. Also, the error from homopolymer can be found, such as 3.08%, 4.06%, and 4.85% errors of tetramer, pentamer, and hexamer, respectively (119). If sequences have longer homopolymer, the error rates is prone to occur more often. Therefore, this study has used 80% of alignment length, which should be good enough to be the cut-off. Interestingly, the result has shown that most samples had the percent of unknown sequences more than that of known sequences. Twelve unknown sequences were randomly chosen to map with non-redundant nucleotide sequences, and there were several types of mapping including matching in continuous parts, matching with patched characteristic and unreported alignment. The percent of alignment length of known and unknown sequences were related to the average read length. It is possible that short read length may cause the error of assembly more often than long read length, and it affected alignment length. However, the result has indicated that read length did not affect the percent alignment length. It is possible that these unknown DNA came from uncultured gut bacteria or unreported species. In congruent with the previous study, 78.8% of unseen DNA has been disclosed in metagenomics in gut bacteria of 124 health subjects (120).

To categorize the DNA types of DNA pool, the known sequences from blastn were subdivided into DNA type profiles to scheme the composition of DNA pool from plasmid purification methods. Gratefully, very few human DNA were contaminated in DNA pool by plasmid purification both with and without enzyme treatment. This study has used the reverse osmosis water to lyse human cell because we anticipated that bacterial cells tolerate to water than human cells can. In fact, bacteria usually confront with osmolarity changes, and they overcome these calamity by had several mechanisms including the solute collection under hypertonic condition and the solute efflux under hypotonic status, although water channels or aquaporins that transport water into and out of the cells were found in all organisms including bacteria and human (121, 122). In other gut microbiome studies, they have not separated bacterial cells from stool and have used DNA extraction stool kit or lysed bacterial cells directly (123-125). These previous methods were not suitable for this study, because they caused enormous contamination with chromosomal bacterial and human DNA. In concordant with one study that compared four commercial kits and one manual method for bacterial DNA extraction from human stool, the results showed that each method was sensitive differently in Gram-positive and Gram-negative bacteria, and the bacterial DNA from different patients were dissimilar with the same method (126). In addition, our result showed that known DNA from plasmid purification without enzyme treatment mainly belongs to chromosomal DNA, whereas that from the method with enzyme treatment was more favorable to plasmid DNA. It is indicated that lambda exonuclease can eliminate most linear chromosomal DNA. However, the method with enzyme treatment had one weak point. Their plasmid output had too much depth of coverage. This result came from the effect of phi29 DNA polymerase that was discussed before (107, 111). Moreover, among total DNA sequences, unknown sequences were the most enormous parts of DNA pool. It should be studied further about the types of unknown sequences.

To identify drug resistome collected in human gut microbiome, ResFinder and Pfam database have been used to perform the identification. ResFinder is the database of acquired resistance genes that specifies with reported resistance genes transferred by plasmids, whereas Pfam is protein family database that is suitable for both reported and unreported resistance genes (18, 19). The results from ResFinder

were repeatedly checked with blastn in NCBI as gold standard. The specificity of ResFinder was 100%, where it was compared with blastn (data not shown). The result has shown that the drug resistomes from patients were responsible for most drug resistance including tetracycline, macrolide, lincosamide, streptogramin B, beta-lactams, sulfonamide in enteric bacteria like previous studies (14, 76, 85, 127, 128). Noteworthy, this study has revealed the increased antibiotic resistance genes including fluoroquinolone, rifampicin, fosfomycin and trimethoprim. It is possible that these resistance genes has just emerged, or has been found by the methods in this study. Moreover, resistance genes including tetracycline, aminoglycoside and chloramphenicol resistance genes can be found in the healthy subject. It is probably that these resistance genes were transferred by plasmids among bacteria between hospitals and communities. Furthermore, some acquired resistance genes or proteins do not relate with antibiotic usage. It was probably resulted from a few reasons. Firstly, linkage disequilibrium of resistance genes caused the transfer of linked gene cassettes or the recombination of sequences of mobile genetic elements (129). For another reason, antimicrobial drugs might select the resistant bacteria that had mutations on the chromosome, such as, target gene changes that could not be discovered by this study approach.

For Pfam analysis, translated-protein sequences from more than 100 nucleotide bp have been aligned with protein family databaseby e-value was equal to or less than 1 (120). We cannot know an optimal e-value to be used as a cut-off in this study. The e-value and frequency of total results from Pfam alignment have been plotted with histogram as shown in figure 5.8. The figure shows the distinguished peak for e-value more than 10^{-2} to 1. It is possible that there were mistaken alignments because of too high e-value. Therefore, Pfam has been tested for the specificity by using resistance genes analyzed with ResFinder as gold standard. The results showed that e-value being less than 10^{-6} was appropriate to be a cut-off value, because specificity was nearly highest and few true-aligned hit was lost. Resistance protein sequences have been chosen with resistance protein family index that was constructed by pfam data. This index has collected only resistance proteins that were not the changed target mechanism. This is the good point because resistance proteins involving target change always locate on chromosomal DNA. Total resistance genes

were about 1% of total genes and most of them belonged to unknown hits. It is indicated that resistance genes were possibly pooled in uncultured or unknown gut bacteria.

When we have compared the pfam results of DNA between plasmid purification with and without enzyme treatment from the same patient, more resistance translated proteins from plasmid purification without enzyme treatment have been found than that with enzyme treatment. It is indicated that resistance genes may be carried on chromosomal fragments, sheared large plasmids, large circular plasmid because lambda exonuclease digests lineared DNA and phi29 DNA polymerase biased to amplified closed circular DNA (22, 107, 111). In congruent with the other result, by plasmid purification without enzyme treatment, when predicted resistance proteins from known hits were matched with DNA types, most were carried on genomic DNA that could not be determined to be chromosomal or plasmid DNA.

Moreover, predicted resistance proteins have been subcategorized into antimicrobial drug classes. Predicted resistance proteins were responsible to the resistance of several antimicrobial drug classes including treated and non-treated antimicrobial drugs. For examples, p4, who has been treated with beta-lactams and aminoglycoside, had multiple drug resistant genes to both used drugs and other unused drugs including glycopeptides, tetracyclins, macrolides, chloramphenicol, trimethoprim, bacitracin, macrolide, lincosamide, streptogramin B, fusiric acids, rifamycins, and polymyxins. Surprisingly, several resistance proteins were also found in the healthy subject. It is possible that these similar genes are community-acquired drug resistance proteins, which is described in the next paragraph, or they are other genes belonging to the similar protein family such as yeast casein kinase and aminoglycoside phosphotransferase APH(3′)-IIIa, D-Ala-D-Ala ligase DdlB and D-Ala-D-Lac ligase VanA, and *Streptomyces* DD-peptidase and TEM-1 β-lactamase (52). Although Pfam possibly caused some errors, it might predict unreported resistance genes.

From the resistance profile from ResFinder and Pfam, the resistance genes from ResFinder have been chosen to determine the molecular epidemiology of resistance plasmids among gut microbiomes in hospitalized patients because of the more precise results of these database. The resistance genes found were:

1) *bla*$_{\text{OXA-1}}$ expresses class D β-lactamase which has serine residue at active site. It hydrolyzes beta-lactam rings of aminopenicillin and is resistant to clavulanic acid (130). Firstly, it was found on Tn*2603* carried on plasmid in *E.coli*, and had the same recombinational hot spot with *aadA* on Tn*21* (131). Moreover, it was found in NDM-1 producing *K. pneumoniae* ST14 from one patient in India and ST340 from one patient in Oman (132). It was located on the same gene cassette *aac(6')-Ib-cr*, which confer resistance to aminoglycoside and fluoroquinolone, in *E. coli* from patients in Portugal (133). In this study, it was found in gut microbiome of p4 who admitted in General Internal Medicine ward, and it had 99.86 % identity with *bla*$_{\text{OXA-1}}$ in *E. coli* from Canada.

2) *bla*$_{\text{TEM-124}}$ was found in the genomic DNA of *Morganella morganii* from Italy, and was poorly studied. It was in the Class A β-lactamase that was mostly susceptible for clavulanic acid and *bla*$_{\text{TEM-1}}$ that is its precursor is found on plasmid in *E.coli* (134). Like *bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM-1}}$ was found in NDM-1 producing *K. pneumoniae* ST14, and was carried on the same cassette with *aac(6')-Ib-cr* (132, 133). Amino acid substitutions of *bla*$_{\text{TEM-1}}$ at position 104, 164, 238, and 240 confered the ESBL property, and at position 69 and 276 conferred the resistance to clavulanic acid (135, 136). The *bla*$_{\text{TEM-124}}$ changes from *bla*$_{\text{TEM-1}}$ with Q4K, E102K, M180T and amino acid deletion at position 246. Although all substitutions does not probably affect the ESBL property, amino acid change from position 246 may cause the insensitivity to clavulanic acid. In this study, gut microbiome plasmids of p4 who stayed in General Internal Medicine ward had 99.03% with *bla*$_{\text{TEM-124}}$ in *M. morganii* from Italy.

3) *arr3* encodes rifampin ADP-ribosyltransferase confers resistance to rifampicin. Recently, it was also reported on the integrons: *intI-aac(6')-Ib-cr-catB3 and arr3* in clinical *K. pneumoniae* and *intI-aacA4-cr-bla*$_{\text{OXA-1}}$*-catB3-arr3* in *Aeromonas* spp. from wastewater (137, 138). Interestingly, it was also found on pNDM-1-Saitama belonging to IncFII plasmid and carrying *bla*$_{\text{NDM-1}}$, *sul1*, *aph*, and *dfrA14* in *K. pneumoniae* (139). In this study, gut microbiome plasmids of p4 present 100% with *arr-3* in *E. coli* from China; also, had the same identity with *arr-6* in *Pseudomonas putida* from Brazil. The *arr-6* was reported in some integrons: *intI-bla*$_{\text{VIM-2}}$*-aadB-arr-6* in *P. aeruginosa* and integron carrying *bla*$_{\text{KPC-2}}$, *dhfrXVb* and *arr-6* in *P. putida* (140-142).

4) *fosA* is the gene encoding fosfomycin-modifying enzyme. This enzyme transfers metallo-glutathione to C1 of fosfomycin particularly although its structure were similar to glyoxalate I (143). Some studies showed that fosA was present on plasmid in Staphylococci, *E. coli*, and other enteric bacteria from patients (144, 145). In this study, the four genes that were found in gut microbiome of p4 had more than 98% of identity with *fosA* in *Klebsiella* spp. from USA.

5) *aac(6′)-Ii* is the gene encoding aminoglycoside N-acetyltransferase. This enzyme affects amino group on the position 6′, and is responsible for the resistance to tobramycin, netilmicin, sisomicin, dibekacin, amikacin, and kanamycin that two latters were provided in Thai hospitals (146). This gene is found on clinical *E. faecium* chromosome, and mostly related to *aac(6′)-Ia* which was found on Tn*21*-like transposon (147). In this study, *aac(6′)-Ii* together with flanking regions are identical between p5 and p10 that live in different wards, and, compared with *E. faecium* from France, both have one nucleotide substitution that cause amino acid change from valine to glutamine.

6) *aac(6′)-aph(2″)* encodes the aminoglycoside-modifying enzyme that has both N-acetyltransferase and O-phosphotransferase activity. It transfers acetyl group from Acetyl-Co A to the amino group on position 6′, and phosphate group from ATP to the hydroxyl group on position 2″, respectively. This enzyme leads bacteria to be resistant to tobramycin, netilmicin, isepamicin, dibekacin, kanamycin, gentamicin, and amikacin that three latter is used in Thai hospital (146). This enzyme was reported in MSSA, MRSA, coagulase-negative staphylococci, enterococci and *S. agalactiae* from human (148, 149). Also, it was found in *Lactobacillus* and *Pediococcus* from pigs and pet (149). This study show *aac(6′)-aph(2″)* in p10, and it had 100% identity with *E. faecalis* from France.

7) *aph(3′)-III* is the gene encoding aminoglycoside O-phosphotransferase. This enzyme transfer phosphate to hydroxyl group on the position 6′, and is responsible for the resistance to isepamicin, gentamicin B, amikacin and kanamycin that two latter are provided in Thai hospitals (146). This gene often located in *aadE-sat4-aph(3′)-III* cluster into the Tn*5405* , and this cluster sometimes linked with *vanA* and *ermB* gene in *E. faecium* (150, 151). Moreover, *aph(3′)-III* were found on 47.2 kB plasmid in *Campylobacter coli* colonizing in gut (152). This study reveals high similar

aph(3')-III and flanking regions in p1 and p5 in General Medicine ward, p10 in ICU Medicine ward and control.

8) *ant(6)-Ia* (the other name is *aadE*) encodes the aminoglycoside-O-nucleotidyltransferase . It transfers adenosine phosphate from ATP to hydroxyl group of the position C6 of aminoglycoside that correspond to the resistance to kanamycin, lividomycin, neomycin, and amikacin (146, 153). This gene usually arranges in the *aadE-sat4-aph(3')-III* cassette into the Tn*5405* and *aadE-aph(3')-III* into the Tn*5404*, which was able to located on plasmid, and was found in staphylococci, *C. coli* and *E. faecium* from different origins including outpatients, hospitalized patients, pigs, poultry, and sewage (150, 151, 154-157). In this study, *ant(6)-Ia* together with flanking regions were identical among p1, p5 and p10, and had 100% identity with *E. faecium* from Germany.

9) *aac(6')-Ib-cr* is the ciprofloxacin-resistance variant of *aac(6')-Ib,* which encode aminoglycoside acetyltransferase, by W102R and Y179D amino acid changes (158). In the first report, it was located in the integron in pHSH10-2 from clinical *E. coli* in USA, and this integron originated from that in pHSH2 contained *aac(6')-Ib*, $bla_{\text{OXA-30}}$, *catB3*, *arr3*, *qacEΔ1*, *sul1*, *qnrA* and *ampR* (158). In addition, *aac(6')-Ib-cr-*$bla_{\text{OXA-30}}$*-catB3-arr3* was found in *qnrB10*-bearing intergron from clinical enterobacteria in Argentina (159). Moreover, *aac(6')-Ib-cr* was found on the same plasmid with *qnr* and $bla_{\text{CTX-M-24}}$ from *E. coli* and *K. pneumoniae* in China in 2008, and found in *E. coli* plasmid from pigs in the same country in the same year (159-161). This study has revealed *aac(6')-Ib-cr* in p4 from General Medicine Ward with 100% identity with *K. pneumoniae* from Argentina.

10) *ermB* that combined with *ermA* and *ermC* encodes 23S rRNA methylase that confers the resistance to macrolide, lincosamide, and streptogramin B (162). This gene was often reported with mobile genetic elements or gene cassettes. For examples, IS*1216V-ermB-vatE* spread among enterococci from both human and animal origins, *ermB* linked with IS*1182* and *aadE-sat4-aphA3* on Tn*5405* among *E. faecium* from different ecological origins, and *ermB* was found on Tn*1545* and Tn*917* on clinical *S. pneumoniae* (151, 162, 163). Also, this gene was reported in MSSA, MRSA and *E. faecium* from 24 European university hospitals (164). In this study, *ermB* together with flanking regions among p1, p5, p10, p16, and *Enterococcus* sp.

from Switzerland had high homology, but their downstream regions of p16, and *Enterococcus* sp. were different from others.

11) *msrC* is responsible for the resistance to macrolide, lincosamide, and streptogramin B, and encodes the protein that functions as an efflux pump of the ABC transporter family (165). It confers the intrinsic resistance in *E. faecium*, but responds to the acquired resistance in some staphylococci (166). It was reported among *E. faecium* from bovine milk, among enterococci, *Lactobacillus fermentum* and *Pediococcus pentosaceus* from fermented food, and among clinical enterococci, streptococci and staphylococci from different continents (165-169). In this study, *msrC* has been found from p1, and had 99.1% identity with that of *E. faecium*.

12) *lnuB* encodes the lincosamide nucleotidyltransferase enzyme that confer clindamycin resistance (170). It was found in several Gram-positive bacteria including enterococci and streptococci from both animals and patients (170-173). This study has determined the identical *lnuB* and flanking regions among different patients within the same ward, and between the different wards; also, they have had the same substitution when they have been compared with *E. faecium* from France. Although lnuB in patients had more than 99% identity with the reference, their flanking regions have so differed.

13) *tetL* and *tet40* encode the proteins that are the members of 27 different tetracycline efflux pump proteins (7 groups) (174, 175). The channel is composed of the water-filled channel and 9-14 transmembrane helices. *tetL* was found on pTHT15, and classified into Group II whereas *tet40* was not indicated the source, and belonged to Group4 (174). *tet40* was reported that located on plasmid in the organic pig gut bacteria from the United Kingdom; also, *tetL* was found in plasmid among *E. faecalis* and *E. coli* from meat (174, 176, 177). In this study, *tetL* and flanking regions are identical among p1, p5 and p10, and had more than 99% identity with reference *S. agalactiae*. *tetL* of p10 has had two nucleotide subtitutions, and has caused P47L and V94L amino acid change compared with p1, p5 and reference. Besides, *tet40* has been found in control, and had 100% identity with uncultured bacterium from UK.

14) *tetM, tetO, tetW* and *tetU* belong to the same group of ribosomal protection proteins (174). They have the paralogous structure with EF-Tu and EF-G GTPases that are important for translation process (174). *tetW* was reported on the

same plasmid with *tet40* in the organic pig gut microbiome (176). *tetM* was prevalent among both clinical and non-clinical bacteria including *S. agalactiae*, *E. faecalis*, *Neisseria*, *Clostridium*, *Campylobacter*, *Gardnerella*, *Mycoplasma*, and *Ureaplasma*, and found on the plasmids and conjugative transposons in *E. faecalis, Eikenella corredens*, *Kingella denitrificans*, and *N. meningitidis* from intestinal tract (176-179). *tetO* on the similar plasmid was found in *Campylobacter*, *Streptococcus*, and *Enterococcus* from 11 hospitals (180). *tetU* was identified on plasmids from clinical *E. faecium*, and had the highest homology with *tetM* (181). This study showed *tetW* in the individual p16 that had 99.31% homology with *Streptococcus suis* from Italy, and *tetO* in the control that had 99.76% identity with *S. mutans* from USA. *tetM* has been found in p5 and p6; however, they had some differences. *tetM* in p5 has been closest with *E. faecalis* from Switzerland with 99.7% homology, but that in p6 had the highest homology with *S. aureus* from Netherlands. Also, their flanking regions have been more different. *tetU* among p1, p5 and p10 had more than 80% identity with *E. faecium* from USA. *tetU* has been various within p5 and has differed from p6; also, their flanking regions have diverged. However, *tetU* and flanking regions from p1 were identical with one *tetU* variant from p5.

15) *sul1* encodes the sulfonamide-insensitive dihydropteroate synthase variant that is not bound with sulfonamides, because it has the better affinity with PABA (182). It was generally found in class 1 integron with other resistance genes on plasmids in several bacteria including Gram-positive bacteria in animals and Gram-negative bacteria in both the same source and hospitalized human around the world (182-184). In this study, *sul1* is found in one patient in the General Medicine ward, and had 100% identity with that of *E. coli* from France.

16) *dfrA5*, *dfrA12*, and *dfrG* express the trimethoprim-resistance dihydrofolate reductases that have the same characteristics like *sul* gene, but their substrate is the dihydrofolic acid (182). *dfrA5* was reported on the integrons in *Salmonella* spp. and *E. coli* in both human and food animals from Europe (185-187). *dfrA12* was found in the integron on plasmid in pathogenic *Vibrio cholerae* from India, and pathogenic *E. coli* in patients and animals from Korea and USA (188-191). *dfrG* was prevalent on transposable elements among clinical Gram-positive bacteria such as *S. pyogenes*, *S. aureus*, and *Listeria monocytogenes* (192-194). This study has

found *dfrA5* in p4 that has closed with that of *Enterobacteriacae* in Sweden with 100% identity, two genes of *dfrA12* in p6 that has 98.71% and 100% homology with *Salmonella* Typhimurium from Japan, and *dfrG* in p10 is identical with that of *S. aureus* from Japan.

17) *catA2*, *catB3*, and *catS* encode the chloramphenicol acetyltransferase that turn chloramphenicol to 3-acetyl and 1,3 acetyl chloramphenicol (195). *catA2* was reported in $bla_{\text{CTX-M}}$ plasmid from uropathogenic *K. pneumoniae* from USA, in the integron in clinical *Salmonella* Infantis from Spain, and in *E. coli* in chicken from China (196-198). *catB3* was carried with the integron on plasmid among clinical *Enterobacter aerogenes* and *E. cloacae* , the integron on conjugative plasmid IncFI among *Salmonella* Typhimurium from hospitalized patients, and the plasmid in *E. coli* from chicken in Vietnam (199-201). *catS* was found on streptococcal chromosomes and enterococcal plasmids from France(195). In this study, *catB3, catA2*, and *catS* in p4, p6, and control have the 99.75%, 95.86%, and 83.16% identity with that of *Salmonella* Typhimurium from Italy, *E. coli* from UK, and *S. pyogenes* from France, respectively.

When the epidemiological data of resistance genes were considered, resistance genes spread was divided into three groups. Firstly, the individual resistance genes, such as bla_{OXA} and $bla_{\text{TEM,}}$ need more investigation, because sequencing outputs were too low to detect all complete resistance genes. However, some genes such as bla_{OXA} and bla_{TEM} had been found in Siriraj Hospital, and the full resistance gene sequences are needed for comparison. Secondly, the similar resistance genes and flanking regions, such as *ant(6)-Ia* and *ermB*, indicated these resistance genes probably spread in hospital and community, because some resistance genes in hospitalized patients have been reported in both food animals and clinical patients. Investigation in environmental bacteria should be further performed to find such genes in the environment of the community. Also, *aph(3')-III* spread in both patients and healthy volunteer. It meant that it pre-existed in community. Last, the spread of the similar resistance genes that had different flanking regions, such as *aac(6')-Ii* and *tetU*. It meant that these genes spread widely from somewhere before hospitalization.

Finally, resistance genes have been related with plasmid type in order that find the causative plasmid for resistance genes spreading. This study used sequence-

based rep typing that has been applied from rep typing with hybridization and PCR. The sequences of hybridization and PCR target has been chosen to construct sequence-based rep typing. The result has showed that none sequences carried both resistance genes and rep-type sequence. However, some present resistance genes have ever been reported in some rep-types of plasmid that have been found patients such as *aph(3')-III* and repHI2; *aac(6')-Ib-cr* and repHI2, FIIA, N, FIB, FIA; $bla_{\text{OXA-1}}$ and repN; *sul1* and repN and U (62, 89). Obviously, some resistance genes can be found more than one plasmid type, because these plasmids have the overlapping host; for example, repHI, repFI, and repFIIA have the same narrow host range in only *Enterobacteriaceae*, and repN that is boarder host-range plasmid can be transferred into *Enterobacteriaceae*. Although no evidence could prove how plasmids spread resistance genes, several genes had ever been reported in the same gene cassette such as *intI-aacA4-cr-*$bla_{\text{OXA-1}}$*-catB3-arr-3* that might be translocated onto plasmid.

CHAPTER VII
CONCLUSION

In this study, we use metagenomic approach to identify resistance plasmids among hospitalized patients' gut microbiomes. Although resistance gene transfer by plasmid among patients' gut microbiome was unclear, several resistance genes that were reported on mobile genetic elements or plasmids have been present in patients. Some genes, such as *aac(6')-Ii*, were found only in hospitals from other countries, and in this study, have spread among patients. It is probably that someone carrying the genes has been admitted into our hospital and has been the resistance origin. Others, such as *aph(3')-III*, were present in both hospitalized human and animals, and have transferred among patients in this study. It is indicated that this resistance gene was selected under the antimicrobial drug treatment among domestic animals and human. There are the resistance gene transmissions between animals and human, and somebody having resistance bacteria has transferred their genes to hospital, when they have to be admitted. This phenomenon points that we should treat patients with antibiotic wisely and appropriately, and we must control the antimicrobial drug usage in food animals.In addition, the appropriate practice of hand washing and the investigation of resistance gene spread should still continued.

Although the plasmid types may be present with some resistance genes that have been found in this study, the relationship between resistance genes and such plasmid types has not been clearly correlated, because not many sequences have been reported for their co-existent for both resistance genes and plasmid signature. There were some limitations about plasmid purification methods, and high throughput sequencing for gut microbiome in this study. Firstly, the poor plasmid purification produced too much contaminated chromosomes, low amount of plasmid, and plasmid size bias. Gut bacterial community is large and complex, and its plasmids are very various with their properties, such as plasmid superhelical density, copy number of plasmid, and plasmid size. Therefore, gut plasmids were difficult to be separated with the traditional methods including anion-exchange chromatography, CsCl-EtBr

gradient, and enzyme treatment. Secondly, high-throughput sequencing produced low output for gut microbiome. In general, chromosome size of *E. coli* is 4.6 mbp/cell, and the approximately amount of bacterial cells in this study were about 10^{11} cells. It meant that there were 4.6×10^{11} mbps of chromosomal DNA. If *E. coli* contains about 10 kbp/copy of plasmid and has 20 copies, there are 2×10^{10} mbps of plasmid DNA for sequencing. However, no simple and single run of any next-generation sequencing platforms produce high enough output and quality for resistance plasmid metagenomic study in gut microbiome. *454* sequencing had the longest read, but give the lowest output. Illumina had the highest output, but give the shortest read that is not suitable for plasmid assembly. PacBio give longest reads, but had too high error rate. Future study needs better plasmid extraction methods and higher outputs of DNA sequencing to disclose enormous gut microbiome plasmids and reveal resistance gene catalogue in Siriraj Hospital.

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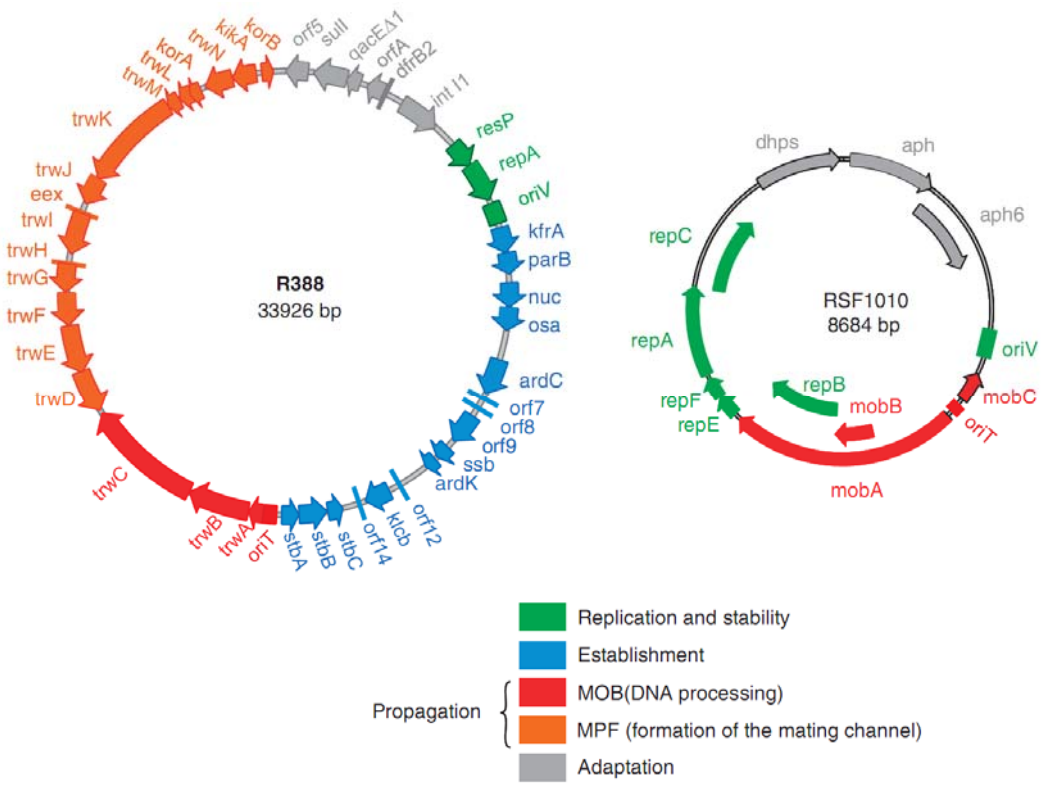
APPENDICES

APPENDIX A

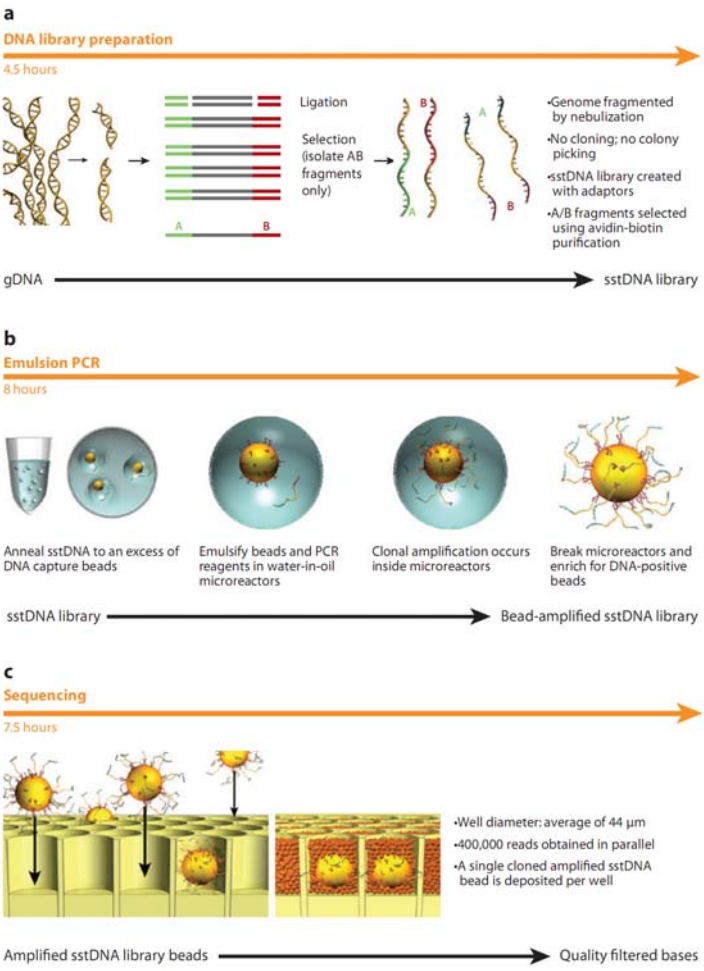
Genetic organization of a conjugate plasmid and a mobilizable plasmid



Plasmid genetic organization (62): The left is a conjugative plasmid, R388, and the right is a mobilizable plasmid, RSF1010. R388 is composed of five modules including replication and stability, establishment, MOB (DNA processing for transfer), MPF (mating-pair formation) and adaptation. RSF1010 lacks of MOB and MPF modules that is required for conjugation.

APPENDIX B

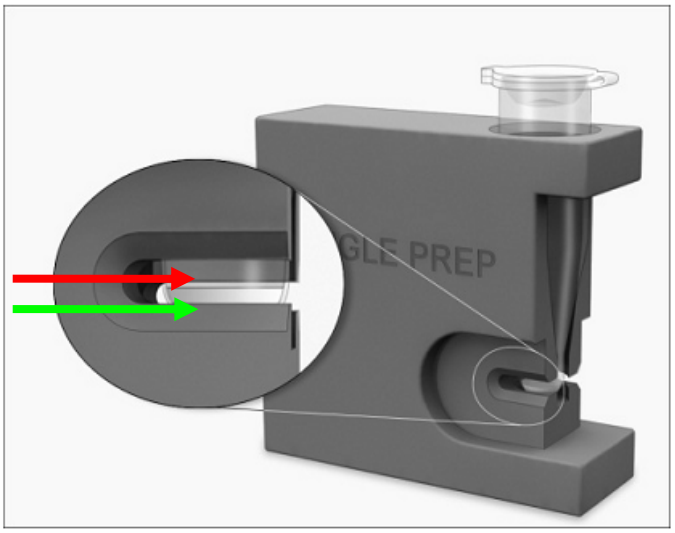
Sequencing pipeline by Roche 454 pyrosequencing



454 Pyrosequencing pipeline: there are three steps. Firstly, DNA sample is fragmented to 700-1,000 base pairs, and is tagged with adapter. Secondly, DNA single molecule with adapter is linked with microbead, and amplified in microemulsion part to enriched-DNA bead. Last, a DNA bead is deposited in microwell, and DNA is sequenced with pyrosequencing reaction. Base calling filters the qualified bases.

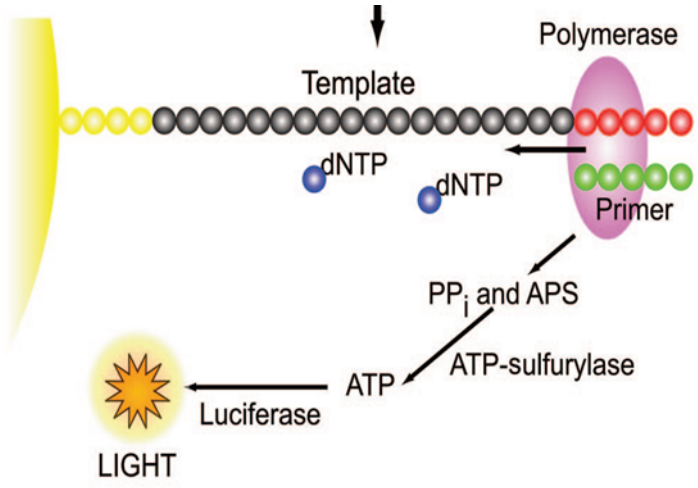
Upper and lower line of GS Junior Bead counter

Upper line = 2,000,000 beads
lower line = 500,000 beads



GS Junior bead counter (Roche): Before sequencing with GS Junior, DNA-enriched beads are between 500,000 – 2,000,000 beads that are suitable for sequencing.

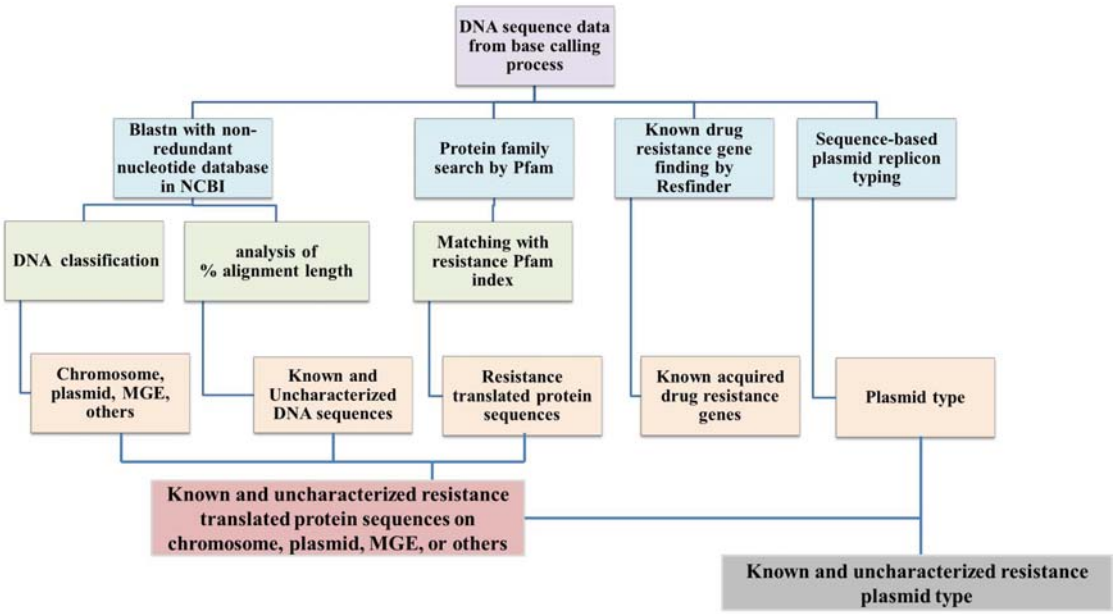
Pyrosequencing reaction in Gs Junior 454 sequencer



Sequencing of 454 platforms are pyrosequencing (Roche): The clonal DNA on beads is incorporated with nucleotides by DNA polymerase, and after that, pyrophosphate (PPi) is released. In the next step, PPi reacts with adenosine 5' phosphosulfate (APS) to ATP by accelerating of ATP sulfurylase. ATP is cofactor of luciferase in changing luciferin to oxyluciferin, and light which is detected by a charge coupled device (CCD) camera. The emitted light is proportional to the amount of incorporated nucleotides.

APPENDIX C

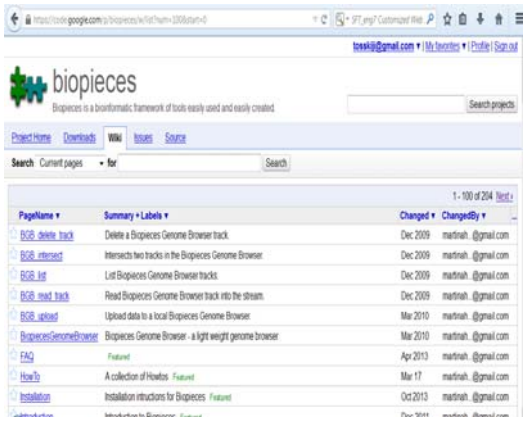
Work flow for bioinformatics analysis

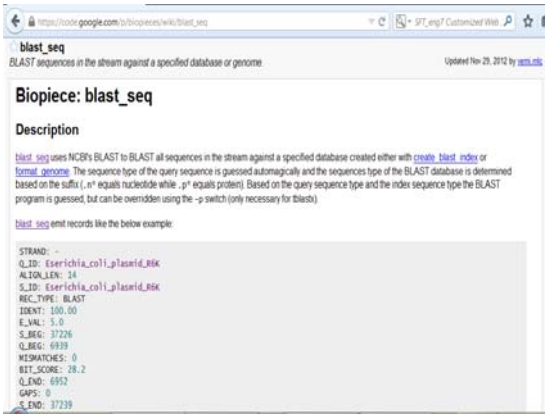


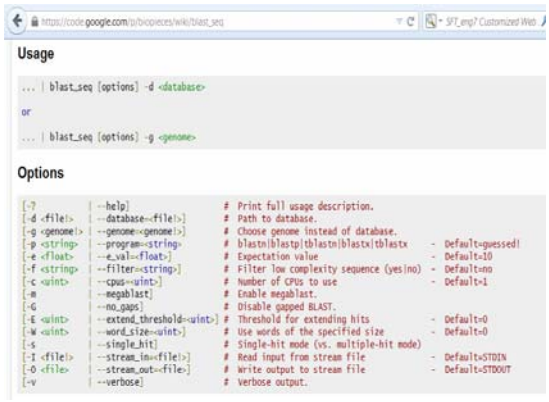
The work flow of bioinformatics analysis: after base calling, DNA sequences were classified to each DNA type, analyzed to known or uncharacterized sequences, matched with acquired resistance genes, translated and searched resistance translated proteins, and matched each plasmid replicon type. All results were integrated to identify resistance plasmid.

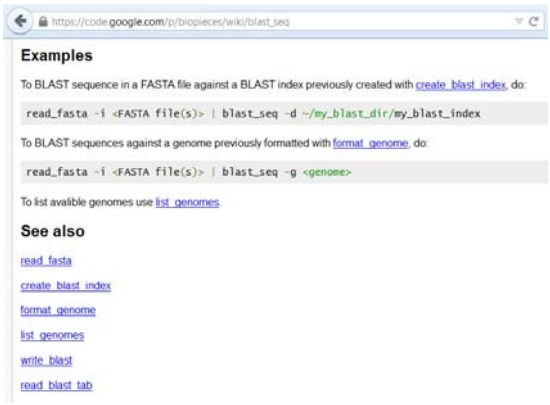
APPENDIX D

Biopieces: bioinformatic framework



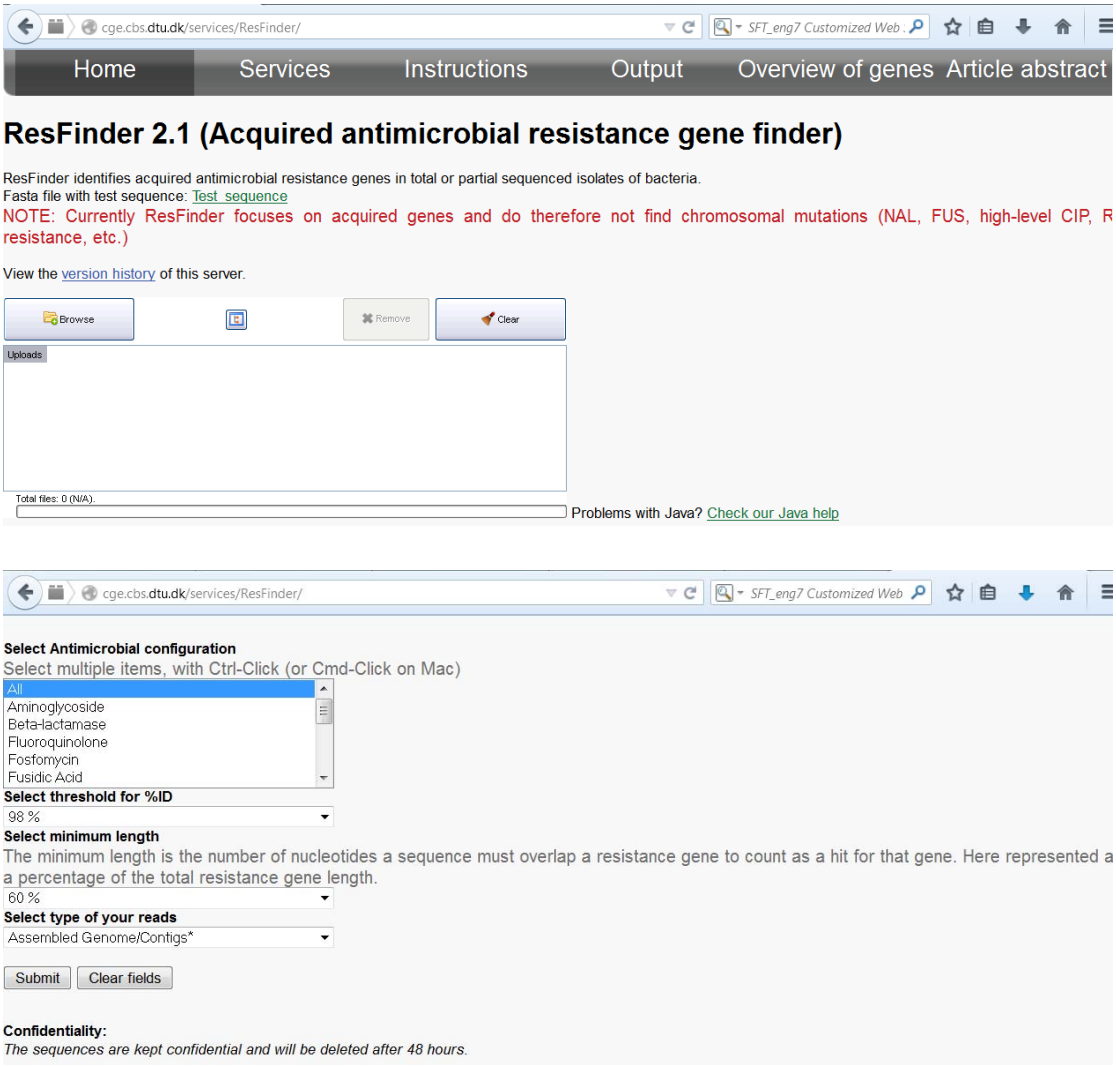






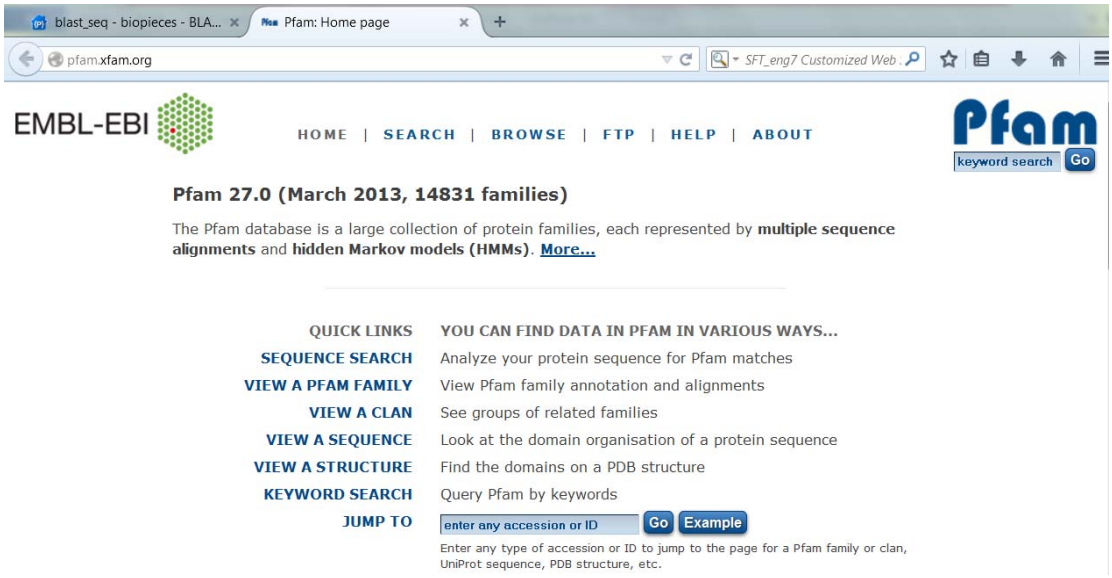
Biopieces is the bioinformatic tools that teach bioinformatic beginner how to command pseudocode to manage enormous DNA or protein data. This tool is free for all user, and is available at https://code.google.com/p/biopieces/w/list?num=100&start=100.

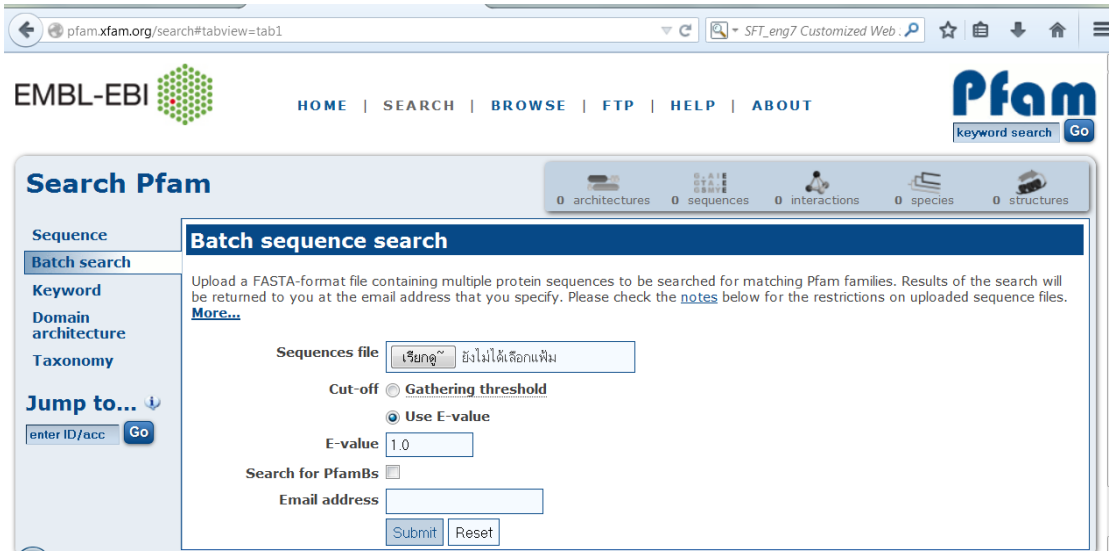
Resfinder: acquired antimicrobial resistance gene searching tool



Resfinder is a webpage that contain acquired resistance database (19). The database contained most acquired resistance gene except target mutation gene, and resistance genes are often found in plasmid. This tool is available at http://cge.cbs.dtu.dk/services/ResFinder/.

Pfam: protein family database





Pfam is a tool that collects a large batch of protein families. It also input up to 5000 translated protein sequence in one searching, and the output of analysis is sent to users' email. E-value of analysis is indicated by users. Pfam is available at http://pfam.xfam.org.

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