การดื้อควิโนโลนผ่านทาง *qnrA* INTEGRON CASSETTE ใน *ESCHERICHIA COLI*

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QUINOLONE RESISTANCE VIA *qnrA* INTEGRON CASSETTE IN *ESCHERICHIA COLI*

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วีร์ วินิจสร : การดื้อควิโนโลนผ่านทาง *qnrA* INTEGRON CASSETTE ใน *ESCHERICHIA COLI*. (QUINOLONE RESISTANCE VIA *qnrA* INTEGRON CASSETTE IN *ESCHERICHIA COLI*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. ดร. พิณทิพย์ พงษ์เพ็ชร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. นพ. ดร. ชาญวิทย์ ตรีพุทธรัตน์, 173 หน้า.

การศึกษานี้ทำการศึกษาคุณลักษณะของยีน *qnrA* ที่เกี่ยวข้องกับการดื้อต่อยาควิโนโลน ในเชื้อ *Escherichia coli* (*E. coli*) ที่แยกได้จากผู้ป่วยในโรงพยาบาล 2 แห่งในกรุงเทพมหานครโดยแห่งหนึ่งเป็นโรงพยาบาลมหาวิทยาลัยและอีกแห่งเป็นโรงพยาบาลเอกชนขนาดตติยภูมิ โดยการใช้เทคนิค Polymerase Chain Reaction (PCR) พบว่าเชื้อ *E. coli* ที่สร้างเอนไซม์ extended spectrum beta-lactamase (ESBL) 100 ไอโซเลต (ดื้อต่อยาควิโนโลน 83 ไอโซเลต) จากโรงพยาบาลมหาวิทยาลัยเป็นเชื้อที่มียีน *qnrA* ถึง 8 ไอโซเลต ในขณะที่เชื้อ *E. coli* ทั้ง 90 ไอโซเลต ที่ดื้อต่อยาควิโนโลนจากโรงพยาบาลเอกชนขนาดตติยภูมิไม่มียีนชนิดนี้ การมี ESBL และ class 1 integron (*intI1*) สัมพันธ์กับการที่เชื้อมียีน *qnrA* เนื่องจากพบว่าเชื้อ *E. coli* ที่มียีน *qnrA* ทุกไอโซเลต สร้างเอนไซม์ ESBL และมียีน *intI1* นอกจากนี้พบว่าเชื้อที่มียีน *qnrA* 4 ไอโซเลต ที่ดื้อต่อยาซิโปรฟลอกซาซิน นั้นมีการเปลี่ยนแปลงลำดับนิวคลีโอไทด์ 2 ตำแหน่ง (double mutations; S83L และ D87N) ที่บริเวณ quinolone resistance determining region (QRDR) ของยีน *gyrA* ในขณะที่เชื้อที่มียีน *qnrA* อีก 4 ไอโซเลต ที่ไม่ดื้อต่อยาซิโปรฟลอกซาซิน นั้นจะไม่มีการเปลี่ยนแปลงลำดับนิวคลีโอไทด์ที่บริเวณ QRDR ของยีน *gyrA* การนำยีน *qnrA* มาทำการตรวจหาลำดับนิวคลีโอไทด์ (DNA sequencing) พบว่ายีน *qnrA* ที่พบในการศึกษานี้เป็นยีน *qnrA* subtype 1 (*qnrA1*) และผลจากการตรวจสอบตำแหน่งของยีน *qnrA* โดยการทำ Southern blot hybridization แสดงให้เห็นว่ายีนนี้จากเชื้อ *E. coli* 3 ใน 8 ไอโซเลต อาจอยู่ในโครโมโซมและ/หรือพลาสมิดที่มีขนาดใหญ่ โดยยีนนี้ integrate เข้าโครโมโซมผ่านทาง transposon ร่วมกับ class 1 integron แม้ว่าเชื้อที่มียีน *qnrA* จะมีการดื้อต่อยาฟลูออโรควิโนโลน (ซิโปรฟลอกซาซินและนอร์ฟลอกซาซิน) ในระดับต่ำ จากการศึกษา time kill study ชี้ให้เห็นว่าเชื้อ *E. coli* มีกลไกอื่นในการดื้อต่อยาฟลูออโรควิโนโลน โดยเฉพาะอย่างยิ่ง การเปลี่ยนแปลงลำดับนิวคลีโอไทด์ 2 ตำแหน่ง (double mutations; S83L และ D87N) ที่บริเวณ QRDR ของยีน *gyrA* โดยยีน *qnrA* จะเสริมให้เชื้อดื้อยาฟลูออโรควิโนโลนเพิ่มขึ้นสูงกว่าเชื้อที่ไม่มียีนชนิดนี้มาก และสามารถพบ regrowth ของเชื้อ แม้ในความเข้มข้นของยาฟลูออโรควิโนโลนในระดับสูงมาก

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต.....

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KEYWORDS: *qnrA* / QUINOLONE / PMQR / *ESCHERICHIA COLI* / ESBL / CLASS 1 INTEGRON / QRDR / *gyrA*

WEE WINISSORN: QUINOLONE RESISTANCE VIA *qnrA* INTEGRON CASSETTE IN *ESCHERICHIA COLI*. ADVISOR: ASSOC. PROF. PINTIP PONGPECH, Ph. D., CO-ADVISOR: ASST. PROF. CHANWIT TRIBUDDHARAT, M.D., Ph. D., 173 pp.

This study determined the characteristics of *qnrA* genes in *Escherichia coli* (*E. coli*) isolated from two hospitals in Bangkok, Thailand (one university hospital and one tertiary private hospital). Polymerase Chain Reaction (PCR) showed a presence of 8 *qnrA* positive isolates out of 100 extended-spectrum beta-lactamase (ESBL) producing *E. coli* isolated (quinolone resistant *E. coli* 83 isolates) from the university hospital, whereas no *qnrA* was detected in 90 quinolone resistant *E. coli* isolated from the tertiary private hospital. All *qnrA* positive isolates associated with the ESBL and class 1 integron (*intI1*). Double mutations in the quinolone resistance determining region (QRDR) in *gyrA* (resulted in S83L and D87N) 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. DNA sequencing analysis confirmed positive result for *qnrA1*. The result from Southern blot hybridization showed that *qnrA* genes were either located in the chromosome and/or large plasmids (3 out of 8 isolates). This indicated that the *qnrA* could be integrated into the chromosome via transposon elements along with class 1 integrons. The result of time kill study showed that the *qnrA* positive isolate indeed conferred low-level resistance to ciprofloxacin and norfloxacin, and they were able to show higher level of fluoroquinolone resistance and increasing the chance of regrowth only when associated with other resistant mechanisms, particularly the mutations in the QRDR (S83L and D87N).

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LIST OF ABBREVIATIONS

AAC(6')-Ib-cr	Aminoglycoside acetyltransferase cr variants
ATCC	American Type Culture Collection
BHI	Brain heart infusion
bp	base pair
bla	beta-lactamase gene
ºC	degree Celsius
CAMB	cation-adjusted Mueller-Hinton II broth
CDC	Centers for Disease Control and Prevention, United States
C. freundii	*Citrobacter freundii*
CLSI	Clinical and Laboratory Standards Institute
CTX-M	Active on cefotaxime, first isolated at Munich
DNA	deoxyribronucleic acid
dNTPs	deoxynucleotide triphosphates
E. coli	*Escherichia coli*
E. cloacae	*Enterobacter cloacae*
ESBL	extended spectrum beta-lactamase
e.g.	exempli gratia
et al.	et alii (and other people)

FOX	Active on cefoxitin
gyrA	gyrase sub unit A gene
IMP	Active on imipenem
intI1	integrase gene of class 1 integron
K. oxytoca	*Klebsiella oxytoca*
KPC	*K. pneumoniae* carbapenemase
K. pneumoniae	*Klebsiella pneumoniae*
LB	Luria Bertani
LPS	lipopolysaccharides
MHB	Mueller-Hinton II broth
ml	milliliter
NCCLS	National Committee for Clinical Laboratory Standards
NSS	Normal Saline Solution
ORF	open reading frame
OXA	Active on oxacillin
PCR	Polymerase Chain Reaction
P. mirabilis	*Proteus mirabilis*
PMQR	plasmid-mediate quinolone resistance
qnr	quinolone resistant gene
QRDR	Quinolone Resistance Determining Region

S. algae	*Shewanella algae*
SHV	Sulfhydryl variable
TEM	Temoniera
TSA	Tryptic Soy Agar
UTIs	Urinary tract infections
V	volt
VEB	Vietnamese extended spectrum beta-lactamase
µg	microgram
µl	microliter
%	percent

CHAPTER I

INTRODUCTION

Escherichia coli is the most important species that belongs to the *Enterobacteriaceae* family. It is commonly considered as normal flora in the human intestine. Nevertheless, *E. coli* is the most common pathogen found in human bacterial infections including community-acquired and hospital-acquired infections including septicemia, gastrointestinal tract and genito-urinary tract infections (Wang *et al*., 2001; Schaechter, 2009).

A widely use of 2nd generation fluoroquinolones, such as, ciprofloxacin and norfloxacin (Oliphant *et al*., 2002) for the treatment of gastrointestinal tract and urinary tract infections resulted in a successful treatment against *E. coli* infections. Nevertheless, resistance to fluoroquinolones was increasing worldwide (Graninger *et al*., 1996; McDonald *et al*., 2001).

Fluoroquinolone resistance in *Enterobacteriaceae* is mostly-mediated by chromosomal mutations in genes coding for DNA gyrase and topoisomerase IV, leading to target alterations. Specifically, double mutations in the Quinolone Resistance Determining Region (QRDR) in *gyrA* gene have been reported to be associated with high fluoroquinolone resistance (Conrad *et al*., 1996; Poirel *et al*., 2005). However, the first plasmid-mediated quinolone resistance (PMQR) determinant, *qnrA*, was discovered

in a clinical isolate of *Klebsiella pneumoniae* from United States in 1998 (Wu *et al*., 2007). Although, *qnrA* genes confer low-level resistance to fluoroquinolone, when associated with other resistant mechanisms, they are able to facilitate the selection of higher-level quinolone resistance (Martinez-Martinez *et al*., 1998; Jacoby, 2005; Rodriguez-Martinez *et al*., 2007).

In addition, *qnrA* has been identified in complex *sul1*-type integrons of the In*4* family of class 1 integrons (Nordmann *et al*., 2005; Cano *et al*., 2009). Integrons are genetic elements that can capture resistance genes for various classes of antibiotics including beta-lactams, aminoglycosides, chloramphenicols, rifampicins, quinolones, trimethoprim and sulfonamides, which mostly linked to mobile genetic elements (MGEs) such as tranposons and conjugative plasmids. MGEs have long been demonstrated in the spread of antibiotic resistance genes among Gram-negative bacteria (Hall *et al*., 1993; Hall *et al*., 1995; Hall, 1997; Mazel, 2006; Poirel *et al*., 2009; Cambray *et al*., 2010).

Recently, *qnrA* genes were commonly found in ESBL producing *E. coli* (Jacoby, 2005; Strahilevitz *et al*., 2009) capable of hydrolyzing extended spectrum beta-lactam antibiotics such as 3rd generation cephalosporins (Bradford, 2001). ESBL producing *E. coli* possessing both *qnrA* and *gyrA* mutations, therefore, limit the choice of antibiotic treatment leading to the treatment failure.

The aim of this study is to determine the characteristics of *qnrA* genes in *E. coli* clinical isolates including their genotypes and hidden phenotypes in relationship with other antibiotic resistance determinants using time kill study, which may provide insight information for a possible clinical resistance.

CHAPTER II

OBJECTIVES

The objectives of this study: To determine

1. The prevalence of *qnrA* genes in the clinical isolates from a university hospital in comparison to those in a tertiary private hospital.
2. The characteristics of *qnrA* genes in *E. coli* clinical isolates including their genotypes and hidden phenotypes in relationship with other antibiotic resistance determinants.

CHAPTER III

LITERATURE REVIEWS

1. *Escherichia coli*

1.1 General characters

Escherichia coli (*E. coli*) is a Gram-negative facultative non-spore-forming motile rod. This species belongs to the *Enterobacteriaceae* family. *E. coli* is a part of common normal flora in the intestine of mammals, whereas, some of which cause human and animal infections of the digestive and urinary tracts, blood, and central nervous system (Schaechter, 2009).

E. coli has distinctive colony morphology on certain laboratory media such as MacConkey agar. Although it may appear as a non-lactose fermenter or as a mucoid colony, *E. coli* normally produces a dry pink colony (lactose positive) with a surrounding pink area of precipitated bile salts on MacConkey agar.

1.2 Taxonomy of *E. coli*

E. coli has been recognized as an important human pathogen since its discovery in 1885 by Dr. Theodor Escherich through his work on bacteria in infant stools. *E. coli* is usually motile, lactose-, lysine decarboxylase- and indole-positive, and is suggested to be mostly commensal microorganisms (Chaudhuri *et al*., 2012).

E. coli is sub-clarified mainly by their different antigenic composition. The relevance of taxonomic are more than 170 different types of O (lipopolysaccharide or somatic) antigens and 80 types of K (capsular) antigens. The other antigenic properties that are used to sub-clarify individual strains are H (flagellar) antigens and F (fimbrial) antigens.

Quantitative approaches to defining taxonomic relationships have been based on patterns of isozymes of metabolically important enzymes, protein composition of the outer membrane, and increasingly genomic sequences (Nataro *et al*., 1998; Schaechter, 2009; Chaudhuri *et al*., 2012).

1.3 Ecology of *E. coli*

E. coli is the facultative bacteria of the human colonic flora. *E. coli* inhabits not only in the large intestine of human but also in the small intestine and mostly large intestine. This microorganism typically inhabits the infant gastrointestinal tract within hours of life, delivery after birth (Nataro *et al*., 1998; Schaechter, 2009). How the microorganisms are transmitted to the neonate is not clear, this may occur during passage through the birth canal or, shortly after birth, via the fecal-oral route transmittion. *E. coli* generally remains harmless while limited to the intestinal lumen; however, in the immune-compromised host, or when gastrointestinal barriers are injured, *E. coli* can cause infection (Nataro *et al*., 1998; Schaechter, 2009).

1.4 Major structure of *E. coli* cell associate with drug resistance

This topic mentions to details of the structures associated with drug resistance including fimbriae or pilli that could transfer DNA such as drug resistance gene via conjugation. Outer membrane proteins, that some of which may be reduced or absent an outer membrane leading to reduced drug accumulation as above mentioned.

1.4.1 Fimbriae or Pili

Fimbriae have been associated with host tissue adhesion of important pathogenic *E. coli*. *E. coli* harbors one or two types of fimbriae including type 1 or common pilli and conjugative or sex pili. First, the most common adhesins found in both mutual and pathogenic *E. coli* are type 1 fimbriae. Second, sex pili or conjugate pilli are encoded on the plasmids including F or R plasmids. Bacterial conjugation is a process of horizontal gene transfer that involves cell to cell contact, in which a conjugative plasmid is transmitted from a donor to a recipient cell through a specialized conjugative pilus (Houdt *et al*., 2005; Schaechter, 2009).

1.4.2 Outer Membrane of *E. coli*

The outer membrane of *E. coli* is an asymmetric bilayer of phospholipid and lipopolysaccharides (LPS). The major outer membrane proteins include the pore forming proteins called porins including OmpC, OmpF, and PhoE. A lack of expression of OmpF related to increase in the resistance to some quinolones. Furthermore, a decreased

expression of OmpF results in a decrease in susceptibility to many classes of antimicrobial agents such as beta-lactams, tetracyclines and chloramphenicol (Lugtenberg *et al*., 1983; Ruiz, 2003; Pages *et al*., 2008; Delcour, 2009; Schaechter, 2009).

1.5 Diseases due to *E. coli*

E. coli is the normal flora of humans and many kinds of animals. However, several strains have been reported that cause infections of the gastrointestinal system and outside of the gastrointestinal system. *E. coli* is a common and important cause of urinary tract infections (UTIs). Furthermore, they are important cause of systemic infections in humans. The systemic infections include bacteremia, septicemia and hospital-acquired pneumonia. *E. coli* is also a leading cause of neonatal meningitis. Uropathogenic *E. coli* (UPEC) primarily causes community-acquired UTIs with an estimated 20% of women over the age of 18 years suffering from at least one UTI in their lifetime. UPEC is responsible for 70–95% of community-UTIs and 50% of nosocomial-UTIs, therefore increased morbidity, mortality, and medical expenses. Recurrent or relapsing UTIs are particularly problematic in many individual persons. Pathogenic *E. coli* including enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, and uropathogenic *E. coli* are divided into groups according to the clinical feature

and their known virulence factors including toxins, adhesions, invasins, antiphagocytic surface components, and others (Schaechter, 2009; Pitout, 2012).

1.6 Pathogenic *E. coli*

1.6.1 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) is able to colonize in the small intestine, where it produce and deliver plasmid-encoded heat-labile (LT) and/or heat-stable (ST) enterotoxins. Both toxins act by changing the net fluid transport activity in the gut from absorption to secretion. LT is structurally similar to cholera toxin and activates the adenylate cyclase cyclic adenosine mono phosphate system, whereas ST works on guanylate cyclase. The intestinal mucosa is not clearly damaged, the watery stool does not contain white or red blood cells, and no inflammatory process occurs in the gut wall. Gut cells activated by LT or cholera toxin remain in that status until they die, whereas the effects of ST on guanylate cyclase are put off when the toxin is washed away from the cell. These strains are the most frequent cause of traveler's diarrhea. ETEC infections are classically associated with acute watery diarrhea. Same as clinical cholera, these infections are asymptomatic or mildly symptomatic to severe extensive cholera-like watery diarrhea caused to rapid dehydration and prostration within a few hours (Schaechter, 2009; Fleckenstein *et al.*, 2010).

1.6.2 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) is among the most important pathogens infecting children worldwide and is one of the main causes of persistent diarrhea. These strains have historically been recognized not by the possession of a specific virulence factor, but by the presence of certain O:H antigens and identified as EPEC by agglutination tests. EPEC induce a characteristic histopathology known as the attaching and effacing (A/E) lesion, which is characterized by the intimate attachment of bacteria to the epithelial surface and effacement of host enterocyte microvilli. There are three stages in EPEC pathogenesis. First, initial adherence to the host cell, second, production and translocation of bacterial proteins through a needle complex via a type III secretory system, and finally, intimate bacterial attachment with pedestal formation. The major virulence proteins participated by all EPEC are encoded on a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE). Among the non-LEE encoded proteins is bundle-forming pilus (BFP), a type IV pilus, encoded on a plasmid, called EPEC adherence factor (EAF). The BFP is responsible for the localized adherence (LA) phenotype of EPEC, characterized by the ability of *E. coli* to form micro-colonies on the surface of the intestinal epithelial cells (Bouzari *et al*., 2000; Ochoa *et al*., 2008).

1.6.3 Enterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E. coli* (EHEC) have been recognized as a cause of serious illness and mortality in outbreaks of food-borne illness that involve a large variety of foods. EHEC compose a limited number of serotypes that cause a characteristic non febrile bloody diarrhea known as hemorrhagic colitis. After the first outbreak in 1982, *E. coli* O157:H7 has become the most widely known EHEC. This serotype is the most common in United States, whereas other serotypes, particularly O26, are found with greater frequency all over the world. Serotype O157 would cause hemorrhagic colitis and other severe symptoms than other pathogenic *E. coli*. EHEC have 2 special characteristics of pathogenic importance. First, they produce high levels of 2 related cytotoxins including Shiga-like toxins (SLT) I and II because the protein synthesis-inhibitory action and binding specificity resemble to *Shigella* toxin. The SLT are cytotoxic for endothelial cells in culture. Second, they possess a gene highly homologous to the EPEC attaching and effacing pathogenicity island. In combination on above mention, the proteins encoded by this gene and the SLT reasonable damage the gut mucosa in a manner characteristic of hemorrhagic colitis (Schaechter, 2009; Viazis *et al*., 2011).

1.6.4 Uropathogenic *E. coli* (EPEC)

Urinary tract infections (UTIs) are among the most common infectious diseases and causing significant morbidity and mortality in United States. The majority of UTIs are

cause rising infections of the urinary tract caused by uropathogenic *E. coli* (UPEC) leading to asymptomatic bacteriuria, cystitis and acute pyelonephritis. UPEC have been reported to express several of virulence factors, e.g. siderophores, adhesins, toxins, invasins and capsules, which enable the adaptation to the hostile environment of the urinary tract, and the circumvention of the host immune defenses. The ability of UPEC to produce UTIs begins with type 1 pilli, which are important for periurethral colonization and for attachment to epithelial cells in the bladder. The presence of P pilli is particularly important in upper UTIs, which extend to the renal pelvis and kidney to cause acute pyelonephritis (Sorsa *et al*., 2004; Kenneth, *et al*., 2010).

2. Fluoroquinolones

2.1 Structure

Quinolones are potent, board spectrum antimicrobial agents, good bioavailability, oral and intravenous formulation and potentially low incidence of adverse reaction. The quinolone was discovered since 1962 and derived from quinine. The addition of a fluorine molecule at position C-6, which one the earliest changes this structure and dramatic increase antibacterial potency that called fluoroquinolone. The basic quinolone structure has been developed by two major groups: quinolones and naphthyridones. The presence of nitrogen at position 8 distinguishes the naphthyridones, while a carbon associated with this position distinguishes the

quinolones (Chu *et al*., 1989; Ball, 2000; Andersson *et al*., 2003; Drlica *et al*., 2009) as showed in Figure 3.1

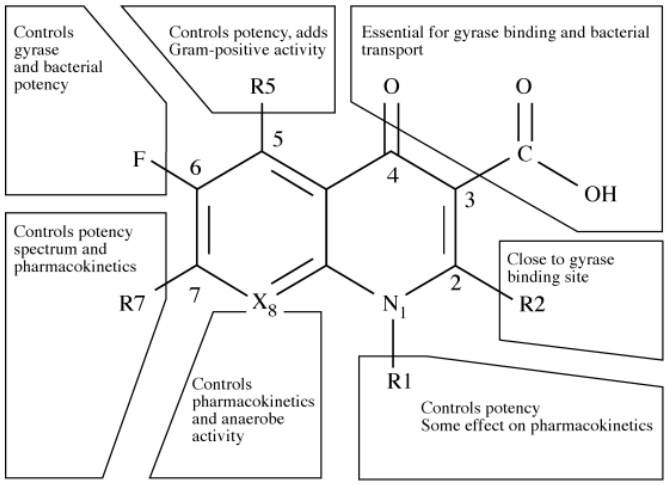

Figure 3.1 Structures of the quinolone and napthyridone cores. In X molecules, where X is a carbon atom, that core is a quinolone (norfloxacin, ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin, gatifloxacin), where X is a nitrogen atom that core is a naphthyridone (nalidixic acid, gemifloxacin). (Andersson *et al*., 2003)

Nalidixic acid was first naphthyridone to be developed, however its efficacy was limited to the treatment of Gram-negative urinary tract infections (UTIs). Nalidixic acid was replaced by norfloxacin and had much better activity and pharmacokinetic properties and became to quinolone. The structure of norfloxacin differs from nalidixic acid the addition of a fluorine molecule at position C-6, replacement of a methyl at position C-7 by a piperazinyl ring and exchange of the nitrogen at position 8 by a carbon. The addition of piperazinyl ring at the position C-7 improves the activity against

Gram-negative bacteria. Latterly, a cyclopropyl group was replaced to the position N-1 of norfloxacin structure and increased potency of this drug. The best illustrated is ciprofloxaxin, which was introduced in 1983 as showed in Figure 3.2 In addition, the N-1-cyclopropyl substituent is by far the optimal group and the most potent of the quinolones in vitro against *Enterobacteriaceae* and *Pseudomonas aeruginosa*. While addition of azabicyclo groups at position C-7 that showed significant anti-Gram-positive activity, marked lipophilicity and half-lives of >10 hours such as moxifloxacin (Chu *et al*., 1989; Ball, 2000; Andersson *et al*., 2003; Drlica *et al*., 2009) as showed in Figure 3.3

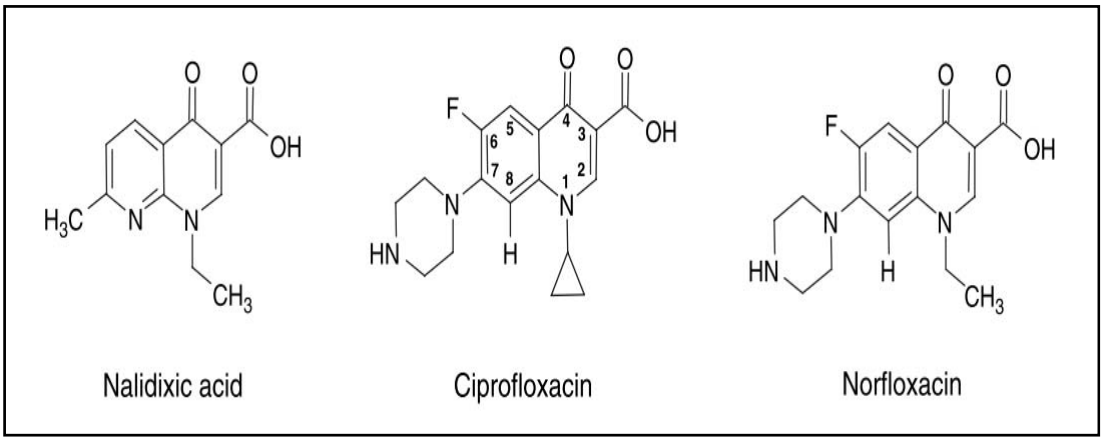

Figure 3.2 The structures of nalidixic acid, ciprofloxacin and norfloxacin, respectively. (Drlica *et al*., 2009)

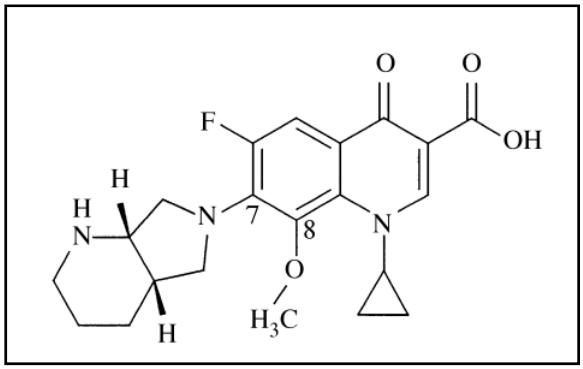

Figure 3.3 Modification of quinolone structures: moxifloxacin. Azabiocyclo rings at position 7 are found in moxifloxacin. A methoxy group at position 8 is found in moxifloxacin and gatifloxacin. (Andersson *et al*., 2003)

2.2 Fluoroquinolone mechanism of action

Fluoronquinolones enter bacterial cell through porins or directly through the lipid and cytoplasmic membrane and target DNA topoisomerases. The primary target for fluoroquinolone is DNA gyrase (topoisomerase II) in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria. DNA gyrase is a tetrameric enzyme composed of two A and two B subunits that encoded by the *gyrA* and *gyrB* genes, respectively. DNA gyrase is responsible for introducing negative supercoils into DNA and for relieving topological stress arising from the translocation of transcription and replication complexes along DNA. Topoisomerase IV is an A_2B_2 as well as DNA gyrase that encoded *parC* and *parE*, respectively. ParC and ParE are highly homologous to GyrA and GyrB, respectively. Topoisomerase IV is primarily involved in decatenation, the unlinking of replicated daughter chromosomes. Fluoroquinolones act by binding to

DNA gyrase/topoisomerase IV-DNA complex. Complex formation is responsible for the inhibition of DNA replication and the bacteriostatic action of the quinolones. Their lethal action is thought to be a separate event from complex formation, and to arise from the relapse of free DNA ends from quinolone-DNA gyrase-DNA complexes. (Drlica *et al*., 1997; Hooper, 2000; Hawkey, 2003; Ruiz, 2003; Nordmann *et al*., 2005)

2.3 Mechanisms of resistance to fluoroquinolones

Resistances to quinolones/fluoroquinolones have been a clinical problem ever since nalidixic acid was introduced in 1962. The fluoroquinolones have been used to treat a great diversity of infections including genitourinary tract infections, gastro-intestinal tract infections, sexually transmitted diseases, skin and soft tissue infections, osteomyelitis and respiratory tract infections. Furthermore, quinolones/fluoroquinolones are among the most important antimicrobial agents that used widely for treatment of bacterial infections both in human and veterinary medicine. The result of their widely used led to an escalating rate of fluoroquinolones resistance. In the United States, the use of fluoroquinolones in 1990s decade increase approximately 40%, with a doubling in the rate of resistance to ciprofloxacin among Gram-negative bacteria. Approximately 60% of *E. coli* strains isolated hospital acquired infections in Beijing, China were resistant to ciprofloxacin, during 1997-1999. The results as above that showed fluoroquinolone resistance were found in every part of the world. However, the future

usefulness of these drugs is threatened by the increasing rate of fluoroquinolone resistant bacteria. Certainly, fluoroquinolone resistannce is found widely in clinical pathogens and thus the major public health concern. Paradoxical, this currently high fluoroquinolones resistance is largely contrary to what was predicted in the early stage of fluoroquinolone development since early 1980s. Because fluoroquinolone agents are xenobiotic and have not been found to occur naturally, that expected fluoroquinolone resistance would occur rarely. In the present, two main mechanism of resistance to fluoroquinolones have been recognized: alteration in the drug targets and decreased drug accumulation as a result of porin reduction and/or the overexpression of efflux pump system. Both of these mechanisms are chromosomally mediated resistance to quinolone. Furthermore, the *qnr* plasmids (plasmid mediated quinolone resistance, PMQR) are integron associated and carry multiple resistant determinants rendering resistance to several classes of antimicrobial agents such as beta-lactams and aminoglycosides. The integron carrying the *qnr* genes have the potential for horizontal transfer of fluoroquinolone resistance genes. The *qnr* plasmids that produce the Qnr protein, which protects the quinolone targets, have been reported in the study from United State, Europe and East Asia. Although, *qnr* confer low-level resistance, when found in the presence of other mechanism, this gene is able to facilitate the selection of higher-level quinolone resistance of the pathogen. (Graninger *et al*., 1996; Martinez-

Martinez *et al*., 1998; McDonald *et al*., 2001; Oliphant *et al*., 2002; Neuhauser *et al*., 2003; Ruiz, 2003; Jacoby, 2005; Li, 2005)

2.3.1 Target alteration

The targets of fluoroquinolone action are two essential bacterial enzymes DNA gyrase and topoisomerase IV. DNA gyrase composed of A_2B_2 subunits that encoded by the *gyrA* and *gyrB* genes, respectively. While topoisomerase IV is A_2B_2 subunits as well those encoded by the *parC* and *parE* genes, respectively. DNA gyrase is more susceptible to inhibition by fluoroquinolones than topoisomerase IV in Gram-negative bacteria whereas topoisomerase IV is normally the primary target, and DNA gyrase is intrinsically less susceptible in Gram-positive bacteria. Hence, resistance mutations appear first in *gyrA* in Gram-negative bacteria, while they appear first in *parC* in Gram-positive bacteria. Target alterations described in the GyrA of *E. coli* in the termed of the "quinolone resistance determining region" (QRDR), that between positions 67 and 106. Mutations in codon 67, 81, 82, 83, 84, 87 and 106 of *gyrA* have been observed of fluoroquinolone resistance in *E. coli* but the highlight for mutations at amino acid positions 83 and 87 as showed in Table 3.1 The most common mutations comprise Ser83Phe, Ser83Leu and Asp87Asn of the *E. coli* GyrA.

Table 3.1 Mutations described in GyrA and GyrB subunits of quinolone-resistant strains of *E. coli*. (Ruiz, 2003)

Codon[a]	Wild amino acid	Mutations described
GyrA		
51[b]	Ala	Val
67[b]	Ala	Ser
81	Gly	Cys, Asp
82[b]	Asp	Gly
83	Ser	Leu, Trp, Ala, Val
84	Ala	Pro, Val
87	Asp	Asn, Gly, Val, Tyr, His
106[b]	Gln	Arg, His
GyrB		
426	Asp	Asn
447	Lys	Glu

[a]Mutations in other codons, such as codon 93, have been described, but their role in development of resistance to quinolones remains unclear.
[b]Only described in mutants obtained *in vitro*.

In study by Weigel and colleagues from Centers for Disease Control and Prevention (CDC), USA in 1998, in *E. coli*, a single point mutation in *gyrA* results in decreased susceptibility to fluoroquinolones, and high-level resistance is associated with double amino acids substitutions in the GyrA. Although additional factors, such as mutations in the ParC subunit of topoisomerase IV and decreased intracellular drug accumulation, have been showed to play a complementary role by increasing the level of resistance, *in vitro* studies with *E. coli* suggest that the first step in selection for decreased susceptibility to fluoroquinolones is an alteration of Ser-83. In GyrB of *E. coli*, amino acid substitutions resulting in resistance to quinolones have been reported at position 426 and confer resistant to all quinolones, whereas those at position 447 in

increase level resistance to nalidixic acid, but more susceptibility to fluoroquinolones. In the *parC* gene of *E. coli* and other bacteria, the most common substitutions appear at codons 80 and 84. For another substitution at codon 78 in *E. coli* has been reported in clinical isolates as showed in Table 3.2 In the *parE* of *E. coli*, only one substitution at Leu445His has been reported in a single quinolone resistant *in vitro* mutant of *E. coli*. Furthermore, this mutation only resembles to affect the MIC of quinolones when presence with mutation in *gyrA* (Yoshida *et al*., 1990; Yoshida *et al*., 1991; Breines *et al*., 1997; Weigel *et al*., 1998; Friedman *et al*., 2001; Ruiz, 2003; Jacoby, 2005; Li, 2005).

Table 3.2 Mutations described in ParC and ParE of the quinolone-resistant strains of *E. coli*. (Ruiz, 2003)

Codon	Wild amino acid	Mutations described
ParC		
78	Gly	Asp
80	Ser	Ile, Arg
84	Glu	Lys, Val, Gly
ParE		
445	Leu	His

2.3.2 Decreased drugs accumulation and efflux resistance mechanism

Decreased fluoquinolones accumulation may be associated via two factors: the bacterial impermeability increase and/or the overexpression of efflux pumps. Fluoloquinolones may cross the outer membrane in two different ways: through specific porins or by diffusion through the phospholipid bilayer. The degree of diffusion of

fluoroquinolones is greatly associated with, and dependent of hydrophobic level that may diffuse through the phospholipid bilayer. All fluoroquinolones may cross the outer membrane through the porins, but only those with a greater level of hydrophobicity may diffuse through the phospholipid bilayer. Thus alterations in the composition of porins and/or lipopolysaccharides may alter susceptibility profiles. In lipopolysaccharide defective mutants that increased susceptibility to hydrophobic quinolones such as nalidixic acid have been described, without alterations in the level of resistance to hydrophilic quinolones such as norfloxacin and ciprofloxcaxin. Fluoroquinolones reach to the targets by cross the cell wall and cytoplasmic membrane in Gram-positive bacteria or traverse an addition outer membrane in Gram-negative bacteria. Gram-negative bacteria could regulate membrane permeability by altering outer membrane porin protein expression that from channels for passive diffusion such as outer membrane protein. The outer membrane protein of *E. coli* possesses three main porins were OmpA, OmpC and OmpF. Particularly, a decrease expression of OmpF is related to increase resistance to some fluoroquinolones sush as norfloxacin, but does not affect the MIC of others such as sparfloxacin or tosufloxacin. Furthermore, a decreased expression of OmpF results in a decrease in susceptibility to a variety of antimicrobial agents such as beta-lactams, tetracyclines and chloramphenicol. In addition, both Gram-negative and Gram-positive bacteria have nonspecific, energy dependent efflux systems, some of which are expressed constitutively and others of which are controlled

by global regulatory systems or are inducible by mutation. In *E. coli*, the AcrAB-TolC efflux pump plays a major role in quinolone efflux and has multiple controls. Mutations in *acrR* (a repressor of *acrAB*) increase pump activity. Contrary, mutations that inactivate *marR* (a repressor of *marA*) allow MarA to activate *acrAB*, *tolC* and a gene that decreases translation of *ompF*, thus collectively decreasing influx and increasing efflux of quinolones. Target alterations and efflux activation are often found together in resistant clinical isolates. Certainly, in *E. coli*, in the absent of the AcrAB efflux pump, *gyrA* mutations hardly increase the MICs of all quinolones. Even with a functional efflux system, single mutation in *gyrA* produce generate only a modest increment in resistance such that they would be considered to be clinically susceptible such as the MIC of ciprofloxacin is $\leq$1 µg/ml. Only with a second mutation in *gyrA* is a clinical level of resistance such as MIC of ciprofloxacin $\geq$4 µg/ml reached. In general, the more resistant clinical isolate, it contains the more quinolone resistance associated mutations. (Hirai *et al*., 1986; Aoyama *et al*., 1987; Cohen *et al*., 1989; Mitsuyama *et al*., 1992; Ruiz, 2003; Jacoby, 2005; Li, 2005)

2.3.3 Plasmid-mediated quinolone resistance (PMQR)

qnrA

There have been reports describing the presence of quinolone resistance gene on plasmids. In 1987, Munshi and colleagues reported transfer of resistance and a 30

kb plasmid to *E. coli* from a nalidixic acid resistant strain of *Shigella dysenteriae*. In retrospect, the supposed transconjugants were probably resistant mutants, because they were selected with nalidixic acid and had no other plasmid-mediated resistances. Serial transfer of the plasmid was not attemped, and its elimination with acridine orange to show a role in resistance was unsuccessful (Munshi *et al*., 1987; Martinez-Martinez *et al*., 1998; Ruiz, 2003). The discovery of PMQR in 1998 was made serendipitously by Martinez-Martinez and colleagues, who study of the properties of a plasmid, pMG252 that gave multi-resistance to beta-lactam antibiotics. The plasmid came from a ciprofloxacin resistant strain of *Klebsiella pneumoniae* (*K. pneumoniae*) isolated in July 1994, from urine specimen of a patient at the University of Alabama at Birmingham, USA in 1994. A quinolone was included as a control in a study of the ability of pMG252 to increase resistance to beta-lactam antibiotics in porin-deficient strain of *K. pneumoniae*. Unexpected finding, the effect of the plasmid was increased the quinolone MICs 4 to 16 fold in this porin-deficiency strains (such as 4 to 32 μg/ml or 0.5 to 4 μg/ml for ciprofloxacin), but even in a *K. pneumoniae* or an *E. coli* with intact porins, pMG252 increased the quinolone MICs from 8 fold to 64 fold (such as 0.004 to 0.125 μg/ml for ciprofloxacin against intact porin, *K. pneumoniae* strain or 0.008 to 0.25 μg/ml for ciprofloxacin against intact porin, *E. coli* strain). Because MICs in the parent strains were initially very low, MICs in the pMG252 derivatives reached only 0.12-0.25 μg/ml for ciprofloxacin and 4 to 32 μg/ml for nalidixic acid. Plasmid pMG252 had a broad host

range and could be transfer by conjugation from *E. coli*, *Citrobacter freundii*, *Shigella* Typhimurium and *Pseudomonas aeruginosa* (Tran *et al*., 2002; Strahilevitz *et al*., 2009). Although the basic level of resistance produced by the plasmid was low, it facilitated selection high resistance. The frequency of spontaneous mutations to ciprofloxacin or nalidixic acid resistance was more than 100 times higher for *E. coli* J53 with pMG252 than plasmid free *E. coli* J53. The plasmid did not have a general mutator effect since the frequency of other genetically defined mutations was equivalent to that of plasmid-free *E. coli*. Plasmid present in such mutants conferred parental low level quinolone resistance when transfer to another *E. coli*, which indicates that mutations responsible for higher resistance were on the host chromosome, not the plasmid (Martinez-Martinez *et al*., 1998; Strahilevitz *et al*., 2009). Subsequent cloning of the gene responsible for this phenotype revealed it to be the 657-nucleotide ORF (open reading frame) and the protein which it encoded was name Qnr, for quinolone resistance protein. Recently, this protein has been renamed QnrA1 (Tran *et al*., 2002; Strahilevitz *et al*., 2009).

A *Klebsiella oxytoca* isolate from Anhui Province, China, was reported to carry a variant of *qnrA* differing from the originally detected gene by four amino acids. This variant was designated *qnrA2*. While searching for a chromosomal orthologue of *qnrA* in the genome sequence of environmental organisms, Poirel and colleagues, proposed the hypothesis that this novel resistance determinant could derive from an environmental,

human, or animal Gram-negative bacteria. They found and identified three additional variants (*qnrA3*, *qnrA4*, and *qnrA5*) of this gene in *Shewanella algae* (*S. algae*), which is a Gram-negative species that widely distributed in marine and freshwater environments. These genes (*qnrA3*, *qnrA4*, and *qnrA5*) differ from the original *qnrA1* in two to four codons. In addition, this report indicates that Gram-negative species were not only of the veterinary world but also of the environment and may be a reservoir for emerging antibiotic resistance genes spreading in human pathogens, as suspected (Poirel *et al*., 2005; Strahilevitz *et al*., 2009). At the same time, *qnrA3* was also detected in *Samonella* Enteritidis (Cheung *et al*., 2005; Strahilevitz *et al*., 2009). Cambau and colleagues investigated for *qnrA* determinants among clinical isolates of *Enterobacteriaceae* with quinolone resistant or extended-spectrum beta-lactamase, that found *qnrA6* in a *Proteus mirabilis* clinical isolate, which five amino acids differed from the original gene (Cambau *et al*., 2006; Strahilevitz *et al*., 2009). More recently, *qnrA7* was detected in *S. algae* isolate in 2010 (Zhou *et al*., 2010).

qnrS

At the beginning of October 2003, a single clone of *Shigella flexneri* 2b caused an outbreak of food poisoning occurred in Aichi Prefecture, Japan. One of eight strains of this clone was resistant to quinolone. This strain had a novel quinolone resistance gene on conjugative plasmid. Cloning identified an ORF of 657 bp encoding a 218

amino acid protein of the pentapeptide repeat family. This protein shares only 59% amino acid identity with QnrA1 and was named QnrS (Hata *et al*., 2005; Strahilevitz *et al*., 2009). In the cause of investigated for *qnr* genes among clinical non-Typhi *Salmonella* isolates from the United States, a *qnrS* variants (*qnrS2*) was detected on a plasmid from *Salmonella enterica* serovar Anatum that coded for a protein that was 92.2% identical in amino acid sequence to QnrS1 (Gay et al., 2006; Strahilevitz *et al*., 2009). At the same time, *qnrS2* was found on a plasmid isolated from activated sludge basin of a wastewater treatment plant in Germany (Bonemann *et al*., 2006; Strahilevitz *et al*., 2009). The *qnrS*, which recovered from veterinary *E. coli* clinical isolates in Guangdong, China, and found to be differed from *qnrS1* in one codon was renamed *qnrS3* (Strahilevitz *et al*., 2009). Recently, *qnrS4* was detected in *Salmonella* isolate from Denmark, in 2008 (Torpdahl *et al*., 2008; Strahilevitz *et al*., 2009).

qnrB

The PMQR gene from *K. pneumoniae*, which could transfer low level quinolone resistance but were negative by Polymerase Chain Reaction (PCR) for *qnrA*, coded for a 214 or 226 amino acid (depending on which potential initiation codon was taken as the start) and was termed *qnrB*. QnrB protein shares 43% and 44% amino acid identities with QnrA and QnrS, respectively (Jacoby *et al*., 2006; Cattoir *et al*., 2008; Strahilevitz *et al*., 2009; Wang *et al*., 2009). The repertoire of *qnrB* variants is wider than that of *qnrA*

and *qnrS*. The first *qnrB* variant, *qnrB2* was found in the first survey among several isolates of *Enterobacteriaceae* from the United States that differs from *qnrB1* in five codons (Jacoby *et al*., 2006; Strahilevitz *et al*., 2009). At about the same time, the surveys in the United States have identified *qnrB3* and *qnrB4* among isolates of *Enterobacteriaceae* and *qnrB5* in *Salmonella enterica* serotype Berta isolates, these differed from *qnrB1* in two, fourteen and six codons, respectively (Robicsek *et al*., 2006; Strahilevitz *et al*., 2009). The *qnrB6* was found among several cephalosporin resistant isolates of *Enterobacteriaceae* in Western China, and which differed from the original *qnrB1* by two amino acids. The *qnrB7* was identified in *Enterobacter cloacae* and *qnrB8* in *Citrobacter freundii*, these genes differed from *qnrB1* four and eleven amino acids, respectively (Cattoir *et al*., 2008; Strahilevitz *et al*., 2009). The *qnrB19* was found in an *E. coli* isolated from Columbia that differed from the original *qnrB1* by six amino acids. The *qnrB10* was discovered in *Citrobacter freundii* and *qnrB12* identified in *Citrobacter werkmanii*, these genes differed from *qnrB1* by five and eight amino acid, respectively (Quiroga *et al*., 2007; Kehrenberg *et al*., 2008; Strahilevitz *et al*., 2009). The *qnrB9*, *qnrB11*, *qnrB16*, *qnrB17* and *qnrB18* were all found among different isolates of *C. freundii* (Strahilevitz *et al*., 2009).

qnrC

A clinical strain of *Proteus mirabilis* from Shanghai, China was isolated from an outpatient with a urinary tract infection. This strain transferred low level quinolone resistance and negative by PCR for the known *qnr* gene including *qnrA*, *qnrS* and *qnrB*. Plasmid pHS10 in of this strain, which, upon conjugation, increased the MIC of ciprofloxacin, carried a 666 bp gene, designated *qnrC1*, coding for a 221 amino acid. *qnrC* had 60%, 45%, 59%, and 32% nucleotide identity with *qnrA1*, *qnrB1*, *qnrS1*, and *qnrD*, respectively (Strahilevitz *et al*., 2009; Wang *et al*., 2009).

qnrD

In the previous study, four isolates of *Salmonella enterica* obtained from human in the Henan Province, China showed reduced susceptibility to ciprofloxacin that was transferable on a small plasmid of about 4.3 kb, which in *E. coli* conferred a 32 fold increase in the MIC of ciprofloxacin. This plasmid was negative for known *qnr* including *qnrA*, *qnrB*, and *qnrS*, and other PMQR genes such as *aac(6')-Ib-cr* and *qepA*. The plasmid encoded a 214 amino acid pentapeptide repeat protein and designated QnrD. The *qnrD* showed 48% similarity to *qnrA1*, 61% similarity to *qnrB1*, and 41% similarity to *qnrS1* (Cavaco *et al*., 2009; Strahilevitz *et al*., 2009).

2.3.4 Additional PMQR determinants

Aminoglycoside acetyltransferase cr variants: AAC(6')-Ib-cr

Several years after the discovery of QnrA, Robicsek and colleagues was investigating the phenomenon of inequality in the level of quinolone resistance transferred with different *qnr* plasmids. Wild type *E. coli* (absent *qnrA*) strains have an MIC of ciprofloxacin of about 0.008 µg/ml, and *qnrA* plasmids determine an MIC of cipofloxacin were usually 0.25 µg/ml for *E. coli*. They found *qnrA*-bearing *E. coli* isolates from Shanghai provided four-fold higher levels of ciprofloxacin resistance (1.0 µg/ml). In addition, they found that high-level resistance not increased by expression of *qnrA*, but associated with other mechanism. The gene responsible for this increase resistance was an aminoglycoside acetyltransferase, *aac(6')-Ib*, which confers resistance to tobramycin, amikacin, and kanamycin. Sequencing showed the important variants of *aac(6')-Ib* in two codons changes, Trp102Arg and Asp179Tyr, which responsible for the ciprofloxacin resistance phenotype. This variant of AAC(6')-Ib showed ability to acetylate cipofloxacin at the amino nitrogen on its piperazinyl substituent and designated AAC(6')-Ib-cr, for ciprofloxacin resistance. The increase in MIC conferred by AAC(6')-Ib-cr is smaller than that conferred by Qnr proteins, AAC(6')-Ib-cr was effected for ciprofloxacin and norfloxacin only, which both have piperzinyl ring at position C-7. Other quinolones

lacking piperazinyl substituted were unaffected (Robicsek *et al*. 2006; Strahilevitz *et al*., 2009; Rodriguez-Martinez *et al*., 2010).

QepA

The novel efflux pump QepA was found to be encoded on plasmid pHPA, discovered in an *E. coli* strain from a urine specimen from an inpatient in Hyogo Prefecture, Japan, in 2002. This plasmid displayed a multiple-resistance profile for aminoglycosides, fluoroquinolones, and broad-spectrum beta-lactam antibiotics. Yamane and colleagues found that *qepA* cloned into pSTV28 increased the MICs of several compounds in an *E. coli* transconjugant. The MICs of nalidixic acid, cipofloxacin, and norfloxacin increased 2, 32 and 64 fold, respectively. Since its discovery, a variant of *qepA* possessing two amino acid substitutions has been found. This variant designed QepA2 conferred a phenotype similar to that of the original QepA determinant (now renamed QepA1) (Yamane *et al*., 2007; Cattoir *et al*., 2008; Strahilevitz *et al*., 2009).

2.3.5 Mechanism of Qnr action

The Qnr proteins belong to the pentapeptide repeat family, which is defined by a series of tandem five amino acid repeats with the recurrent general motif approximately represented by the sequence A(D/N)LXX and more precisely represented by [Ser, Thr, Ala, or Val][Asp or Asn][Leu or Phe][Ser, Thr, or Arg][Gly] (Robicsek *et al*. 2006;

Strahilevitz *et al*., 2009). A characteristic feature of the Qnr proteins is that they are formed by two domains of pentapeptide repeats separated by a single amino acid, usually glycine as showed in Figure 3.4

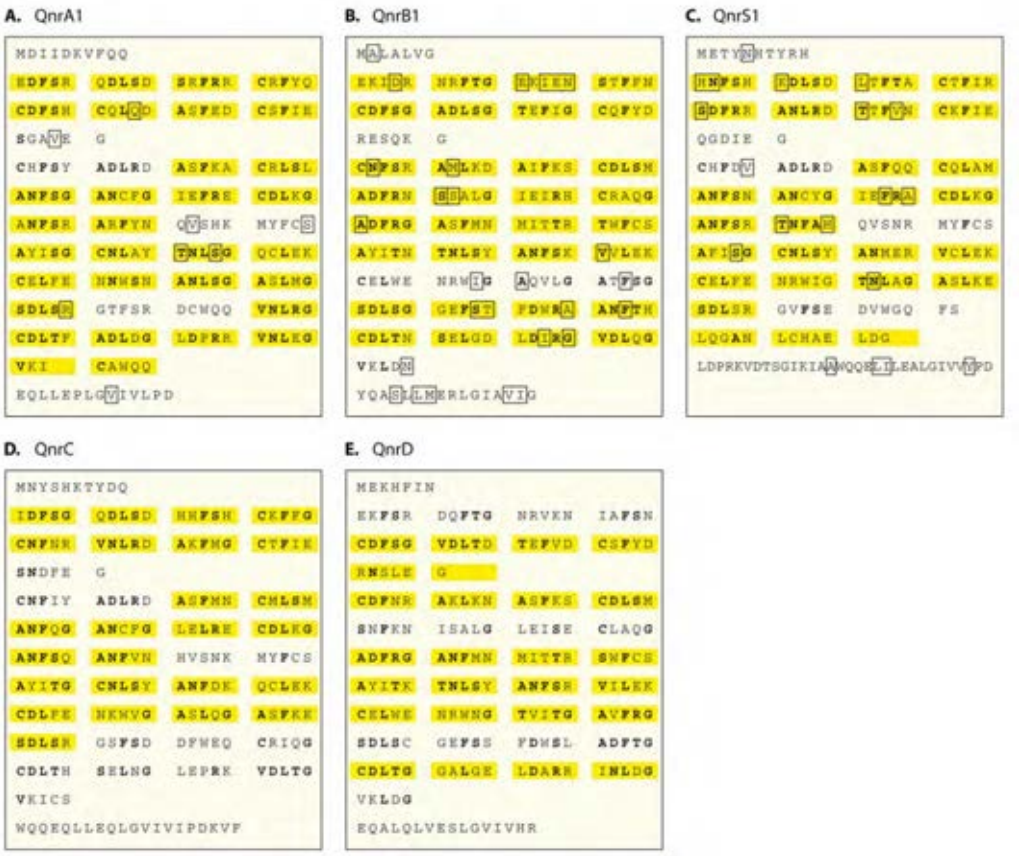

Figure 3.4 Amino acid sequence of Qnr displayed to emphasize the pentapeptide repeating unit with a consensus sequence of S/T/A/V/C-D/N-L/F-S/T/R-G. (A) QnrA1. (B) QnrB1. (C) QnrS1. (D) QnrC. (E) QnrD. No QnrC or QnrD variants have been described yet; yellow highlighting indicates the pentapeptide repeat according to Pfam website platform analysis; boldface type indicates residues that conform to the pentapeptide amino acid motif; boxed areas are amino acid changes in some Qnr variants (Strahilevitz *et al*., 2009).

To date, more than 500 proteins are known to contain such pentapeptide repeat motifs; however, the function of nearly all of these proteins is unknown. Three pentapeptide repeat proteins are of particular interest because they confer some level of quinolone resistance. The detailed mechanism of action of the PMQR protein (Qnr) is still unknown (Robicsek *et al*. 2006; Strahilevitz *et al*., 2009). McbG is a pentapeptide repeat protein sharing 19.6% amino acid identity with Qnr. It protects DNA gyrase against the effect of a microcin (Garrido et al., 1988; Strahilevitz *et al*., 2009). Microcin B17 (MccB17), is a bacterial poison that, like the quinolones, inhibits DNA gyrase (though at a different site from that of the quinolones). Organisms producing MccB17 also produce McbG, which protects them from the effect of this toxin, and *mcbG* has been found on resistant plasmids in clinical isolates (Heddle *et al*., 2001; Jacoby *et al*., 2003; Park *et al*., 2007; Strahilevitz *et al*., 2009). MfpA, a pentapeptide repeat protein having 18.9% amino acid similarity to QnrA, has been more thoroughly studied. The mfpA gene was first identified on the chromosome of *Mycobacterium smegmatis*. When expressed on a multi-copy plasmid, this gene resulted in an increase in the MIC of ciprofloxacin for this microorganism of between four-fold and eight-fold, and the inactivation of the gene on the *M. smegmatis* chromosome resulted in increased ciprofloxacin susceptibility. Subsequently, a variant of this gene found in *Mycobacterium tuberculosis* was showed to inhibit the activity of DNA gyrase by directly interacting with the enzyme. Both the three-dimensional structure of this MfpA variant and its charge distribution closely

resemble those of DNA. Thus, this protein is thought to inhibit gyrase through competition with DNA for binding. This interaction has also been proposed to underlie the fluoroquinolone resistance that the gene confers: DNA gyrase bound to MfpA will not participate in the quinolone-gyrase-cleaved DNA complex that is deleterious for cells (Hegde *et al*., 2005; Robicsek *et al*. 2006). The mechanism by which QnrA protects DNA gyrase has also been studied. As expected from its pentapeptide repeat structure, QnrA did not seem to effect a change in quinolone accumulation, outer membrane porins or drug inactivation; none of these are the mechanism of Qnr activity (Tran *et al*., 2002; Robicsek *et al*. 2006; Strahilevitz *et al*., 2009). The direct effect of QnrA on quinolone inhibition of DNA gyrase activity has been studied using a DNA supercoiling assay. In this assay, the inhibition of gyrase-mediated DNA supercoiling caused by ciprofloxacin was reversed in a dose-dependent manner by purified QnrA protein (tagged with histidine residues to facilitate purification). QnrA-His_6 alone, however, did not itself affect DNA supercoiling, nor did it, by contrast with MfpA, inhibit gyrase-mediated DNA supercoiling, although a Qnr variant can do so at very high concentrations. QnrA did, however, reduce gyrase binding to DNA as would be predicted if it formed, like MfpA, a DNA-like structure. In addition, QnrA has now been showed to bind gyrase (both subunits and holoenzyme) directly, as well as *E coli* topoisomerase IV, which it likewise protects from quinolone inhibition. QnrA binding to gyrase or topoisomerase IV, which inhibits the gyrase-DNA interaction, could

account, at least in part, for the protection against quinolones by minimising opportunities for these agents to stabilise the lethal gyrase-DNA-quinolone cleavage complex. How QnrA might compete with DNA for gyrase binding without substantially inhibiting gyrase activity *in vitro* is not yet known (Tran *et al*., 2002; Tran *et al*., 2005a; Tran *et al*., 2005b; Robicsek *et al*. 2006).

2.3.6 Resistance activity of Qnr

In general, the acquisition of a *qnrA* bearing plasmid will not contribute a wild type bacteria fluoroquinolone insusceptible according to CLSI clinical breakpoints. The extent to which QnrA protects *Enterobacteriaceae* clinical isolates against fluoroquinolones has usually been determined by measuring the difference in quinolone MICs for the *E. coli* strain with and without a *qnrA* bearing plasmid (Strahilevitz *et al*., 2009). The first report of a *qnrA* plasmid found that the MIC of ciprofloxacin increased range from 0.008 µg/ml to 0.25 µg/ml in the *E. coli* J53 transconjugant, with a range from 0.125 µg/ml to 2.0 µg/ml for other *qnr* plasmid transconjugants of this strain (Wang *et al*., 2003; Wang *et al*., 2004; Strahilevitz *et al*., 2009). A study of Wang and colleagues assessed the quinolone resistance conferred by 17 clinical *qnrA* bearing plasmids as showed in Table 3.3 Donor bacteria originally harboring these plasmids all had exhibited higher levels of resistance to quinolones than the transconjugants, suggesting that additional mechanisms determinants of quinolone resistance

frequently coexist with that of *qnrA*. Compared to plasmid-free J53, the MIC_{90} of ciprofloxacin increased 125-fold. The agent for whom the loss of activity was least pronounced was nalidixic acid (four fold to eight fold increases in MIC). Also noteworthy is the finding that *qnrA*-encoding plasmids from *K. pneumoniae* isolated from USA yielded transconjugants with very similar quinolone susceptibilities, whereas *qnrA*-encoding plasmids from Chinese *E. coli* varied in ciprofloxacin susceptibilities by 16 fold. The *qnr* can supplement other resistant determinants via altered quinolone target enzyme, efflux pump activation or deficiency outer membrane porin channels. The higher level resistance to all quinolone donor compared to transconjugant strain reflected especially additional chromosomal resistance mutations (Wang *et al*., 2003; Wang *et al*., 2004; Robicsek *et al*. 2006; Strahilevitz *et al*., 2009).

Table 3.3 *In vitro* activity of quinolones and newer quinolones against transconjugants containing *qnr* and donor strains. MIC 50 = MIC for 50% of strains; MIC 90 = MIC for 90% of strains. Adapted from Wang and colleagues 2004 (Wang *et al*., 2004)

Agents	*E. coli* J53 MIC (μg/ml)	MIC (μg/ml)			
		Transconjugants (n=17)		Donors (n=15)	
		MIC50	MIC90	MIC50	MIC90
AM-1121	0.008	0.5	0.5	16	≥64
BAYy3118	0.004	0.125	0.125	4	16
Ciprofloxacin	0.008	0.25	1	16	128
Levofloxacin	0.015	0.5	0.5	32	≥32
Moxifloxacin	0.03	0.5	1	32	≥64
Nalidixic acid	4	16	32	≥256	≥256
Sitafloxacin	0.008	0.125	0.125	4	8

MIC studies assessed the effect of a resistant gene on growth inhibition by an antimicrobial agent. There are other indices by which the effect of a resistant gene can be assessed. A time kill study has examined the bactericidal activity of ciprofloxacin and ofloxacin in the presence of QnrA. Despite the fact that QnrA protects against quinolone growth inhibition, it did not block the bactericidal activity of these quinolones at concentrations of twice MIC or greater (Mammeri et al., 2005; Robicsek *et al*. 2006; Strahilevitz *et al*., 2009). Another measure of resistant gene effect is a change in the mutant prevention concentration (MPC). The MPC is the lowest concentration of quinolone required to prevent the growth of quinolone resistant mutants from a starting

inoculum of about 10^{10} bacteria (defined a large inoculum is used to ensure the presence of single-step mutants occurring at a low frequency). When the quinolone concentration remains above the MPC, single-step resistant mutants are unlikely to arise. The early finding that QnrA facilitated recovery of mutants with higher levels of quinolone resistance prompted an assessment of its effect on the MPC of ciprofloxacin. The MPC for wild type *E. coli* J53 is 0.125 μg/mL; *E. coli* J53 carrying a *qnrA* plasmid has an MPC more than ten-fold greater as showed in Figure 3.5 Thus, the low level resistance conferred by these mechanisms might not allow a population of bacteria to survive in the presence of a quinolone. However, it substantially enhances the number of strains with resistance mutations that can be selected from the population, as also occurs with chromosomal quinolone resistance mutations (Jacoby, 2005; Robicsek *et al*. 2006; Strahilevitz *et al*., 2009).

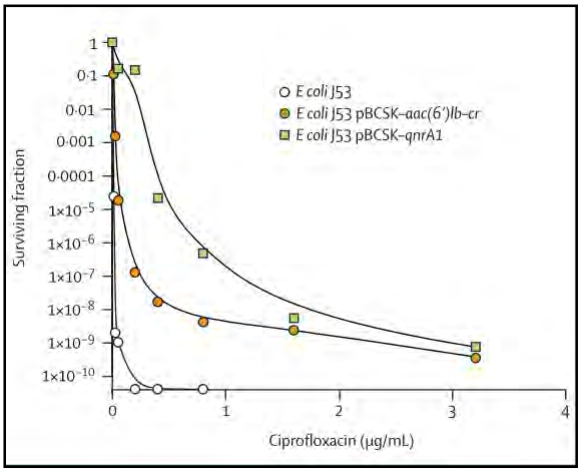

Figure 3.5 Mutant Prevention Concentration (MPC) assay. About 10^{10} organisms and appropriate dilutions were applied to Mueller-Hinton agar plates containing the indicated concentrations of ciprofloxacin. Surviving colonies were counted after incubation for 72 hour at 37°C. The lowest concentration of ciprofloxacin at which no mutant colonies were seen was 0.2 µg/ml for *E. coli* J53 and 3.2 µg/mL for *E. coli* J53 pBCSK-*aac(6')Ib-cr* or *E. coli* J53 pBCSK-*qnrA1* (Robicsek *et al*. 2006).

According, the MPC study by Rodriguez-Martinez and colleagues showed that in the presence of *qnrA1*, mutations in *gyrA* genes were easily selected to produce high levels of quinolone resistance. As showed in the Figure 3.6, MPC of ciprofloxacin against *E. coli DH10B* (wild type strain) and *E. coli DH10BpACYC184* (wild type), were both 0.016 µg/ml but in the presence of *qnrA1* (*E. coli DH10BpACYC184* + *qnrA1* strain), MPC was escalated to 2 µg/ml. Furthermore, in the case of *K. pneumoniae C2* (*gyrA* mutation; Ser83Phe + porin deficiency strain), MPC of ciprofloxacin against this strain was 8 µg/ml but in the presence of *qnrA1*, *K. pneumoniae C2 pMG252* (*gyrA*

mutation; Ser83Phe + porin deficiency + *qnrA1* strain) MPC was escalated to 128 µg/ml as showed in Figure 3.7 (Rodriguez-Martinez *et al*., 2007).

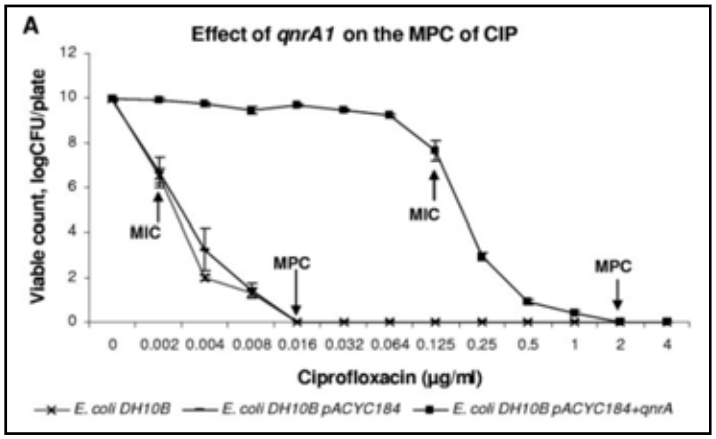

Figure 3.6 MPC of ciprofloxacin against three *E. coli* strains. *E. coli DH10B* (wild type strain), *E. coli DH10BpACYC184* (wild type) and *E. coli DH10BpACYC184* + *qnrA1* strain (Rodriguez-Martinez *et al*., 2007).

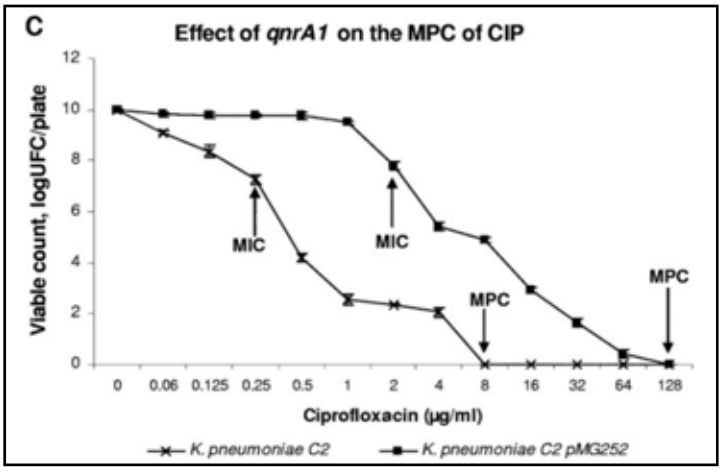

Figure 3.7 MPC of ciprofloxacin against two *K. pneumoniae* strains. *K. pneumoniae C2* (*gyrA* mutation; Ser83Phe + porin deficiency strain) and *K. pneumoniae C2 pMG252* (*gyrA* mutation; Ser83Phe + porin deficiency + *qnrA1* strain) (Rodriguez-Martinez *et al*., 2007).

2.3.7 QnrA plasmid

Genes for quinolone resistance have been found on plasmids varying in size and incompatibility specificity such as A/C_2, H12 and FII (Strahilevitz *et al*., 2009), indicating that the spread of multiple plasmids has been responsible for the dissemination of this resistance around the world. *qnrA1* plasmids can vary in size from 20 to 320 kb (Cambau *et al*., 2006; Lavilla *et al*., 2008; Strahilevitz *et al*., 2009) and belong to at least three plasmid incompatibility (Inc) groups (Poirel et al., 2007; Strahilevitz *et al*., 2009). The *qnrA1* is usually associated with insertion sequence common region 1 (IS*CR1*; formerly orf513) (Toleman *et al*., 2006; Strahilevitz *et al*., 2009). IS*CR1*, which may have mobilized the nearby sequence and a truncated 3' conserve sequence from one integron to the 3' conserve sequence of another integron through rolling circle transposition, thus facilitating the formation of complex class 1 integrons associated with IS*CR1*. IS*CR1* were found to be associated with several antimicrobial resistance genes, including the PMQR determinant *qnr* as well as genes encoding resistance to chloramphenicol, trimethoprim, aminoglycosides and beta-lactams. However, lacking the 59 base elements required for site-specific recombination, these IS*CR1*-linked genes could not have been acquired as gene cassettes. It was hypothesized that these antimicrobial resistant genes were added to the 3' conserve sequence of the class 1 integron (*intI1*) through co-mobilization with the nearby IS*CR1* from other integrons using rolling circle transposition and homologous

recombination (Toleman *et al*., 2006; Chen *et al*., 2009). The *qnrA1* IS*CR1* complex is inserted in turn into a *sul1*-type integron containing several other resistant gene cassettes as showed in Figure 3.8

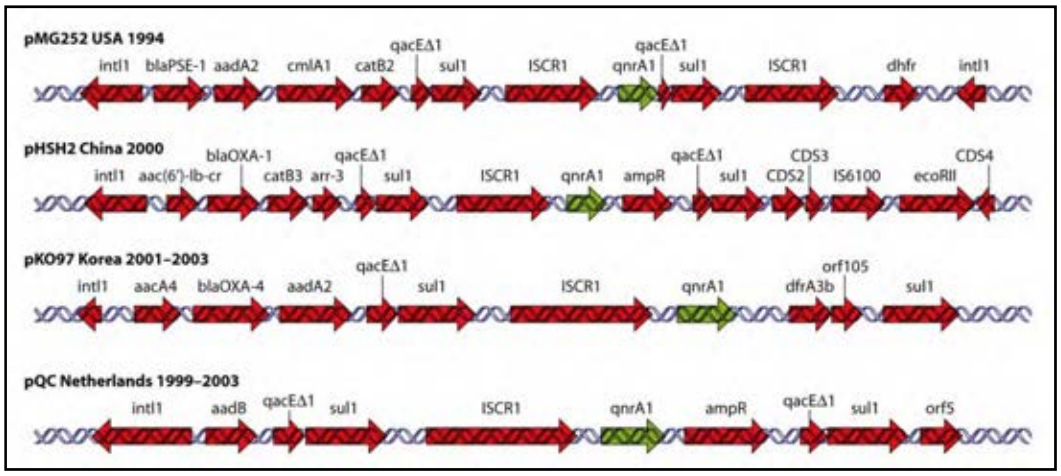

Figure 3.8 Genetic environment of *qnrA1*. (Strahilevitz *et al*., 2009)

2.3.8 Chromosomal *qnrA* genes

After a total of 48 Gram-negative clinical and environmental species were screened that included the *Enterobacteriaceae*, *Aeromonadaceae*, *Pseudomonadaceae*, *Xanthomonadaceae*, *Moraxellaceae*, and *Shewanellaceae* families, variants of *qnrA* (*qnrA3*) were located on the chromosome of *Shewanella algae*. *S. algae* is widely distributed in aquatic environments and is rarely implicated in human infections. These data suggest that *S. algae* is a possible reservoir of *qnrA* (Poirel *et al*., 2005; Rodriguez-Martinez *et al*., 2010)

2.3.9 Clinical relevance of *qnrA*

The discovery of PMQR especially *qnrA* could explain how exquisitely susceptible wild type *Enterobacteriaceae* have provided to develop high-level quinolone resistance. In the face of clinical concentrations of quinolone, microorganisms with even a single resistance mutation can survive, and give rise to increasingly resistant mutants. A study by Robicsek and colleagues studied 313 ceftazidime resistant *Enterobacteriaceae* isolates obtained in the United States from 1999 to 2004. The *qnr*-bearing isolates were likely to be susceptible by the criteria of CLSI as not susceptible (Robicsek, 2006a; Robicsek *et al*., 2006b). These susceptible microorganisms, carrying a *qnr* gene but lacking sufficient chromosomal mutations to qualify as resistant, that are clinically troublesome. Such microorganisms, *in vitro*, graciously develop resistance. CLSI breakpoints were developed based on data that most non resistant *Enterobacteriaceae* strains had very low MICs of quinolones. If *qnr* bearing plasmids widely penetrate a population of pathogens, the practice of exposing such microorganisms to quinolones could stimulate the rapid development of resistance. This phenomenon has already been documented in a clinical setting; a sensitive of *E. coli* harboring *qnrA* isolate but no classic quinolone resistance mutations was found to develop chromosomal mutations and subsequent high level resistance after 5 days of norfloxacin therapy (Poirel et al., 2006; Robicsek, 2006).

In most of the cases, the *qnrA* gene was easily transferable by conjugation whereas in rare cases it was not (Martinez-Martinez *et al*., 1998; Jacoby *et al*., 2003; Wang *et al*., 2003; Rodriguez-Martinez *et al*., 2003; Wang *et al*., 2004; Mammri *et al*., 2005; Nazik *et al*., 2005; Nordmann *et al*., 2005; Poirel *et al*., 2005). According the study of Poirel and colleagues found that for 11 clinical isolates of *Enterobacteriaceae* from Thailand that harbored *qnrA*, quinolone resistance could be transferred to transconjugants but was present at very low levels or not at all in the donors (Poirel *et al*., 2005). The expression of the QnrA determinant may vary after its transfer in reference strains suggesting heterogeneous expression of quinolone resistance determinants (Wang *et al*., 2003; Wang *et al*., 2004; Nordmann *et al*., 2005; Poirel *et al*., 2005).

The emergence of PMQR determinants in *Enterobacteriaceae* isolates may accommodation further the efficacy of quinolones that are, together with beta-lactams and macrolides, that the most generally prescribed antibiotics for treating human infections. This novel mechanism of resistance may be important for the treatment not only of nosocomial-acquired but also of community-acquired infections. Nevertheless, it remains to be determined if QnrA determinants in *Enterobacteriaceae* really accommodation the clinical efficacy of fluoroquinolones in the absence of additional chromosomally-mediated quinolone resistance determinants. Present knowledge on Qnr

determinants indicates that they are more various than previously expected. Their prevalence and the prevalence of their association with ESBL-encoding genes remain to be determined whereas Asian isolates seem already to be an important reservoir of Qnr determinants. Identification of *qnrA* genes embedded in integrons argues for their recent emergence in clinical isolates since an increase in integron prevalence in multidrug-resistance in *Enterobacteriaceae* has been reported recently. That may increase the opportunity for antibiotic resistance genes transfer among Gram-negative bacteria (Schmitz *et al*., 2001; Nordmann *et al*., 2005).

3. Integrons

The emergence and spread of antibiotic resistant organisms has been taking place, even though primarily slowly, since the introduction of antibiotic therapies. Penicillin resistant *Staphylococcus aureus* were first detected in the late 1940s and the first evidenced multidrug resistant isolates were *Shigella* strains found in Japan in the mid-1950s. These bacteria were subsequently shown to contain plasmids, and then called R-factors, that could transfer the resistances to antibiotic sensitive cells, therefore spreading the resistance genes to new microorganisms. Plasmids harboring one or more antibiotic resistance genes and the common association of antibiotic resistance with plasmids were detected in every part of the world. The next process performed with the intension to understand how the resistance

genes could be acquired was the discovery of transposons. These mobile genetic elements provided an demonstration for the finding that plasmids were present in clinical isolates from the pre-antibiotic era, but do not carry antibiotic resistance genes. If the resistance genes are part of mobile units, such as transposons, that can relocate from one DNA molecule (chromosome or plasmid) to another, it is possible for them to be picked up by plasmids. In addition, if the plasmid is conjugative or mobilization, the resistance genes can then be moved by these vehicles into other microorganisms. This combination is a powerful one, and has doubtless been assisted by the strong selective pressure of antibiotic use, not only in human medicine, but also in agriculture and aquaculture (Hall *et al*., 1998).

However, granted the already large number of different resistance genes that were known, the discovery of transposons simply shifted the question of how new antibiotic resistance genes were acquired, from how they were acquired by plasmids to how they were acquired by transposons. In the case of composite transposons such as Tn*5* (*neo*, *ble*, *str*), Tn*9* (*cat Al*) and Tn*10* (*tet 13*) where the resistance genes are flanked by two copies of an insertion sequence (IS) it is possible to envisage that the ISs move by chance to locations on either side of the resistance genes to form the transposon. However, relatively few resistance genes are found in composite transposons and it is now known that a substantial

proportion of the antibiotic resistance genes found in Gram-negative microorganisms are part of much smaller mobile genetic units known as gene cassettes. These novel mobile elements usually include only a single gene and a recombination site that confers mobility (Hall *et al*., 1991; Recchia *et al*., 1995; Hall *et al*., 1998).

Gene cassettes are usually found integrated at a specific location in a companion element known as an integron, and it is the integron that encodes the enzyme responsible for cassette movement. One or more gene cassettes and an integron can combine to form integrons containing different arrays of antibiotic resistance genes. Indeed, arrays of several cassettes, each containing a determinant for resistance to a different antibiotic or group of antibiotics, have been found in integrons isolated from multidrug resistant bacteria. Because so many different resistance genes are found in cassettes, it is possible to form a vast number of different arrays and this feature clearly contributes to multidrug resistance (Stokes *et al*., 1989; Hall *et al*., 1995; Hall *et al*., 1998).

In addition, the integrons are themselves mobile elements, namely transposons or defective transposon derivatives, and sometimes they are found within other transposons. Transposition enables integrons to move onto plasmids and hence across species boundaries. As a consequence, the resistance genes found

in gene cassettes and thus associated with integrons are now widely distributed amongst the *Enterobacteriaceae* family and are also found in *P. aeruginosa* (Hall *et al*., 1998).

3.1 Structure of integron

All integrons are composed of a stable platform, which contains the functional elements required for the system operation, associated with a variable array of discrete gene cassettes encoding accessory functions as showed in Figure 3.9

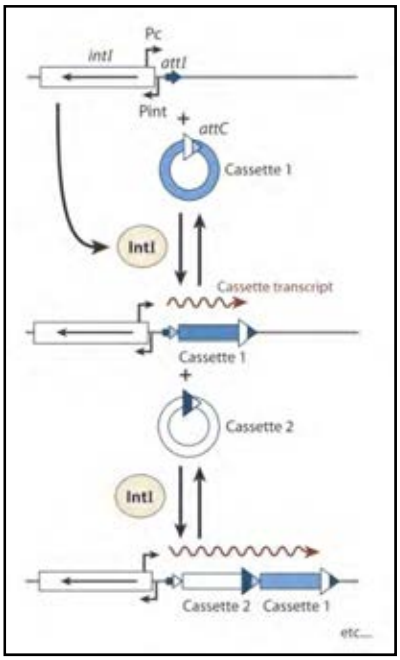

Figure 3.9 Integron-mediated gene capture and model for cassette exchange. Outline of the process by which circular gene cassettes are repeatedly inserted at the specfic *attI* site in an integron downstream of the strong promoter Pc. *intI*, integrase encoding gene; IntI, integrase IntI (Cambray *et al*., 2010).

3.2 The Functional Platform

The principal component of the integron functional platform is the *intI* gene, which encodes a site specific tyrosine recombinase. This enzyme catalyzes the specific excision and integration of dedicated and discrete genetic elements, known as gene cassettes. The integration cassette essentially occurs at a specific locus lying immediately adjacent to *intI*, referred to as the primary recombination site *attI*. A large majority of cassettes are promoterless. The expression of the genes they carry is then ensured by a dedicated Pc promoter, which is embedded either in the *intI* gene or in the *attI* site and oriented toward the integration point. By itself, the functional platform is stable and non-mobile (Stokes *et al*., 1989; Collis *et al*., 1993; Collis *et al*., 1995; Cambray *et al*., 2010; Jove *et al*., 2010).

3.3 The Cassette Array

Successive integration at the *attI* site results in the streamlined assembly of different gene cassette arrays. This cassette array constitutes the variable part of the integron. Gene cassettes are minimal functional elements intended to be mobilized by the integrase of integrons. They are generally constituted by a single open reading frame (ORF) immediately followed by a recombination site termed *attC* (formerly called 59-base element), which is specifically recognized by IntI. As mentioned above, cassette borne genes are most generally promoterless, and their expression is hence

conditioned by the proximity of an external promoter, essentially Pc promoter. Accordingly, the ORFs in cassettes are usually oriented toward the *attC* site. The excision of cassettes by the IntI integrase leads to nonreplicative, covalently closed circular intermediates (Collis *et al*., 1992; Collis *et al*., 1993; Recchia *et al*., 1994; Cambray *et al*., 2010)

3.4 Mobile integrons

Mobile integrons contain only a few cassettes that appear to be of heterogeneous origins and were probably collected successively in different genomics backgrounds. The longest array identified is composed of eight cassettes. The heterogeneity of the cassettes is attested by the inconsistent codon usage of their encoded ORFs and by the sequence and size diversity of their *attC* sites. Contrasting with their apparent heterogeneous origins, cassettes associated with a mobile integrons display a striking functional homogeneity, as they are mostly involved in antibiotic resistance. A pool of greater than 130 different cassettes harboring various antibiotics resistance genes has been identified in mobile integrons (based on a 98% nucleotide identity threshold). Together, these cassettes provide resistance to most classes of antibiotics including beta-lactams, all aminoglycosides, chloramphenicol, trimethoprim, rifampicin, erythromycin, fosfomycin, lincomycin, quinolones, and antiseptics of the quaternary ammonium compound.

At present, 5 classes of mobile integrons are known to have a role in the dissemination of antibiotic resistance genes. These classes have been historically defined based on the sequence of the encoded integrases, which show 40 to 58% identity. All 5 classes are physically linked to mobile DNA elements, such as insertion sequences (IS), transposons and conjugative plasmids, these elements can serve as vehicles for the intra-species and inter-species transmission of genetic material. Only the first 3 classes of mobile integrons are historical classes that are involved in the multiple antibiotic resistance phenotypes, all 5 classes have been associated with antibiotic resistance determinants (Mazel, 2006; Cambray *et al*., 2010).

Class 1 integrons are associated with functional and non functional transposons derived from Tn*402* that can be embedded in larger transposons, such as Tn*21*. These elemenst are found extensively in clinical isolates, and most of the known antibiotic resistance gene cassettes belong to this it. They are detected in 22 to 59% of Gram-negative clinical isolates, and they have also been occasionally identified in Gram-positive bacteria. These elements confer resistance to many classes of antibacterial agents such as beta-lactams, all aminoglycosides, chloramphenicol, trimethoprim, rifampicin, erythromycin, fosfomycin, lincomycin and antiseptics of the quaternary ammonium compound. This class is not associated with resistance genes have been recovered in environmental bacteria (Mazel, 2006; Cambray *et al*., 2010).

Class 2 integrons are exclusively associated with Tn*7* derivatives and show a dozen different cassette arrays. The integrase gene of class 2 integrons, *intI2*, generally contains a nonsense mutation in codon 179 that yields a non functional protein, which can be rescued by a single mutation. However, it is not known whether the cassette recombination in the different Tn*7* derivatives is mostly due to occasional natural suppression of the ochre179 codon, leading to an active integrase, or due to the trans-acting recombination activity of another IntI, such as IntI1, which has been showed to recognize and recombine the *attI2* site of class 2 integrons. Furthermore, class 2 integrons encoding a functional integrase have been isolated on two occasions. In the first instance, it was associated with four non antibiotic resistance gene cassettes. These data suggest that mobile integron are not specifically dedicated to antibiotic resistance but are likely to be broadly involved in mediating bacterial adaptation. The prevalence of resistance functions probably results from biased sampling focused on clinically relevant environment and reflects the evolutionary success of integrons in these settings (Mazel, 2006; Cambray *et al.*, 2010).

Class 3 integrons are also thought to be located in a transposon and are less prevalent than class 2. This class is not associated with resistance genes have been recovered in environmental bacteria as well as class 1 integron. The other 2 classes of mobile integrons, class 4 and class 5, have been identified through their involvement in

the development of trimethoprim resistance in *Vibrio* species. The class 4 integron is embedded in a subset of the integrative and conjugative element SXT found in *Vibrio cholerae*. The class 5 integron is located in a compound transposon carried on the pRSV1 plasmid of *Alivibrio salmonicida* (Mazel, 2006; Cambray *et al*., 2010).

3.5 Relationship of *qnrA* and integron

qnrA genes has been identified and located in In*4* family class 1 integrons, In*36* and In*37*, which are also knows as complex *sul1*-type integrons because of the presence of duplicate *qacE*Δ*1* and *sul1* genes which genes surround a sequence encoding orf513 (now called IS*CR1*) as showed in Figure 3.10 This relationship is supported *in vitro* susceptibility results on transconjugants containing plasmids harboring *qnrA*, demonstrating that all transconjugants were resistant to sulfamethoxazole. The *qnrA* gene was not associated with a 59-base element as a form of a gene cassette as found in common class 1 integrons. Structural comparison of *qnrA* integrons cassettes showed fluctuation both in the upstream and downstream-*qnrA* located DNA sequences. This suggests that the process that had led to *qnrA* gene insertion in the *sul1*-type integron may fluctuate. Interestingly, no ESBL gene was located inside any *qnrA* via *sul1*-type integrons. This observation indicates that co-localization of *qnrA* and ESBL genes on the same plasmids probably results from

unrelated genetic events (Tran *et al*., 2002; Jacoby *et al*., 2003; Wang *et al*., 2003; Wang *et al*., 2004; Mammeri *et al*., 2005; Nordmann *et al*., 2005; Poirel *et al*., 2005).

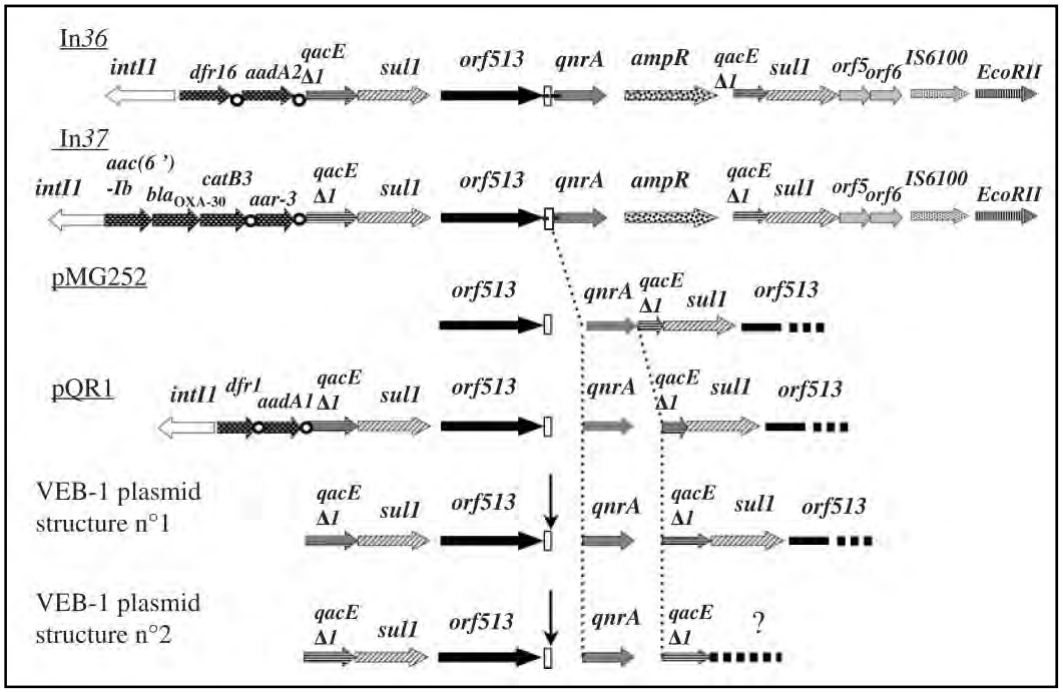

Figure 3.10 Schematic comparisons of *sul1*-type integrons that contain *qnrA* genes. The compared structures are those of *E. coli* isolates from China (In*36* and In*37*), a *K. pneumoniae* isolate from the USA (pMG252), an *E. coli* isolate from France (pQR1), enterobacterial isolates from Thailand and France (VEB-1 plasmid structure no. 1) and other enterobacterial isolates from Thailand (VEB-1 plasmid structure no. 2). The vertical rectangle indicates the right-hand boundary of the CR1 element and the vertical arrow indicates a 2 bp deletion. The question mark indicates unknown DNA sequences but different from those reported above. Dotted vertical lines indicate the absence of a DNA fragment between the *qnrA* genes, *qacE*Δ*1* in pMG252 (Nordmann *et al*., 2005).

4. **Extended spectrum beta-lactamases (ESBLs)**

Extended spectrum beta-lactamases (ESBL), which hydrolyse extended spectrum cephalosporins and are inhibited by clavulanic acid, are spreading among *Enterobacteriaceae*. The presences of ESBL are found in various members of the *Enterobacteriaceae*, particularly *K. pneumoniae* and *E. coli*. ESBL are also detected in non-fermentative Gram-negative bacteria such as *P. aeruginosa* and *Acinetobacter baumannii* (Jacoby *et al*., 2005). The utilizable therapeutic regimens for the treatment of ESBL associated infections are limited by drug resistance associated by the ESBL, along with frequently observed co-resistance to various classes of antibiotics, including cephamycins, fluoroquinolones, aminoglycosides, tetracyclines and trimethoprim/sulfamethoxazole. Important clinical data considering the effectiveness of different regimens for ESBL associated infections are limited. Although certain cephalosporins may appear active *in vitro* studies, associated clinical outcomes are often suboptimal. The beta-lactam antibiotic plus beta-lactamase inhibitor combinations may be of value, but the supporting evidence is weak. Carbapenems are considered to be alternative choice, and could be more effective than fluoroquinolones for serious infections. Tigecycline and polymyxins have substantial antimicrobial activity against ESBL producing *Enterobacteriaceae* (Falagas *et al*., 2009).

4.1 Detection of ESBL in laboratories

In the laboratory, the detection of ESBL could be based on the phenotypic or genotypic characteristic of the enzymes. The phenotypic tests are routinely used in clinical diagnostic laboratories, whereas the genotypic tests are mainly used in reference or research laboratories. The phenotypic tests for ESBL detection involve screening and confirmatory tests. The screening test consists of testing for resistance to cefpodoxime, cefotaxime, ceftazidime, ceftriaxone, or aztreonam. The confirmatory test is based on the synergistic between the above antimicrobial agents and clavulanic acid. Several methods including the double disk synergy test, the combination disk method or specific ESBL Etests can be used in this considerate. Poor sensitivity of these tests may be observed when the evaluated ESBL producing organism additionally produces a beta-lactamase not inhibited by clavulanic acid, such as an AmpC beta-lactamase or metallo-beta-lactamase. Methods to vanquish this limitation include the use of cefepime, which is a weak substrate for most AmpC beta-lactamases, the use of chromogenic agar, cloxacillin containing agar, or the addition of EDTA to inactivate metallo-beta-lactamases. The genotypic tests for the detection of ESBL originally consist of PCR amplification of the specific genes. Considering the TEM and SHV type ESBL, additional molecular techniques, such as sequencing or restriction fragment length polymorphism, are required for the identification of specific point mutations that differentiate these enzymes from parent enzymes without ESBL activity. Although technically challenging,

the genotypic methods have the advantage of identification of the specific type of ESBL present in an isolate, particularly, usefulness for epidemiology. In addition, they may be detected the low level resistance, and performed without prior culture of the microbiological specimen (Wiegand *et al*., 2007; Pitout *et al*., 2008; Drieux *et al*., 2008; Falagas *et al*., 2009).

4.2 Classification of ESBLs

Two major classification for categorizing beta-lactamase enzymes are Ambler classes (molecular class, A to D), based on amino acid sequence homology, and Bush-Jacoby-Medeiros groups (class 1 to 4), based on substrate and inhibitor profile as showed in Table 3.4 A "family portrait" shows the structural similarity of class A, C, and D serine beta-lactamases as showed in Figure 3.11 These three classes have serine at their active site. Only class B beta-lactamases ("a class apart") are metallo-beta-lactamases which possess either a single Zn^{2+} ion or a pair of Zn^{2+} ions coordinated to His/Cys/Asp residues in the active site (Drawz *et al*., 2010).

Table 3.4 Beta-lactamase classification schematics (Drawz *et al.*, 2010).

Ambler class	Bush-Jacoby-Medeiros class	Preferred substrates	Inhibited by clavulanate	Representative enzyme(s)
A (serine penicillinases)	2a	Penicillins	+	PC1 from *S. aureus*
	2b	Penicillins, narrow-spectrum cephalosporins	+	TEM-1, TEM-2, SHV-1
	2be	Penicillins, narrow-spectrum and extended-spectrum cephalosporins	+	SHV-2 to SHV-6, TEM-3 to TEM-26, CTX-Ms
	2br	Penicillins	−	TEM-30, SHV-72
	2c	Penicillins, carbenicillin	+	PSE-1
	2e	Extended-spectrum cephalosporins	+	FEC-1, CepA
	2f	Penicillins, cephalosporins, carbapenems	±	KPC-2, SME-1, NMC-A
B (metallo-β-lactamases)	3	Most β-lactams, including carbapenems	−	IMP-1, VIM-1, CcrA, and BcII (B1); CphA (B2); L1(B3)
C (cephalosporinases)	1	Cephalosporins	−	AmpC, CMY-2, ACT-1
D (oxacillinases)	2d	Penicillins, cloxacillin	±	OXA-1, OXA-10
Not classified	4			

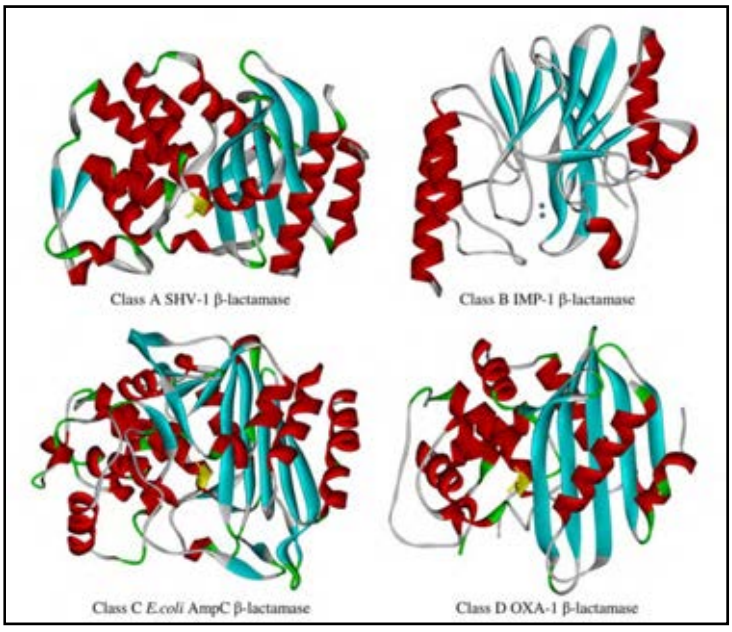

Figure 3.11 "Family portrait" of beta-lactamase enzymes including class A SHV-1, class B IMP-1, class C *E. coli* AmpC and class D OXA-1. For classes A, C, and D, the active-site serine is showed in yellow; for class B, the two Zn^{2+} ions are showed (Drawz *et al.*, 2010).

4.2.1 Class A serine beta-lactamases

In general, class A enzymes are susceptible to the commercially available beta-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam, although the *K. pneumoniae* carbapenemase (KPC) may be an important exception to this generalization. The first plasmid-mediated beta-lactamase was identified in *E. coli* in 1963 that was named TEM after the patient from whom it was isolated. TEM-1 is the most generally encountered beta-lactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1. TEM-1 is able to hydrolyze penicillins and early cephalosporins such as cephalothin and cephaloridine. SHV, another common beta-lactamase found primarily in *K. pneumoniae*, was named from the term “sulfhydryl reagent variable”. Early studies of SHV-1 showed that *p*-chloromercuribenzoate inhibited the hydrolysis of cephaloridine but not that of benzylpenicillin. TEM and SHV are general beta-lactamases found in *E. coli* and *K. pneumoniae* isolates, that responsible for urinary tract, hospital-acquired respiratory tract, and bloodstream infections. Furthermore, SHV-1 and TEM-1 share 68% sequence homology, and the size of the active site of SHV-1 and TEM-1 are the same, which may have important structural implications, especially related to the substrate profiles of SHV variants. Although bla_{TEM} and bla_{SHV} may be detected on plasmids, other class A enzymes are encoded on the chromosome such as bla_{PenA} from *Burkholderia pseudomallei* or on integrons such as bla_{VEB-1} from *P. aeruginosa* and *Acinetobacter*

baumannii (Datta *et al*., 1965; Livermore, 1995; Tzouvelekis *et al*., 1999; Bradford, 2001; Drawz *et al*., 2010).

4.2.2 Class A ESBL

The increasing number of beta-lactamases in *E. coli* and *K. pneumoniae* as well as the other pathogens such as *Haemophilus influenzae* and *Neisseria gonorrhoeae*, induced to the development of extended-spectrum cephalosporins with an oxyimino side chain such as carbapenems, cephamycins, and monobactams. Later, the introduction of the penems and cephems in the early 1980s, these agents were effective against many beta-lactam resistant organisms. Nevertheless, selective pressure quickly fostered the emergence of ESBL, which could hydrolyze many of the oxyimino-cephalosporins. Other ESBLs, such as CTX-M, originated by plasmid transfer from preexisting chromosomal ESBL genes from *Kluyvera* spp., which typically are nonpathogenic commensal organisms. CTX-M ESBLs now represent important enzymes detected in isolates from the community and are the most commonly isolated ESBLs in many parts of the world, particularly Europe. ESBL hydrolyze penicillins, narrow and extended spectrum cephalosporins. In contrast, ESBL could not efficiently degrade cephamycins, carbapenems, and beta-lactamase inhibitors. ESBL showing a significant risk to public health and to hospitalized patients in intensive care units, when infection with an ESBL may lead to significant morbidity and mortality. The SHV, TEM, and CTX-M

families are the majority of ESBL (Bonnet, 2004; Paterson *et al*., 2005; Drawz *et al*., 2010).

4.2.3 Class A serine carbapenemases

Class A serine carbapenemases include the non-metallo-carbapenamase of class A such as IMI, SME, and KPC. The members of this group of beta-lactamases could hydrolyze carbapenems as well as cephalosporins, penicillins, and aztreonam. These enzymes have been identified primarily in *E. cloacae*, *Serratia marcescens*, and *K. pneumoniae*, which often carry multiple resistance determinants. The *bla* gene for *E. cloacae* and *S. marcescens* are typically detected on the chromosome, while the *K. pneumoniae* carbapenemase bla_{KPC} gene is harbored on plasmids containing Tn*4401*. MICs of carbapenems in carbapenemase-expressing strains could vary from moderately increased (2 to 4 μg/ml) to resistant ($\geq$32 μg/ml) (Nordmann *et al*., 1993; Nordmann *et al*., 2002; Queenan *et al*., 2007; Nordmann *et al*., 2009; Drawz *et al*., 2010)

4.2.4 Class B metallo-beta-lactamases

Class B enzymes are Zn^{2+}-dependent beta-lactamases such as IMP-1 and VIM-1 that show a hydrolytic mechanism different from that of the serine beta-lactamases of classes A, C and D. Microorganisms producing these enzymes normally show resistance to penicillins, cephalosporins, carbapenems and beta-lactamase inhibitors. Interestingly, the hydrolytic profile of metallo-beta-lactamases does not typically include

aztreonam. These enzymes likely develop separately from the other Ambler classes or molecular classes, which have serine at their active site. The *bla* genes of these enzymes are located on the chromosome, plasmid, and integrons *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* provide class B enzymes encoded by mobile genetic elements (Bush *et al*., 1995; Massova *et al*., 1998; Ikonomidis *et al*., 2005; Walsh *et al*., 2005; Hujer *et al*., 2006; Corvec *et al*., 2008; Drawz *et al*., 2010).

4.2.5 Class C serine cephalosporinases

The sequence of the *ampC* gene from *E. coli* was reported in 1981. It differed from the sequence of penicillinase-type beta-lactamases such as TEM-1 but, like them, had serine at its active site. AmpC belong to class C in the Ambler structural classification, while in the functional classification of Bush-Jacoby-Medeiros groups, they were assigned to group 1. Class C AmpC beta-lactamases include CMY-2, P99, ACT-1 and DHA-1, which are normally encoded by *bla* genes located on the bacterial chromosome, although plasmid-borne AmpC enzymes are becoming more prevalent. Microrganisms expressing the AmpC beta-lactamase are typically resistant to penicillins, beta-lactam/beta-lactamase inhibitor combinations, and cephalosporins, including cefoxitin, ceftriaxone, and cefotaxime. These enzymes poorly hydrolyze cefepime and are inhibited by cloxacillin, oxacillin, and aztreonam. Production of chromosomal AmpC in Gram-negative bacteria is at a low level (“repressed”) but can be

“derepressed” by induction with certain beta-lactams, particularly cefoxitin. The selection of mutant bacterial populations is concern significant that are genetically derepressed for AmpC production, which can cause a dramatic increase in MICs during the course of beta-lactam therapy such as after 14 days of ceftazidime therapy, an isolate of *P. aeruginosa* with MICs increased from 1 to 32 μg/ml was selected (Livermore, 1987; Bennett *et al*., 1993; Bush *et al*., 1995; Hanson *et al*., 1999; Philippon *et al*., 2002; Juan *et al*., 2005; Babic *et al*., 2006; Jacoby, 2009; Drawz *et al*., 2010).

4.2.6 Class D serine oxacillinases

Class D beta-lactamases were originally categorized as “oxacillinases” because of their ability to hydrolyze oxacillin at a rate of at least 50% of that of benzylpenicillin, in contrast to the relatively slow hydrolysis of oxacillin by classes A and C. OXA beta-lactamases can also confer resistance to penicillins, cephalosporins, extended-spectrum cephalosporins (OXA-type ESBL), and carbapenems (OXA-type carbapenemases) in microorganisms. In general, OXA enzymes are resistant to inhibition by clavulanic acid, sulbactam, and tazobactam, except some of which such as OXA-2 and OXA-32 are inhibited by tazobactam but not sulbactam and clavulanic acid, and OXA-53 is inhibited by clavulanic acid (Naas *et al*., 1999; Poirel *et al*., 2002; Mulvey *et al*., 2004; Drawz *et al*., 2010).

4.3 *qnrA* associated ESBL and epidemiology of these genes

qnrA genes were commonly found in ESBL producing microorganisms, particularly, *Enterobacteriaceae* family. Genes for ESBL and AmpC are often found on the *qnrA* plasmid. The *qnrA1* was discovered in a *K. pneumoniae* isolate from Alabama on plasmid pMG252 also expressing the uncommon FOX-5 beta-lactamase. Plasmids carrying *qnrA1* and FOX-5 have subsequently been found in specimens from Delaware, Kentucky, New York, North Carolina, and Tennessee (Rodriguez-Martinez *et al*., 2003; Wang *et al*., 2004; Strahilevitz *et al*., 2009) and from as far away as Brazil in *E. coli* isolates (Castanheira *et al*., 2007; Strahilevitz *et al*., 2009). In *E. coli* isolates from Canada and France, *qnrA1* is associated with VEB-1 beta-lactamase (Mammeri *et al*., 2005; Poirel *et al*., 2006), while *qnrA1* is associated with $bla_{\text{VEB-1}}$ from Turkey found in *C. freundii* isolate (Nazic *et al*., 2005), In Southeast Asia, *Enterobacteriaceae* isolates including *E. coli*, *K. pneuminiae* and *Enterobacter sakazakii* from Thailand, *qnrA1* is associated with $bla_{\text{VEB-1}}$ as well (Poirel *et al*., 2005), while the *Enterobacteriaceae* isolates including *E. coli*, *K. pneuminiae*, *Klebsiella oxytoca*, *Enterobacter cloacae* and *C. freundii* from widely part of the world include Australia, China, France, Hungary, Netherland, South Korea, Spain, Taiwan, United Kingdom, *qnrA1* is linked with ESBL including SHV-5, SHV-7, SHV-12 and various CTX-M family (Wang *et al*., 2003; Corkill *et al*., 2005; Nazic *et al*., 2005; Nordmann *et al*., 2005; Cambau *et al*., 2006; Paauw *et al*., 2006; Rodriguez-Martinez *et al*., 2006; Wu *et al*., 2007; Jiang *et al*., 2008; Lavilla *et al*.,

2008; Szabo *et al*., 2008; Tamang *et al*., 2008; Strahilevitz *et al*., 2009). Through the late 2008, the several publications in the peer-reviewed journals reported over 20,960 isolates in the worldwide that were screened for PMQR genes. The average prevalence of *qnrA*, *qnrS*, *qnrB* and *aac(6')-Ib-cr* in this data base were 1.5%, 2.4%, 4.6% and 10.8%, respectively. Although, *E. coli* is the most common was tested for PMQR genes. Nevertheless, in the huge majority of surveys, *qnr* was more prevalence among *Enterobacter* spp. and *Klebsiella* spp. than in *E. coli* isolates while *aac(6')-Ib-cr* is the most common found among *E. coli* isolates more than the other *Enterobacteriaceae* isolates (Strahilevitz *et al*., 2009).

CHAPTER IV

MATERIALS AND METHODS

1. Bacterial isolates

1.1 Clinical isolates

A total of 190 clinical isolates of *E. coli* from one university hospital and one tertiary private hospital were included in this study as showed in Table 4.1. The 100 ESBL producing *E. coli* isolates were collected from the university hospital during the period of January to February 2004. Among 100 isolates from the university hospital, 83 isolates were resistant, 2 isolates were intermediate and 15 isolates were susceptible to quinolone (ciprofloxacin). The 90 quinolone resistant *E. coli* isolates based on resistant criteria to ciprofloxacin (zone diameter $\leq$ 15 mm) and resistant to norfloxacin (zone diameter $\leq$ 12 mm) collected from the tertiary private hospital during the period of June to October 2010, were also included in this study. Among 90 isolates from the tertiary private hospital, 52 isolates were ESBL producing *E. coli* and 38 isolates were non-ESBL producing *E. coli*. All isolates in this study derived from blood, pus, sputum or urine. Identification, disk diffusion test and detection of ESBL producing *E. coli* isolates had been confirmed by routine laboratory procedure of the university hospital and the tertiary

private hospital, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010).

Table 4.1 The total number of *E. coli* isolates from 2 hospitals in this study.

E. coli isolates	ESBL screening (%)		Susceptibility test of quinolone (%)			Total
	ESBL	Non-ESBL	Susceptible	Intermediate	Resistant	
University hospital	100	-	15 (15)	2 (2)	83 (83)	100
Tertiary private hospital	52 (57.78)	38 (42.22)	-	-	90	90

1.2 Control strain

E. coli ATCC 25922 was used as a control strain in this study.

2. Detection of *qnrA* genes by Polymerase Chain Reaction (PCR)

2.1. Genomic DNA extraction

The total genomic DNA extraction of *E. coli* clinical isolates were extracted using the Puregene Yeast/Bact. Kit B (Gentra systems Co., Ltd., USA) according to the manufacturer's instructions. Five hundred microliters of bacterial overnight culture was added into 1.5 ml microcentrifuge tube and centrifuged at 16,000 x g for 1 minute to pellet the cells. The supernatant was carefully removed as much as possible by a pipette. The pellet was resuspended with 300 µl of Cell Lysis Solution and incubated at

80ºC for 5 minutes to lyse the cells. The cell lysate was added with 1.5 µl of the RNase A Solution, was mixed by inverting the tube 25 times and incubated at 37ºC for 60 minutes. Sample was cool down at room temperature. The 100 µl of Protein Precipitation Solution was added to the cell lysate and vortexed vigorously at high speed for 20 seconds, and then centrifuged at 16,000 x g for 3 minutes. The precipitated proteins could form a tight pellet. The supernatant containing DNA was poured into a clean 1.5 ml microcentrifuge tube containing 300 µl of 100% isopropanol and mixed by inverting gently 50 times. The DNA was pelleted by centrifuge at 16,000 x g for 1 minute. The supernatant was discarded and the microcentrifuge tube was drained by inverting on a clean absorbent paper. The DNA pellet was washed with 300 µl of 70% ethanol by inverting the tube several times, centrifuged at 16,000 x g for 1 minute and the supernatant was discarded. The microcentrifuge tube was carefully drained on a clean absorbent paper to remain the pellet in the tube and allowed to air dried for 15-20 minutes. The DNA pellet was rehydrated with 50 µl of DNA Dehydrate Solution and incubated at 60ºC for 1 hour. The tube was tapped periodically to aid in dispersing the DNA and was stored at -20ºC until the DNA was used.

2.2 PCR amplification of *qnrA* genes

All 190 isolates were screened for the presence of *qnrA* genes by PCR method with the primer sets to detect *qnrA1* to *qnrA6* (position 30 to 49 and 589 to 608) as

showed in Table 4.2 One microliter of genomic DNA was added to a reaction mixture containing 1x PCR buffer, 2mM $MgCl_2$, 200 µM deoxynucleoside triphosphate, 1 µM each primer, and 1 unit of DyNazyme™ II DNA polymerase (Applied Finnzymes OY, Finland). The amplification condition for *qnrA* were pre-denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, and final extension at 72°C for 7 min.

Table 4.2 Primer used in this study

Primer	Sequence	PCR product size (bp)	Reference
qnrA-F	5'-AGAGGATTTCTCACGCCAGG-3'	579	Cattoir *et al.*,2007
qnrA-R	5'-TGCCAGGCACAGATCTTGAC-3'		
*gyrA*6	5'-CGACCTTGCGAGAGAAAT-3'	626	Weigel *et al.*, 1998
*gyrA*631R	5'-GTTCCATCAGCCCTTCAA-3'		
intI1-F	5´-AAGGATCGGGCCTTGATGTT-3´	471	Pongpech *et al.*, 2008
intI1-R	5´-CAGCGCATCAAGCGGTGAGC-3´		

2.3 Analysis of PCR product

Amplification product were analyzed by running the PCR product (PCR product 6 µl and loading dye 2 µl) in 1% agarose gel in 1x TBE buffer. A 1 kb plus DNA ladder (Invitrogen, USA) was used as a DNA size marker. Agarose gel was run in TBE buffer at a constant 100 volts for 30 minutes.

The agarose gel was stained in the ethidium bromide solution (1 µg/ml) for 8 minutes. Then the gel was destained in deionized water for 10 minutes and photographed the gel under the UV transluminator.

2.4 Identification of *qnrA* subtype.

***qnrA* cloning**

A PCR product of *qnrA* gene was cloned into a plasmid vector, pSC-A-amp/kan using StrataClone PCR Cloning Kit® (Agilent Technologies, Inc., Canada) according to the manufacturer's instructions. First step, the ligation reaction mixture was prepared by combining 3 µl of StrataClone Cloning Buffer, 2 µl of PCR product and 1 µl StrataClone Vector Mix amp/kan. The ligation reaction mixture was incubated at room temperature for 5 minutes and taken the reaction on ice. All of the cloning reaction mixture was added into StrataClone SoloPack competent cells and mixed gently. The transformation mixture was incubated on ice for 20 minutes and heated-shock at 42°C for 45 seconds. After that the mixture was incubated on ice for 2 minutes. Then 250 µl of LB broth (pre-warm 42°C) was added into the mixture to recover the cell at 37°C with agitation for 2 hours. Fifty microliters and 125 µl of the transformation mixture were plated on LB plate containing 50 µg/ml of kanamycin that has been spreaded with 40 µl of 2% X-gal. The plate was incubated at 37°C overnight. The white colonies were selected for the insertion of PCR products from the blue white screen (a technique that show for the

detection of successful ligations in vector base gene cloning by using the presence of x-gal, if the ligation was successful, the colonies will be white, if not, the colonies will be blue).

Preparation of plasmid DNA

Several transformants were extracted for recombinant plasmids by High-Speed Plasmid Mini Kit® (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's instructions to find a positive *E. coli* clone carrying pSC-A-amp/kan recombinant plasmid inserted with the *qnrA* gene. In the first step or the Harvesting step, white colony was picked into the microcentrifuge tube containing LB broth with the 50 µg/ml kanamycin and incubated at 37°C overnight in shaking water bath. Then 1 ml of bacterial culture was transferred to a microcentrifuge tube, centrifuged at 16,000 x g for 1 minute and the supernatant was discarded. Step 2 or the Re-suspension step, 200 µl of PD1 Buffer (RNase A added) was added and resuspended the cell pellet by vortex. Step 3 or the Lysis step, 200 µl of PD2 Buffer was added and the tube was left at room temperature for 2 minutes. Step 4 or the Neutralization step, 300 µl of PD3 Buffer was added, mixed immediately by inverting the tube 10 times and centrifuged at 16,000 x g for 3 minutes. Step 5 or the DNA Binding step, the supernatant from Step 4 was added to the PD Column in a 2 ml collection tube and centrifuged at 16,000 x g for 30 seconds. The flow-through was discarded and placed the PD Column back in the 2 ml collection

tube. Step 6 or the Wash step, 400 µl of W1 Buffer was added into the PD Column, centrifuged at 16,000 x g for 30 seconds, discarded the flow-through and placed the PD Column back in the 2 ml Collection Tube. Six hundred microliters of Wash Buffer was added into the PD Column and centrifuged at 16,000 x g for 30 seconds. The flow through was discarded and placed the PD Column back in the 2 ml collection tube. The PD Column in the 2 ml Collection Tube was centrifuged at 14-16,000 x g again for 3 minutes to dry the column matrix. Step 7 or the DNA Elution step, the dried PD Column was transferred to a new microcentrifuge tube and added 50 µl of Elution Buffer into the center of the column matrix. The microcentrifuge tube was stood for at least 2 minutes to allow the Elution Buffer to be completely absorbed and centrifuged at 16,000 x g for 2 minutes to eluted the DNA.

Restriction enzyme analysis

The transformed clone was analyzed in order to find transformant clones, which carried recombinant plasmid; restriction analysis was performed with *Eco*RI digestion. StrataClone® PCR cloning vector pSC-A-amp/kan contains *Eco*RI flanked PCR product insertion site as showed in Figure 4.1, which used for screening the clones with specific insert orientation. *Eco*RI (New England BioLabs, Inc., USA) digestion was performed with the following reaction as showed in Table 4.3. The digestion reaction was incubated at 37°C for 1 hour. The reaction was stopped by heating at 60°C for 20 minutes and

observed the generated fragment size by 1% agarose gel electrophoresis. A recombinant plasmid, which generated the theoretical DNA fragment verified *qnrA* gene by DNA sequencing with M13-20 and M13 reverse primer at First BASE Laboratories, Malaysia in order to confirmed the correct insertion position and identified *qnrA* subtype. The BLAST program was used to compare the nucleotide and protein sequences to those available on the internet at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

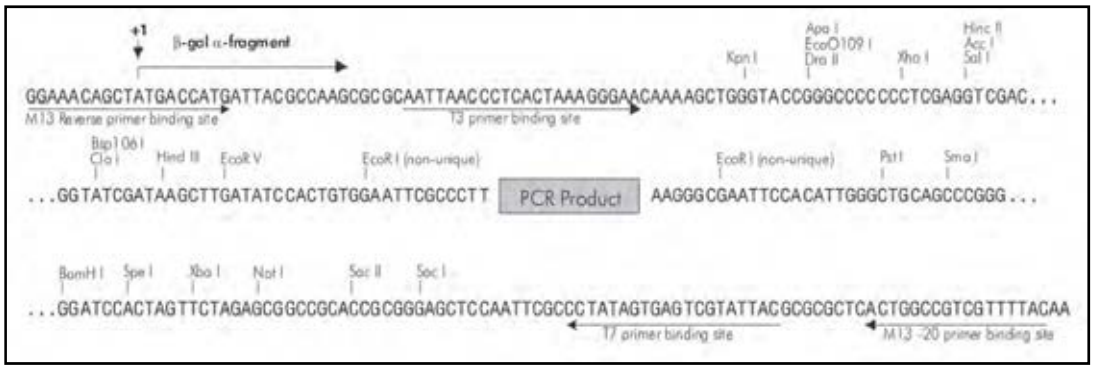

Figure 4.1 pSC-A-amp/kan PCR cloning vector PCR product insertion site region used in cloning experiment.

Table 4.3 Recipes for digestion reaction for *Eco*RI restriction analysis.

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

3. Detection of *gyrA* mutation by PCR and DNA sequencing

The presence of mutations in the QRDR of *gyrA* gene were investigated in all *qnrA* positive isolates by PCR and sequencing using primer (position 6 to 23 and 614 to 631) showed in Table 4.2. The amplification condition for *gyrA* were pre-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, and final extension at 72°C for 10 min. Amplification product were analyzed by agarose gel electrophoresis. The PCR product of *gyrA* gene was cloned into a plasmid vector, pSC-A-amp/kan by using StrataClone PCR Cloning Kit® (Agilent Technologies, Inc., Canada) and selected the clone number for plasmid DNA analysis of the PCR fragment insertion. Several transformants were extracted for recombinant plasmids by High-Speed Plasmid Mini Kit® (Geneaid Biotech Ltd., Taiwan). A recombinant plasmid; restriction analysis was performed with *Eco*RI digestion. A recombinant plasmid, which generated the theoretical DNA fragment verified *gyrA* gene by DNA sequencing with M13-20 and M13 reverse primer at First BASE Laboratories, Malaysia in order to confirmed the correct insertion position and identified *gyrA* gene. The BioEdit program was used for DNA and amino acid alignment. All sequences of *gyrA* genes in the *qnrA* positive isolates aligned to compare with positive control of *gyrA* mutation (S83L and D87N) isolate and wild type QRDR (negative control) isolate (these 2 isolates kindly provided by Asst. Prof. Dr. Chanwit Tribuddharat).

4. Detection of class 1 integron integrase (*intI1*) gene by PCR

The presence of *IntI1* genes were investigated in all *qnrA* positive isolates by PCR using primer described in Table 4.2. The amplification condition for *IntI1* were pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, and final extension at 72°C for 7 min. Amplification product were analyzed by agarose gel electrophoresis and double checked by Southern blot hybridization.

5. Location of *qnrA* and *intI1* gene analysis by Southern blot hybridization

The locations of *qnrA* and *intI1* genes were determined by Southern blot hybridization using genomic DNA of all *qnrA* plus *intI1* positive isolates including genomic DNA, *Eco*RI-digested genomic DNA (New England BioLabs, Inc., USA) and *Bam*HI-digested genomic DNA (New England BioLabs, Inc., USA). *Eco*RI and *Bam*HI digestion were performed with the following reaction as showed in Table 4.4 and Table 4.5, respectively. The reaction was stopped by heating at 60°C for 20 minutes. The DNA was analyzed by agarose gel electrophoresis. PCR product using *qnrA* and *intI1* primer were used for making specific hybridization probe. The specific probe consisted of the 579 bp fragment for *qnrA* and the 471 bp for *intI1* gene. Specific probe was labeled by using High Prime DNA Labeling Kit (Roche Applied Science, Germany). Detection was done by using CDP-Star detection module (Amersham Pharmacia Biotech, Ltd., UK).

Table 4.4 Recipes for digestion reaction for *Eco*RI enzyme

Reagents	Volume (µl)
10x NEBuffer 4	2
*Eco*RI	1
Genomic DNA	5
Sterile water	12

Table 4.5 Recipes for digestion reaction for *Bam*HI enzyme

Reagents	Volume (µl)
10x NEBuffer 4	2
*Bam*HI	1
Genomic DNA	5
Sterile water	12

Labeling the probe

Specific probe was labeled by using High Prime DNA Labeling Kit (Roche Applied Science, Germany) according to the manufacturer's instructions. DNA template (16 µl of PCR product) was added into the reaction tube. The DNA was denatured by heating in a heat box at 100°C for 10 minutes and chilled quickly in ice. The 4 µl of Fluorescein-High Prime was added into the denatured DNA, mixed and centrifuged briefly. The probe was incubated at 37°C for overnight. The 2 µl 0.2 M EDTA (pH 8.0) was added to stop reaction.

The gel was prepared according to the method of Brown, 2004. Briefly, the gel was incubated in depurination solution (0.25 M HCl) for 20 minutes and shaken slowly at room temperature. Then, the gel was rinsed by distilled water. The gel was then incubated in denaturation solution (1.5 M NaCl/0.5 M NaOH) for 25 minutes and shaken slowly at room temperature and was rinsed by distilled water. The gel was then incubated in neutralization solution (1.5 M NaCl/0.5 M Tris-Cl pH 7) for 30 minutes and shaken slowly at room temperature and was rinsed again by distilled water.

In order to set up the transfer by dawnward capillary transfer, a stack of paper towels was made 2 to 3 cm high in a glass dish. The towels should be slightly wider than the gel. As showe in Figure 4.2, four pieces of Whatman 3MM filter paper were placed on top of the paper towels. A fifth filter paper was wet with transfer buffer and placed on top. A piece of Nytran® positive charge nylon membrane (Schleicher & Schuell Inc., USA) was cut just large enough to cover the exposed surface of the gel and wet the membrane. The membrane was placed on the top filter paper. Remove bubbles by rolling a glass pipet over the surface of the membrane. The gel was placed on the membrane and removed bubbles. Three pieces of Whatman 3MM paper were soaked and cut to the same size as the gel with transfer buffer (20x SSC) and place on top of the gel. Two larger pieces of Whatman 3MM paper together were placed and soak in transfer buffer. The glass dish containing transfer buffer was placed on a support and

used the two pieces of soaked Whatman 3MM paper to form a bridge between the gel and the reservoir. A light plastic cover was placed over the top of the stack to reduce evaporation. Leave at room temperature for overnight. The paper towels and filter papers were removed and recovered the membrane. The membrane was left to dry.

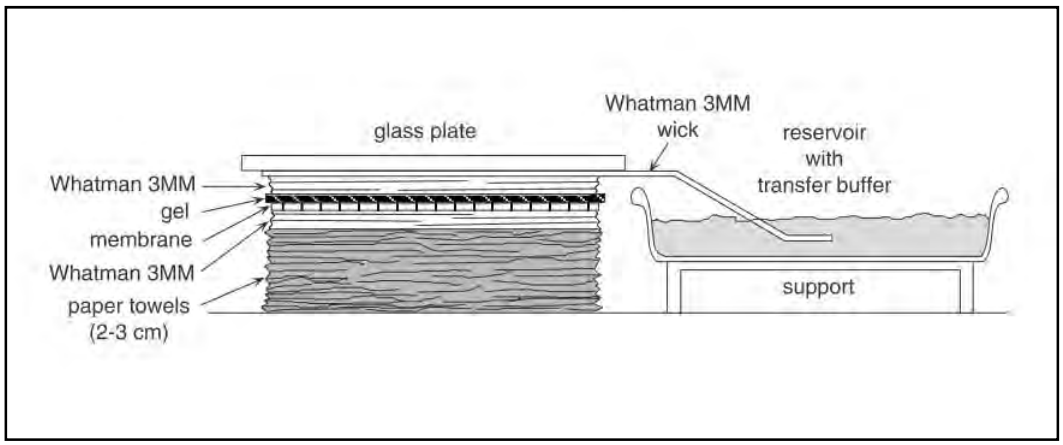

Figure 4.2 The transfer pyramid setup for Southern blotting via downward capillary transfer (Brown, 2004).

The hybridization step was performed according to the method of Wheeler *et al*., 2000. Briefly as followed: the membrane was incubated with 30 ml of the pre-hybridization buffer at 60°C for 30 minutes and was then incubated with 30 ml of the hybridization buffer at 60°C for overnight. The membrane was washed with 50 ml of the stringency I (1x SSC/0.1% SDS) at 60°C for 15 minutes and then was washed with 50 ml of the stringency II (0.5x SSC/0.1% SDS) at 60°C for 15 minutes.

In order to detect the hybridization, the membrane was blocked with 30 ml of the blocking solution (5% skim milk/buffer A) at room temperature for 1 hour and then with 30 ml of the anti-fluorescien-Alkaline phosphatase (AP) conjugated (AP conjugated/BSA/buffer A) at room temperature for 1 hour. After that, the membrane was washed three times with 100 ml of 0.3% tween 20 at room temperature for 15 minutes per each washing step. The membrane was placed on the Saran wrap. Four hundred microliters of CDP-star solution was added on the membrane and left for 3 minutes and then transferred to the new saran wrap. The wrapped membrane was placed in the film cassette. In the dark, a sheet of autoradiography film (FUJIFILM Corporation, Japan) was placed on the top of the membrane before closing the film cassette. The film was exposed for 5, 10, 20, 30, 60, 90 and 120 minutes, respectively and then removed for film development.

6. Transfer of *qnrA* gene

Transconjugation experiment was performed to examine the ability of horizontal gene transfer of *qnrA* gene. The experiments were performed for all 8 *qnrA* positive isolates. Streptomycin resistant *E. coli* TOP'10 were used as the recipient strain and MacConkey agar plate containing ciprofloxacin 0.128 µg/ml (kindly provided by Siam Pharmaceutical Co., Ltd., Thailand) and streptomycin 64 µg/ml (kindly provided by M&H Co., Ltd., Thailand) were used as a selective marker. Donor (*qnrA* harboring isolates)

and recipient were grown separately in 3 ml of BHI broth at 37 °C in shaking incubator. Cell suspensions of donor and recipient were mixed at various donor:recipient ratio such as 1:1, 1:5, 1:10. The mixtures were incubated in shaking incubator at 37 °C for overnight. The 40 µl of mixtures were spreaded on MacConkey agar containing ciprofloxacin 0.128 µg/ml and streptomycin 64 µg/ml, and incubated at 37 °C for overnight. The colonies of transconjugants were observed.

Transformation experiments by chemical reaction were performed for *qnrA* positive isolates for which no transconjugant were obtained. Plasmid DNA was extracted from the donors strain into chemical competent *E. coli* TOP'10 (Invitrogen, USA). The 40 µl vial of competent *E. coli* TOP'10 (Invitrogen, USA) cell was thawed on ice. Twenty microliters of plasmid extracted from donor was pipetted directly into the vial of competent cells and mixed by tapping gently. The mixture was incubated on ice for 30 minutes and continued to incubate by heated-shock at 42ºC for 30 seconds. Finally, the mixture was incubated on ice for 2 minutes. The 250 µl of BHI broth (pre-warm at 42ºC) was added into the mixture which was then incubated at 37ºC for 1-2 hour with shaking incubator. The mixture was spread on MacConkey agar containing ciprofloxacin 0.128 µg/ml and 0.256 µg/ml, and incubated at 37 °C for overnight. The colonies of transformants were observed.

Transformation experiments by electroporation modified from the method by Sambrook *et al.*, 2001 were performed for *qnrA* positive isolates for which no transformant by chemical reaction were obtained. Transformant were selected on MacConkey agar plate containing ciprofloxacin 0.128 µg/ml and 0.256 µg/ml. A single colony of *E. coli* was inoculated into 50 ml LB broth. The culture was then inoculated in shaking incubator at 37 °C for overnight. The 25 ml of culture was inoculated into 500 ml of LB broth and grown at 37 °C with shaking to an OD_{600} of 0.4. The culture was rapid by kept in an ice bath for 15 minutes and transferred again to a centrifuge bottle. The culture was then centrifuged at 1,000 x g, 4°C for 15 minutes. The supernatant was discarded and the cell pellet was resuspended in 500 ml sterile ice-cold water. The cell suspension was then centrifuged at 1,000 x g, 4°C for 20 minutes. The supernatant was discarded and the cell pellet was resuspended in 250 ml of ice-cold 10% glycerol. The centrifugation step was repeated twice. But 10 ml and 1 ml of ice-cold 10% glycerol were added to resuspended the cell pellet, respectively. A 1:100 dilution of the cell suspension was measured at the OD_{600}. The cell suspension was diluted to a concentration of 2-3 × 10^{10} cells/ml (1.0 OD_{600} = 2.5 × 10^{8} cells/ml) with ice-cold 10% glycerol. The eletrocompetent cells were stored at -70°C until used. The 50-200 µl of competent cell suspension was thawed, added into the cuvette and mixed with 2-4 µl of plasmid DNA. The eletroporation cuvette was then kept on ice. The electroporation apparatus was set to deliver an electric pulse of 25 µF, 2.5 kV and 200 ohm resistance.

The cell was immediately resuspended in 1 ml of LB broth and grown at 37ºC for 1 hour with shaking incubator. The suspension was spread on MacConkey agar containing ciprofloxacin 0.128 µg/ml and 0.256 µg/ml, and incubated at 37 °C for overnight. The colonies of transformants were selected.

7. Antimicrobial susceptibility test

Disk diffusion test of all *qnrA* positive isolates in this study had been performed according to the CLSI guidelines (CLSI, 2010). The tested antibiotics with the amount of drug per disk were in parenthesis were amikacin (30 µg), ampicillin (10 µg), ampicilin/sulbactam (10/10 µg), amoxicillin/clavulanic acid (20/10 µg), cefepime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg), piperacillin/tazobactam (100/10 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg). *E. coli* ATCC 25922 was used as a quality control strain. The size of the zone of inhibition was interpreted by referring to table of zone diameter breakpoints of the CLSI guidelines (CLSI, 2010) as showed in the Table 4.6.

Table 4.6 Zone diameter breakpoints interpretive standard antimicrobial agents of CLSI guidelines (CLSI, 2010).

Antimicrobial disk/Concentration	Zone of inhibition (mm.)		
	Susceptible	Intermediate	Resistant
amikacin (30 µg)	≥17	15-16	≤14
ampicillin (10 µg)	≥17	14-16	≤13
ampicilin/sulbactam (10/10 µg)	≥15	12-14	≤11
amoxicillin/clavulanic acid (20/10 µg)	≥18	14-17	≤13
cefepime (30 µg)	≥18	15-17	≤14
cefotaxime (30 µg)	≥26	23-25	≤22
ceftazidime (30 µg)	≥21	18-20	≤17
ceftriaxone (30 µg)	≥23	20-22	≤19
ciprofloxacin (5 µg)	≥21	16-20	≤15
gentamicin (10 µg)	≥15	13-14	≤12
imipenem (10 µg)	≥16	14-15	≤13
meropenem (10 µg)	≥16	14-15	≤13
piperacillin/tazobactam (100/10 µg)	≥21	18-20	≤17
trimethoprim/sulfamethoxazole (1.25/23.75 µg)	≥16	11-15	≤10

Minimum Inhibitory Concentration (MICs) of ciprofloxacin and norfloxacin (kindly provided by Siam Pharmaceutical Co., Ltd., Thailand) against 4 selected tested *E. coli* strains and control *E. coli* ATCC 25922 were determined by the broth macrodilution method with cation-adjusted Mueller-Hinton II (CAMB) broth (Difco Laboratories, USA) according to method of CLSI guidelines (CLSI, 2010). The 5 tested strains were

categorized into 5 groups (1 strain in each group): *qnrA* positive with *gyrA* mutations (S83L and D87N) strain, *qnrA* negative with *gyrA* mutations (S83L and D87N) strain (kindly provided by Asst. Prof. Dr. Chanwit Tribuddharat), *qnrA* positive with wild type QRDR strain, *qnrA* negative with wild type QRDR strain (kindly provided by Asst. Prof. Dr. Chanwit Tribuddharat) and *E. coli* ATCC 25922. The MICs was interpreted by MIC interpretive standard of the CLSI guidelines (CLSI, 2010) as showed in the Table 4.7.

Table 4.7 MIC interpretive standard of antimicrobial agents of CLSI guidelines (CLSI, 2010).

Antimicrobial agents	MIC interpretive standard		
	Susceptible	Intermediate	Resistant
ciprofloxacin	$\leq$1	2	$\geq$4
norfloxacin	$\leq$4	8	$\geq$16

The broth macrodilution method was modified from CLSI, 2010, procedure. Briefly, the antimicrobial concentrations used in initial stock solution were prepared 10 folds greater than the desired final concentration. The maximum concentration was diluted 2 folds until the lowest concentration was obtained. The 1 ml of drug in each concentration was transferred into 8 ml of CAMB for working media preparation (final volume of working media = 9 ml) before adding 1 ml of the standardized inoculum. Four *E. coli* tested strains and *E. coli* ATCC 25922 were selected from TSA plate and transferred to the tube containing 7 ml of 0.85% normal saline solution (NSS). The turbidity of culture was adjusted to a 0.5 McFarland standard solution (1-2 × 10^8 cfu/ml).

The suspension was diluted 10 folds to make 1-2 × 10^7 cfu/ml. The suspension was diluted 10 folds to make 1-2 × 10^6 cfu/ml of the bacterial inoculums. The 1 ml of inoculum was transferred to working media and incubated at 37 °C for 16 to 20 hour. The MIC was observed and interpreted the result of the tested.

Minimum Bactericidal Concentration (MBCs) were determined by method recommended by Rodriguez-Martinez. (Rodriguez-Martinez *et al*., 2008)

8. Time kill study

All 5 selected *E. coli* strains from the previous MICs determination study were included in this study. The bactericidal activity of ciprofloxacin and norfloxacin (kindly provided by Siam Pharmaceutical Co., Ltd., Thailand) were determined by the time kill method described in the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 1999). Time kill assays were conducted on Mueller-Hinton II (MHB) broth (Difco Laboratories, USA) at drug concentrations of 1xMIC, 2xMIC, 4xMIC and 8xMIC for each strain.

Time kill procedure

The antimicrobial concentrations used in initial stock solution were prepared 10 folds greater than the desired final concentration. The maximum concentration was diluted 2 folds until the lowest concentration. The 1 ml of drug in each concentration was transfer into 8 ml of MHB for prepare working media before adding the standardized inoculum (final volume of working media = 9 ml). The *E. coli* strains were selected from TSA plate and transferred to the tube containing 7 ml of 0.85% NSS. The turbidity of culture was adjusted to a 0.5 McFarland standard solution (1-2 × 10^8 cfu/ml). The suspension was diluted 10 folds to make 1-2 × 10^7 cfu/ml of the bacterial inoculum. The 1 ml of inoculum was transferred to working media and incubated at 37 °C in shaking water bath for 24 hour. Finally, the initial inoculum for each isolate which was in the range 5 × 10^5 to 5 × 10^6 cfu/ml. The samples were collected for culture at the time 0, 2, 4, 6, 8 and 24 hour after the microorganism was exposed to antimicrobial concentrations including the growth control group (no antimicrobial). The 0.5 ml of the collected samples was diluted 10 fold in 0.85% NSS and 20 µl of each dilution was dropped on TSA plates which were then incubated at 37 °C for 16 to 20 hour. The quantity of survival bacteria in each group was calculated to obtain the killing curves data. Killing curves were constructed by Microsoft Excel 2007 and GraphPad Prism 3.0 program at each time interval. The $\log_{10}$ change of the viable cell counts (number of colonies × 50 × dilution factor) compared to the starting inoculum was determined. The results were

analyzed by determining the number of isolates which yields in the $\log_{10}$ number of cfu/ml at 2, 4, 6, 8 and 24 hour compared to the viable cell count at 0 hour. Bactericidal activity was defined as a $\geq$99.9% (a $\geq 3\log_{10}$) reduction and bacteriostatic activity was defined as a 0 to $<3\log_{10}$ reduction in the numbers of cfu/ml from that of the starting bacterial concentration. The regrowth defined as an increase in growth of $\geq 2\log_{10}$ cfu/ml after 6 hour of incubation (Gunderson *et al*., 2004).

CHAPTER V

RESULTS

1. Detection of *qnrA* genes by PCR

1.1 Genomic DNA extraction

Genomic DNA of *E. coli* clinical isolates were successfully extracted by the Puregene Yeast/Bact. Kit B (Gentra systems Co., Ltd., USA) and obtained large amount of DNA as showed in Figure 5.1

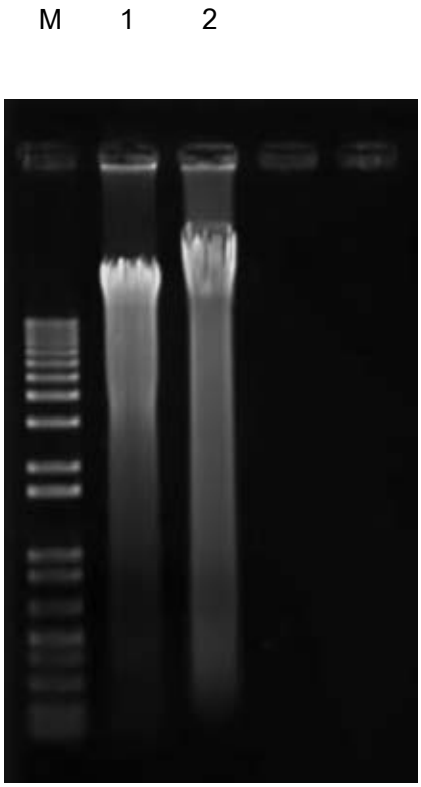

Figure 5.1 Genomic DNA of *E. coli* isolates. Lane M, 1 kb plus DNA ladder; Lane 1-2, *E. coli* isolates.

1.2 PCR amplification of *qnrA* genes and analysis of PCR product

qnrA genes were found in 8 out of 190 tested *E. coli* clinical isolates (4.21%). PCR product was obtained at the size of 579 bp as showed in Figure 5.2. From the university hospital isolates, *qnrA* were detected in 8% (8/100) of 100 ESBL producing *E. coli* isolates, whereas no *qnrA* was detected from 90 quinolone resistant *E. coli* isolated from the tertiary private hospital. All 8 *qnrA* positive isolates, 37.5% (3/8) were susceptible, 12.5% (1/8) were intermediate, and 50% (4/8) were resistant to ciprofloxacin.

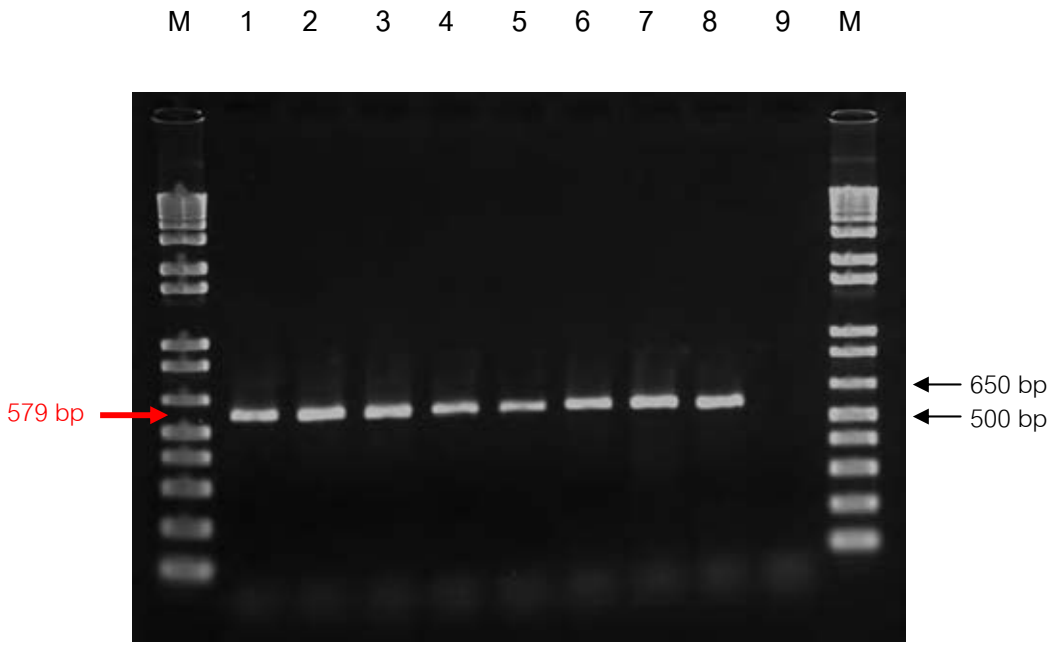

Figure 5.2 PCR with the specific primers to *qnrA* generating 579 bp PCR products. Lane M, 1 kb plus DNA ladder; Lane 1-2, 4-8, positive samples; Lane 3, No. 67 carrying *qnrA*, positive control (kindly provided by Asst. Prof. Dr. Chanwit tribuddharat); Lane 9, negative control.

1.3 Identification of *qnrA* subtype

After PCR product of *qnrA* gene was successfully cloned into a plasmid vector. The *qnrA* recombinant plasmid was extracted from the transformed cells and analyzed by *Eco*RI restriction analysis. The result of successful recombinant plasmid were showed in Figure 5.3

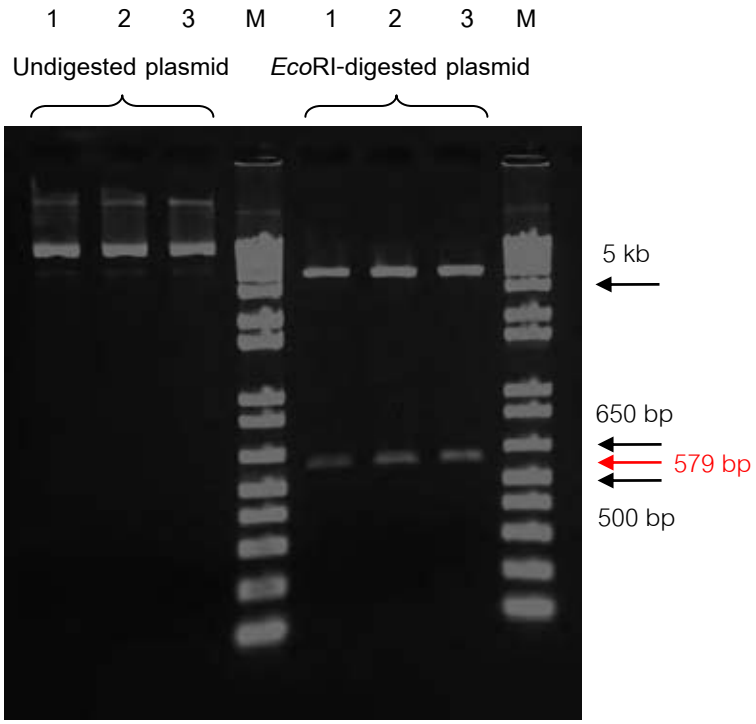

Figure 5.3 The successful *qnrA* recombinant plasmids. Lane M, 1 kb plus DNA ladder; Lane 1-3 *qnrA* recombinant plasmids.

A recombinant plasmid was verified *qnrA* variant by DNA sequencing analysis. That confirmed positive result for *qnrA1* (≥99% nucleotides identity to *E. coli* pGD011 QnrA1, EU195836) as showed in Figure 5.4

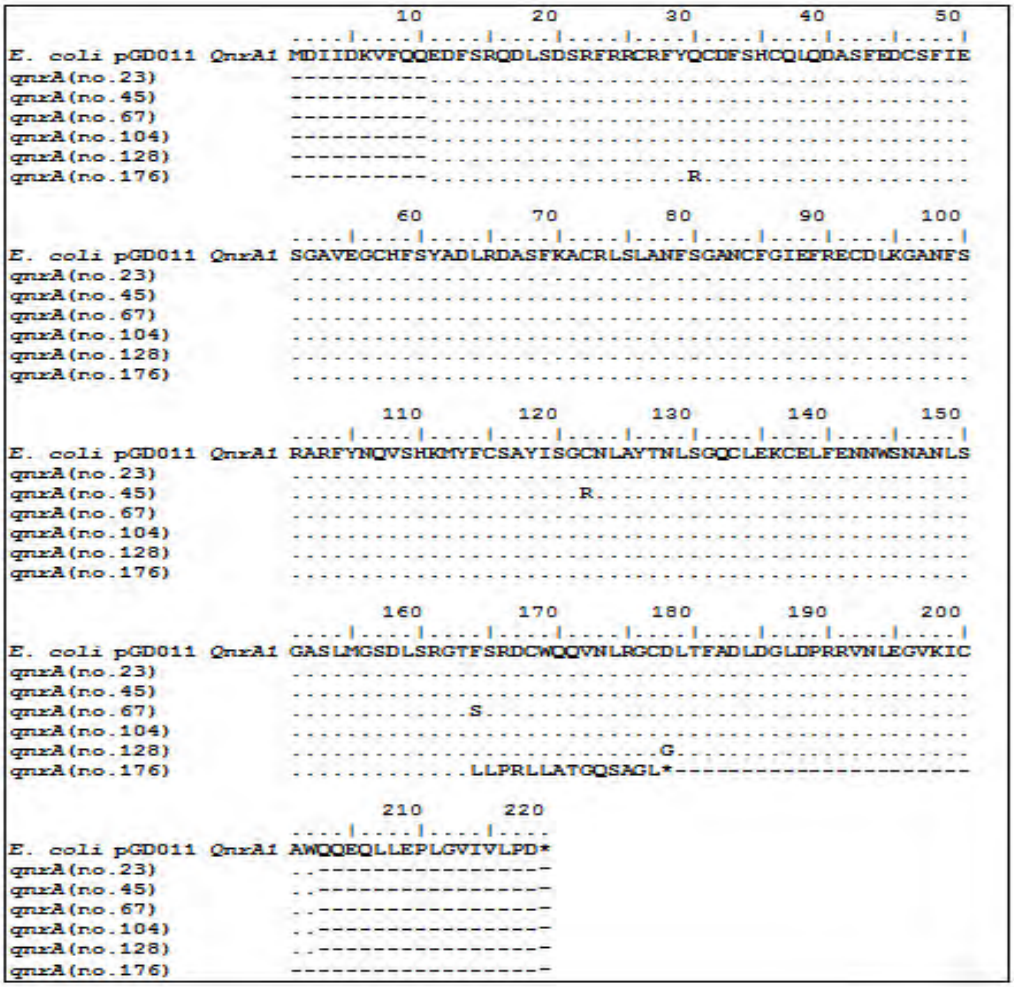

Figure 5.4 Amino acid sequencing of QnrA homologs confirm a positive result for *qnrA1* (≥99% nucleotides identity to *E. coli* pGD011 QnrA1, EU195836).

2. Detection of *gyrA* mutation by PCR and DNA sequencing

2.1 PCR amplification of *gyrA* genes and analysis of PCR product

All *qnrA* positive isolates were amplified for *gyrA* gene by PCR and investigated the relationship of *qnrA* and *gyrA* mutations. PCR product was obtained at the size of 626 bp as showed in the Figure 5.5

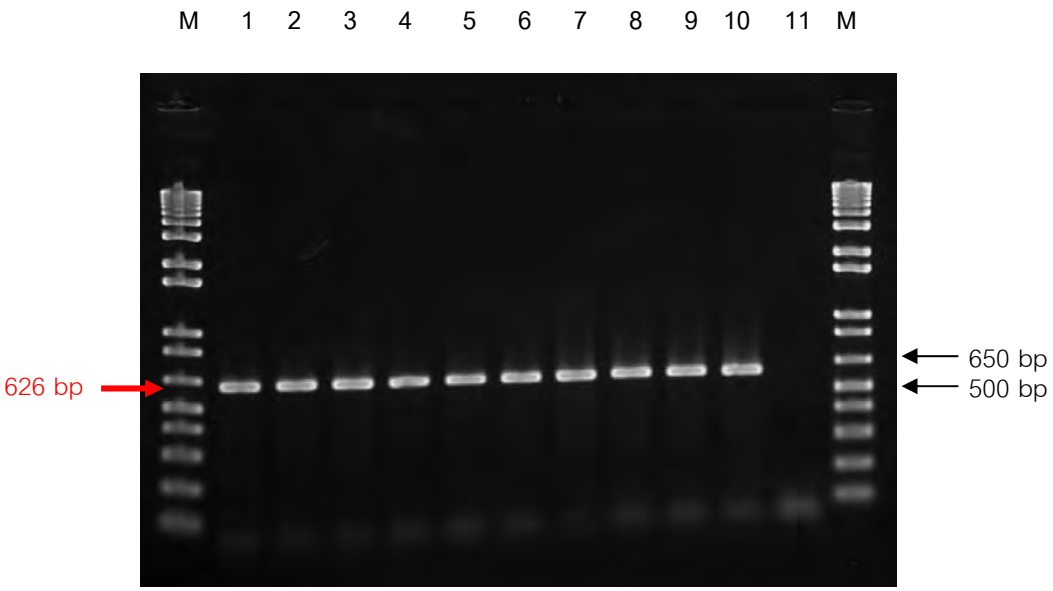

Figure 5.5 PCR with specific primers to *gyrA* generating 626 bp PCR products. Lane M, 1 kb plus DNA ladder; Lane 1-8, samples (*qnrA* positive isolates); Lane 9, No. 98 *gyrA* mutations (S83L and D87N) positive isolate as positive *gyrA* mutations control; Lane 10, No. 137 wild type QRDR and *qnrA* negative isolate as negative *gyrA* mutations control; Lane 11, negative control

After PCR product of *gyrA* gene was successfully cloned into a plasmid vector. The *gyrA* recombinant plasmid was extracted from the transformed cells and analyzed by *Eco*RI restriction analysis. The results of successful recombinant plasmid were showed in Figure 5.6

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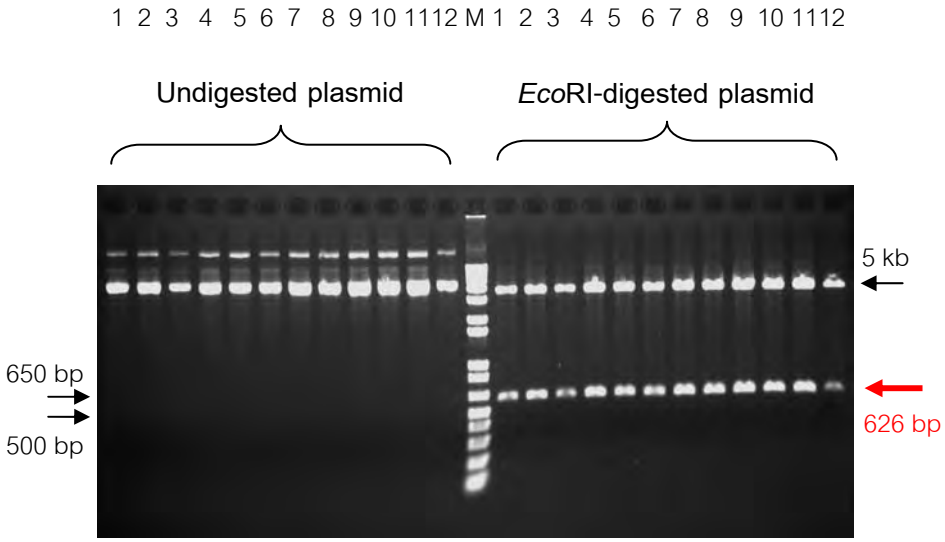

Figure 5.6 The successful *gyrA* recombinant plasmid. Lane M, 1 kb plus DNA ladder; Lane 1-12 *gyrA* recombinant plasmids

A recombinant plasmid was verified *gyrA* genes by DNA sequencing analysis. That confirmed positive result for *gyrA* genes (≥99% nucleotides identity to *E. coli* strain C20137-14 GyrA (*gyrA*) gene, partial codons, DQ447132.1) as showed in Figure 5.7 All sequences of *gyrA* genes from 8 *qnrA* positive isolates were aligned to compare with positive control of *gyrA* mutation (S83L and D87N) isolate and wild type QRDR (negative control) isolate for detect the mutations of *gyrA* gene by BioEdit program, that showed double mutations in the QRDR region (S83L and D87N in *gyrA* gene) were found in 4 out of 8 (50%) *qnrA* positive isolates as showed in Figure 5.8

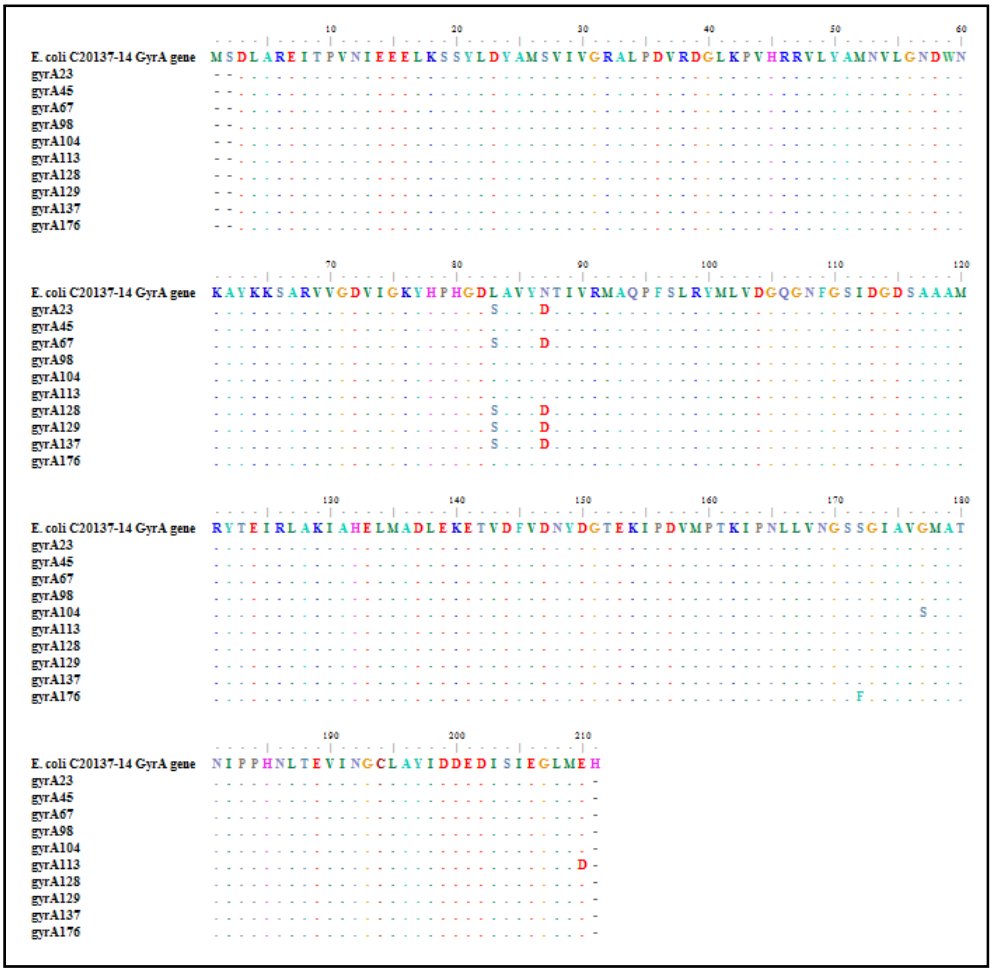

Figure 5.7 Amino acid sequence of GyrA homologs confirm a positive result for *gyrA* genes (≥99% nucleotides identity to *E. coli* strain C20137-14 GyrA (*gyrA*) gene, partial codons, DQ447132.1)

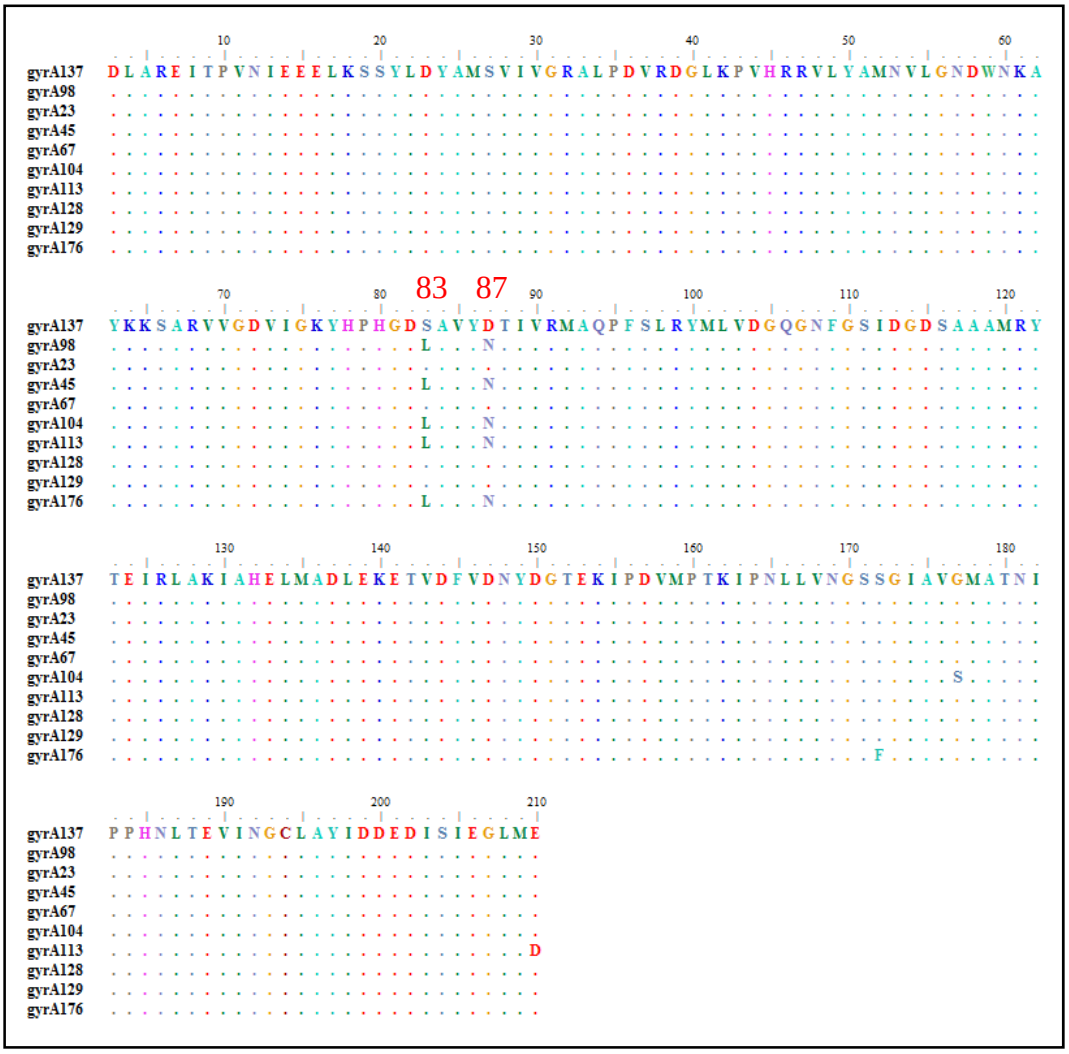

Figure 5.8 Amino acid sequences of GyrA among the *qnrA* positive isolates (*gyrA*23, 45, 67, 104, 113, 128, 129 and 176) aligned to compare with positive control of *gyrA* mutations (S83L and D87N; *gyrA*98) isolate and wild type QRDR (negative control; *gyrA*137) isolate.

3. Detection of class 1 integron integrase (*intI1*) genes by PCR

3.1 PCR amplification of *intI1* genes and analysis of PCR product

Class 1 integrons (*intI1* genes) were found in all *qnrA* positive isolates (100%, 8/8). PCR product was obtained at the size of 471 bp in the length as showed in Figure 5.9 and double checked these genes by Southern blot hybridization.

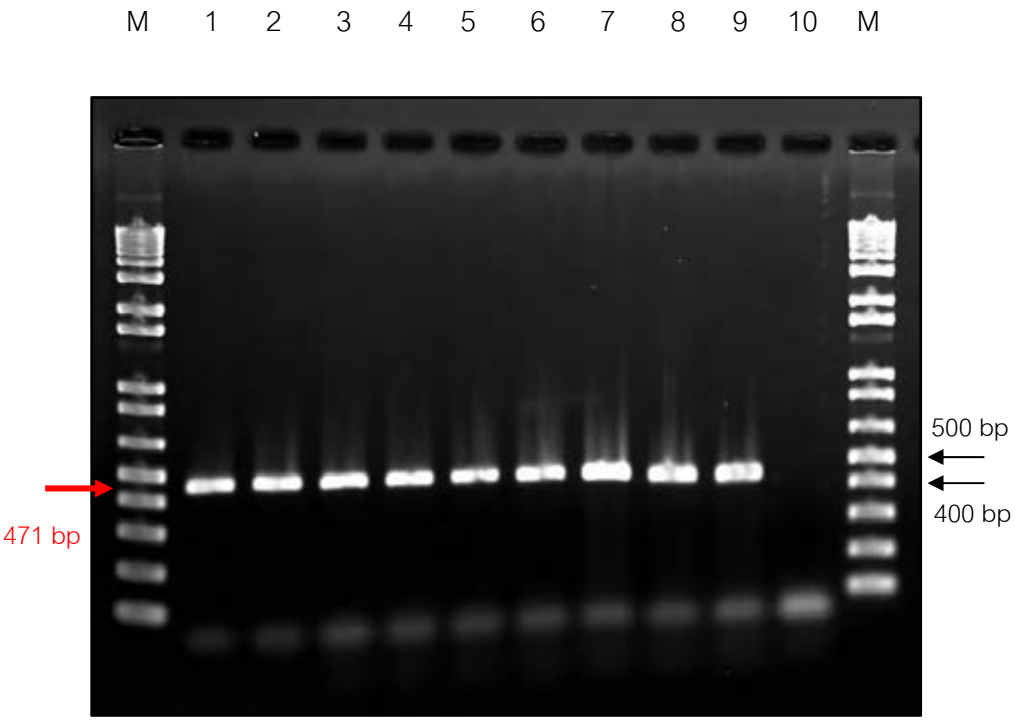

Figure 5.9 PCR with specific primers to *intI1* generating 471 bp PCR product. Lane M, 1 kb plus DNA ladder; Lane 1-8, positive samples (*qnrA* positive isolates); Lane 9, PA 67 possessed integron carrying $bla_{IIMP-15}$, positive control (kindly provided by Asst. Prof. Dr. Chanwit Tribuddharat); Lane 10, negative control.

4. Location of *qnrA* and *intI1* genes analysis by Southern blot hybridization

The locations of *qnrA* and *intI1* genes were analyzed by Southern blot hybridization using genomic DNA of all *qnrA* plus *intI1* positive isolates being tested were categorized into 3 groups for each gene, genomic DNA, *Eco*RI-digested genomic DNA (New England BioLabs, Inc., USA) and *Bam*HI-digested genomic DNA (New England BioLabs, Inc., USA).

The results of Southern blot analysis of *qnrA* genes were showed in Figure 5.11. For all *qnrA* genes, the hybridization signals were obtained at migration position of the chromosomal DNA indicated that *qnrA* genes were likely to locate on the chromosomes and large plasmids (3/8 isolates, Lane 1, 4 and 5) in the experiment using genomic DNA. In this experiment, all genomic DNA fragments including chromosomal, large plasmid and small plasmid DNAs were undigested DNA. After the genomic DNA extraction step, circular chromosomal DNA would be broken to linear DNAs, thus, the circular DNAs of large plasmids migrated slower than fragments of chromosomal DNAs. Then large plasmid hybridization signals were visualized above the chromosomal hybridization signals. In *Eco*RI-digested genomic DNA experiment, the specific *qnrA* probe could not hybridize some *Eco*RI digested DNAs due to a star activity, but in lane 14, the specific *qnrA* probe could hybridize the small digested DNA fragment with strong intensity suggesting of a fragment from plasmid origin (the plasmid

genes commonly have higher copy numbers than that the chromosomal genes resulting in increased hybridization signals with the specific probe). In the *Bam*HI-digested genomic DNA experiment, *qnrA* genes were likely to locate on the chromosome as same interpretation as in the genomic DNA experiment. There was autolysis of DNA in lane 10 and 18 with no specific restriction digestion as showed in Figure 5.10, so the hybridization results were not obtained for this isolate.

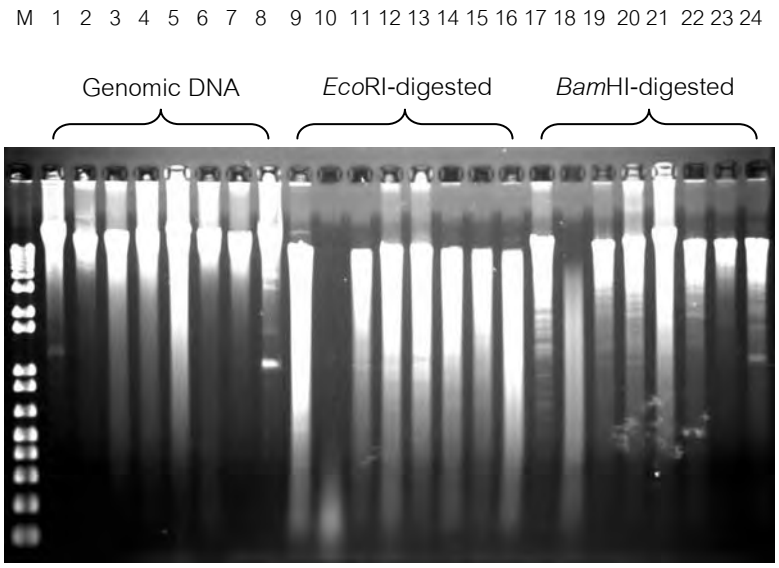

Figure 5.10 Electrophoresis before Southern blotting of *qnrA* genes, genomic DNA of all *qnrA* positive isolates also harbor with *intI1*. 1) Genomic DNA experiment, 2) *Eco*RI-digested genomic DNA experiment and 3) *Bam*HI-digested genomic DNA experiment. Lane M, 1 kb plus DNA ladder; Lane 1-8, 9-16 and 17-24; all 8 *qnrA* positive isolates also harbor with *intI1*.

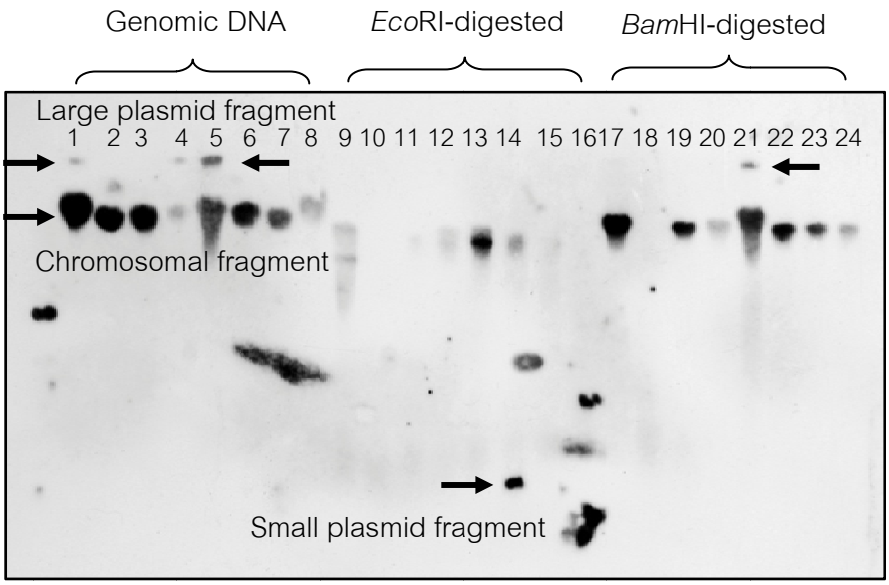

Figure 5.11 Southern blot hybridization result with *qnrA* gene probe. 1) Genomic DNA experiment, 2) *Eco*RI-digested genomic DNA experiment and 3) *Bam*HI-digested genomic DNA experiment. Lane 1-8, 9-16 and 17-24; all 8 *qnrA* positive isolates. Arrow head shows location of *qnrA* genes fragments.

The results of Southern blot analysis in *intI1* genes as showed in Figure 5.13, all of them were likely to locate in the chromosome as well as large plasmid (1/8 isolates, Lane 4) in genomic DNA experiment and the specific *intI1* probe could not hybridize these genes in *Eco*RI-digested genomic DNA experiment as well as *qnrA* genes. In the *Bam*HI-digested genomic DNA experiment, the specific *intI1* probe could hybridize these genes with two positive bands in a single isolate (5/8 isolates, lanes 20-24 of Figure 5.13) showing that there were two integrons in each isolate, since here was no *Bam*HI restriction site in this gene. There was autolysis of DNA in lane 10 and 18 with no specific restriction digestion as showed in Figure 5.12, so the hybridization results were not obtained for this isolate as well as *qnrA* gene.

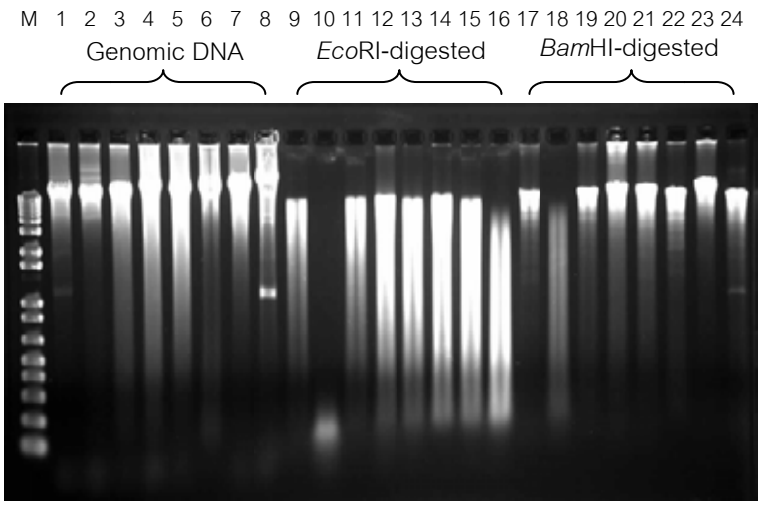

Figure 5.12 Electrophoresis before Southern blotting of *intI1* gene, genomic DNA of all *qnrA* positive isolates also harbor with *intI1*. 1) Genomic DNA experiment, 2) *Eco*RI-digested genomic DNA experiment and 3) *Bam*HI-digested genomic DNA experiment. Lane M, 1 kb plus DNA ladder; Lane 1-8, 9-16 and 17-24; all 8 *qnrA* positive isolates also harbor with *intI*.

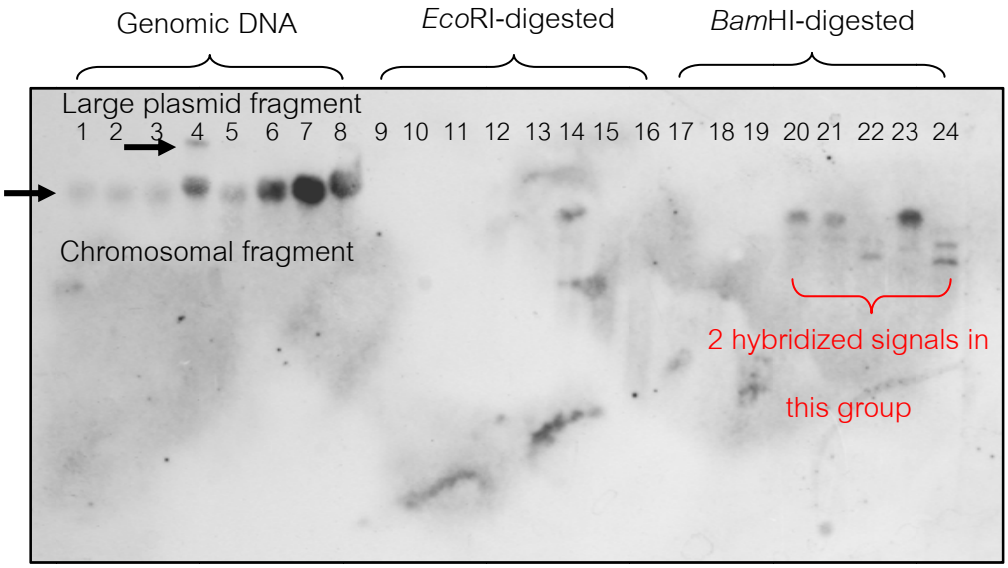

Figure 5.13 Southern blot hybridization result with *intI1* gene probe. 1) Genomic DNA experiment, 2) *Eco*RI-digested genomic DNA experiment and 3) *Bam*HI-digested genomic DNA experiment. Lane 1-8, 9-16 and 17-24; all 8 *intI1* positive isolates (all 8 *qnrA* positive isolates found along with *intI1* genes). Arrow head shows location of *intI1* genes fragments and the red braces shows 2 hybridized signals in *Bam*HI-digested experiment.

5. Transfer of *qnrA* gene

Conjugation and transformation methods by both chemical reaction and electroporation were failed, that it was very likely *qnrA* genes could locate in chromosome. For conjugation, *qnrA* genes could be found on chromosome and large plasmid (3 isolates, Lane 1, 4 and 5) in the results of Southern blot analysis (genomic DNA experiment) in Figure 5.11 as showed the MIC of streptomycin against these isolates (donors) were 128 to 512 µg/ml and were similar to the MIC of this drug against the recipient *E. coli* TOP'10 strain (>256 µg/ml), causing the difficulty in selection of transconjugant. In addition, transformation of *qnrA* genes by chemical reaction and electroporation could not be performed because the plasmids from these isolates were too large ($\geq$12 kb) to be transformed as showed in Figure 5.14

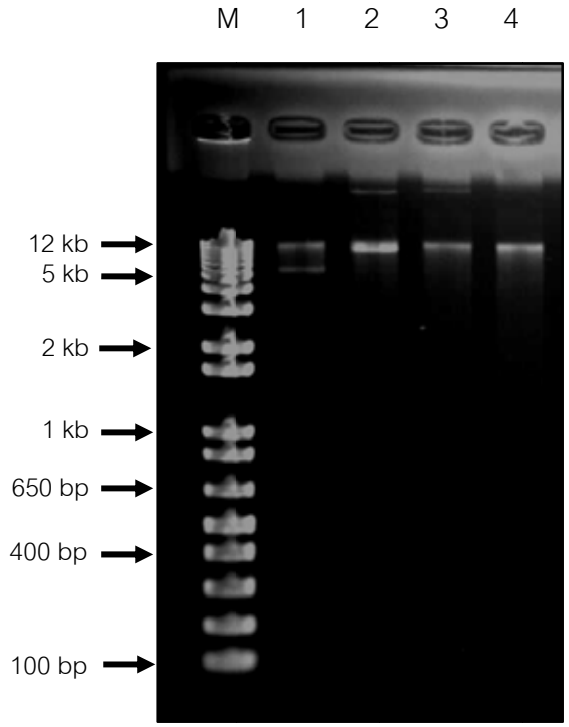

Figure 5.14 Plasmid extracts of *qnrA* positive isolates. Lane M, 1 kb plus DNA ladder; Lane 1-3, *qnrA* positive isolates which found these gene on both chromosome and large plasmid; Lane 4, *qnrA* positive isolate which found this gene on chromosome only.

6. Antimicrobial susceptibility test

In disk diffusion method, all *qnrA* positive isolates, were resistant to ampicillin, ampicillin/sulbactam, cefotaxime, and ceftazidime, while 50% (4/8) were resistant to ciprofloxacin, 25% (2/8) amikacin, 62.5% (5/8) amoxicillin/clavulanic acid, 62.5% (5/8) gentamicin, 75% (6/8) ceftriaxone, 87.5% (7/8) trimethoprim/sulfamethoxazole, and 12.5% (1/8) piperacillin/tazobactam, respectively. Seven of eight (87.5%) *qnrA* positive isolates were susceptible to cefipime. All eight *qnrA* positive isolates, (100%) were susceptible to imipenem and meropenem (as showed in Table 5.1). Focus on

ciprofloxacin, 4 out of 8 *qnrA* positive isolates 50% were resistant to ciprofloxacin and these isolates have double mutations in the QRDR region (S83L and D87N in *gyrA*), while the isolates without double mutations still susceptible to this drug (as showed in Table 5.2). The strain with posture *qnrA* without *gyrA* mutations was intermediate to ciprofloxacin. In fact the inhibition zone of this isolate was 20 mm. which nearby the susceptible breakpoint of ciprofloxacin (≥21 mm).

Table 5.1 The antimicrobial susceptibility pattern by disk diffusion test among all *qnrA* positive isolates.

Isolates	Antimicrobial agents													
	AM	AmC	SAM	SXT	CTX	CAZ	CRO	AN	GM	TZP	FEP	CIP	IPM	MEM
No. 23	R	I	R	R	R	R	R	S	R	S	I	S	S	S
No. 45	R	R	R	R	R	R	R	S	I	S	S	R	S	S
No. 67	R	R	R	S	R	R	R	R	R	S	S	S	S	S
No.104	R	I	R	R	R	R	R	S	R	S	S	R	S	S
No.113	R	R	R	R	R	R	R	R	R	I	S	R	S	S
No.128	R	R	R	R	R	R	I	S	I	S	S	I	S	S
No.129	R	R	R	R	R	R	R	I	R	R	S	S	S	S
No.176	R	I	R	R	R	R	I	S	S	S	S	R	S	S

AM = ampicillin, AmC = amoxicillin/clavulanic acid, SAM = ampicillin/sulbactam, SXT = trimethoprim/sulfamethoxazole, CTX = cefotaxime, CAZ = ceftazidime, CRO = ceftriaxone, AN = amikacin, GM = gentamicin, TZP = piperacillin/tazobactam, FEP = cefipime, CIP = ciprofloxacin, IPM = imipenem and MEM = meropenem

S = susceptible, I = intermediate and R = resistant

Table 5.2 Conclusion data of all *qnrA* positive isolates. S = Serine, L = Leucine, D = Aspartic acid and N = Asparagine.

Isolates	ESBL	*qnrA*	*intI1*	*gyrA* mutations		Disk diffusion test (CIP)	Zone of inhibition (mm.)
				S83L	D87N		
No. 23	+	+	+	-	-	S	24
No. 45	+	+	+	+	+	R	6
No. 67	+	+	+	-	-	S	26
No. 104	+	+	+	+	+	R	6
No. 113	+	+	+	+	+	R	8
No. 128	+	+	+	-	-	I	20
No. 129	+	+	+	-	-	S	21
No. 176	+	+	+	+	+	R	6

CIP = ciprofloxacin

S = susceptible, I = intermediate and R = resistant

In MICs determination, *E. coli* clinical strains being tested were categorized into 5 groups (1 isolate in each group), as showed in Table 5.3. The *qnrA* negative with *gyrA* mutations (S83L and D87N in QRDR) strain is highly resistant to ciprofloxacin and norfloxacin (MIC, 32 and 64 µg/ml), *qnrA* positive with wild type QRDR strain confer low level resistance to ciprofloxacin and norfloxacin (MIC, 0.12 and 1 µg/ml), respectively but associated with double mutations in *gyrA* genes such as *qnrA* positive with *gyrA* mutations (S83L and D87N) strain, which may increase level of resistance to ciprofloxacin (MIC, 64 µg/ml), while *qnrA* negative with wild type QRDR was susceptible to ciprofloxacin and norfloxacin (MIC, 0.03 and 0.12 µg/ml) as well as wild type ATCC 25922 strain (MIC, 0.008 and 0.06 µg/ml), respectively. The MIC of these drugs against wild type ATCC 25922 strain was in the range of quality control (0.004-0.015 µg/ml for

ciprofloxacin and 0.03-0.12 µg/ml for norfloxacin). The MICs of ciprofloxacin and norfloxacin against *qnrA* positive with wild type QRDR strain were two and three folds dilution higher than those against *qnrA* negative with wild type QRDR strain, respectively. The MICs of ciprofloxacin and norfloxacin against *qnrA* positive with wild type QRDR strain were both four folds dilution higher than wild type ATCC 25922 strain.

The MBCs of ciprofloxacin and norfloxacin against *qnrA* positive with wild type QRDR strain were four and three folds higher than the MICs, respectively. The MBCs of ciprofloxacin and norfloxacin against *qnrA* positive with wild type QRDR strain were one and five folds higher than that against *qnrA* negative with wild type QRDR strain, respectively. The MBCs of ciprofloxacin and norfloxacin against *qnrA* positive with wild type QRDR strain were four and seven folds higher than that against Wild type *E. coli* ATCC 25922 strain, respectively.

Table 5.3 MICs and MBCs of ciprofloxacin and norfloxacin against 5 different types of *E. coli* clinical strains.

Strains	MIC (µg/ml)		MBC (µg/ml)	
	Ciprofloxacin	Norfloxacin	Ciprofloxacin	Norfloxacin
qnrA positive with *gyrA* mutations (S83L and D87N) strain	64	64	>256	>256
qnrA negative with *gyrA* mutations (S83L and D87N) strain	32	64	>64	>256
qnrA positive with wild type QRDR strain	0.12	1	2	8
qnrA negative with wild type QRDR strain	0.03	0.12	1	0.25
Wild type *E. coli* ATCC 25922 strain	0.008	0.06	0.12	0.06

7. Time kill study

Five different types *E. coli* clinical strains as in previous study were included in the time kill study. It was found that 0.03-0.06 µg/ml (4xMIC and 8xMIC) of ciprofloxacin showed bactericidal activity against wild type *E. coli* ATCC 25922 after two hour of incubation, 0.015 µg/ml (2xMIC) of ciprofloxacin showed bactericidal activity from the fourth to the eighth hour, while 0.008 µg/ml (1xMIC) of this drug showed slightly inhibitory effect as showed in Figure 5.15

The 0.25 µg/ml (8xMIC) of ciprofloxacin showed bactericidal activity against *qnrA* negative with wild type QRDR strain after four hours of incubation. For the longer incubation period (6 hours), the bactericidal activity of 0.12 µg/ml of ciprofloxacin (4xMIC) against this strain was observed while the 0.06 µg/ml (2xMIC) showed bacteriostatic activity at all times of the experiment. The 0.03 µg/ml (1xMIC) showed slightly inhibitory effect as showed in Figure 5.15

For *qnrA* positive with wild type QRDR strain, the bactericidal activity of 0.5-1 µg/ml (4xMIC and 8xMIC) of ciprofloxacin against *qnrA* positive strain was observed at the longer incubation period (6 hours) while 0.25 µg/ml (2xMIC) showed bactericidal activity at the eighth hour of incubation. The 0.12 µg/ml (1xMIC) showed slightly inhibitory effect. Moreover, the regrowth was observed in 0.12 µg/ml (1xMIC) at twenty four hour as showed in Figure 5.15

For *qnrA* negative with *gyrA* mutations (S83L and D87N) strain, all ciprofloxacin concentrations (32-256 µg/ml) showed bacteriostatic activity as showed in Figure 5.15

For *qnrA* positive with *gyrA* mutations (S83L and D87N) strain, only 512 µg/ml (8xMIC) of ciprofloxacin showed bactericidal activity after four hour of incubation while the other concentrations (64-256 µg/ml) showed bactericidal activity during the sixth to eighth hour, furthermore regrowth was observed in 64-128 µg/ml (1xMIC and 2xMIC) at twenty-four hour as showed in Figure 5.15

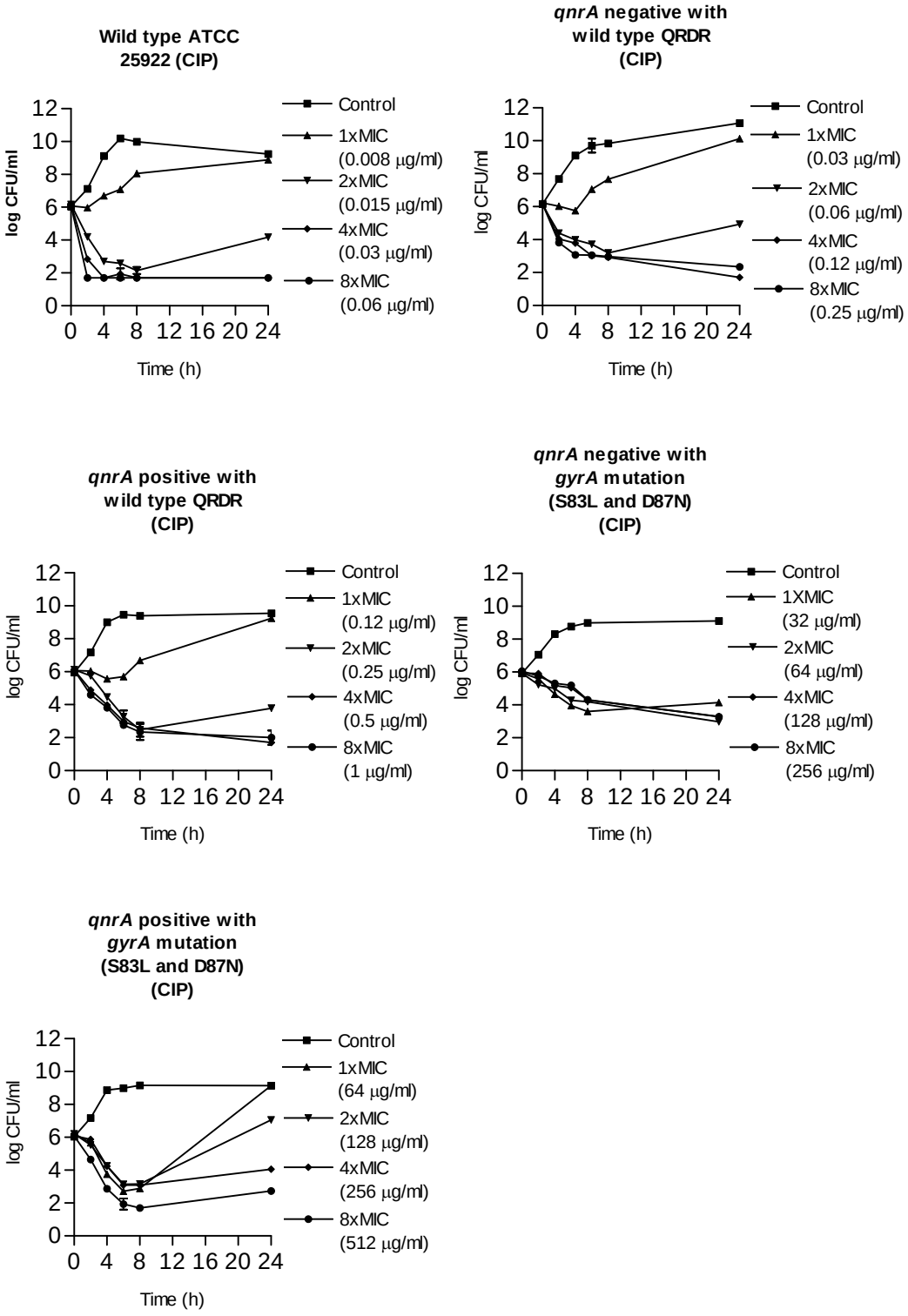

Figure 5.15 Time kill curves showed antibacterial activity of ciprofloxacin against 5 groups of *E. coli* strains, CIP = ciprofloxacin, h = hours.

For the antibacterial activity of norfloxacin, all norfloxacin concentrations (0.06-0.5 µg/ml) showed bactericidal activity against wild type *E. coli* ATCC 25922 after eight hour of incubation as showed in Figure 5.16

The 1 µg/ml (8xMIC) of norfloxacin showed bactericidal activity against *qnrA* negative with wild type QRDR strain after eight hour of incubation, while 0.5 µg/ml (4xMIC) showed bactericidal activity at twenty-four hour. The 0.25 µg/ml (2xMIC) showed bacteriostatic activity at all times of the experiment. In addition, regrowth was observed in 0.12 µg/ml (1xMIC) at twenty fourth hours as showed in Figure 5.16

All norfloxacin concentrations (1-8 µg/ml) showed bactericidal activity against *qnrA* positive with wild type QRDR strain at twenty-four hours of incubation as showed in Figure 5.16

For *qnrA* negative with *gyrA* mutations (S83L and D87N) strain, 512 µg/ml (8xMIC) showed bactericidal activity against *qnrA* negative with *gyrA* mutations (S83L and D87N) strain after two hour of incubation, while the other concentrations (64-256 µg/ml) showed slightly inhibitory effect. Moreover, regrowth was observed in 64 µg/ml (1xMIC) at twenty fourth hours as showed in Figure 5.16

For *qnrA* positive with *gyrA* mutations (S83L and D87N) strain, only 512 µg/ml (8xMIC) showed bactericidal activity against *qnrA* positive and *gyrA* mutation (S83L and D87N) strain during the second to eighth hour of incubation The 128 µg/ml (2xMIC) showed bacteriostatic activity at all times of the experiment. The 256 µg/ml (4xMIC) showed bactericidal activity at eight hour of incubation only. In addition, regrowth was observed in 128 µg/ml (2xMIC) at twenty fourth hours and 64 µg/ml (1xMIC) showed slightly inhibitory effect as showed in Figure 5.16

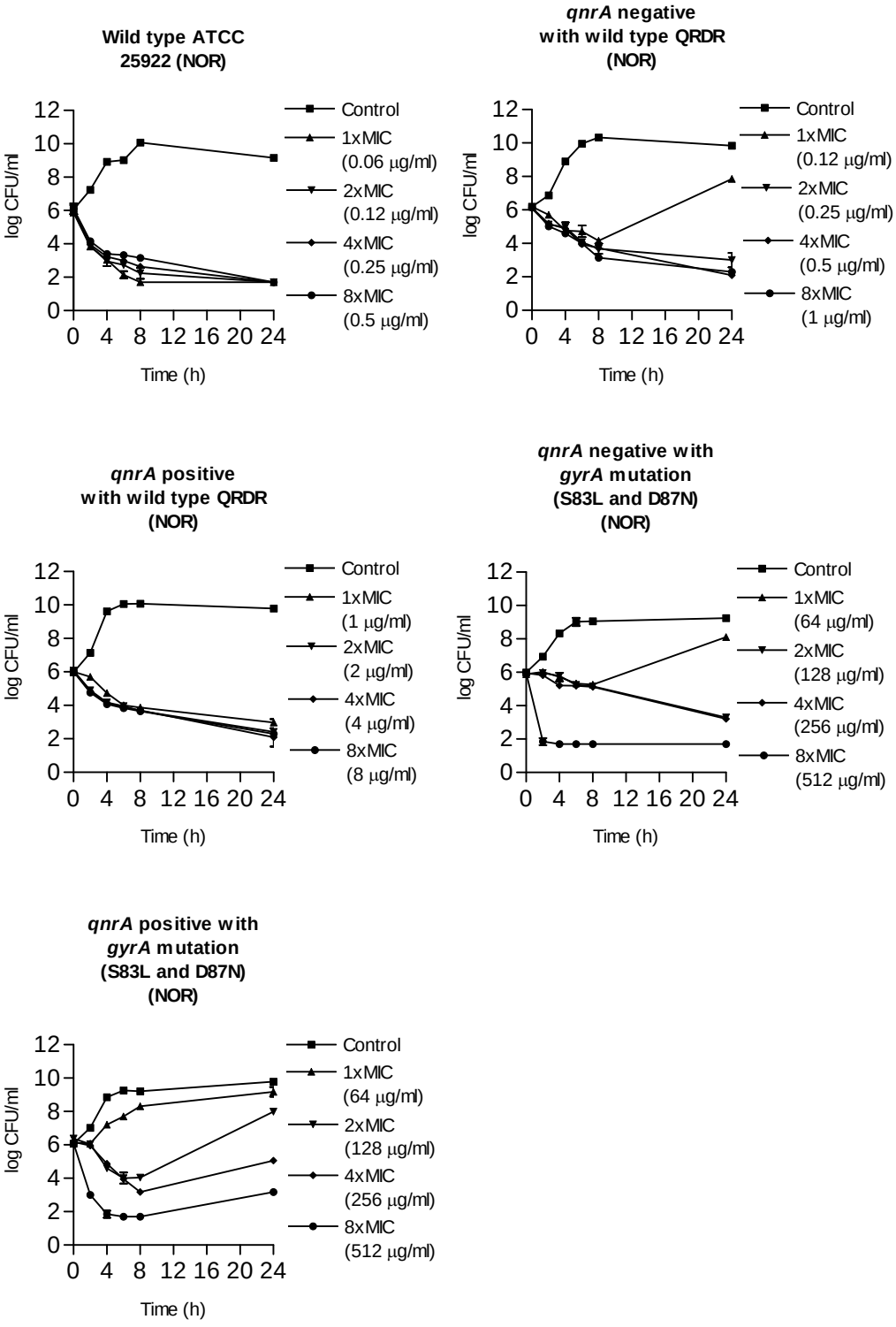

Figure 5.16 Time kill curves showed antibacterial activity of norfloxacin against 5 groups of *E. coli* strains, NOR = norfloxacin, h = hours

CHAPTER VI

DISCUSSION

E. coli is the most common pathogen usually found anywhere including in Thailand. The report from National Antimicrobial Resistance Surveillance Center, Thailand (NARST) demonstrated that *E. coli* was the number one of the top ten isolates from all regions of Thailand in 2009 (NARST, 2009). Furthermore, *E. coli* was also the number one of the top ten recovered from several specimens in the year 2009 including blood (19%), pus (18%), tissues (16%) and urine (44%)(NARST, 2009a; NARST, 2009b; NARST, 2009c; NARST, 2009d). In addition, the antibiogram (percentage of susceptible organisms) from NARST in 2007 showed that *E. coli* isolates were 52 and 51% susceptible to ciprofloxacin and norfloxacin, respectively (NARST, 2007), which indicated the decrease in susceptibility to fluoroquinolones. Several previous reports suggested that *qnrA* genes were commonly found in *Enterobacteriaceae* including *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Enterobacter* spp, *Proteus mirabilis*, *Citrobacter* spp, and *Salmonella* spp, thus, the high prevalence of these genes were from *E. coli* and *K. pneumoniae* isolated from many parts of the world including North America, South America, Europe, and Asia (Jacoby, 2005; Robicsek *et al*., 2006; Strahilevitz *et al*., 2009). However, the study on PMQR genes in Thailand is still limited; only one study by Poirel and colleagues, which showed that 11 out of 23 bla_{VEB-1}-positive enterobacterial

isolates (48%) including *E. coli*, *K. pneumoniae* and *Enterobacter sakazakii* from Bangkok, Thailand were also *qnrA* positive (Poirel *et al*., 2005). According to this study, 10 out of 100 ESBL producing *E. coli* were also positive for bla_{VEB-1} genes (kindly information provided by Asst. Prof. Dr. Chanwit Tribuddharat). The *qnrA* genes were also detected in 4 out of these 10 strains. There are limited numbers of research on PMQR genes in Thailand as well as in the other countries in Southeast Asia. These genes have been reported from this region in only two studies included *qnrB* genes detected in *K. pneumoniae* isolates from Singapore and PMQR genes including *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr* and *qepA* detected in *Enterobacteriaceae* isolates from Ho Chi Minh City in Vietnam (Le *et al*., 2009; Teo *et al*., 2009). In contrary, there have been several publications on PMQR genes from the East Asia including China, Japan, and South Korea (Strahilevitz *et al*., 2009; Rodriguez-Martinez *et al*., 2010).

Among 100 ESBL producing *E. coli* isolates from a university hospital in this study, 83% (83/100) were resistant to quinolone, while from 90 quinolone resistant *E. coli* isolates from a tertiary private hospital, 57.78% (52/90) were ESBL producing *E. coli*. These primarily data suggested that ESBL and quinolone resistance phenotype might be associated or concomitant. This study attempted to find the prevalence of *qnrA* genes in a university hospital in comparison to those in a tertiary private hospital. It was demonstrated that the prevalence of *qnrA* genes in ESBL producing *E. coli* isolates from

the university hospital was 8% (8/100). In contrast, no *qnrA* was detected neither from non-ESBL nor ESBL producing *E. coli* isolates from the tertiary private hospital. Due to the fact that the university hospital in this study was a well-recognized university hospital with approximately 4 times larger in size (2,400 vs. 550 beds) than the selected tertiary private hospital in this study, and the extensive usage of antimicrobial agents is presently very common in the university hospital (Pongpech *et al*., 2008). Furthermore, high prevalence of both ESBL genes and high *intI1* genes, a marker of multiple antibiotic resistance class 1 integron in this selected university hospital have been reported (Pongpech *et al*., 2008), thus, the selected university hospital may have clonal expansion or endermic clone of multiple drug resistance (MDR) higher than the selected tertiary private hospital. As mentioned above, the *qnrA* genes are likely to be more prevalent there. According to several previous studies, *qnrA* genes were usually detected from the university hospitals or teaching hospitals worldwide including China, France, Greece, South Korea, Sweden, Taiwan, Thailand, and United States (Martinez-Martinez *et al*., 1998; Girlich *et al*., 2001; Wang *et al*., 2003; Poirel *et al*., 2005; Cambau *et al*., 2006; Poirel *et al*., 2006; Wu *et al*., 2007; Xu *et al*., 2007; Tamang *et al*., 2008; Fang *et al*., 2009; Kim *et al*., 2009; Galani *et al*., 2010). Strahilevitz *et al* (2009) reported that among 20,960 isolates of *Enterobacteriaceae* from all over the world the average display rate of *qnrA* was 1.5% (Strahilevitz *et al*., 2009), while the prevalence of *qnrA* from ESBL producing *E. coli* in this study was 8%. DNA sequencing analysis confirmed

positive results for a *qnrA1* variant as well as the *qnrA1* variants among *Enterobacteriaceae* isolates from Thai patients in 2005 by Poirel and colleagues (Poirel *et al*., 2005). These primarily data suggested that most of *qnrA* detected in Thailand were *qnrA1* variant.

Target alteration of DNA gyrase and topoisomerase IV particularly double mutations in QRDR region in *gyrA* gene conferred high-level resistance to fluoroquinolones similar to the decrease in drug accumulation in the bacterial cells from the activation of efflux pump and/or porin deficiency (Weigel *et al*., 1998; Uchida *et al*., 2010). The data from this study showed that double mutations in the QRDR region (S83L and D87N) were found in 4 out of 8 (50%) *qnrA* positive isolates. This primary data suggested that *qnrA* and double mutations in *gyrA* gene were not concomitant. These 4 isolates were resistant to ciprofloxacin, while the isolates without double mutations (S83L and D87N) were still susceptible to this drug. According to several previous studies, S83L and D87N were a common type of double mutations in QRDR region in *gyrA* genes and could be detected all over the world including Argentina, China, Germany, Philippines, Saudi Arabia, Singapore, Spain, Sri Lanka, Taiwan, Thailand, United Kingdom, United States, and Vietnam (Yoshida *et al*., 1990; Conrad *et al*., 1996; Everett *et al*., 1996; Weigel *et al*., 1998; Tavio *et al*., 1999; Uchida *et al*., 2010).

Several previous studies reported that *qnrA* genes identified and located in In*4* family of class 1 integrons, In*36* and In*37*, which were also known as complex *sul1*-type integrons because of the presence of duplicate *qacE*Δ*1* and *sul1* genes and surrounded by a sequence encoding an IS*CR1* (formerly orf513). The *qnrA* gene was not associated with a 59-base element as a form of a gene cassette found in common class 1 integrons. Interestingly, no ESBL gene was located with any *qnrA* in the *sul1*-type integrons. This observation indicated that the co-localization, if any, of *qnrA* and ESBL genes on the same plasmids would probably be resulted from unrelated genetic events (Tran *et al*., 2002; Jacoby *et al*., 2003; Wang *et al*., 2003; Wang *et al*., 2004; Mammeri *et al*., 2005; Nordmann *et al*., 2005; Poirel *et al*., 2005). Due to the above observation, the primary association between *qnrA* and *intI1* (class 1 integron) genes were investigated. The result showed that all *qnrA* positive isolates produced ESBL and were also positive for *intI1* genes confirming that *qnrA* associated with ESBL and class 1 integron as indicated in the previous studies (Jacoby, 2005; Nordmann *et al*., 2005; Cano *et al*., 2009; Strahilevitz *et al*., 2009). These data also suggested that ESBL producing *E. coli* possessed both *qnrA* and class 1 integron which increased the opportunity to spread the antibiotic resistance genes among Gram-negative bacteria.

Although, *qnrA* is a plasmid-mediated quinolone resistance (PMQR) gene, the result from Southern blot hybridization in this study showed that *qnrA* and *intI1 genes*

were likely to locate on the chromosome as well as on the large plasmids. This indicated that the *qnrA* and *intI1* genes could be integrated into the chromosome by mobile genetic elements, such as, transposon associated with class 1 integrons. The chromosomal location of *qnrA* in class 1 integrons may facilitate a spread of this antibiotic resistance gene (*qnrA*) via vertical and horizontal genes transfer for the intraspecies and interspecies. The locations of *qnrA* genes in this study were similar to chromosomal location of *qnrA3* in *Shewanella algae* reported by Poirel and colleagues in 2005 (Poirel *et al*., 2005).

The *qnrA* gene transfer either by conjugation or transformation methods either by chemical reaction or electroporation was not successful suggesting the chromosomal locations of *qnrA* genes and/or the *qnrA* plasmid was not conjugative. Furthermore, the probability transfer DNA by conjugation in Gram-negative bacteria required the optimum donors:recipients ratios because the most conjugative plasmids isolated from strains of bacteria in nature is repressed such that only 1 of 1,000 to 10,000 cells carrying the conjugative plasmid is able to transfer it. In some instance, transient derepression occurs on transfer to a recipient cell, the detection of plasmid transfer by conjugation which required a period of several hours or longer for matings and with periodic dilution of the mating mixture to maintain appropriate bacterial densities (Provence *et al*., 1994). ESBL producing *E. coli* harboring *qnrA* isolates in this study may spread this gene to

other Gram-negative bacteria via a mobile genetic element, such as, transposons with class 1 integrons. Although, the *qnrA* plasmid may not conjugate and unable to initiating conjugation, but, they can transfer only with the assistance of conjugative plasmid. Since the conjugative plasmids contain both *tra* (transfer gene) and *bom* (basis of mobility) genes, while, non conjugative plasmid contain only *bom* gene that cannot synthesize the donor pili and thus cannot mediate interaction of donor and recipients cell required for conjugation to occur. Thus the non conjugative plasmid that contains the *bom* locus is present in a cell that also contains the conjugative plasmid and when conjugation is allowed to proceed, recipient cells are found to contain both the conjugative and non conjugative plasmids. This results from the interaction of the non conjugative plasmid with the *tra* gene products encoded by the conjugative plasmid. The *tra* gene is able to perform in *trans* and mediate the conjugation of plasmids that contain *bom* gene (Provence *et al*., 1994). Several studies on the chromosomal *bla* gene by Nordmann and colleagues demonstrated that when the hybridization signals of these genes were obtained at chromosomal position of migration, indicating a likely chromosomal location of these genes, the conjugation and transformation of these genes were usually failed (Bellais *et al*., 2000; Nordmann *et al*., 2000; Mammeri *et al*., 2002; Mammeri *et al*., 2003a; Mammeri *et al*., 2003b; Heritier *et al*., 2004). Furthermore, three *qnrA* genes in this study were likely to locate on both the chromosome and the large plasmid. According to the study by Poirel and colleagues, several

Enterobacteriaceae clinical isolates from Thailand harbored *qnrA* which could be rarely transferred to transconjugants (Poirel *et al*., 2005; Robicsek *et al*., 2006). Although the explanation for this observation is not clear, it is possible that the plasmid is not conjugative or *qnrA* is lost during the process of conjugation (Martinez-Martinez *et al*., 1998; Jacoby *et al*., 2003; Wang *et al*., 2003; Rodriguez-Martinez *et al*., 2003; Wang *et al*., 2004; Mammeri *et al*., 2005; Nazic *et al*., 2005; Nordmann *et al*., 2005; Poirel *et al*., 2005).

All *qnrA* positive isolates were resistant to ampicillin, ampicillin/sulbactam, cefotaxime, and ceftazidime, while 25% (2/8), 37.5% (3/8) and 100% (8/8) of these isolates were susceptible or intermidiate to ceftriaxone, amoxicillin/clavulanic acid, and cefepime, respectively. The resistance to all of the above beta-lactam antibiotics except for cefepime could possibly be due to AmpC beta-lactamase encoded by *bla* genes located on the bacterial chromosome (Jacoby, 2009; Drawz *et al*., 2010). However, the *bla* gene in *qnrA* positive isolates in this study should be further investigated. In several previous studies, *qnrA* was usually associated with ESBL and AmpC beta-lactamase including SHV-5, SHV-7, SHV-12, and various CTX-M family, and FOX-5 (Rodriguez-Martinez *et al*., 2003; Wang *et al*., 2003; Wang *et al*., 2004; Corkill *et al*., 2005; Nazic *et al*., 2005; Nordmann *et al*., 2005; Cambau *et al*., 2006; Paauw *et al*., 2006; Rodriguez-Martinez *et al*., 2006; Wu *et al*., 2007; Jiang *et al*., 2008; Lavilla *et al*., 2008; Szabo *et al*.,

2008; Tamang *et al*., 2008; Strahilevitz *et al*., 2009). Eighty eight percent (7/8) of *qnrA* positive isolates were resistant to trimethoprim/sulfamethoxazole. According to the previous studies, *in vitro* susceptibility of the transconjugants containing plasmids harboring *qnrA*, showed that all transconjugants were resistant to sulfamethoxazole (Wang *et al*., 2004). Furthermore, sulfamethoxazole alone is a bacterostatic agent (AMA, 1986), while a combined trimethoprim/sulfamethoxazole is bactericidal agent (Masters *et al*., 2003). For the reason, some of the isolates were still susceptible to trimethoprim/sulfamethoxazole, while they were all resistant to sulfamethoxazole. These data which were reminiscent of a previous study, suggest that the *qnrA* determinant may be expressed at variable levels, possibly depending on host functions (Martinez-Martinez *et al*., 2003; Poirel *et al*., 2005). In addition, all *qnrA* positive isolates were susceptible to imipenem and meropenem indicating that carbapenems can be an alternative choice for the treatment of infections by ESBL producing *E. coli* containing both *qnrA* and double *gyrA* mutations (S83L and D87N).

From the MICs determination, *qnrA* positive with wild type QRDR isolate conferred low-level resistance to ciprofloxacin and norfloxacin (MIC, 0.12 and 1 µg/ml), respectively. Even though the isolate lost the susceptibility in some degree, the drug concentrations were still lower than the clinical breakpoint level. The MIC of ciprofloxacin against this isolate was similar to that of a *qnrA* transconjugant in the previous studies

that were 0.12-0.25 µg/ml (Wang *et al*., 2003; Wang *et at*., 2004; Strahilevitz*et al*., 2009), suggesting that this isolate may have a single *qnrA* resistance determinant as well. From the previous studies, a single *gyrA* mutation (S83L) isolates without other quinolone resistance mechanisms (wild type *gyrB*, *parC*, and *parE* genes) were usually required 2 µg/ml (range 0.25-2 µg/ml) of ciprofloxacin for the inhibition, which showed that these isolates were intermediate resistant to ciprofloxacin (Everett *et al*., 1996; Weigel *et al*., 1998). The double mutations in *gyrA* isolates were showed to be highly resistant to ciprofloxacin (>4 µg/ml) and were usually associated with other chromosomal resistance mechanisms, particularly, *parC* mutation (Everett *et al*., 1996; Heisig, 1996; Vila *et al*., 1996; Weigel *et al*., 1998; Bagel *et al*., 1999). In this study, double mutations in *gyrA* (S83L and D87N) alone without *qnrA* were highly resistant to ciprofloxacin (32 µg/ml), indicating that this isolate should be associated with other resistant mechanisms such as *parC* mutation. The isolate with double mutations in *gyrA* genes (S83L and D87N) and associated with *qnrA* became very high resistant to ciprofloxacin (MIC, 64 µg/ml). It should be noted that the sole *qnrA* positive isolate could confer low-level resistance to fluoroquinolone, and might be problematic clinically.

In the MBC determination, the MBCs of ciprofloxacin and norfloxacin against *qnrA* positive isolate were four (0.12 to 2 µg/ml) and three folds (1 to 8 µg/ml) dilution higher than the MICs, respectively. MIC is a concentration for only inhibition of an

organism, not to kill them all. Thus, single-step resistant mutants may be rising. Although, the isolate with *qnrA* alone conferred low level resistant to ciprofloxacin and norfloxacin (0.12 and 1 µg/ml, respectively), but, MBCs against this isolate were (2 and 8 µg/ml, respectively) at the intermediate level of resistance. Thus, the isolate with *qnrA* gene might be a problem with fluoroquinolone treatment in the future.

The study on the effect of *qnrA* on the bactericidal activity of fluoroquinolones is still limited; two *in vitro* studies and one *in vivo* study in mice model (Memmeri *et al*., 2005; Rodriguez-Martinez *et al*., 2008; Briales *et al*., 2011). Nevertheless, the bactericidal activities against the isolate with *qnrA* alone and *qnrA* positive isolate harboring other resistant determinants, particularly double mutations in *gyrA* gene have not been reported previously. This study attempted to investigate the bactericidal activity against *qnrA* with and without double mutations in *gyrA* gene, and double mutations in *gyrA* gene alone, in each individual isolate. This study suggested that double mutations in *gyrA* (S83L and D87N) isolate played an important role in fluoroquinolone resistance, because bacteriostatic activity could be observed in all drug concentrations of ciprofloxacin (32-256 µg/ml) and norfloxacin (64-256 µg/ml). The bactericidal activity of 4xMIC and 8xMIC of ciprofloxacin against *qnrA* negative with wild type QRDR isolate and *qnrA* positive with wild type QRDR isolate (0.12-0.25 µg/ml and 0.5-1 µg/ml, respectively) were observed at the sixth hour of incubation, while 1x MIC

(0.03 μg/ml and 0.12 μg/ml, respectively) showed slightly inhibitory effect. According to the previous studies, 4xMIC of ciprofloxacin or higher concentrations showed bactericidal activity or a marked reduction against *E. coli* harbored *qnrA* plasmid after four to eight hours of incubation (Mammeri *et al*., 2005; Briales *et al*., 2010). The *qnrA* negative with wild type QRDR isolate could have the other resistant mechanisms, such as, increased AcrAB efflux pump, and/or porin deficiency, particularly OmpF (Jacoby, 2005; Nordmann *et al*., 2005); however, *qnrA* positive isolate was less affected by bactericidal activities of fluoroquinolones than wild type *E. coli* ATCC 25922. The combination mechanisms, such as *qnrA* positive and double mutations in *gyrA* (S83L and D87N) isolate, showed that *qnrA* supported double mutations in *gyrA* (S83L and D87N) in decreasing bactericidal activities of these drugs and increasing the chance of regrowth (the regrowth occurred in 2xMIC, both 128 μg/ml at twenty fourth hours). This isolate was highly resistant to ciprofloxacin and norfloxacin with MICs of 64 μg/ml. The MIC levels were much higher than peak serum concentration that could be accomplished during drug monotherapy, and the increased doses could also lead to the increased drug toxicities (Dan *et al*. 1987; Boy *et al*., 2004). Ciprofloxacin could decrease the bacterial amount more than norfloxacin, even though these drugs had the similar structures. The side chain of position N-1 in norfloxacin was ethyl group, while cyclopropyl group was in ciprofloxacin. Cyclopropyl was the optimal group for this position and could be beneficial to ciprofloxacin in having the most potent activities

against the members of *Enterobacteriaceae* and *Pseudomonas aeruginosa in vitro* study (Chu *et al*., 1989; Domagala, 1994; Andersson *et al*., 2003). The MIC_{90} of ciprofloxacin against *E. coli* was two folds dilution less than norfloxacin (MIC_{90}, 0.03 mg/L and 0.12 mg/L) (Anderssone*t al*., 2003).

The results from this study indicated that *qnrA1* gene in *E. coli* clinical isolates from Thailand was associated with ESBL gene and class 1 integrons. Moreover 50% of *qnrA* positive isolates with double mutations in the QRDR region (S83L and D87N) were resistant to ciprofloxacin. The ESBL producing *E. coli* with both *qnrA* and double mutations in *gyrA* could limit the choices of antibiotic treatment, even though these isolates were susceptible to carbapenems. The *qnrA1* and *intI1* genes could be integrated into the chromosome by mobile genetic elements, such as, transposon associated with class 1 integrons. Furthermore, 5 *qnrA1* positive isolates carrying two integrons in each isolate may increase the opportunity for antibiotic resistance gene transfer among Gram-negative bacteria. Although *qnrA1* conferred low-level resistance to ciprofloxacin and norfloxacin, when associated with other resistant mechanisms, particularly, *gyrA* mutations (S83L and D87N), it could confer higher-level of fluoroquinolone resistance. Genetic vehicle of *qnrA* positive isolates in this study included complex *sul1*-type integrons and *qnrA* linked with any *bla* gene which must be further investigated.

CHAPTER VII

CONCLUSION

The prevalence of *qnrA* genes is 8% (8/100) in ESBL producing *E. coli* isolates (quinolone resistant *E. coli* 83 isolates) from the university hospital; in contrast, no *qnrA* was detected neither from non-ESBL nor ESBL producing *E. coli* isolates from the tertiary private hospital. All *qnrA* positive isolates were also positive for *intI1* genes confirming that *qnrA* gene were associated with ESBL and class 1 integron. Double mutations in the QRDR region of *gyrA* genes (S83L and D87N) were found in 4 out of 8 (50%) *qnrA* positive isolates. These 4 isolates were resistant to ciprofloxacin, while the isolates without double mutations were still susceptible to this drug. This primary data suggested that *qnrA* and double mutations in *gyrA* gene were not concomitant. In addition, all *qnrA* positive isolates were susceptible to imipenem and meropenem indicating that carbapenems can be an alternative choice for the treatment of infections by ESBL producing *E. coli* containing *qnrA* and double *gyrA* mutations (S83L and D87N).

The result from Southern blot hybridization showed that *qnrA* and *intI1 genes* were likely to locate on the chromosome as well as on the large plasmids. This indicated that the *qnrA* and *intI1* genes could be integrated into the chromosome by mobile genetic elements. Interestingly, in the *Bam*HI-digested genomic DNA experiment, the

specific *intI1* probe gave two positive signals from a single isolate (5/8 isolates), this demonstrated the presence of two integrons in each *qnrA* positive isolate and wide spread of antibiotic resistance genes in Gram-negative bacteria.

From MIC determination, *qnrA* positive with wild type QRDR strain showed low-level resistance to ciprofloxacin and norfloxacin (MIC, 0.12 and 1 µg/ml, respectively), but when associated with double mutations in *gyrA* genes (S83L and D87N), they became highly resistant to ciprofloxacin (MIC, 64 µg/ml). It should be noted that the sole *qnrA* positive isolate could confer low-level resistance to fluoroquinolone, and might be problematic clinically.

Time kill experiment in this study suggested that the double mutations in *gyrA* gene (S83L and D87N) isolate played an important role in fluoroquinolone resistance, because bacteriostatic activity could be observed in all drug concentrations of ciprofloxacin (32-256 µg/ml) and norfloxacin (64-256 µg/ml). The longer incubation period (6 hours) has showed the bactericidal activity of 4x MIC and 8x MIC (0.5-1 µg/ml) of ciprofloxacin against *qnrA* positive with wild type QRDR strain, while 1x MIC (0.12 µg/ml) showed slightly inhibitory effect. The combination mechanisms, such as *qnrA* positive with *gyrA* mutations (S83L and D87N), showed that *qnrA* supported *gyrA* mutations (S83L and D87N) in decreasing bactericidal activities of these drugs and increasing the chance of regrowth (the regrowth occurred in 2x MIC, both 128 µg/ml at

twenty fourth hour). This strain was highly resistant to ciprofloxacin and norfloxacin with MICs of 64 µg/ml. The MIC levels were much higher than peak serum concentration that could be accomplished during drug monotherapy, and increased doses could also lead to increased drug toxicities.

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APPENDICES

APPENDIX A

THE RESULTS OF DISK DIFFUSION AND TIME KILL TESTED IN THIS STUDY

Table A-1 Inhibition zone size and antimicrobial susceptibility pattern of all *qnrA* positive isolates, *qnrA* negative and double mutation in *gyrA* isolate, and *qnrA* negative and wild type QRDR isolate.

Isolates	Inhibition zone size (mm.)/Antimicrobial susceptibility pattern													
	AM		AMC		SAM		SXT		CTX		CAZ		CRO	
No.23	6	R	14	I	10	R	6	R	6	R	12	R	6	R
No.45	6	R	12	R	8	R	6	R	14	R	10	R	14	R
No.67	6	R	13	R	10	R	21	S	20	R	12	R	19	R
No.104	6	R	14	I	10	R	6	R	15	R	12	R	13	R
No.113	6	R	13	R	6	R	6	R	14	R	6	R	12	R
No.128	6	R	12	R	6	R	6	R	22	R	11	R	20	I
No.129	6	R	10	R	6	R	6	R	8	R	12	R	8	R
No.176	6	R	15	R	7	R	6	R	20	R	12	R	20	I
No.98	6	R	17	I	16	S	20	S	6	R	11	R	6	R
No.137	6	R	10	R	6	R	20	S	13	R	6	R	13	R

AM = ampicillin, AmC = amoxicillin/clavulanic acid, SAM = ampicillin/sulbactam, SXT = trimethoprim/sulfamethoxazole, CTX = cefotaxime, CAZ = ceftazidime, CRO = ceftriazone, S = susceptible, I = intermediate and R = resistant

Table A-1: (cont.)

Isolates	Inhibition zone size (mm.)/Antimicrobial susceptibility pattern													
	AN		GM		TZP		FEP		CIP		IMP		MEM	
No.23	21	S	8	R	26	S	16	I	24	S	30	S	31	S
No.45	18	S	13	I	22	S	21	S	6	R	28	S	30	S
No.67	13	R	11	R	24	S	25	S	26	S	26	S	30	S
No.104	18	S	6	R	24	S	23	S	6	R	30	S	31	S
No.113	14	R	8	R	20	I	20	S	8	R	26	S	26	S
No.128	17	S	13	I	21	S	26	S	20	I	27	S	26	S
No.129	16	I	6	R	14	R	18	S	21	S	26	S	28	S
No.176	19	S	15	S	24	S	26	S	6	R	24	S	26	S
No.98	13	R	20	S	25	S	18	S	6	R	25	S	30	S
No.137	10	R	6	R	20	I	24	S	28	S	21	S	28	S

AN = amikacin, GM = gentamicin, TZP = piperacillin/tazobactam, FEP = cefipime, CIP = ciprofloxacin, IPM = imipenem and MEM = meropenem

S = susceptible, I = intermediate and R = resistant

Table A-2 Log viable cell count of ciprofloxacin against wild type *E. coli* ATCC 25922.

Time (hour)	Control		1xMIC (0.008 mg/ml)		2xMIC (0.015 mg/ml)		4xMIC (0.03 mg/ml)		8xMIC (0.06 mg/ml)	
0	6.041393	6.041393	6.041393	6.113944	6.190332	6.176091	6.176091	6.176091	6.041393	6.130334
2	7.130334	7.130334	5.954243	6	4.146128	4.255272	2.740363	2.929419	1.69897	1.69897
4	9.09691	9.161368	6.812913	6.60206	2.653213	2.740363	1.69897	1.69897	1.69897	1.69897
6	10.19033	10.20412	7.176091	7	2.69897	2.477121	2.176091	1.69897	1.69897	1.69897
8	9.875061	10.11394	7.954243	8.176091	2	2.30103	1.69897	1.69897	1.69897	1.69897
24	9.278753	9.217484	8.812913	8.954243	4.176091	4.176091	1.69897	1.69897	1.69897	1.69897

Table A-3 Statistic analysis of ciprofloxacin against wild type *E. coli* ATCC 25922 by GraphPad Prism 3.0 program.

Tukey's Multiple Comparison Test	P value	95% CI
Control vs 1xMIC (0.008 mg/ml)	P > 0.05	-1.315 to 4.296
Control vs 2xMIC (0.015 mg/ml)	P < 0.001	2.152 to 7.763
Control vs 4xMIC (0.03 mg/ml)	P < 0.001	3.144 to 8.755
Control vs 8xMIC (0.06 mg/ml)	P < 0.001	3.388 to 8.999
1xMIC (0.008 mg/ml) vs 2xMIC (0.015 mg/ml)	P < 0.05	0.6618 to 6.273
1xMIC (0.008 mg/ml) vs 4xMIC (0.03 mg/ml)	P < 0.01	1.653 to 7.264
1xMIC (0.008 mg/ml) vs 8xMIC (0.06 mg/ml)	P < 0.001	1.898 to 7.509
2xMIC (0.015 mg/ml) vs 4xMIC (0.03 mg/ml)	P > 0.05	-1.814 to 3.797
2xMIC (0.015 mg/ml) vs 8xMIC (0.06 mg/ml)	P > 0.05	-1.570 to 4.041
4xMIC (0.03 mg/ml) vs 8xMIC (0.06 mg/ml)	P > 0.05	-2.561 to 3.050

Table A-4 Log viable cell count of ciprofloxacin against *qnrA* negative with wild type QRDR strain.

Time (hour)	Control		1xMIC (0.03 mg/ml)		2xMIC (0.06 mg/ml)		4xMIC (0.12 mg/ml)		8xMIC (0.25 mg/ml)	
0	6.146128	6.176091	6.243038	6.230449	6.09691	6.079181	6.217484	6.190332	6.09691	6.217484
2	7.778151	7.60206	5.954243	6.09691	4.352182	4.431364	4.041393	4.041393	3.778151	3.875061
4	9	9.230449	5.740363	5.778151	3.977724	4	3.69897	3.875061	3.021189	3.146128
6	9.39794	10	7.041393	7.079181	3.845098	3.60206	3	3.060698	3	3.113943
8	9.845098	9.845098	7.69897	7.653213	3.190332	3.20412	2.929419	2.90309	3	2.954242
24	11.07918	11.07918	10.14613	10.11394	5.041393	4.845098	1.69897	1.69897	2.39794	2.30103

Table A-5 Statistic analysis of ciprofloxacin against *qnrA* negative with wild type QRDR strain by GraphPad Prism 3.0 program.

Tukey's Multiple Comparison Test	P value	95% CI
Control vs 1xMIC (0.03 mg/ml)	P > 0.05	-0.9242 to 4.491
Control vs 2xMIC (0.06 mg/ml)	P < 0.001	1.835 to 7.251
Control vs 4xMIC (0.12 mg/ml)	P < 0.001	2.611 to 8.026
Control vs 8xMIC (0.25 mg/ml)	P < 0.001	2.649 to 8.064
1xMIC (0.03 mg/ml) vs 2xMIC (0.06 mg/ml)	P < 0.05	0.05142 to 5.467
1xMIC (0.03 mg/ml) vs 4xMIC (0.12 mg/ml)	P < 0.01	0.8272 to 6.243
1xMIC (0.03 mg/ml) vs 8xMIC (0.25 mg/ml)	P < 0.01	0.8650 to 6.281
2xMIC (0.06 mg/ml) vs 4xMIC (0.12 mg/ml)	P > 0.05	-1.932 to 3.484
2xMIC (0.06 mg/ml) vs 8xMIC (0.25 mg/ml)	P > 0.05	-1.894 to 3.521
4xMIC (0.12 mg/ml) vs 8xMIC (0.25 mg/ml)	P > 0.05	-2.670 to 2.746

Table A-6 Log viable cell count of ciprofloxacin against *qnrA* positive with wild type QRDR strain.

Time (hour)	Control		1xMIC (0.12 mg/ml)		2xMIC (0.25 mg/ml)		4xMIC (0.5 mg/ml)		8xMIC (1 mg/ml)	
0	5.954243	5.954243	6	6.146128	6.176091	6.079181	6.041393	6.146128	6	6.146128
2	7.176091	7.176091	6.041393	6.041393	5.653213	5.845098	4.875061	4.90309	4.653213	4.544068
4	8.875061	9.130334	5.477121	5.653213	4.544068	4.39794	3.954242	4	3.740363	3.90309
6	9.544068	9.39794	5.69897	5.69897	3	3.544068	3.021189	2.954242	2.69897	2.845098
8	9.39794	9.39794	6.778151	6.60206	2.176091	2.778151	2.477121	2.69897	2	2.69897
24	9.544068	9.544068	9.146128	9.361728	3.69897	3.875061	1.69897	1.69897	1.69897	2.30103

Table A-7 Statistic analysis of ciprofloxacin against *qnrA* positive with wild type QRDR strain by GraphPad Prism 3.0 program.

Tukey's Multiple Comparison Test	P value	95% CI
Control vs 1xMIC (0.12 mg/ml)	P > 0.05	-0.8419 to 4.583
Control vs 2xMIC (0.25 mg/ml)	P < 0.01	1.398 to 6.823
Control vs 4xMIC (0.5 mg/ml)	P < 0.001	2.006 to 7.431
Control vs 8xMIC (1 mg/ml)	P < 0.001	2.109 to 7.534
1xMIC (0.12 mg/ml) vs 2xMIC (0.25 mg/ml)	P > 0.05	-0.4727 to 4.952
1xMIC (0.12 mg/ml) vs 4xMIC (0.5 mg/ml)	P < 0.05	0.1356 to 5.560
1xMIC (0.12 mg/ml) vs 8xMIC (1 mg/ml)	P < 0.05	0.2389 to 5.664
2xMIC (0.25 mg/ml) vs 4xMIC (0.5 mg/ml)	P > 0.05	-2.104 to 3.321
2xMIC (0.25 mg/ml) vs 8xMIC (1 mg/ml)	P > 0.05	-2.001 to 3.424
4xMIC (0.5 mg/ml) vs 8xMIC (1 mg/ml)	P > 0.05	-2.609 to 2.816

Table A-8 Log viable cell count of ciprofloxacin against *qnrA* negative with *gyrA* mutations (S83L and D87N) strain.

Time (hour)	Control		1XMIC (32 mg/ml)		2xMIC (64 mg/ml)		4xMIC (128 mg/ml)		8xMIC (256 mg/ml)	
0	5.929419	5.929419	5.929419	5.977724	5.954243	5.90309	5.929419	5.954243	6.09691	6.021189
2	7.060698	7.041393	5.69897	5.477121	5.230449	5.230449	5.812913	6	5.778151	5.740363
4	8.322219	8.30103	4.653213	4.653213	4.977724	5	5.230449	5.130334	5.322219	5.30103
6	8.653213	8.90309	3.929419	4	4.255272	4.30103	5.021189	5.060698	5.176091	5.230449
8	9	8.977724	3.60206	3.60206	4.146128	4.230449	4.30103	4.30103	4.30103	4.322219
24	9.041392	9.190331	4.113944	4.190332	2.90309	3.060698	3.30103	3.30103	3.352183	3.217484

Table A-9 Statistic analysis of ciprofloxacin against *qnrA* negative with *gyrA* mutations (S83L and D87N) strain by GraphPad Prism 3.0 program.

Tukey's Multiple Comparison Test	P value	95% CI
Control vs 1XMIC (32 mg/ml)	P < 0.001	1.614 to 5.140
Control vs 2xMIC (64 mg/ml)	P < 0.001	1.667 to 5.193
Control vs 4xMIC (128 mg/ml)	P < 0.001	1.321 to 4.847
Control vs 8xMIC (256 mg/ml)	P < 0.001	1.278 to 4.804
1XMIC (32 mg/ml) vs 2xMIC (64 mg/ml)	P > 0.05	-1.710 to 1.816
1XMIC (32 mg/ml) vs 4xMIC (128 mg/ml)	P > 0.05	-2.056 to 1.470
1XMIC (32 mg/ml) vs 8xMIC (256 mg/ml)	P > 0.05	-2.099 to 1.427
2xMIC (64 mg/ml) vs 4xMIC (128 mg/ml)	P > 0.05	-2.109 to 1.417
2xMIC (64 mg/ml) vs 8xMIC (256 mg/ml)	P > 0.05	-2.152 to 1.374
4xMIC (128 mg/ml) vs 8xMIC (256 mg/ml)	P > 0.05	-1.806 to 1.720

Table A-10 Log viable cell count of ciprofloxacin against *qnrA* positive with *gyrA* mutations (S83L and D87N) strain.

Time (hour)	Control		1xMIC (64 mg/ml)		2xMIC (128 mg/ml)		4xMIC (256 mg/ml)		8xMIC (512 mg/ml)	
0	6.09691	5.977724	6.30103	6.113944	6.243038	6.176091	6.021189	6.176091	6.041393	6.09691
2	7.190332	7.161368	5.740363	5.60206	5.477121	5.60206	5.845098	5.90309	4.60206	4.69897
4	8.845098	8.90309	3.740363	3.778151	4.30103	4.230449	4.176091	4.322219	2.929419	2.812913
6	8.954243	9.041392	2.60206	2.845098	3.176091	3.09691	3.09691	3.09691	1.69897	2.176091
8	9.130334	9.176091	2.929419	2.845098	3.176091	3.146128	3.079181	3.113943	1.69897	1.69897
24	9.060698	9.243038	9.079182	9.217484	7.113944	7.021189	4.060698	4.041393	2.778151	2.69897

Table A-11 Statistic analysis of ciprofloxacin against *qnrA* positive with *gyrA* mutations (S83L and D87N) strain by GraphPad Prism 3.0 program.

Tukey's Multiple Comparison Test	P value	95% CI
Control vs 1xMIC (64 mg/ml)	P < 0.05	0.5377 to 5.793
Control vs 2xMIC (128 mg/ml)	P < 0.01	0.7072 to 5.963
Control vs 4xMIC (256 mg/ml)	P < 0.01	1.193 to 6.448
Control vs 8xMIC (512 mg/ml)	P < 0.001	2.276 to 7.532
1xMIC (64 mg/ml) vs 2xMIC (128 mg/ml)	P > 0.05	-2.458 to 2.797
1xMIC (64 mg/ml) vs 4xMIC (256 mg/ml)	P > 0.05	-1.973 to 3.283
1xMIC (64 mg/ml) vs 8xMIC (512 mg/ml)	P > 0.05	-0.8893 to 4.366
2xMIC (128 mg/ml) vs 4xMIC (256 mg/ml)	P > 0.05	-2.142 to 3.113
2xMIC (128 mg/ml) vs 8xMIC (512 mg/ml)	P > 0.05	-1.059 to 4.197
4xMIC (256 mg/ml) vs 8xMIC (512 mg/ml)	P > 0.05	-1.544 to 3.711

Table A-12 Log viable cell count of norfloxacin against wild type *E. coli* ATCC 25922.

Time (hour)	Control		1xMIC (0.06 mg/ml)		2xMIC (0.12 mg/ml)		4xMIC (0.25 mg/ml)		8xMIC (0.5 mg/ml)	
0	6	6.113944	5.875061	5.875061	6.255272	5.875061	5.90309	5.929419	6.342422	5.875061
2	7.243038	7.255272	3.929419	3.740363	3.875061	3.875061	3.929419	4	4.130334	4.20412
4	9	8.845098	2.778151	3.30103	2.929419	3	3.230449	3.230449	3.39794	3.39794
6	9.079182	8.977724	2	2.30103	2.69897	2.778151	2.954242	3.021189	3.30103	3.361728
8	10.09691	10.0607	1.69897	1.69897	2	2.477121	2.653213	2.60206	3.146128	3.176091
24	9.09691	9.217484	1.69897	1.69897	1.69897	1.69897	1.69897	1.69897	1.69897	1.69897

Table A-13 Statistic analysis of norfloxacin against wild type *E. coli* ATCC 25922 by GraphPad Prism 3.0 program.

Tukey's Multiple Comparison Test	P value	95% CI
Control vs 1xMIC (0.06 mg/ml)	P < 0.001	3.084 to 7.648
Control vs 2xMIC (0.12 mg/ml)	P < 0.001	2.870 to 7.434
Control vs 4xMIC (0.25 mg/ml)	P < 0.001	2.729 to 7.293
Control vs 8xMIC (0.5 mg/ml)	P < 0.001	2.489 to 7.053
1xMIC (0.06 mg/ml) vs 2xMIC (0.12 mg/ml)	P > 0.05	-2.496 to 2.068
1xMIC (0.06 mg/ml) vs 4xMIC (0.25 mg/ml)	P > 0.05	-2.637 to 1.927
1xMIC (0.06 mg/ml) vs 8xMIC (0.5 mg/ml)	P > 0.05	-2.877 to 1.687
2xMIC (0.12 mg/ml) vs 4xMIC (0.25 mg/ml)	P > 0.05	-2.423 to 2.141
2xMIC (0.12 mg/ml) vs 8xMIC (0.5 mg/ml)	P > 0.05	-2.663 to 1.901
4xMIC (0.25 mg/ml) vs 8xMIC (0.5 mg/ml)	P > 0.05	-2.522 to 2.042

Table A-14 Log viable cell count of norfloxacin against *qnrA* negative with wild type QRDR strain.

Time (hour)	Control		1xMIC (0.12 mg/ml)		2xMIC (0.25 mg/ml)		4xMIC (0.5 mg/ml)		8xMIC (1 mg/ml)	
0	6.230449	6.176091	6.146128	6.255272	6.041393	6.09691	6.079181	6.161368	6.041393	6.255272
2	6.90309	6.845098	5.69897	5.69897	5.113944	5.146128	5.130334	5.217484	5.079181	4.977724
4	8.90309	8.90309	4.90309	4.653213	4.778151	5.176091	4.954243	4.812913	4.60206	4.60206
6	9.954243	9.954243	4.977724	4.477121	4.113944	4.041393	3.954242	3.954242	4	4
8	10.32222	10.32222	4.079181	4.255272	3.477121	3.90309	3.778151	3.653213	3.190332	3.09691
24	9.845098	9.845098	7.845098	7.875061	2.69897	3.30103	2.176091	2	2.30103	2.30103

Table A-15 Statistic analysis of norfloxacin against *qnrA* negative with wild type QRDR strain by GraphPad Prism 3.0 program.

Tukey's Multiple Comparison Test	P value	95% CI
Control vs 1xMIC (0.12 mg/ml)	P < 0.05	0.5263 to 5.697
Control vs 2xMIC (0.25 mg/ml)	P < 0.001	1.608 to 6.778
Control vs 4xMIC (0.5 mg/ml)	P < 0.001	1.776 to 6.946
Control vs 8xMIC (1 mg/ml)	P < 0.001	1.894 to 7.065
1xMIC (0.12 mg/ml) vs 2xMIC (0.25 mg/ml)	P > 0.05	-1.504 to 3.667
1xMIC (0.12 mg/ml) vs 4xMIC (0.5 mg/ml)	P > 0.05	-1.336 to 3.835
1xMIC (0.12 mg/ml) vs 8xMIC (1 mg/ml)	P > 0.05	-1.217 to 3.953
2xMIC (0.25 mg/ml) vs 4xMIC (0.5 mg/ml)	P > 0.05	-2.417 to 2.753
2xMIC (0.25 mg/ml) vs 8xMIC (1 mg/ml)	P > 0.05	-2.299 to 2.872
4xMIC (0.5 mg/ml) vs 8xMIC (1 mg/ml)	P > 0.05	-2.467 to 2.704

Table A-16 Log viable cell count of norfloxacin against *qnrA* positive with wild type QRDR strain.

Time (hour)	Control		1xMIC (1 mg/ml)		2xMIC (2 mg/ml)		4xMIC (4 mg/ml)		8xMIC (8 mg/ml)	
0	5.875061	6.079181	6	6	6.041393	6.09691	6.146128	5.954243	6	6
2	7.146128	7.146128	5.69897	5.69897	4.90309	4.90309	4.778151	4.845098	4.778151	4.778151
4	9.544068	9.69897	4.69897	4.778151	4.176091	4.176091	4.176091	4.079181	4.041393	4.113944
6	10.07918	10.04139	4.079181	3.954242	3.954242	3.954242	3.954242	3.875061	3.778151	3.90309
8	10	10.14613	3.954242	3.778151	3.69897	3.653213	3.69897	3.69897	3.69897	3.60206
24	9.812913	9.778152	3.113943	2.845098	2.544068	2.30103	2.477121	1.69897	2.30103	2.30103

Table A-17 Statistic analysis of norfloxacin against *qnrA* positive with wild type QRDR strain by GraphPad Prism 3.0 program.

Tukey's Multiple Comparison Test	P value	95% CI
Control vs 1xMIC (1 mg/ml)	P < 0.001	1.987 to 6.470
Control vs 2xMIC (2 mg/ml)	P < 0.001	2.337 to 6.820
Control vs 4xMIC (4 mg/ml)	P < 0.001	2.422 to 6.905
Control vs 8xMIC (8 mg/ml)	P < 0.001	2.429 to 6.912
1xMIC (1 mg/ml) vs 2xMIC (2 mg/ml)	P > 0.05	-1.892 to 2.591
1xMIC (1 mg/ml) vs 4xMIC (4 mg/ml)	P > 0.05	-1.807 to 2.676
1xMIC (1 mg/ml) vs 8xMIC (8 mg/ml)	P > 0.05	-1.799 to 2.683
2xMIC (2 mg/ml) vs 4xMIC (4 mg/ml)	P > 0.05	-2.156 to 2.326
2xMIC (2 mg/ml) vs 8xMIC (8 mg/ml)	P > 0.05	-2.149 to 2.334
4xMIC (4 mg/ml) vs 8xMIC (8 mg/ml)	P > 0.05	-2.234 to 2.249

Table A-18 Log viable cell count of norfloxacin against *qnrA* negative with *gyrA* mutations (S83L and D87N) strain.

Time (hour)	Control		1xMIC (64 mg/ml)		2xMIC (128 mg/ml)		4xMIC (256 mg/ml)		8xMIC (512 mg/ml)	
0	6.041393	5.929419	5.977724	5.812913	6	5.778151	5.977724	5.954243	5.977724	5.929419
2	6.90309	6.977724	6.041393	6.021189	5.929419	6	5.929419	5.69897	2	1.69897
4	8.322219	8.342422	5.544068	5.90309	5.875061	5.69897	5.146128	5.278754	1.69897	1.69897
6	9.190331	8.845098	5.30103	5.361728	5.255272	5.230449	5.176091	5.20412	1.69897	1.69897
8	8.977724	9.146128	5.243038	5.278754	5.176091	5.161368	5.176091	5.079181	1.69897	1.69897
24	9.30963	9.176091	8.113943	8.113943	3.255272	3.352183	3.255272	3.176091	1.69897	1.69897

Table A-19 Statistic analysis of norfloxacin against *qnrA* negative with *gyrA* mutations (S83L and D87N) strain by GraphPad Prism 3.0 program.

Tukey's Multiple Comparison Test	P value	95% CI
Control vs 1xMIC (64 mg/ml)	P > 0.05	-0.3177 to 4.392
Control vs 2xMIC (128 mg/ml)	P < 0.05	0.5157 to 5.226
Control vs 4xMIC (256 mg/ml)	P < 0.01	0.6541 to 5.364
Control vs 8xMIC (512 mg/ml)	P < 0.001	3.309 to 8.019
1xMIC (64 mg/ml) vs 2xMIC (128 mg/ml)	P > 0.05	-1.522 to 3.188
1xMIC (64 mg/ml) vs 4xMIC (256 mg/ml)	P > 0.05	-1.383 to 3.327
1xMIC (64 mg/ml) vs 8xMIC (512 mg/ml)	P < 0.01	1.271 to 5.981
2xMIC (128 mg/ml) vs 4xMIC (256 mg/ml)	P > 0.05	-2.217 to 2.493
2xMIC (128 mg/ml) vs 8xMIC (512 mg/ml)	P < 0.05	0.4378 to 5.148
4xMIC (256 mg/ml) vs 8xMIC (512 mg/ml)	P < 0.05	0.2995 to 5.010

Table A-20 Log viable cell count of norfloxacin against *qnrA* positive with *gyrA* mutations (S83L and D87N) strain.

Time (hour)	Control		1xMIC (64 mg/ml)		2xMIC (128 mg/ml)		4xMIC (256 mg/ml)		8xMIC (512 mg/ml)	
0	6.079181	6.079181	6.146128	6.146128	6.380211	6.380211	6.176091	6.176091	6.09691	6.09691
2	7.021189	7.021189	6.079181	6.079181	6.041393	6.041393	5.954243	5.954243	3	3
4	8.812913	8.90309	7.20412	7.20412	4.544068	4.653213	4.90309	4.845098	1.69897	2
6	9.255273	9.255273	7.69897	7.69897	4.255272	3.778151	3.90309	4	1.69897	1.69897
8	9.190331	9.230449	8.278753	8.352182	4.079181	4	3.176091	3.176091	1.69897	1.69897
24	9.875061	9.69897	8.977724	9.380211	7.954243	8.041392	5.060698	5.060698	3.20412	3.161368

Table A-21 Statistic analysis of norfloxacin against *qnrA* positive with *gyrA* mutations (S83L and D87N) strain by GraphPad Prism 3.0 program.

Tukey's Multiple Comparison Test	P value	95% CI
Control vs 1xMIC (64 mg/ml)	P > 0.05	-1.515 to 3.378
Control vs 2xMIC (128 mg/ml)	P < 0.05	0.4099 to 5.302
Control vs 4xMIC (256 mg/ml)	P < 0.01	1.057 to 5.949
Control vs 8xMIC (512 mg/ml)	P < 0.001	3.001 to 7.894
1xMIC (64 mg/ml) vs 2xMIC (128 mg/ml)	P > 0.05	-0.5215 to 4.371
1xMIC (64 mg/ml) vs 4xMIC (256 mg/ml)	P < 0.05	0.1254 to 5.018
1xMIC (64 mg/ml) vs 8xMIC (512 mg/ml)	P < 0.001	2.070 to 6.962
2xMIC (128 mg/ml) vs 4xMIC (256 mg/ml)	P > 0.05	-1.799 to 3.093
2xMIC (128 mg/ml) vs 8xMIC (512 mg/ml)	P < 0.05	0.1450 to 5.037
4xMIC (256 mg/ml) vs 8xMIC (512 mg/ml)	P > 0.05	-0.5020 to 4.391

APPENDIX B

Reagents

1. Reagent for agarose gel electrophoresis

1.1 10x Tris borate buffer (TBE)

Tris base	108	g
Boric acid	55	g
0.5 M EDTA pH 8.0	40	ml

Adjusted volume to 1,000 ml with distilled water then sterilized by autoclaving at 121˚C 15 minutes.

1.2 Ethidium bromide (10 mg/ml)

Ethidium bromide	50	mg
Distilled water	5	ml

All ingredients were mixed and stored at 4˚C in the bottle with covered in aluminum foil.

1.3 1% agarose gel

Agarose ultrapure	0.4	g
1x TBE	40	ml

2. Reagents for Southern blot hybridization

2.1 Depurination solution

Stock solution (200 ml of 2 N HCl). 33 ml of Conc. HCl was mixed 167 ml of distilled water.

Working solution (700 ml of 0.25 M HCl). 87.5 ml of 2 N HCl was mixed with 612.5 ml of distilled water.

2.2 Denaturation solution (1.5 M NaCl/0.5 M NaOH)

Stock solution (1,000 ml of 3 M NaCl). 175.32 g of NaCl was mixed with distilled water, adjusted volume to 1,000 ml and autoclaved at 121˚C 15 minutes.

Stock solution (1,000 ml of 1 M NaOH). 40 g of NaOH was mixed with distilled water, adjusted volume to 1,000 ml

Working solution (1.5 M NaCl/0.5 M NaOH). 350 ml of 3 M NaCl and 1 M NaOH were mixed together.

2.3 Neutralization solution (1.5 M NaCl/0.5 Tris pH 7.0)

Stock solution (1,000 ml of 1 M Tris pH 9.5) 121.14 g of Tris was mixed with distilled water, adjusted volume to 1,000 ml and autoclaved at 121˚C 15 minutes.

Working solution (1.5 M NaCl/0.5 Tris pH 7.0). 350 ml of 3 M NaCl and 1 M Tris pH 9.5 were mixed together and adjusted pH to 7.0

2.4 Hybridization buffer 30 ml (5x SSC/0.1% SDS/1:20 liquid block)

7.5 ml of 20x SSC and 10% SDS 0.3 ml were mixed with distilled water 20.7 ml. 1.5 ml of liquid block solution was added in this solution and mixed together.

2.5 Stringency wash solution I 50 ml (1x SSC/0.1% SDS)

2.5 ml of 20x SSC and 0.5 ml of 10% SDS were mixed with 47 ml of distilled water.

2.6 Stringency wash solution II 50 ml (0.5x SSC/0.1% SDS)

1.25 ml of 20x SSC and 0.5 ml of 10% SDS were mixed with 48.25 ml of distilled water.

2.7 Buffer A pH 9.5 (300 mM NaCl/100 mM Tris pH 9.5)

100 ml of 3 M NaCl and Tris pH 9.5 were mixed with 800 ml of distilled water and adjusted pH to 9.5

2.8 Blocking solution (5% skim milk/Buffer A)

1.5 g of skim milk was added in 3o ml of Buffer A and mixed together.

2.9 Anti-fluorescein-AP conjugate solution 30 ml

Anti-fluorescein-AP conjugate 6 µl, Bovine serum albumin (BSA) were added in Buffer A, mixed and adjusted by Buffer A to 30 ml.

2.10 Washing solution (0.3% tween 20 in Buffer A)

0.9 ml of tween 20 was added in Buffer A 300 ml and mixed together.

BIOGRAPHY

Mr. Wee Winissorn was born on September 9th, 1982 in Bangkok, Thailand. My Hometown is Rayong province. I graduated with the Bachelor of Pharmacy Degree (Pharmaceutical care) from the Faculty of Pharmacy, Huachiew Chalermprakiet University in 2006. I started to work as a pharmacist in Kasemrad Hospital Rattanathibeth, Nonthaburi during March to September, 2006, and then I worked in Phyathai 2 Hospital, Bangkok until 2009. Consequently, I have enrolled for the Master's degree of Science in Pharmacy (Microbiology) at the department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University since November 2009.