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Plasmid-mediated quinolone resistance determinants in Enterobacteriaceae isolated in Ho Chi Minh City, Vietnam

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Abstract

Fluoroquinolones are amongst the most effective antimicrobials used to treat infectious diseases caused by members of the Enterobacteriaceae. The rapid spread of fluoroquinolone resistant Enterobacteriaceae threatens the future effectiveness of this widely used group of antimicrobial compounds. Fluoroquinolone resistance in Enterobacteriaceae in Vietnam highlights an approaching crisis in antimicrobial therapy of Enterobacteriaceae infections as fluoroquinolone resistant infections may require expensive alternative treatments. Furthermore, a lack of alternative drugs for treating fluoroquinolone resistant bacteria also contributes to the difficulty in treating such infections. The aim of this study was to investigate the prevalence and mechanisms of fluoroquinolone resistance in commensal Enterobacteriaceae isolated from two different populations resident in Ho Chi Minh City; hospitalized patients and healthy volunteers. I investigated the prevalence of mutations in the DNA gyrase and the topoisomerase gene, the target of the fluoroquinolones and the main resistance mechanisms in Enterobacteriaceae. Additionally, I investigated the spectrum of plasmid-mediated quinolone resistance (PMQR) determinants, a more contemporary mechanism of fluoroquinolone resistance. By using whole plasmid sequencing, bioinformatics and a miniaturized DNA nanoarray, an association of PMQR determinants with other antimicrobial resistance genes including bla_{LAP-2}, which confers resistance to β-lactam antimicrobials, was found. Moreover, diverse patterns of antimicrobial resistance between isolates from hospital and community populations were revealed. An increase in the quantity of PMQR determinants isolated from rectal swabs of children treated with antimicrobials was also demonstrated using a gene specific real-time PCR. I surmise that variety antimicrobial classes might contribute to an increase in copy number and co-selection of PMQR determinants in Ho Chi Minh City. Fluoroquinolone resistance in Enterobacteriaceae has increased over time and this class of antimicrobial is still commonly used to treat infection caused by such organisms. Understanding fluoroquinolone resistance mechanisms and their relationship to other antimicrobial resistance mechanisms in Enterobacteriaceae should play an important role in the control of the spread of antimicrobial resistance genes and aid treatment strategies for clinicians in Vietnam.

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Declaration

The work described in this thesis is my own work and was carried out in the Oxford University Clinical Research Unit – Vietnam. The sequencing of the 3 plasmids were carried out at the Sanger Institute, UK and the DNA miniaturized experiments of 32 plasmids were carried out at the Veterinary Laboratories Agency, Weybridge, Surrey, UK. This thesis has not been submitted for a degree or other qualification to this or any other university.

The Blind Men and the Elephant

It was six men of Indostan
To learning much inclined,
Who went to see the Elephant
(Though all of them were blind),
That each by observation
Might satisfy his mind

The First approached the Elephant,
And happening to fall
Against his broad and sturdy side,
At once began to bawl:
"God bless me! but the Elephant
Is very like a wall!"

The Second, feeling of the tusk,
Cried, "Ho! what have we here
So very round and smooth and sharp?
To me 'tis mighty clear
This wonder of an Elephant
Is very like a spear!"

The Third approached the animal,
And happening to take
The squirming trunk within his hands,
Thus boldly up and spake:
"I see," quoth he, "the Elephant
Is very like a snake!"

The Fourth reached out an eager hand,
And felt about the knee.
"What most this wondrous beast is like
Is mighty plain," quoth he;
" 'Tis clear enough the Elephant
Is very like a tree!"

The Fifth, who chanced to touch the ear,
Said: "E'en the blindest man
Can tell what this resembles most;
Deny the fact who can
This marvel of an Elephant
Is very like a fan!"

The Sixth no sooner had begun
About the beast to grope,
Than, seizing on the swinging tail
That fell within his scope,
"I see," quoth he, "the Elephant
Is very like a rope!"

And so these men of Indostan
Disputed loud and long,
Each in his own opinion
Exceeding stiff and strong,
Though each was partly in the right,
And all were in the wrong!

So oft in theologic wars,
The disputants, I ween,
Rail on in utter ignorance
Of what each other mean,
And prate about an Elephant
Not one of them has seen!

John Godfrey Saxe's (1873)

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Abbreviations

ACT	Artemis Comparison Tool
ARIs	acute respiratory infections
ART	Artemis
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
CDS	coding sequences
CIP	ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
cfu	colony-forming unit
DNA	Deoxyribonucleic acid
ddNTP	dideoxyribonucleotide triphosphate
dNTP	deoxyribonucleotide triphosphate
DTCS	Dye terminator cycle sequencing
E. coli	*Escherichia coli*
ESBL	Extended spectrum beta-lactamase
HTD	the Hospital for Tropical diseases
Inc groups	Incompatibility group
IS element	insertion sequence element
kp	kilobase pair

K. pneumoniae	*Klebsiella pneumoniae*
LB	Luria-Bertani
MIC	minimum inhibitory concentration
MDR	multi-drug resistance
NA	Nalidixic acid
NCBI	National Centre for Biotechnology Information
OD	Optical Density
ORF	open reading frame
PCR	polymerase chain reaction
PhHV	Phocine herpesvirus
PMQR	Plasmid-mediated quinolone resistance
RAPD	Random Amplified Polymorphic DNA
R plasmids	Resistance plasmids
S. aureus	*Staphylococcus aureus*
Spp.	Species
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
Tm	melting temperature
WHO	World Health Organisation

1. Introduction

1.1. The Enterobacteriaceae family

The Enterobacteriaceae (named because this group of bacteria largely inhabit the gastrointestinal tract of animals) is the largest family of medically important Gram-negative bacilli. This heterogeneous group currently consists of 44 genera and 176 described species (Brenner *et al.,* 2005). The Enterobacteriaceae are ubiquitous and are found worldwide in soil, water, vegetation and constitute a substantial fraction of intestinal flora in animals and humans (Blood and Curtis 1995). The genera that belong to this bacterial family have been classified on the basis of their biochemical properties, antigenic structures and characteristic signatures found within their nucleic acid (variation compared by DNA hybridization and sequencing) (Holt *et al.,* 1994; Pham *et al.,* 2007). Despite the overall complexity of the Enterobacteriaceae, more than 95% of enteric isolates considered to be medically important belong to 10 genera, constituting less than 25 species (NHS 2010).

The Enterobacteriaceae family is traditionally divided into two subcategories, the coliforms and the noncoliforms (NHS 2010).

i. The coliforms include *Escherichia coli* (*E. coli*) and other Gram-negative enteric flora that ferment lactose rapidly (within 48 hours), such as *Klebsiella*, *Enterobacter*, *Hafnia*, *Serratia* and *Citrobacter*.
ii. The noncoliforms are generally non-lactose-fermenting or slow lactose-fermenting bacteria that are either commensal flora or pathogens, such as *Proteus*, *Morganella*, *Providencia* and *Edwardsiella*.

Although a limited number of exceptions occur, this distinction (coliforms and non-coliforms) has considerable practical importance in laboratory, clinical and epidemiologic terms.

1.1.1. Biochemical and microbiological identification of Enterobacteriaceae

Members of the family Enterobacteriaceae range from 0.3 to 1.0 x 1.0 to 6.0 µm in size (Murray *et al.,* 1998). They are non-spore forming bacilli and the majority demonstrate motility. The genera found within the Enterobacteriaceae share several key properties; they ferment glucose, reduce nitrates to nitrites and are oxidase-negative (Murray *et al.,* 1998). Morphology and microscopic staining characteristics alone are insufficient to precisely identify the specific enteric bacilli. As a result, a battery of biochemical tests are commonly used to identify the principal genera and species (Blood and Curtis 1995).

1.1.2. Enterobacteriaceae diseases in humans

The Enterobacteriaceae are common agents of disease in humans. Various species of the family Enterobacteriaceae are commonly associated with pneumoniae (*Klebsiella*), urinary tract infections (*Citrobacter*, *Escherichia*, *Enterobacter*), bacteremia (*Klebsiella*, *Serratia*, *Morganella*) and meningitis (*Morganella*, *Citrobacter*) (Murray *et al.,* 1998). The Enterobacteriaceae are also recognized as a major cause of nosocomial infections (Gaynes and Edwards 2005). Gaynes and colleagues analysed data from the National Nosocomial Infections Surveillance System in the United States of America (USA) in 2003 and revealed that Gram-negative bacilli were associated with 23.8% of bloodstream infections, 65.2% of pneumonia episodes, 33.8% of surgical site infections and 71.1% of urinary tract infections in Intensive Care Units (ICUs) in the USA (Gaynes and Edwards 2005).

Enterobacteriaceae are estimated to be responsible for approximately 100,000 deaths annually in the USA and account for about half of all clinically significant bacteria isolated in hospital laboratories (WHO 2002b). The family Enterobacteriaceae are generally transmitted via the fecal/oral route, which can occur as a result of inappropriate hand washing or by consuming contaminated water or food (Murray *et al.,* 1998). Some clinically relevant genera of Enterobacteriaceae are shown in Table 1.1 (WHO 2002b).

Table 1.1 Medically important species of the family Enterobacteriaceae.

Species	Main infections
Escherichia	Urinary tract infections
Shigella	Shigellosis
Salmonella	Typhoid fever, paratyphoid fever, foodborne illness
Enterobacter	Urinary tract infections and respiratory tract infections
Klebsiella	Pneumonia, urinary tract infections and septicemia
Serratia	Lower respiratory tract infections, urinary tract infections, surgical wounds and skin tissue infections
Proteus	Wound and urinary tract infections
Morganella	Sepsis, meningitis, urinary tract infections and skin infections
Providencia	Urinary tract infections
Citrobacter	Urinary tract infections, infant meningitis and sepsis
Edwardsiella	Gastroenteritis and wound infections

1.1.3. Treatment of infections caused by members of the Enterobacteriaceae

The appropriate therapeutic options for the treatment of infections cause by Enterobacteriaceae in the latter part of the 20th century were later generation cephalosporins and fluoroquinolones. However, as with many older antimicrobial agents, cephalosporins and fluoroquinolones have become significantly less effective in the early part of the 21st century. There has been an increase in resistance to both cephalosporins (generally due to a dramatic increase and dissemination of Extended spectrum Beta-lactamase (ESBL) encoding genes) and fluoroquinolones (Denton 2007) (Figure 1.1).

Effective treatment selection is further compounded for clinicians in developing countries, where the infectious disease burden is higher than that in the west and the living conditions may not be as advanced as of those in industrialized countries. Furthermore, antimicrobial resistance levels are, in general, higher in developing countries than in developed countries, potentially due to uncontrolled antimicrobial usage. Additionally, the selection of appropriate antimicrobials may also be limited by cost, as the price of new antimicrobial is often prohibitive (Okeke *et al.,* 2005).

At present, treatment options for infections caused by multi-drug resistant (MDR) members of Enterobacteriaceae, which also exhibit resistance or borderline resistance to cephalosporins and fluoroquinolones, are limited. Currently, carbapenems as well as temocillin and colistin are amongst the available alternatives for treatments of infections cause by MDR members of the Enterobacteriaceae. (Denton 2007).

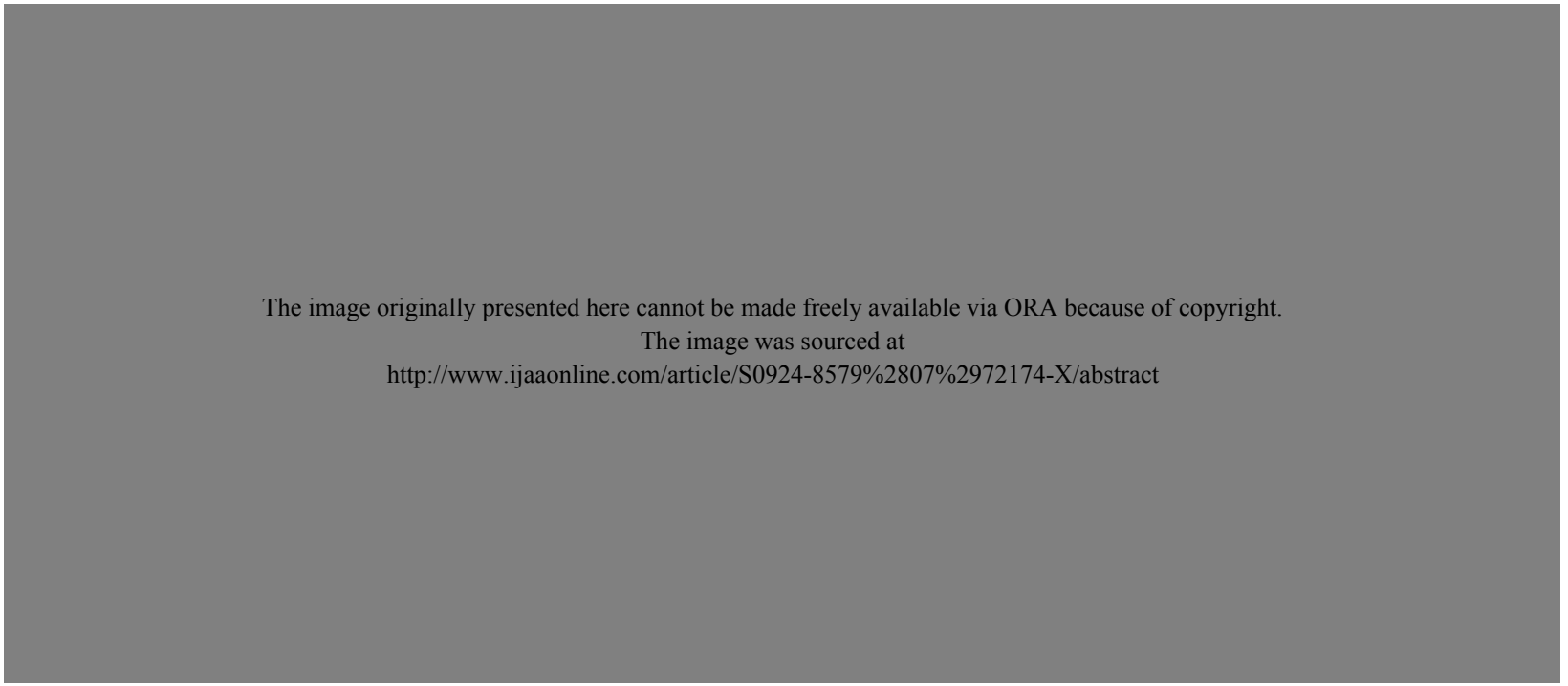


Figure 1.1 Resistance to third-generation cephalosporins and fluoroquinolones in *E. coli* isolates in Europe (2005).

The figure was adapted from Denton and colleagues (Denton 2007).

1.1.4. Hospital-acquired (nosocomial) infections

Some patients when admitted to hospital actually acquire more severe infections during treatment due to the hospital environment and the patient's general ill-health. Cross-infection from patient to patient or from hospital staff to patients is a constant hazard. Infections are considered to be nosocomial if they occur within 48 hours or more after admission to hospital or within 30 days after discharge. Nosocomial infections are estimated to occur in approximately 5% of all patients admitted to hospital in USA (WHO 2002b). In some clinical wards, such as intensive care units, up to 10% of the patients can acquire a nosocomial infection. There are approximately 2 million nosocomial infections each year in the USA, leading directly or indirectly to 80,000 deaths (WHO 2002b). Hospital infections are generally caused by microorganisms which are normal commensal flora in either patients or hospital staff. These organisms are maintained within the hospital environment and have a pathogenic potential, which may infect susceptible patients (Donskey 2004; Stiefel and Donskey 2004).

1.1.4.1. The hospital environment

Infectious diseases can be spread easily and rapidly in a hospital environment for several reasons (Murray *et al.,* 1998):

i. Many patients may have weakened immune systems because of underlying disease and cannot resist a further infection (compromised hosts).

ii. There are often multiple patients within the same room or the same ward; this close proximity has obvious implications for increasing the number of opportunities for cross-infection.

iii. Hospital staff move from patient to patient; direct contact with different patients increases the probability of transferring of pathogens.

iv. Many medical procedures, such as hypodermic injection, spinal lumbar puncture and removal of tissue samples (biopsy) or fluids (drawing blood), breach the skin barrier and carry with them the risk of directly introducing pathogens into the patient.

v. Some therapeutic drugs, such as steroid used for controlling inflammation, increase susceptibility to infection. In addition, the use of antimicrobials to control infection can select for antimicrobial-resistant organisms.

1.1.4.2. Nosocomial pathogens

Nosocomial pathogens can infect several sites; of particular relevance are the urinary tract, the blood and the lower respiratory tract (Figure 1.2). One of the most important and widespread hospital pathogens is *Staphylococcus aureus* (*S. aureus*) (Wang, F. D. *et al.,* 2008; Alvarez *et al.,* 2010). *S. aureus* is the most common cause of pneumonia and the third most common cause of blood infections and is also particularly problematic in nurseries (Mori *et al.,* 2001; Miyachi *et al.,* 2007). *Enterococcus* species, *Pseudomonas aeruginosa*, *Candida albicans*, *E. coli* and *Klebsiella pneumoniae* are also particularly common agents of nosocomial infection (Ikaheimo *et al.,* 1993; Benca *et al.,* 2007; Amin and Wareham 2009; Taneja *et al.,* 2010).

Figure 1.2 Nosocomial infections.

Relative frequency of pathogens based on body site. These data are taken from the National Nosocomial Infections Surveillance, which was conducted by the Center for Disease Control and Prevention (CDC) in the USA in 2000 (Nester *et al.,* 2004).

1.1.5. Community-acquired infections

A community-acquired infection is an infection contracted outside of a health care setting or an infection present on admission. Community-acquired infections are often distinguished from nosocomial infections by the types of organisms that affect patients who are recovering from a disease or injury. Community-acquired respiratory infections commonly involve strains of *Haemophilus influenzae* or *Streptococcus pneumoniae* and are usually more sensitive to antimicrobials than hospital-acquired bacteria (Mosby Inc. 2009). Community-acquired infections include a wide range of diseases, such as pneumonia, urinary tract infections, skin and soft tissue infections and meningitis, of which community-acquired pneumonia is considered the most common disease (Goossens and Sprenger 1998; Murray *et al.,* 1998).

Community-acquired pneumonia is one of the most frequently diagnosed infectious diseases globally and is associated with significant morbidity and mortality, particularly in children and elderly (Fung and Monteagudo-Chu 2010). Aetiology of community-acquired pneumonia is different among geographical regions; *Legionella* spp., *Coxiella burnetti*, *K. pneumoniae*, *Burkholderia pseudomallei*, Gram-negative enteric bacilli and *Mycobacterium tuberculosis* are the main aetiology of community-acquired pneumonia of countries bordering the Mediterranean Sea, North-west Spain, South Africa, Southeast Asia, Italy and non-industrialised countries, respectively (Lim *et al.,* 2009). Cephalosporins and fluoroquinolones are considered the most useful agents for treatment of community-acquired pneumonia (Dryden *et al.,* 2009). For severe pneumonia, the British Thoracic Society guidelines recommend intravenous co-amoxiclav plus clarithromycin. Alternative

recommended regimens are benzylpenicillin plus levofloxacin or a cephalosporin plus clarithromycin (Lim *et al.,* 2009).

1.1.6. Intestinal flora

The intestinal (gut) microflora of animals consist of an enormous, hugely diverse microbial population which constitutes the largest reservoir of microbial flora in humans (Donskey 2004). It is estimated that more than four hundred species can be detected within the intestinal microflora population, of which the predominant members are obligate anaerobic bacteria (Kmet and Piatnicova 2010). The majority of bacteria in gut flora belong to the genera *Bacteroides*, *Clostridium*, *Fusobacterium* and *Peptostreptococcus* (Madigan *et al.,* 2003), where species from the genus *Bacteroides* alone constitute about 30% of all bacteria in the gut population (Moore and Holdeman 1974). *E. coli* and aerobic streptococci are maintained as subdominant flora with approximately 6 to 8 log colony forming unit (CFU) per gram of feces (Tancrede 1992). Whilst science is attempting to unravel the complex bacterial populations within the gastrointestinal tract of humans, the delicate relationship and ecological balance between bacterial species that constitute the "normal flora" of the intestine are still poorly understood.

The intestinal flora of mammals is responsible for a wide variety of beneficial functions for the hosts, thus mammals and the intestinal microflora have a vital symbiotic relationship. The intestinal flora catalyse metabolic reactions which result in the enzymatic breakdown of food and nutrients. Furthermore, the enterobacterial flora play an important role as a major defense mechanism, protecting the body against potential colonization by invading pathogens. The commensals (such as

members of the family Enterobacteriaceae) prevent this colonization by competing for nutrients and for attachment sites (Tancrede 1992).

The natural equilibrium of the normal flora is disturbed by the effect of antimicrobial agents, diet or due to infection. It has been suggested that exposure to antimicrobial agents will increase the prevalence of resistance to antimicrobials among many fecal bacteria (Sorum and Sunde 2001). Fluit and colleagues studied the difference in proportion of MDR Enterobacteriaceae in intestinal microflora of 99 children in the Netherlands with chronic active otitis media after they were treated with trimethoprim/sulfamethoxazole or placebo (van der Veen *et al.,* 2009). After 6 weeks of follow-up, 32 (91%) children in the trimethoprim/sulfamethoxazole group carried trimethoprim/sulfamethoxazole-resistant Enterobacteriaceae versus 10 (21%) of children in the placebo group [rate differences: 70 (95% CI: 55; 85)] (van der Veen *et al.,* 2009). Diet also plays an important role in the balance of bacteria in the intestinal microflora. Hentges and colleagues carried out a study about the effect of high-beef diet on the fecal bacterial flora of humans living in the USA. Ten volunteers completed a 4-month diet series consisting of 1 month each of a control diet, a meatless diet, a high-beef diet and the same control diet. During the 4th week of each diet, three stool specimens collected from each volunteer were analysed for content of facultative, aerobic and anaerobic bacteria. The result showed that the ratio of mean counts of anaerobic to facultative and aerobic organisms was approximately 15:1 during the meatless diet and 34:1 during the high-meat diet (Hentges *et al.,* 1977).

1.1.7. Escherichia coli

Members of the genus *Escherichia* are almost universal inhabitants of the intestinal tract of humans and warm-blooded animals. Some strains of *Escherichia* are pathogenic, causing diarrhea in infants. Some are opportunistic pathogens, causing urinary tract infections in older persons or in immuno-suppressed individuals (Murray *et al.,* 1998). The genus *Escherichia* consists of five species, of which, *E. coli* is the most common and clinically important species. *E. coli* is associated with a variety of diseases, including meningitis, gastroenteritis, urinary tract infections and sepsis (Murray *et al.,* 1998). *E. coli* are frequently found to be the cause of sepsis, neonatal meningitis, urinary tract infections and gastroenteritis. *E. coli* is one of the most common Gram-negative bacilli isolated from septic patients and is responsible for causing of more than 80% of all-community-acquired urinary tract infections and most hospital-acquired infections globally (Murray *et al.,* 1998). It is also a prominent cause of gastroenteritis in developing countries. Most infections of *E. coli* (with the exception of neonatal meningitis and gastroenteritis) are endogenous, meaning the host's normal microbial flora are able to establish infection when the host's immune defenses are compromised (Murray *et al.,* 1998).

1.1.8. Klebsiella pneumoniae

Klebsiella, named after the German microbiologist Edwin Klebs, is a genus of Gram-negative, rod shaped, non-motile bacilli belonging to the Enterobacteriaceae. To date, seven species of *Klebsiella* have been described: *K. pneumoniae*, *K. ozaenae*, *K. rhinoscleromatis*, *K. oxytoca*, *K. planticola*, *K. terrigena* and *K. ornithinolytica*. *Klebsiella* are commonly found in the respiratory tract, urinary tract and also constitute a fraction of the intestinal flora (Murray *et al.,* 1998). In certain conditions

Klebsiella can cause pneumonia, urinary tract infections, ankylosing spondylitis (degenerative inflammatory arthritis) and septicemia (Murray *et al.,* 1998). *K. pneumoniae* is the most commonly referred to species of the genus *Klebsiella*. In addition to the aforementioned general characteristics of *Klebsiella*, *K. pneumoniae* is an encapsulated, lactose fermenting and facultative anaerobe. *K. pneumoniae* is found in soil, water and on vegetables. In humans, it can be found on the skin, and in the pharynx and gastrointestinal tract. It is the most clinically relevant member of the *Klebsiella* genus and can cause both nosocomial and community infections (Murray *et al.,* 1998). *Klebsiella* infections are more likely to occur in people with weakened immune systems, such as those addicted to alcohol, diabetics or patients in hospital that have another underlying infection. It is uncommon for *K. pneumoniae* to cause lower respiratory tract infections in healthy people (Murray *et al.,* 1998). However, *K. pneumoniae* can cause pneumonia in people who have been hospitalized (hospital-acquired pneumonia). The disease can often be severe, resulting in destruction of the lungs, and can be fatal (Craven 2006). The most common symptom associated with *K. pneumoniae* pneumonia is severe cough with sputum secretion.

There is a geographical association of community-acquired *K. pneumoniae* infections. Studies published since 1990 show that only a small number of patients (around 2 to 4%) admitted to a hospital in Spain with severe community-acquired pneumonia had *K. pneumoniae* as the etiologic agent (Alkhayer *et al.,* 1990; British Thoracic Society 1992; Rello *et al.,* 1993; Almirall *et al.,* 1995; Rello *et al.,* 1996). In contrast, *K. pneumoniae* associated with community-acquired pneumonia in Africa and Asia accounts for 29% and 62% of all cases, respectively (Ko *et al.,* 2002). Moreover, *K. pneumoniae* is also associated with urinary tract infections and

abdominal infections. It is also the second most common bacterial pathogen, after *E. coli*, causing urinary tract infections in the elderly (Franco 2005).

1.2. (Fluoro)quinolones: the structures, mode of action and resistance mechanisms

The quinolones are a family of synthetic broad-spectrum antimicrobials with significant bactericidal effects on most members of the Enterobacteriaceae, including *E. coli* and *Klebsiella*. First generation quinolones were synthesized in 1962 and were introduced into clinical use in 1964 in the form of nalidixic acid (Ball 2000). Nalidixic acid was used primarily in the treatment of diarrhoea and urinary tract infections. A number of other related drugs were synthesized in the 1960s and 1970s with a superior antibacterial spectrum. These quinolones included oxolinic acid, cinoxacin, pipemidic acid, flumequine, rosoxacin and miloxacin. Even in minimal concentrations, quinolones inhibit a wide variety of Gram-positive and Gram-negative bacteria (Bellido and Pechere 1989).

1.2.1. Structures of (fluoro)quinolones

Nalidixic acid, one of the first generation quinolones, was introduced with a 1,8-naphthyridine and contains 2 nitrogen atoms (Figure 1.3). A major advance in antimicrobial chemotherapy came with the synthesis of the second generation of quinolones by substituting a fluorine at position 6 (Figure 1.3), hence the name of fluoroquinolone, which increased activity against Gram-negative bacteria. These early compounds were most potent against Gram-negative organisms and *Streptococcus pneumoniae,* compared to former antimicrobial groups such as penicillin (Van Bambeke *et al.,* 2005). As a result, fluoroquinolones were used in the treatment of respiratory tract infections. Of these second-generation

fluoroquinolones, ciprofloxacin and ofloxacin are the most widely used today (Van Bambeke *et al.,* 2005).

The third generation fluoroquinolones were modified by the presence of an alkyl-substituted piperazine or pyrolidine at position 7, and of a methoxy at position 8 (Figure 1.3). The result of the modification was further improvement in activity against Gram-positive bacteria along with a significant improvement in anti-anaerobe activity compared to the second-generation fluoroquinolones. The relationship of fluoroquinolone structure and activity is shown in Figure 1.4. Figure 1.4 shows that the variation of different groups in important positions results in increased activity of fluoroquinolones against both Gram-negative and Gram-positive organisms. The fluorine at position R6 plays an important role in activity of fluoroquinolones against Gram-negative organisms and *Streptococcus pneumoniae*. Moreover, naphthyridones at position X8 increases activity of fluoroquinolones against anaerobes or alkyl at position R7 increase the activity to Gram-positive organisms.

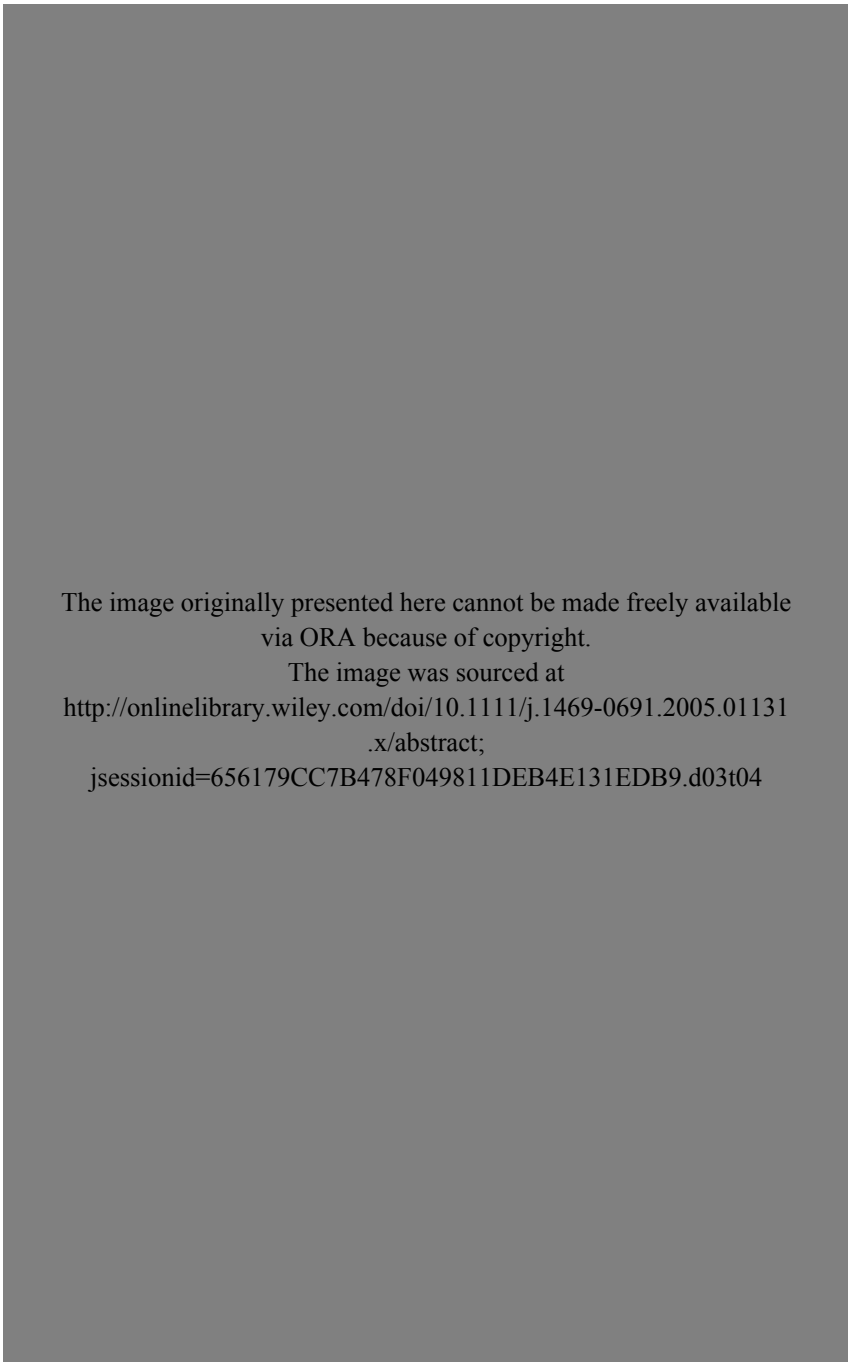


Figure 1.3 The pharmacophore and structure of the main quinolones.

This figure shows the pharmacophore and structures of mains quinolones that have been approved for human use. The names in bold refer to compounds in large-scale clinical use in Europe. Names in italic refer to compounds for which commercialization has been suspended or severely reduced because of side-effects. The figure was adapted from Van Bambeke and colleagues (Van Bambeke *et al.,* 2005).

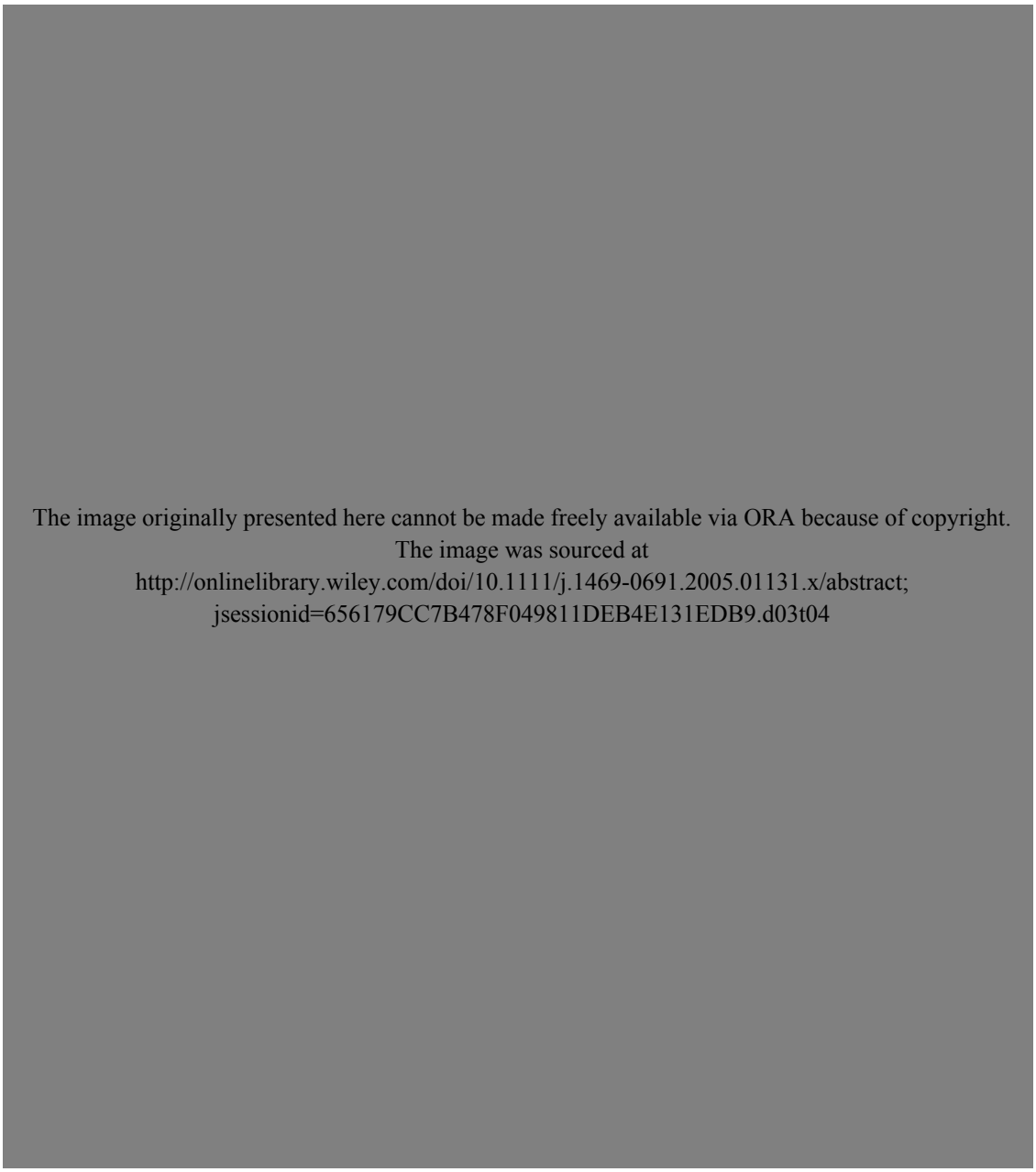


Figure 1.4 Structure – property relationship in quinolones.

The figure was adapted from Van Bambeke and colleagues (Van Bambeke *et al.,* 2005).

1.2.2. Gyrase and topoisomerase IV as targets for fluoroquinolones

Topoisomerases are enzymes that can change the topological formation of the DNA through the breaking and rejoining of DNA strands. There are 2 classes of topoisomerase; type I and type II enzymes (Berger 1998). DNA gyrase and topoisomerase IV are type II topoisomerases and are found in all Enterobacteriaceae (Levine *et al.,* 1998). The DNA gyrase is encoded by 2 individual genes, *gyrA* and *gyrB*; these genes encode 97 kDa protein and 89.9 kDa, respectively. The GyrB subunit is the site for ATP binding and the GyrA subunit contains the tyrosine residue active site required for DNA cleavage and religation. DNA gyrase is unique among the type II topoisomerase in that it is the only known topoisomerase that can convert relaxed duplex DNA into a negatively supercoiled form (Levine *et al.,* 1998).

Topoisomerase IV is comprised of the translated products of the *parC* and *parE* genes in Gram-negative bacteria; *parC* and *parE* encode 83.7 kDa and 70.2 kDa proteins, respectively (Levine *et al.,* 1998). In contrast to the DNA gyrase, topoisomerase IV catalyzes intermolecular DNA decatenation reactions. Topoisomerase IV is the enzyme that unlinks daughter chromosomes following DNA replication and resolves knots and catenenes that result from recombination events (Anderson and Osheroff 2001).

1.2.3. Mechanisms of (fluoro)quinolone action

(Fluoro)quinolones target the prokaryotic type II topoisomerase, i.e. the DNA gyrase and the topoisomerase IV (Drlica 1999). DNA gyrase and topoisomerase IV are essential enzymes that are involved in the transcription and replication process of

DNA of the Enterobacteriaceae (Levine *et al.,* 1998). As a result, any antimicrobials that target either the DNA gyrase or the topoisomerase IV prevent bacterial growth. However, quinolones do not kill bacterial cells by preventing the functions of these essential enzymes. Rather, quinolones increase the cellular concentration of covalently topoisomerase-cleaved DNA complexes (cleavage complexes) that are intermediates in the DNA strand passage reactions of the DNA gyrase and topoisomerase IV (Anderson and Osheroff 2001). When DNA topoisomerases bind to chromosomal DNA to form cleavage complexes, it is trapped by the quinolone antimicrobials. The ternary quinolone-enzyme-DNA complexes then change the transient enzyme-associated breaks to permanent double-stranded breaks in the genetic material. This action subsequently blocks vital DNA processes such as DNA replication and DNA transcription which ultimately induces cell death (Anderson and Osheroff 2001).

1.2.4. Bactericidal activity of fluoroquinolones

In general, all of the currently available quinolones exhibit activity against almost 90% of the Enterobacteriaceae (Bellido and Pechere 1989). Moreover, fluoroquinolones have been used to treat fastidious Gram-negative species such as *Haemophilus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Moraxella catarrhalis* and enteric pathogens such as enterotoxigenic *E. coli* (ETEC), *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio* spp., *Campylobacter jejuni* or *Pseudomonas* spp. or Gram-positive bacteria such as methicillin-susceptible *Staphylococcus aureus* and *Streptococcus pneumoniae* (Bellido and Pechere 1989). Several factors have been described which can reduce the activity of

fluoroquinolones, such as the size of the bacterial inoculum, exposure to magnesium and low pH (5.5) (Neu 1992).

1.2.5. Pharmacology

All of the fluoroquinolones are absorbed effectively through the gastrointestinal wall (Wolfson and Hooper 1991). However, the degree of absorption differs depending on the type of fluoroquinolone, ranging from 55% to 95% of the total ingested quantity (Eliopoulos 1988). The peak serum concentrations are usually observed one or two hours after consumption. The mean terminal half-lives of the fluoroquinolones ranges from 3 hours (for norfloxacin) to 18 hours (for sparfloxacin). Ciprofloxacin has a half-life of 4 hours (Eliopoulos 1988).

After consumption, fluoroquinolones are widely distributed throughout the body tissues and fluids. Interstitial fluid concentrations can range from 50% to 100% of the peak serum concentrations in the first several hours. Most fluoroquinolones have an extremely large apparent volume of distribution compared with other antimicrobials such as ß-lactams or aminoglycosides. Fluoroquinolones reach particularly high concentrations in salivary glands, nasal mucosa and in bronchial epithelium (Gerding and Hitt 1989).

Fluoroquinolones are cleared via the renal mechanism (ofloxacin and lomefloxacin) or the hepatic system (pefloxacin) or both (norfloxacin, ciprofloxacin, enoxacin, tosufloxacin and sparfloxacin) (Bergan *et al.,* 1988; Eliopoulos 1988; Wolfson and Hooper 1991).

1.2.6. Fluoroquinolone resistance

1.2.6.1. Alterations in the target enzymes (DNA gyrase and topoisomerase IV)

There have been many studies demonstrating that alterations within the target enzymes DNA gyrase and topoisomerase IV reduce the susceptibility to fluoroquinolones (Nishino *et al.,* 1997; Weigel *et al.,* 1998; Chen *et al.,* 2003; Hansen and Heisig 2003; Lee *et al.,* 2005; Hu *et al.,* 2007). These alterations are localized within specific domains called the quinolone resistance-determining region (QRDR). The two most common resistance mutations with the *gyrA* gene are at codon 83 and codon 87 and these substitutions alter serine at position 83 and aspartic acid at position 87 in GyrA. These substitutions cause reduced binding of fluoroquinolones to the gyrase-DNA complex, which allows the bacteria to reach the clinically resistant level (Weigel *et al.,* 1998). Mutations in ParC at position 80 and 84 have also been reported but are less common than those in GyrA (Nishino *et al.,* 1997; Chen *et al.,* 2003; Hansen and Heisig 2003; Lee *et al.,* 2005).

1.2.6.2. Alteration in drug permeability

To reach their targets in the cell cytoplasm, fluoroquinolones must cross the cytoplasmic membrane and the outer membrane (Diver *et al.,* 1990; Bryan and Bedard 1991). As a result, resistance to fluoroquinolones in Gram-negative bacteria is also associated with reductions in number of porins in the membrane and reduced bacterial accumulation of drug. However, porin reduction alone is insufficient to stimulate complete resistance (Bryan and Bedard 1991). It has been shown that resistance caused by reduced accumulation of fluoroquinolones requires the presence and enhanced expression of endogenous efflux systems that actively pump the chemicals out of the cytoplasm (Hooper 1999). In Gram-negative bacteria, the efflux

systems typically have three components: the efflux pump located in the cytoplasmic membrane, an outer membrane protein and a membrane fusion protein, which connects the inner and outer membrane proteins.

1.2.6.3. Plasmid-mediated quinolone resistance determinants

Resistance to fluoroquinolones can also be conferred by antimicrobial resistance genes which are carried on plasmids. Fluoroquinolone resistance conferred by plasmid-borne genes were first described in 1998 in a *K. pneumoniae* strain isolated from a patient with urinary tract infection in the USA (Martinez-Martinez *et al.,* 1998). Since the first report, there have been a number of studies demonstrating the presence of plasmid-mediated quinolone resistance (PMQR) determinants in Enterobacteriaceae and other species (Strahilevitz *et al.,* 2009). There are 3 types of PMQR genes that have been described, these are; *qnr*, *aac(6')-Ib-cr* and *qepA* genes. The characteristics of each gene are detailed in the following sections. The PMQR genes do not confer full resistance (as defined by the CLSI), but they all increase the minimum inhibitory concentration (MIC) to the fluoroquinolones of the strains carrying the genes. As a result, they stimulate other resistance mechanisms to (fluoro)quinolones, such as mutations at the *gyrA* or *parC*, and subsequently result in higher levels of bacterial resistance. Martinez-Martinez and colleagues found that the frequency of spontaneous mutations to ciprofloxacin or nalidixic acid resistance was more than 100 times higher for transconjugants with *qnrA* than strains without *qnrA* (Martinez-Martinez *et al.,* 1998).

1.3. Horizontal gene transfer

1.3.1. Plasmids

Plasmids are typically extra-chromosomal fragments of DNA, usually circular, and exist and replicate autonomously with respect to the chromosome (Funnell and Phillips 2004). Plasmids normally contain genes essential for initiation and control of replication of plasmids and accessory genes that may be useful to their bacterial host such as antimicrobial resistance or virulence genes (Amabile-Cuevas and Chicurel 1992; Bergstrom *et al.,* 2000). Different plasmids are present in cells in a particular number (copy number). Some plasmids are present in the cell in only 1 - 3 copies, whereas others may be present in over 100 copies (del Solar and Espinosa 2000). Copy number of plasmids is controlled by negative regulatory systems that adjust the rate of replication per plasmid copy in response to fluctuations in the copy number. To date, three regulatory mechanisms of plasmid copy number have been studied thoroughly, which are those that involve directly repeated sequences (iterons), those that use antisense RNAs and those that use a mechanism involving an antisense RNA in combination with a protein (del Solar and Espinosa 2000).

Resistance plasmids (R plasmids) are the most widespread and well-studied group of plasmids, which confer resistance to antimicrobials and other inhibitor factors. R plasmids were first discovered in *Shigella* and *E. coli* in Japan in 1950s and early 1960s and were found to carry resistance genes to aminoglycosides, tetracycline, chloramphenicol and sulfonamides (Watanabe 1963; Nikaido 2009). Soon after these R plasmids were isolated, it was shown that the R plasmids could transfer resistance properties to sensitive strains via cell-to-cell contact (Watanabe 1963; Nikaido

2009). Resistance plasmids have since been found throughout the world (Nikaido 2009).

The grouping of plasmids into incompatibility (Inc) groups has been a useful method of classification of plasmids. Incompatibility results from the inability of plasmids to coexist within the same cell over many generations due to shared replication, copy number control, or portioning mechanisms eventually exclude one of the plasmids from the cell (Lawley *et al.,* 2004). So far, there are 27 Inc groups have been recognized in the Enterobacteriaceae, which are B, C, D, FI, FII, FIII, FIV, FV, FVI, FVII, HI, HII, I1, I2, Iγ, J, K, M, N, P, Q, T U, W, X, Y and Com9 (Taylor *et al.,* 2004).

1.3.2. Transposons and insertion sequences

Transposons are DNA elements that can jump, or transpose, from one place in DNA to another in a bacterial genome. The movement by a transposon is called transposition, and the enzymes that promote transposition are called transposases (Snyder and Champness 2003). The frequency of transposition varies from approximately one in 10^3 cell divisions to approximately once in every 10^8 cell divisions, depending upon the type of transposon (Snyder and Champness 2003).

Insertion sequence elements (IS elements) are the simplest type of transposon. They are typically 750 to 2,000 bp long and carry only a gene encoding a transposase that is required for them to move to a different location (Snyder and Champness 2003). Insertion sequence elements carry no selectable genes, therefore, their only function is that of inactivating a gene upon insertion (Snyder and Champness 2003). IS elements are found in both chromosomal and plasmid DNA. To date, more than 700

IS elements have been characterized in bacteria and most are designated by a number identifying its type, e.g. IS1, IS2, IS3 (Snyder and Champness 2003).

Two IS elements of the same type form a larger transposon, called a composite transposon, by bracketing other genes (Snyder and Champness 2003). For example, Tn5 is a composite transposon which consists of genes for kanamycin resistance (Kan^r) and streptomycin resistance (Str^r) bracketed by copies of an IS element called IS50 (Reznikoff 1993). Some composite transposons have the bracketing IS elements in the same orientation, whereas others have IS elements in opposite orientations (Snyder and Champness 2003).

The inverted repeat sequences found at the ends of transposons and transposase enzymes are two essential components for transposition of transposons. The transposase recognizes, cuts and eventually ligates the DNA during transposition (Madigan *et al.,* 2003). To date, two mechanisms of transposition are known, which are called conservative and replicative transposition. In conservative transposition, the transposable element is excised from one location in the chromosome and becomes reinserted at a second location. The copy number of a conservative transposon therefore remains at one. By contrast, replicative transposons are duplicated, and a new copy is inserted at another location. Thus, after the transposition event is completed, one copy of the transposing element remains at the original site and another copy is found at the new site (Madigan *et al.,* 2003).

1.3.3. Conjugation

Bacterial conjugation (mating) is a process of genetic transfer that involves cell-to-cell contact (Madigan *et al.,* 2003). Conjugation is a plasmid-encoded mechanism and a plasmid which can transfer a copy of itself to a new host is called conjugative

plasmid (Lawley *et al.,* 2004). Conjugation is a complicated process that requires products of many genes, including *tra* genes (Snyder and Champness 2003). The products of the *tra* genes are *trans* acting and can act on other plasmids in the same cell. To date, up to 105 *tra* genes have been recognized with a variety of functions in mating pair formation, such as adhesion, transport or activation of the pilin. Each type of incompatibility group plasmid has different *tra* genes with the same function. For example, for function of transport, the gene in the FI Inc plasmids is *traD* while the gene in the HI1 Inc plasmid is *traG* (Lawley *et al.,* 2004).

1.4. Plasmid-mediated quinolone resistance determinants

1.4.1. *qnr* genes

Plasmid-mediated quinolone resistance (PMQR) determinants can be horizontally transferred between bacterial species via conjugation. The first PMQR gene reported was named *qnr* (Martinez-Martinez *et al.,* 1998). The plasmid-borne group of *qnr* genes is now comprised of 5 families, *qnrA* (Martinez-Martinez *et al.,* 1998), *qnrB* (Jacoby *et al.,* 2006), *qnrS* (Hata *et al.,* 2005) and more recently, *qnrC* (Wang, M. *et al.,* 2009) and *qnrD* (Cavaco *et al.,* 2009). These five different genes differ from each other by over 40% in nucleotide sequence. Within each *qnr* gene group, different alleles vary in less than 10% in nucleotide level (Jacoby *et al.,* 2008).

1.4.1.1. qnrA

The *qnrA* gene was discovered by the group of Martinez-Martinez and colleagues while studying pMG252, an MDR plasmid isolated from a *K. pneumoniae* strain (Martinez-Martinez *et al.,* 1998). They found an increase of MIC to nalidixic acid in recipient strains from 4- to 16-fold in the porin-deficient isolates and from 8- to 64-

fold in isolates with intact porins isolates. Subsequent cloning of the gene responsible for this MIC increase revealed a 657 bp open reading frame. The protein encoded was named Qnr, later called QnrA1, for quinolone resistance. To date, there have been 6 variants of *qnrA* genes, named *qnrA1* to *qnrA6*. (Table 1.2).

1.4.1.2. qnrS

In 2003, a clone of *Shigella flexneri* 2b caused a food-borne outbreak of enterocolistis in Japan. One of eight strains causing the outbreak was found to be resistant to ciprofloxacin. This strain was later found to contain a unique conjugative plasmid, which when conjugated into another bacterial isolate transferred quinolone resistance. Cloning and sequences identified an open reading frame encoding a 218 amino acid protein of the pentapeptide repeat family. The identified protein shares 59% amino acid identity with QnrA1 and was named QnrS (Hata *et al.,* 2005), latterly renamed QnrS1. To date, there have been 4 different alleles of *qnrS* described which have been named from *qnrS1* to *qnrS4* (Jacoby *et al.,* 2008) (Table 1.3).

1.4.1.3. qnrB

Another member of the *qnr* family is *qnrB*. The gene *qnrB* was discovered by Jacoby and colleagues while investigating *K. pneumoniae* strains isolated in India (Jacoby *et al.,* 2006). Jacoby *et al*. found that several strains could transfer low-level quinolone resistance but were negative by PCR for the *qnrA* gene. Experimental evidence revealed the PMQR determinant responsible for this phenotype was a 214 amino acid protein and the gene encoding the protein was termed *qnrB1* (Jacoby *et al.,* 2006). The QnrB1 protein shares 43% and 44% amino acid identities with QnrA and QnrS, respectively (Jacoby *et al.,* 2006). Gene *qnrB* has the greatest number of sequence

variants compared to other *qnr* members, with 30 alleles and amino acid differences at 27 out of 214 possible sites (13%) (Jacoby *et al.,* 2008) (Table 1.4).

1.4.1.4. qnrC

The *qnrC* gene was discovered in a *Proteus mirabilis* clinical strain 06-489 from China; 06-489 had the ability to transfer low-level quinolone resistance and was negative by PCR for all the known *qnr* genes. The plasmid pHS10, isolated from the *Proteus mirabilis* 06-489 strain, increased the MIC to ciprofloxacin of the transconjugant *E. coli* J53 with plasmid pHS10 from 0.008 to 0.25µg/ml. The plasmid pHS10 was subsequently found to carry a 666 bp gene, which was later designated *qnrC1* (GenBank accession number EU917444). This *qnrC* gene encoded a 221 amino acid protein, which shared 64%, 42%, 59% and 43% amino acid identities with QnrA1, QnrB1, QnrS1 and QnrD, respectively (Wang, M. *et al.,* 2009).

1.4.1.5. qnrD

In 2009, Cavaco and colleagues found 4 *Salmonella enterica* isolates obtained from humans in China that showed reduced susceptibility to ciprofloxacin. The phenotype was later revealed to transfer on a small plasmid of approximately 4.3 kb (Cavaco *et al.,* 2009). When the plasmid was transferred to *E. coli*, it conferred a 32-fold increase in the MIC to ciprofloxacin. This plasmid was confirmed to be PCR negative for the *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr* and *qepA* genes. The study of Cavaco *et al*. later revealed that the plasmid carried a gene encoding a 214 amino acid pentapeptide repeat protein, which was subsequently designated QnrD. *qnrD* gene (GenBank accession number EU692908) shared 48% homology to *qnrA1*, 61%

homology to *qnrB1* and 32% homology to *qnrS1* at the DNA level (Cavaco *et al.,* 2009).

1.4.1.6. The characteristics of Qnr proteins

The Qnr proteins belong to the pentapeptide repeat family, which are defined by a series of tandem 5 amino acid repeats (Tran and Jacoby 2002). Within the pentapeptide repeats, no position is completely conserved, but each of the residues of an individual pentapeptide exhibits a propensity for a restricted number of amino acids with the recurrent general motif represented by [Ser, Thr, Ala or Val][Asp or Asn][Leu or Phe][Ser, Thr or Arg][Gly] (Bateman *et al.,* 1998; Vetting *et al.,* 2006). Qnr proteins often have a cysteine at position i^{-2} (with position i representing the central amino acid of each repeat) (Strahilevitz *et al.,* 2009)

A characteristic feature of the Qnr protein is that they are formed by two domains of pentapeptide repeats separated by a single amino acid, usually glycine. The primary structures of QnrA, QnrB and QnrS are similar, with 9 pentapeptide repeat units connected by a single glycine, followed by a cysteine, with a variable number of units (22 in QnrS, 28 in QnrA and 29 in QnrB, QnrC and QnrD) (Figure 1.5). The contribution of the Glycine and Cysteine to the function of Qnr is unknown. However, the ability of QnrA and QnrS to protect against quinolone activity declines when Cysteine is substituted by Tyrosine (Cattoir *et al.,* 2007b).

Table 1.2 The amino acid substitutions found in QnrA alleles.

This table was adapted from the website http://www.lahey.org/qnrStudies.

Allele	Substitution at position								GenBank
	39	**54**	**108**	**116**	**127**	**130**	**161**	**213**	**Number**
QnrA1	Q	V	V	S	T	S	R	V	AY070235
QnrA2	R			A	A			I	AY675584
QnrA3	R		I		A				DQ058661
QnrA4	R		I		A	N			DQ058662
QnrA5	R		I		A		C		DQ058663
QnrA6	R	I	I		A		H		DQ151889
QnrA7	R		I		A		H		GQ463707

Table 1.3 The amino acid substitutions found in QnrS alleles.

This table was adapted from the website http://www.lahey.org/qnrStudies.

Allele	Substitution at position																		GenBank
	5	11	12	16	21	31	41	44	61	89	91	102	106	120	148	201	206	207	216	Number
QnrS1	N	H	N	K	L	S	T	V	V	F	A	T	H	S	N	A	L	I	Y	AB187515
QnrS2	R		S	Q	I	C	A	I		L	E	A	N	T	H	S	Q	L	F	DQ485530
QnrS3																				EU077611
QnrS4		R							A											FJ418153

Table 1.4 The amino acid substitutions found in QnrB alleles.

This table was adapted from the website http://www.lahey.org/qnrStudies.

Allele	Substitution at position																																GenBank Number
	2	**7**	**11**	**18**	**20**	**21**	**22**	**27**	**35**	**36**	**55**	**60**	**69**	**74**	**79**	**80**	**87**	**94**	**118**	**129**	**142**	**144**	**147**	**151**	**162**	**163**	**168**	**171**	**186**	**188**	**198**	**202**	**204**	**205**	**212**	**213**	
QnrB1	A	G	D	E	I	E	N	N	L	S	N	M	C	A	S	S	R	A	N	V	I	A	L	F	S	T	A	F	I	G	N	S	L	M	V	I	DQ351241
QnrB2			N												A						M									R				I		DQ351242
QnrB3											K										M															DQ303920
QnrB4	T				V							N			I	N		S			M	T				S		V			S			L		M	DQ303921
QnrB5	T				V										V						M	T									S				I		DQ303919
QnrB6															A						M																EF520349
QnrB7															A						M				T										I		EU043311
QnrB8	T				V							I			V					A	M	T		L		S	T					A					EU043312
QnrB9															A						M														I		EF526508
QnrB10	T				V										V						M	T															DQ631414
QnrB11	T			A	V							I			V						M	T				S			V		S		I	L		M	EF653270
QnrB12	T			A	V							I			V						M	T				S			V		S		I	L			AM774474
QnrB13															A						M								R						I		EU273755
QnrB14						D									A						M													T	I		EU273757
QnrB15							S								A	N					M														I		EU302865
QnrB16															A						M	T													I		EU136183
QnrB17																					M																AM919398
QnrB18						D									A						M																AM919399
QnrB19	T				V										V						M	T									S						EU432277
QnrB20			N												A						M								R								AB379831
QnrB21	T				V							I			V					A	M	T		L		S	T			S	A					FJ611948
QnrB22	T				V					C		N			I	N		S			M	T				S		V		V	S			L		M	FJ981621
QnrB23								Y							A						M														I		FJ981622
QnrB24									M					V	A						M																HM192542
QnrB25					V							I			V				S	A	M	T		L		S	T				S	A			I		HQ172108
QnrB27	T	S			V										A			S			M	T	A			A					S	A					HM439641
QnrB28	T	S			V										V			S			M		A			A					S	A					HM439643
QnrB29														V	A						M																HM439649
QnrB30													S		A						M																HM439650
QnrB31															A		S				M								R						I		HQ418999

Figure 1.5 The amino acid sequence of various Qnr proteins.

These amino acid sequences are 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. 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.

1.4.1.7. Mechanisms of actions of Qnr proteins

Experimental evidence has demonstrated that transconjugants of *E. coli* with a *qnrA* gene do not demonstrate any change in quinolone accumulation, outer membrane porins or their ability to inactivate the antimicrobial (Tran and Jacoby 2002). The function of the Qnr is protection of the DNA gyrase from the quinolones, which is similar to MfpA protein, a fluoroquinolone resistant gene identified in the chromosome of *Mycobacterium smegmatis* (Montero *et al.,* 2001).

It has been shown that QnrA can bind to the DNA gyrase in the absence of relaxed DNA, ciprofloxacin and ATP, indicating that the binding of QnrA to the DNA gyrase does not require the presence of the ternary complex of enzyme, DNA and quinolone (Tran *et al.,* 2005a; Tran *et al.,* 2005b). DNA filter binding assays have shown that DNA binding to the DNA gyrase is decreased when the DNA gyrase is exposed to Qnr (Tran *et al.,* 2005a; Tran *et al.,* 2005b). As a result, it has been proposed that QnrA protects the bacterial cell from the activity of the quinolones by binding to the DNA gyrase or the topoisomerase IV at a site overlapping the DNA binding site. However, it is not clear how QnrA competes with DNA for gyrase binding without functionally inhibiting the gyrase activity *in vitro*.

1.4.1.8. Origins of *qnr* genes

The number, variety and geographical distribution of *qnr* genes have raised questions about bacterial origin of these *qnr* genes and the role of the *qnr* genes prior to protection of the bacteria from fluoroquinolones.

The genome sequences of 48 Gram-negative bacterial species were screened for sequences that exhibited similarity to *qnr* genes. Variants of *qnrA*, *qnrA3* and *qnrA5*,

were found on the chromosome of *Shewanella algae* (Poirel *et al.,* 2005b). The MIC levels to ciprofloxacin, ofloxacin, sparfloxacin and norfloxacin of *Shewanella algae* were 0.12, 0.5, 0.5 and 0.5 μg/ml, respectively, which were observed 4- to 8-fold higher than that of *Shewanella putrefaciens*, an organism closely related to *Shewanella algae* which is lacking a chromosomal *qnrA* gene (Poirel *et al.,* 2005b). These data suggest that *Shewanella algae* is a possible candidate for the original reservoir of *qnrA*. Additionally, two previous studies also described pentapeptide repeat proteins with between 40 and 67% amino acid identity to *qnrA* present in other waterborne *Shewanella* species (Poirel *et al.,* 2005a; Saga *et al.,* 2005).

A study focusing on DNA sequences in a database of microbial populations collected from seawater identified a gene encoding a pentapeptide repeat protein, designated marine metagene Qnr. Marine metagene Qnr demonstrated 88% DNA homology with QnrB5 and QnrB19 (Venter *et al.,* 2004; Sanchez *et al.,* 2008).

A number of *qnrS2* genes have been reported on plasmids carried by various environmental organisms. In water collected from the Seine river, France, Cattoir and colleagues found *Aeromonas ounctata* subspecies *punctata* and *Aeromonas media* strains carrying plasmids that transferred quinolone resistance. These plasmids were then found to carry *qnrS2* (Cattoir *et al.,* 2007a). The *qnrS2* gene has also been found in plasmids from *Aeromonas allosaccharophila* (Picao *et al.,* 2008) and on plasmid pGNB2 in sludge basin bacteria (Bonemann *et al.,* 2006). All four of these plasmids isolated from *Aeromonas ounctata*, *Aeromonas media*, *Aeromonas allosaccharophila* and sludge basin bacteria conferred the increase in MIC to fluoroquinolones.

The aforementioned findings provide support for the hypothesis that the *qnr* genes originated in the chromosome of environmental bacteria and have been transferred to plasmids. Fluoroquinolones have been detected in wastewater effluents and in water from rivers in densely populated regions (Golet *et al.,* 2002). Golet and colleagues investigated the presence of fluoroquinolones in the aqueous compartments of the Glatt Valley Watershed, a densely populated region in Switzerland. Ciprofloxacin and norfloxacin were identified in municipal wastewater effluents and in the receiving surface water of the Glatt River with the concentrations ranging from 54 to 130 ng/L (for ciprofloxacin) and from 30 to 87 ng/L (for norfloxacin) (Golet *et al.,* 2002). Although fluoroquinolones in water are degraded by sunlight, adsorption to sediments or degradation by terrestrial fungus, small quantities of fluoroquinolones may remain in the environment. As a result, it is possible that environmental quinolone accumulation has contributed to the success of these genes (Strahilevitz *et al.,* 2009).

1.4.1.9. The evolution of *qnr* genes

Baquirin and colleagues generated a phylogeny of representative *qnr* alleles from each of the major groups of plasmid *qnr* genes (*qnrA*, *qnrB* and *qnrS*). They also included chromosomal homologues of *qnr* alleles, including chromosomal *qnr* from *Shewanella* species and *Vibrio vulnificus* (Baquirin and Barlow 2008). They found three groups of plasmid *qnr* alleles clustered in three distinct clades. The distances from the most recent common ancestor of the QnrA clade and the extant taxa of that clade are short, which suggests that the diversity within the clade is likely to be recent (Baquirin and Barlow 2008). In contrast, the distances between the most recent common ancestor of the QnrB clade and the extant taxa of that clade are

sufficiently long that it is likely that the mobilization of that clade occurred in the preantibiotic era and that much of the clade diversity is unrelated to clinical antimicrobial usage. Baquirin and colleagues also found evidence that recombination had occurred between *qnrB4* and the ancestor of *qnrB5* and *qnrB6* (Baquirin and Barlow 2008).

1.4.2. aac(6')-Ib-cr

In 2005, Robicseck and colleagues identified a variant of an aminoglycoside-modifying enzyme, *aac(6')-Ib-cr*, which has the ability to modify certain fluoroquinolones (Robicsek *et al.,* 2006a). While investigating the MIC to ciprofloxacin of transconjugants generated from a Chinese population of *qnrA*-bearing clinical *E. coli*, Robicseck and colleagues found two plasmids in separate transconjugants from a single *E. coli* donor, in which one yielding high-MIC transconjugants 1 μg/ml (pHSH10-2) and the other yielding low-MIC transconjugants 0.25 μg/ml (pHSH10-5). Both plasmids were approximately 60 kb in size and Southern hybridization confirmed the presence of *qnrA* in both plasmids. By random transposon mutagenesis of the 2 plasmids pHSH10-2 and pHSH10-5, they found the gene responsible for the elevated MIC to be a variant of aminoglycoside acetyltransferase, *aac(6')-Ib*, which confers resistance to tobramycin, amikacin and kanamycin (Robicsek *et al.,* 2006a). Sequencing showed that this allele was different from other known variants of *aac(6')-Ib* and contained two codon substitutions, Trp102Arg and Asp179Tyr. These substitutions play an important role in the ciprofloxacin resistance phenotype. An acetylation assay showed that the function of this Aac(6')-Ib variant, later called Aac(6')-Ib-cr, is to acetylate ciprofloxacin at the amino nitrogen on its piperazinyl substitution (Robicsek *et al.,* 2006a).

1.4.2.1. Resistance activity of Aac(6')-Ib-cr

The increase in MIC to fluoroquinolones conferred by Aac(6')-Ib-cr is less than that conferred by Qnr proteins (Robicsek *et al.,* 2006a). As predicted by its specific quinolone target, Aac(6')-Ib-cr is only effective against ciprofloxacin and norfloxacin, which have piperazinyl secondary amines. Other quinolones are unaffected by this protein (Robicsek *et al.,* 2006a).

Studies of purified Aac(6')-Ib and its cr variant indicate that the mutant enzyme has only slightly reduced efficiency for the acetylation of kanamycin compared to the Aac(6')-Ib protein (Vetting *et al.,* 2008). The acetylation of ciprofloxacin, although less efficient than that of kanamycin, was sufficient in bacterial cells to produce a reduced-susceptibility phenotype equivalent to that of cells exposed to chemically synthesized N-acetyl ciprofloxacin (Robicsek *et al.,* 2006a). In a molecular model of ciprofloxacin binding to Aac(6')-Ib-cr, the Asp179Tyr mutation had the greatest effect, resulting in interaction with the quinolone rings to enhance drug binding. The Trp102Arg mutation serves to stabilize the positioning of Tyr179. The model is consistent with the magnitude of the effects of the individual mutations, with Asp179Tyr having a partial resistance phenotype, Trp102Arg having little detectable resistance phenotype, and the two mutations together having the full resistance phenotype to ciprofloxacin (Robicsek *et al.,* 2006a).

1.4.2.2. The genetic environment of *aac(6')-Ib-cr* plasmids

aac(6')-Ib-cr is in an integron cassette with an associated *attC* site. As a result, it is found in various integrons and is particularly associated with $bla_{CTX\text{-}M\text{-}15}$ gene (Machado *et al.,* 2006; Cordeiro *et al.,* 2008; Fihman *et al.,* 2008; Pitout *et al.,* 2008; Pomba *et al.,* 2009; Sabtcheva *et al.,* 2009). In addition, *aac(6')-Ib-cr* has been

reported to be associated with other PMQR genes including *qnrA* (Xu *et al.,* 2007; Jiang *et al.,* 2008), *qnrB* (Quiroga *et al.,* 2007; Jiang *et al.,* 2008; Yang *et al.,* 2008; Ma *et al.,* 2009; Pomba *et al.,* 2009), *qnrS* (Jiang *et al.,* 2008; Ma *et al.,* 2009) and *qepA*, another member of PMQR determinants (Ma *et al.,* 2009).

1.4.3. qepA

A novel efflux pump, named QepA, was found on a plasmid (pHPA) in an *E. coli* strain isolated from a urine specimen of a patient in Japan in 2002 (Yamane *et al.,* 2007). The *qepA* gene encodes for a 511 amino acid protein belonging to the 14-transmembrane-segment major facilitator superfamily of transporters. When cloned into an *E. coli*, it increases the MICs to several antimicrobials, including erythromycin, nalidixic acid, ciprofloxacin, norfloxacin (increased 2-, 2-, 32-, 64-fold, respectively) (Yamane *et al.,* 2007). To date, there have two variants of QepA described, QepA1 and QepA2, which differ by 2 amino acids. The *qepA1* gene has been found on 113- and 168 kb IncF1 plasmids from Belgium (Perichon *et al.,* 2007; Perichon *et al.,* 2008) and *qepA2* was also discovered on an IncF1 plasmid in France (Cattoir *et al.,* 2008b).

1.4.4. Epidemiology of plasmid-mediated quinolone resistant determinants

1.4.4.1. Epidemiology of *qnr* and *aac(6')-Ib-cr*

After the initial discovery of *qnrA* in a *K. pneumoniae* strain in 1994, much surveillance has been conducted targeted at detecting the intercontinental spread of this gene. The first broad descriptive study was a survey to detect the *qnrA* by PCR in more than 350 Gram-negative isolates collected in the 1990s from a wide geographic range across the USA (Jacoby *et al.,* 2003). *qnrA* was detected in only 6

isolates (4 *E. coli* and 2 *Klebsiella* spp. isolates). All these 6 strains were from the same centre in Alabama where the original strain had been detected, and all these six isolates were collected between July and December 2004 (Jacoby *et al.,* 2003). A summary of the epidemiology of *qnr* and *aac(6')-Ib-cr* genes is shown in Figure 1.6.

In general, the majority of studies concerning PMQR genes have been localized to narrow geographical regions (mostly in USA and Europe) and a limited range of bacterial genera (mainly within the Enterobacteriaceae). Most studies utilize PCR to examine clinical isolates of the Enterobacteriaceae collected in the 1990s or early 2000s, over time periods ranging from a few months to more than a decade. Through 2008, more than 70 publications in peer-reviewed journals and conference abstracts reported over 20,960 isolates that were tested for PMQR genes (Strahilevitz *et al.,* 2009). *qnr* genes have been found in most of the continents and in the majority of clinically common Enterobacteriaceae. *qnr* genes are more prevalent among *Enterobacter* spp. and *Klebsiella* spp. than *E. coli* (Jeong *et al.,* 2005; Poirel *et al.,* 2005c; Cambau *et al.,* 2006; Robicsek *et al.,* 2006b; Jiang *et al.,* 2008; Lavilla *et al.,* 2008; Minarini *et al.,* 2008; Wang, A. *et al.,* 2008b). Conversely, *aac(6')-Ib-cr* is more common among *E. coli* strains than other members of Enterobacteriaceae (Park *et al.,* 2006; Jiang *et al.,* 2008; Yang *et al.,* 2008; Sabtcheva *et al.,* 2009).

Epidemiological studies have been useful in supporting genetic data indicating a linkage between PMQR genes and other antimicrobial resistance genes, particularly ESBL genes. Various investigators have demonstrated a high *qnr* prevalence (*qnrA* and *qnrB*) among ESBL-positive strains (Park *et al.,* 2007; Wu *et al.,* 2007; Yang *et al.,* 2008). As a result, whether such isolates exhibit resistance to cephalosporins and are carrying both ESBL and PMQR genes and whether PMQR genes can be

effectively treated with fluoroquinolones requires further investigation. A further concern arising from epidemiological studies is the association of *aac(6')-Ib-cr* with $bla_{CTX\text{-}M\text{-}15}$, an ESBL that has emerged worldwide in recent years (Coque *et al.,* 2008; Pitout *et al.,* 2008).

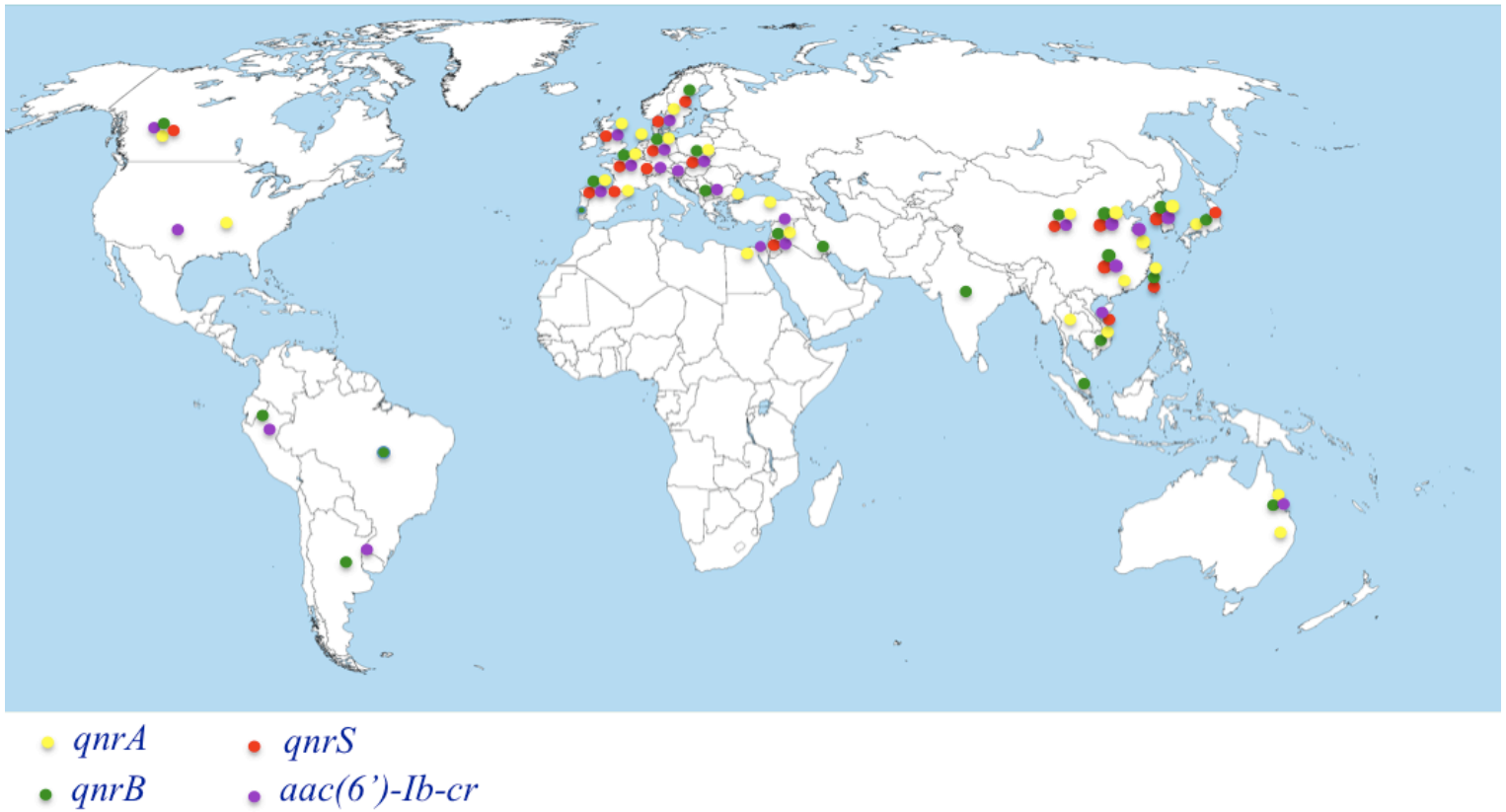


Figure 1.6 The global distribution of plasmid-mediated quinolone resistance determinants (2009).

1.4.4.2. Vietnam and South East Asia

To date, there has been only one study concerning the PMQR determinants in Vietnam. The study investigated the occurrence of the PMQR determinants *qnrA* and *qnrS* among diarrhoeagenic enterobacterial isolates recovered from Hanoi, Vietnam, during the period March 2001 to April 2002. In total, 162 *E. coli* isolates, 28 *Shigella* isolates and 4 *Enterobacter cloacae* isolates were screened for the presence of *qnrA* and *qnrS*, only a single *Enterobacter cloacae* isolate harboured a 50-kb *qnrS*-positive conjugative plasmid (Poirel *et al.,* 2006b).

There has been significant surveillance related to the prevalence and genetic properties of PMQR genes isolated in other parts of Asia. From isolates that were collected before 2005, the majority that harbor a PMQR gene are only positive with *qnrA* gene. The prevalence of *qnrA* genes in these surveillance ranges from 1% to 7% (Wang *et al.,* 2003; Cheung *et al.,* 2005; Poirel *et al.,* 2005c; Robicsek *et al.,* 2006a; Wu *et al.,* 2007; Xu *et al.,* 2007; Jiang *et al.,* 2008; Wu *et al.,* 2008b; Kim, S. Y. *et al.,* 2009). However, almost all of the studies after 2005 demonstrated the presence of all 4 PMQR types (*qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*) (Ahmed *et al.,* 2007; Park *et al.,* 2007; Liu *et al.,* 2008; Shin *et al.,* 2008; Wang, A. *et al.,* 2008a; Wang, A. *et al.,* 2008b; Yang *et al.,* 2008; Cui *et al.,* 2009; Kim, H. B. *et al.,* 2009; Ma *et al.,* 2009).

1.5. Aims of study

The aims of this study were to describe and further investigate the fluoroquinolone resistance prevalence and plasmid-mediated quinolone resistance determinants in Enterobacteriaceae isolated from patients admitted to the tetanus ward of the Hospital for Tropical Diseases and healthy volunteers living in Ho Chi Minh City. The specific questions and aims of this study were as follows:

1. What is the prevalence of antimicrobial resistant Enterobacteriaceae in patients and healthy individuals living in Ho Chi Minh City?

   The aim was to calculate the frequency of carriage of antimicrobial resistant Enterobacteriaceae in patients and healthy individuals living in Ho Chi Minh City. Additionally, I aimed to determine the prevalence of PMQR genes in isolated organisms and their effect on the MIC in wild-type strains with and without additional resistance associated chromosomal mutations.

2. What is the structure and relationship of *qnrS* encoding plasmids?

   To study *qnrS* plasmids, 3 *qnrS1*-containing plasmids originating from hospital and the community were DNA sequenced and annotated. The aim was to use these data to characterize the dominant *qnrS1*-containing mobile element circulating in Enterobacteriaceae isolated in Ho Chi Minh City.

3. What is the antimicrobial resistance pattern of *qnrS1*-harbouring plasmids isolated from hospital and community populations?

I aimed to investigate the association of the *qnrS1* gene with other antimicrobial resistance genes, which may be co-transferred and be contributing to positive selection.

4. What is the effect of antimicrobial therapy in the selection of the gastrointestinal microbial population containing PMQR genes?

   I aimed to assess the copy number of the *qnrA*, *qnrB* and *qnrS* genes before and after the use of antimicrobials in a group of children with acute respiratory infections presenting at the outpatient clinic of a hospital in Ho Chi Minh City, Vietnam.

2. Study subjects and materials and methods

2.1. Study subjects

The strains in this study were collected from 3 distinct study populations; strains obtained from patients admitted to hospital (hospital strains), healthy volunteers from the local community (community strains) and children with suspected acute respiratory infections (ARIs).

2.1.1. Isolation of the hospital strains

One hundred and ninety four patients with severe tetanus infections admitted to the Tetanus intensive care ward at the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City over the period from 26th April to 18th November 2004 and 15th May to 29th December 2005 were enrolled. Swabs from axilla, nose, sputum and anus of all patients were taken on admission and twice weekly from admission to day of discharge. The clinical data of these patients were also collected on the day of admission.

2.1.2. Isolation of the community strains

The community strains were collected from stool samples of 27 healthy adults and 77 children (5 – 14 years old) living in Ho Chi Minh City, who volunteered to participate in typhoid vaccine study M01ZH09 (Tran *et al.,* 2010) in November 2005 and April 2007, respectively; and from nasal and rectal swabs from 100 consecutive one – three day old healthy neonates, born after uncomplicated pregnancies and delivered on a general obstetrics ward of an Obstetric hospital in Ho Chi Minh City from 29th May to 29th June 2006. None of the individuals (including the neonates' mothers) had any known contact with antimicrobials (prescribed or otherwise) for 4

weeks prior to the sample collection. The demographic information from these volunteers was obtained for later statistical analysis.

2.1.3. Organisms isolated from children with acute respiratory infections (ARIs)

Three hundred children less than 16 years of age presenting to the Outpatient Department in Children's Hospital Number 1 in Ho Chi Minh City with ARIs from 8th April to 16th November 2009 were enrolled in this study. Rectal swabs were taken from all enrollees and were submerged in 1 ml of sterile saline and stored at -80°C until analysis.

2.2. General chemicals, reagents and buffers

General laboratory chemicals were purchased from Sigma (Steinheim, Germany), Merck (Hohebrunn, Germany) or BDH (Poole, England) unless otherwise stated. Buffers were prepared as aqueous solutions in distilled water according to standard methods (Sambrook and Russell 2001), and adjusted to the required pH. When required, solutions were sterilized by autoclaving. The following standard buffers were used:

i. Tris-EDTA (TE) buffer: 10 mM Tris, 1 mM EDTA, pH 8.0
ii. Tris-Borate-EDTA (TBE) buffer: 89 mM Tris, 89 mM Boric acid, 2 mM EDTA (pH8)
iii. E buffer: 40 mM Tris, 2 mM Disodium.EDTA (pH 7.9 by glacial acetic acid)
iv. MacConkey media: 1.7% (w/v) peptone, 0.3% (w/v) proteose peptone, 1% (w/v) lactose, 0.5% (w/v) NaCl, 0.0001% (w/v) crystal violet, 0.003%

(w/v) neutral red, 0.15% (w/v) bile salts, 1.35% (w/v) agar, dissolve in 1 L distilled water.

v. Mueller-Hinton (MH) agar: 0.4% (w/v) beef infusion solids, 0.15% (w/v) starch, 1.75% (w/v) casein hydrolysate, 1.5% (w/v) agar, dissolved in 1 L of distilled water.

vi. Brain Heart Infusion (BHI) broth: 1.75% (w/v) calf brain-beef heart infusion, 1% (w/v) pancreatic digest of gelatin, 0.2% (w/v) dextrose, 0.5% (w/v) sodium chloride, 0.25% (w/v) disodium phosphate, dissolved in 1 L distilled water.

2.3. Prevalence of PMQR determinants in Enterobacteriaceae isolated from Ho Chi Minh City

2.3.1. Criteria for isolated strains

2.3.1.1. Patients admitted to hospital (hospital strains)

Three thousand one hundred and twenty three swabs from axilla, nose, sputum and anus of 194 patients were collected and cultured on MacConkey media with and without supplementation of gentamicin (8 µg/ml) or ceftazidime (2 µg/ml) (Sigma-Aldrich, UK). All colony morphologies grown on MacConkey agar supplemented with antimicrobials were Gram stained. All isolates confirmed to be Gram-negative and oxidase negative were identified using API20E system (bioMerieux SA, Marcy-l'Etoile, France).

For hospital strains, only *K. pneumoniae* grown on MacConkey supplemented with gentamicin and *E. coli* or other Enterobacteriaceae (except for *K. pneumoniae*) grown on MacConkey supplemented with ceftazidime and confirmed ESBL positive

were included in the study. Since we focused on nosocomial strains, only the first isolates growing on the selective agars, typically ranging from 2 days to 1 week after admission, were tested.

2.3.1.2. Healthy volunteers (community strains)

One hundred and four anal swabs from 27 healthy adults and 77 healthy children and 100 nasal swabs and 100 anal swabs from 100 healthy neonates were collected and cultured on MacConkey media with and without supplementation of gentamicin (8 µg/ml), ceftazidime (2 µg/ml) or nalidixic acid (16 µg/ml) (Sigma-Aldrich, UK). All colony morphologies grown on MacConkey agar supplemented with antimicrobials were Gram stained. All isolates confirmed to be Gram-negative and oxidase negative were identified using API20E system (bioMerieux SA, Marcy-l'Etoile, France).

2.3.2. Identification of Enterobacteriaceae using the API 20E

The API 20E is a biochemical identification system for the Enterobacteriaceae. The system consists of a plastic strip with 20 microtubes containing dehydrated substrate of 2-ntrophenyl-βD-galactopyranoside (ONPG), arginine (ADH), lysine (LDC), ornithine (ODC), trisodium citrate (CIT), sodium thiosulfate (H2S), urea (URE), tryptophane (TDA), sodium pyruvate (IND), acetoin (VP), gelatin (GEL), glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), sucrose (SAC), melibiose (MEL), amygdalin (AMY) and arabinose (ARA) (Figure 2.1). The procedure was performed according to the manufacturer's recommendations (bioMerieux). Briefly, test substrates were reconstituted after having been filled with test bacterial suspension in sterile saline. After filling, the cupules of arginine (ADH), lysine (LDC), ornithine (ODC), H_2S (H2S), and urea (URE) microtubes

were overlaid with mineral oil. The incubation box was prepared by adding 5 ml of sterile water into the honeycombed wells of the tray to create a humid atmosphere. The strip was then placed into the incubation box and incubated at 37^{o}C. After 24 hours, the strip was read by referring to the interpretation table. Reagents were added into the VP, TDA, IND cupules and the results were recorded. The 21 test results, including oxidase test to detect cytochrome c oxidase, were subsequently converted to a seven-digit profile using the octal coding system following the manufacturer's instructions.

	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
E. coli 25922	-	-	+	+	-	-	-	-	+	-	-	+	+	-	+	+	-	+	-	+	-
K. pneumoniae 70060	-	-	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-

Figure 2.1 Representative biochemical characteristic of *E. coli* and *K. pneumoniae* using API 20E test.

The API20E test strip includes reactions for, ONPG (activate the ß-galactosidase), ADH (dehydrolate of the L- arginine by arginine dihydrolase), LDC (decarboxylate of the L-lysine by lysine decarboxylase), ODC (decarboxylate of the L-ornithine by ornithine decarboxylase), CIT (utilize the citrate), H2S (produce the H_2S), URE (activate the urease), TDA (activate the tryptophan deaminase), IND (produce the Indole, which detected by addition of Kovac's reagent), VP (produce the acetoin, which detected by Voges Proskauer test), GEL (activate the gelatinase) , GLU (ferment of glucose), MAN (ferment of mannose), INO (ferment of inositol), SOR (ferment of sorbitol) RHA (ferment of rhamnose), SAC (ferment of sucrose), MEL (ferment of melibiose), AMY (ferment of amygdalin), ARA (ferment of arabinose) and OX (cytochrome oxidase).

2.3.3. Antimicrobial susceptibility testing

Antimicrobial containing discs and E-test strips were supplied by Difco (UK) and AB Biodisk (Solna, Sweden), respectively.

2.3.3.1. Disc diffusion method

The susceptibility of isolates to antimicrobials was determined using the disc diffusion method on Mueller-Hinton (MH) agar plates, according to Clinical and Laboratory Standards Institute (CLSI) procedures and interpreted following CLSI guidelines (CLSI 2007). Four colonies from the overnight plated culture were suspended in 1 ml of sterile distilled water to produce a suspension, which matches the turbidity of the 0.5 McFarland standard. The inoculum was then spread evenly over the surface of the agar plate by swabbing in three different directions. Before applying discs, the plate was allowed to dry until there was no visible surface moisture. After incubating for 20 hours at 37^{o}C, the diameter of the clear zone of inhibition of growth was measured (CLSI 2007). *E. coli* ATCC 25922 and *K. pneumoniae* 700603 were used as control strains for antimicrobial sensitivity assays. The breakpoints of antimicrobials used in this study for Enterobacteriaceae are shown in Table 2.1.

2.3.3.2. E-test

The minimum inhibitory concentration (MIC) is the most commonly used parameter to describe the efficacy of an antimicrobial agent against a bacterial strain. Here, the MICs to nalidixic acid and ciprofloxacin of all isolates were measured using E-test (AB Biodisk, Sweden). Similar to the disc diffusion method, an MH plate was swabbed with the bacterial suspension and allowed to dry before placing E-test strip

on the surface. The plates with E-test strips were incubated for 20 hours at 37^{o}C. The MIC was then read from the scale where the ellipse edge intersects the strip and read at the point of inhibition of all growth. When growth occurs along the entire strip and no inhibition is seen, the MIC was reported as greater than the highest value on the reading scale. When the ellipse is so large that the zone edge does not intersects the strip, the MIC was read as less than the lowest reading on the scale (CLSI 2007) (Figure 2.2).

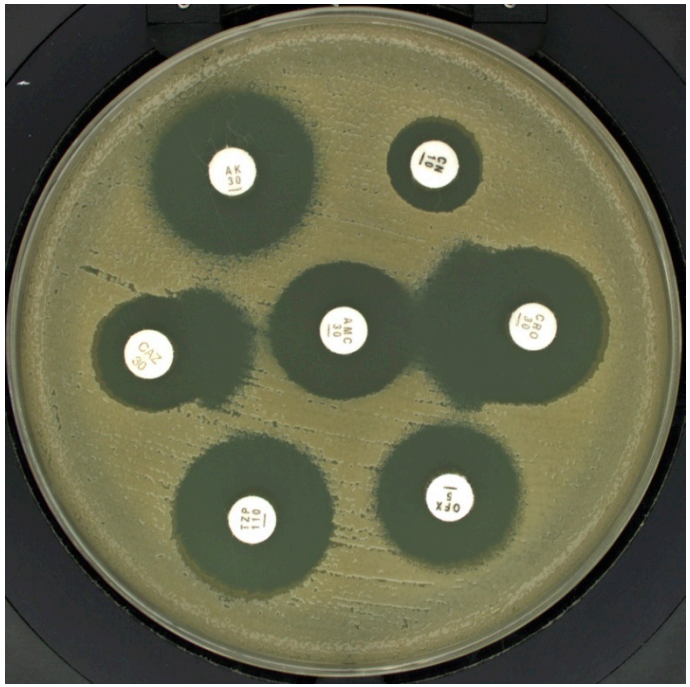
2.3.3.3. Test for ESBL producing

All strains were subjected to phenotypic test to confirm ESBL producing using double discs tests. The plates were inoculated as for the susceptibility test. Discs containing only cefotaxime (30 mg) and ceftazidime (30 mg) and both antimicrobials combined with clavulanic acid (10mg) were applied on the plate, according to CLSI guidelines (CLSI 2007). The isolate was confirmed to produce ESBL if the difference of diameter of inhibition growth zones of cefotaxime and cefotaxime with clavulanic acid or ceftazidime and ceftazidime with clavulanic acid was more than 5 mm (CLSI 2007). *E. coli* ATCC 25922 and *K. pneumoniae* 700603 were used as control strains for antimicrobial sensitivity and ESBL producing test assays (Figure 2.2 and Figure 2.3).

Table 2.1 Zone diameter interpretive standard based on CLSI guidelines.

Antimicrobial Agents	Disc content	Zone diameter, Nearest Whole (mm)			Equivalent MIC Breakpoints (μg/ml)	
		R	I	S	R	S
Amikacin	30 μg	≤ 14	15 – 16	≥ 17	≥ 32	≤ 16
Ampicillin	10 μg	≤ 13	14 – 16	≥ 17	≥ 32	≤ 16
Cefepime	30 μg	≤ 14	15 – 17	≥ 18	≥ 32	≤ 8
Ceftriaxone	30 μg	≤ 13	14 – 20	≥ 21	≥ 64	≤ 8
Chloramphenicol	30 μg	≤ 12	13 – 17	≥ 18	≥ 32	≤ 8
Ciprofloxacin	5 μg	≤ 15	16 – 20	≥ 21	≥ 4	≤ 1
Gentamicin	10 μg	≤ 12	13 – 14	≥ 15	≥ 8	≤ 4
Imipenem	10 μg	≤ 13	14 – 15	≥ 16	≥ 16	≤ 4
Kanamycin	30 μg	≤ 13	14 – 17	≥ 18	≥ 25	≤ 6
Nalidixic acid	30 μg	≤ 13	14 – 18	≥ 19	≥ 32	≤ 8
Piperacillin-tazobactam	100/10 μg	≤ 17	18 – 20	≥ 21	≥ 128/4	≤ 16/4
Tetracycline	30 μg	≤ 14	15 – 18	≥ 19	≥ 16	≤ 4
Ticarcillin	75 μg	≤ 14	15 – 19	≥ 20	≥ 128	≤ 16
Trimethoprim-Sulphamethazole	1.25/23.75 μg	≤ 10	11 – 15	≥ 16	≥ 8/152	≤ 2/38

Zone diameter interpretive standard and equivalent minimal inhibitory concentration (MIC) breakpoints for Enterobacteriaceae of used antimicrobials in this study (CLSI 2007)

a) 

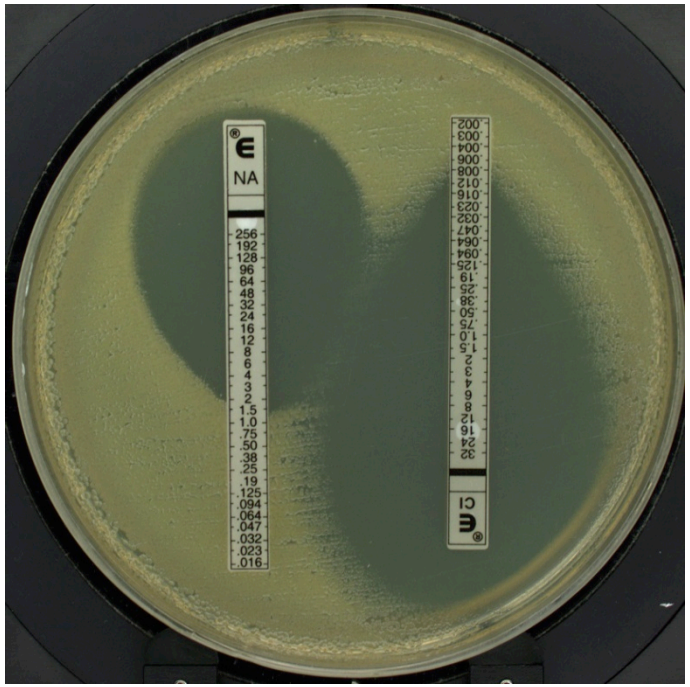
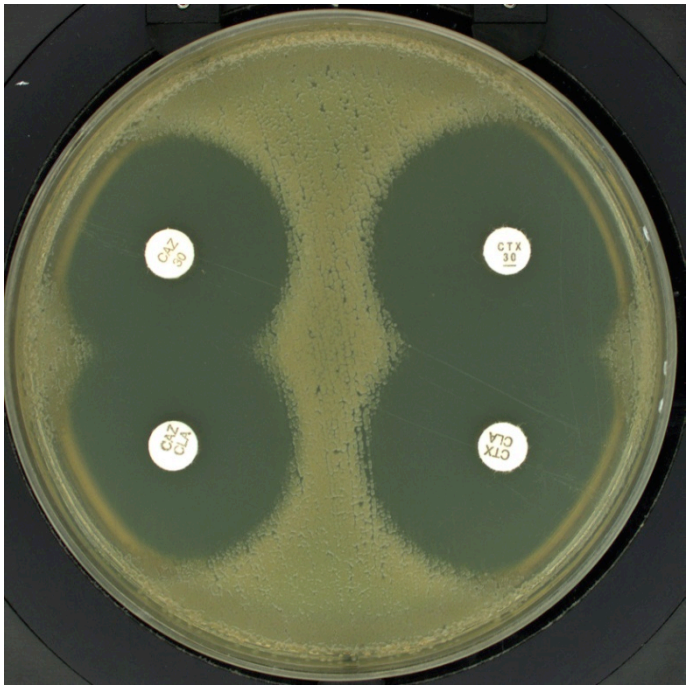
b) 

Figure 2.2 Representative image of a sensitivity test and MIC E-test for examining of antimicrobial agents.

This figure represents the sensitivity test and MIC E-test to Nalidixic acid and Ciprofloxacin. Figure a) shows the sensitivity test of *K. pneumoniae* ATCC 700603 strain to amikacin (AK), gentamicin (CN), ceftriaxone (CRO), ofloxacin (OFX), piperacillin-tazobactam (TZP), ceftazidime (CAZ) and augmentin (AMC). Figure b) shows the MIC E-test of *E. coli* ATCC 25922 strain to Nalidixic acid (MIC = 2 μg/ml) and Ciprofloxacin (MIC = 0.012 μg/ml).

a) 

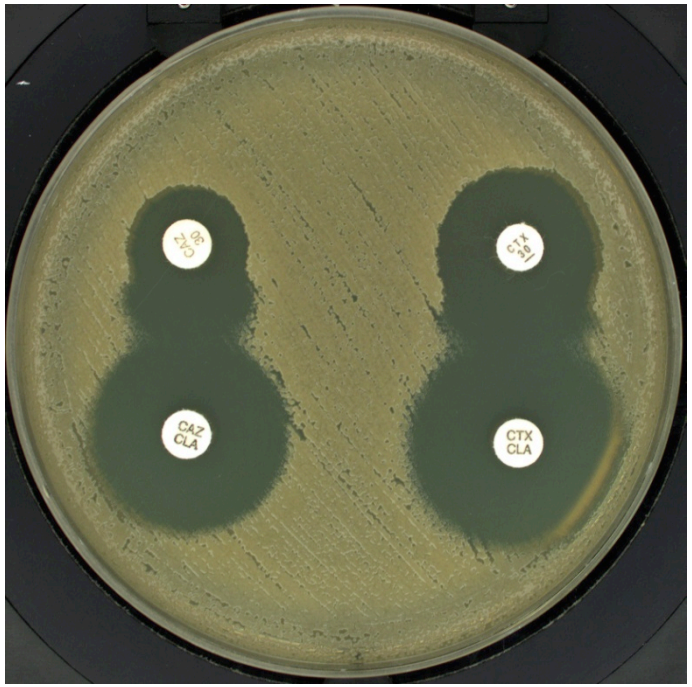
b) 

Figure 2.3 A representative image of an ESBL test using the double-disc method.

This figure represents two control strains for negative and positive ESBL producing, using double-disc test. Figure a) shows the negative strain of ESBL producing (*E. coli* ATCC 25922). The diameter of clear zone for each antimicrobial discs: CAZ: 31 mm; CAZ+CLA: 31 mm; CTX: 34 mm; CTX+CLA: 34mm. Figure b) shows the positive strain of ESBL producing (*K. pneumoniae* ATCC 700603). The diameter of clear zone for each antimicrobial discs: CAZ: 16 mm; CAZ+CLA: 26 mm; CTX: 22 mm; CTX+CLA: 28 mm. The difference of diameter between CAZ and CAZ+CLA or CTX and CTX+CLA is greater than 5 mm, implying a positive ESBL strain.

2.3.4. Bacterial conjugation

Bacterial conjugation was used to examine whether *qnrS*-containing plasmids were conjugative. The donor strains were *E. coli* and *K. pneumoniae* isolates, which contain *qnrS* gene, as confirmed by PCR. The recipient strain was *E. coli* J53Az^R (Wang *et al.,* 2004a), which was resistant to Sodium azide and sensitive to all other antimicrobial agents, strain was kindly provided by Wang and colleagues.

The bacterial conjugation procedure used was adapted from Wang et al. (Wang *et al.,* 2004a). Conjugation was performed in a 1:1 ratio in liquid cultures by static incubation overnight at 37^{o}C. Five millilitres of donor and recipient strains were grown in Luria-Bertani (LB) broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, dissolved in 1 L distilled water). On the following day, 500 µl of each overnight culture of donor and recipient strains were added into 4 ml of LB broth without antimicrobials. The mixture of donor and recipient strains was then incubated overnight at 37^{o}C without agitation. Donor and recipient strains were cultured separately as control. One hundred microlitres of conjugation mixture and control strains were then spread on LB plates (1.5% (w/v) Bacto-agar in LB broth) including 100 µg/ml sodium azide and 0.03 µg/ml of ciprofloxacin or 16 µg/ml of nalidixic acid. Any colony grown on the selective media was subcultured for further analysis such as API20E, antimicrobial sensitivity testing, and plasmid extraction. Transconjugants were stored in Brain Heart Infusion (BHI) broth with 5% (v/v) of glycerol and stored at -20^{o}C freezer.

2.3.5. Genomic DNA extraction from cultured bacterial strains

Genomic DNA from cultured strains was isolated using the Wizard genomic DNA preparation Kit (Promega, USA) according to the manufacturer's instructions. Briefly, a colony was picked and incubated in 2 ml of LB broth with agitation at 37^{o}C overnight. The cultured broth was then centrifuged to pellet the cells in a microcentrifuge at 13,200 rpm for 2 minutes; the pellet was resuspended in lysis buffer. Total DNA was eluted in 100 µl of TE and store at -20^{o}C until required.

2.3.6. DNA quantification by spectrophotometer

Genomic and plasmid DNA were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.), which is a cuvette free spectrophotometer. Distilled water was used as a blank. The concentration of DNA was calculated by the computer based on the following formula:

$c = (A260 \times 50)/0.1$

Where c is the nucleic acid concentration in ng/µl, A260 is the absorbance in AU, and the path length is 0.1 cm. The wavelength-dependent extinction coefficient of double stranded DNA is 50 ng-cm/µl.

2.3.7. Molecular typing using Random Amplification of Polymorphic DNA (RAPD)

RAPD was used to identify clonally related hospital strains and additionally all of the *qnr* or *aac(6')-Ib-cr* positive community strains. We used 3 different primers: ERIC1, ERIC2 and TT3 (Table 2.2), which have been used previously to distinguish the Enterobacteriaceae from other bacterial families. All hospital strains from each

six-month period and all selected community strains were processed simultaneously for each primer, and electrophoresis was performed under identical conditions on the same day. Amplification was performed using a DNA Engine Tetrad 2 (Bio-Rad)

PCR for RAPD was carried out in a total volume of 50 μl, consisting of 5 μl of 10X buffer, 3 μl of $MgCl_2$ 50 mM (for ERIC1 and ERIC2) and 4 μl of $MgCl_2$ (for TT3), 0.5 μl of dNTPs 25 μM, 1.5 μl of primer 10 μM (for ERIC1 and ERIC2) and 3 μl of primer 10 μl (for TT3), 0.15 μl of Bioline Taq (5U/μl), 2 ng of genomic DNA and distilled water to get the total volume of 50 μl.

PCR amplification for primers ERIC1 and ERIC2 was carried out for 5 minutes at 95^{o}C and subsequently for 35 cycles at 95^{o}C for 1 minute, 43^{o}C for 1 minute and 72^{o}C for 2 minutes. For primer TT3, PCR amplification was carried out at 95^{o}C for 5 minutes, 40^{o}C for 5 minutes, 72^{o}C for 5 minutes and followed by 32 cycles of 95^{o}C for 1 minute, 40^{o}C for 1 minute and 72^{o}C for 2 minutes. The final elongation was 72^{o}C for 5 minutes and the reaction was held at 12^{o}C until analysis using 1% agarose gel and electrophoresis at 80 V for 2 hours. The PCR amplifications were visualized by illumination on UV transilluminator and photographed using Quantity One UVtech DNA documentation system.

RAPD patterns were clustered and interpreted by combining the resulting amplification patterns of all three primers and analyzed with BioNumerics software (Applied Maths, Belgium). Two isolates were considered clonally related when they had RAPD patterns that were identical. Isolates of a given species differing by one or two bands for all three primers combined were considered variants of a given type. This was based on similarities in patterns of multiple isolates obtained from individual patients, by visualization and computer based analysis BioNumerics.

Table 2.2 Oligonucleotide primers used in RAPD PCR reaction.

Primers	**Predicted product size (bp)**	**Annealing temperature**	**Primer sequence (5' → 3')**	**Sources of reference**
ERIC1	NA	43^{o}C	ATGTAAGCTCCTGGGGATTCAC	(Versalovic *et al.,* 1991)
ERIC2	NA	43^{o}C	AAGTAAGTGACTGGGGTGAGCG	(Versalovic *et al.,* 1991)
TT3	NA	40^{o}C	GGCGAGGAGCG	(Schultsz *et al.,* 1997)

2.3.8. PCR for plasmid-mediated quinolone resistance determinants and gyrase and topoisomerase IV genes

PCRs for PMQR determinants and gyrase and topoisomerase IV genes were performed to amplify several specific targets, which were *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib*, *qepA*, *gyrA* and *parC* genes. Primer sequences and annealing temperature of each target are detailed in Table 2.4. PCR amplifications using the Bioline Taq polymerase enzyme (Bioline, UK) were carried out as per manufacturers instructions in a total volume of 50 μl. PCRs were performed in 0.2 ml thin wall PCR tubes. The ultra pure water used in the reaction was nuclease free (Sigma) and the dNTPs mixture was supplied in a 25 mM solution (Roche). The quantities used in the PCR reaction mixture are outlined in Table 2.3.

DNA amplifications were performed using the following programme:

i. 1 cycle of 94^{o}C for 5 minutes.
ii. 35 cycles of 94^{o}C for 30 seconds, 55^{o}C* for 30 seconds, and 72^{o}C for 30 seconds.
iii. 1 cycle of 72^{o}C for 5 minutes.
iv. Held at 12^{o}C.

*Annealing temperature was dependent on the Tm of primers; 55^{o}C is an average. The precise annealing temperatures of each gene are listed on Table 2.4.

Table 2.3 PCR mixture for PCR for plasmid-mediated quinolone resistance determinants and gyrase and topoisomerase IV genes.

Reagents	Volume	Final concentration
10X buffer	5 µl	1X
50 mM $MgCl_2$	2.5 µl	25 mM
25 mM dNTPs	0.5 µl	0.25 mM
10 µM primer forward	1 µl	0.2 µM
10 µM primer reverse	1 µl	0.2 µM
5 U/µl Taq polymerase	0.15 µl	0.75 U
DNA template	1 µl	2 ng genomic DNA
H_2O	38.85 µl	-
	50 µl	

Table 2.4 Oligonucleotide primers used in PCR reactions for plasmid-mediated quinolone resistance determinants and gyrase and topoisomerase IV genes.

Target genes	Primers	Predicted product size (bp)	Annealing temperature	Primer sequence (5' → 3')	Sources of reference
Plasmid-mediated quinolone resistance (PMQR) determinants					
qnrA	QnrA-F	627	48°C	TCAGCAAGAGGATTTCTCA	(Robicsek *et al.,* 2005)
	QnrA-R			GGCAGCACTATTACTCCCA	
qnrB	QnrBm-F	264	55°C	GGMATHGAAATTCGCCACTG	(Cattoir *et al.,* 2007c)
	QnrBm-R			TTTGCYGYYCGCCAGTCGAA	
qnrS	QnrS-F	491	48°C	ATGGAAACCTACAATCATAC	(Whichard *et al.,* 2007)
	QnrS-R			AAAAACACCTCGACTTAAGT	
aac(6')-Ib	Aac(6')-Ib-F	482	55°C	TTGCGATGCTCTATGAGTGGCTA	(Park *et al.,* 2006)
	Aac(6')-Ib-R			CTCGAATGCCTGGCGTGTTT	
qepA	QepA_F	199	55°C	GCAGGTCCAGCAGCGGGTAG	(Yamane *et al.,* 2008)
	QepA_R			CTTCCTGCCCGAGTATCGTG	
gyrA* and *parC					
gyrA	GyrA-F	625	55°C	CGACCTTGCGAGAGAAAT	(Weigel *et al.,* 1998)
	GyrA-R			GTTCCATCAGCCCTTCAA	
parC	parC_E_F	395	55°C	AAACCTGTTCAGCGCCGCATT	(Vila *et al.,* 1996)
(*E. coli*)	parC_E_R			GTGGTGCCGTTAAGCAAA	
parC	parC_K_F1	389	55°C	CTGAATGCCAGCGCCAAATT	(Brisse and Verhoef 2001)
(*K. pneumoniae*)	parC_K_R1			TGCGGTGGAATATCGGTCGC	

2.3.9. PCR for sequence of PMQR determinants and gyrase and topoisomerase IV genes

Purified PCR products of PMQR genes, *gyrA* and *parC* genes were subjected to a DNA sequencing reaction. Both strands of all DNA samples that were required were independently sequenced; therefore, two PCR reactions were carried out using the forward and reverse primers, which were the same primers as using in DNA amplifications (Table 2.4). The twenty microlitres of PCR sequencing mixture consisted of 4 µl DTCS master mix, 0.125 µM primer, 20 ng of DNA template and distilled water. PCR was performed with 30 cycles of denaturation at 96^{o}C for 20 seconds, 20 seconds at the specific annealing temperature and extension at 60^{o}C for 2 minutes.

2.3.10. PCR purification

PCR amplicons were purified using the QIAquick PCR purification kit following the manufacturer's instructions (QIAGEN GmbH, Germany).

2.3.11. Sequencing of PMQR determinants and gyrase and topoisomerase IV genes

PCR amplicons (PMQR genes and *gyrA* and *parC* genes) were sequenced using the CEQ DTCS - Quick Start Kit and analyzed using CEQ2000XL software on a CEQ8000 DNA sequencer (Beckman Coulter, USA). A sequencing reaction involves 3 steps, which are preparation of DNA by PCR for sequencing, purification of the PCR amplicon by ethanol precipitation and sequence analysis.

Excess PCR reagents were removed from the PCR products by ethanol precipitation reaction, which included 4 µl of stop solution (3M NaOAc and 100 mM EDTA) and

1µl of 20 mg/ml glycogen (supplied in DTCS Quick Start kit) and 20 µl of a sequencing reaction. The solution was mixed thoroughly before and after the addition of 60 µl of cold 95% ethanol. The DNA pellet was collected after centrifugation at 14,000 rpm for 15 minutes in a microcentrifuge (Eppendorf) and washed twice with 200 µl of cold 70% ethanol. For each wash, the microfuge tube was centrifuged immediately at 14,000 rpm for 2 minutes. The DNA pellet was allowed to dry for 15 minutes before being rehydrated in 40 µl of sample loading solution and was incubated at room temperature for at least 15 minutes (supplied in DTCS Quick Start kit, USA). Finally, the resulting DNA was subjected to an electrophoresis in the CEQ 8000 capillary sequencer, and the result was analyzed using CEQuence Investigator_CEQ2000XL.

2.4. Sequencing of the whole plasmids

2.4.1. Bacterial strains

In 112 *qnrS1*-containing Enterobacteriaceae (37 *E. coli*, 67 *K. pneumoniae* and 8 other species of Enterobacteriaceae) isolated from patients admitted to the hospital and healthy volunteers living in Ho Chi Minh City, we chose 3 *qnrS1*-habouring plasmids to sequence for the whole plasmids. Two of these plasmids were isolated from patients and the other was isolated from healthy volunteer.

2.4.2. Plasmid extraction of plasmids greater than 20 kb in size

To extract highly-purified plasmid DNA greater than 20 kb in size, QIAGEN Plasmid Midi Kit (QIAGEN) was used, according to the manufacture's instructions. This protocol is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low-salt and pH

conditions. RNA, proteins, dyes, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. This plasmid DNA was subsequently stored in TE and kept at -20^{o}C until further molecular analysis.

2.4.3. Whole plasmid sequencing

The three selected *qnrS1*-containing plasmids were sequenced at Sanger Institute (UK) by conventional Sanger sequencing. The principle of this sequencing method was described previously (Parkhill *et al.,* 2001). The plasmid DNA was fragmented by sonication, and several clone libraries were generated using pUC18 plasmids in *E. coli*, using DNA size fractions ranging from 1.0 to 2.5 kb. The whole plasmid sequence was obtained from a number of end sequences derived from these libraries using big dye terminator chemistry on ABI377 automated sequencers. The sequences were assembled, finished and annotated using the program Artemis.

2.4.4. Annotation of plasmids

2.4.4.1. Artemis

Plasmid sequence annotation was analyzed using Artemis software from the Sanger Institute. Artemis is freely available along with relevant manuals at http://www.sanger.ac.uk/resources/software/artemis/. Artemis is a DNA sequence visualization and annotation tool that allows the results of any analysis or sets of analyses to be viewed in the context of the sequence and its six-frame translation (Figure 2.4) (Rutherford *et al.,* 2000; Carver *et al.,* 2008).

In general, to annotate whole plasmid sequences, 4 steps were followed:

i. Predict coding sequences (CDS): using Artemis to define open reading frame (ORF) based on the start and stop codon and the limitation of ORF is more than 100 amino acids.

ii. Discarding unreliable CDSs: based on the GC frame plot and GC content, I discarded CDSs with GC frame plot or low GC content (less than 45%).

iii. Comparing predicted CDSs with predicted CDSs from online gene prediction programme: sequences were analyzed with 2 different programmes: BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Glimmer (www.ncbi.nlm.nih.gov/genomes/MICROBES/glimmer_3.cgi). At the end of this step, reliability of the predicted CDSs was confirmed.

iv. Annotation of CDSs: using FASTA to compare the predicted CDSs with the uniprot database to reveal the name and function of the CDSs.

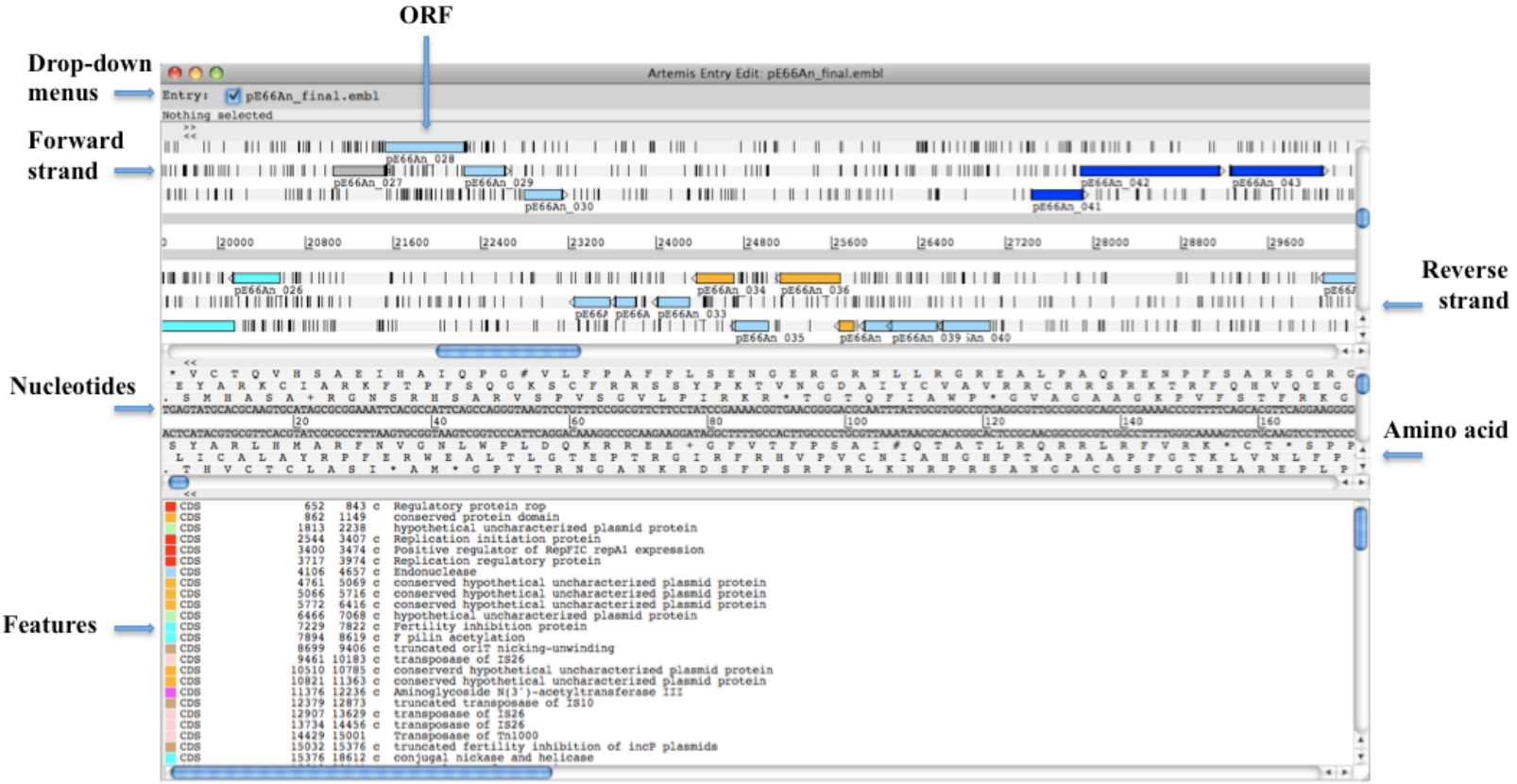


Figure 2.4 A representative screenshot of plasmid annotation using Artemis.

Displayed here is the annotation of plasmids pE66An, isolated from an *E. coli* strain in hospital group.

2.4.4.2. Artemis comparison tools (ACT)

Comparisons of DNA plasmid sequences were performed using Artemis Comparison Tool (ACT). ACT is an interface of Artemis software and has most of the functions of Artemis, but is especially useful for comparing DNA sequences. Using ACT, gene cluster insertion or absence can quickly be visualized. The stringency of the alignment can be adjusted by using sliding bars on the software. The ACT programme also allows the analysis to be visualized ranging from the base pair level to that of the whole genome. This can be achieved by lowering or raising the bar tabs. ACT utilises the same DNA sequence files from Artemis but requires a DNA comparison crunch file (Figure 2.5) (Abbott *et al.,* 2005; Carver *et al.,* 2008).

In the study, the BLAST programme on NCBI (National Center for Biotechnology Information) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare plasmid sequences to obtain crunch files.

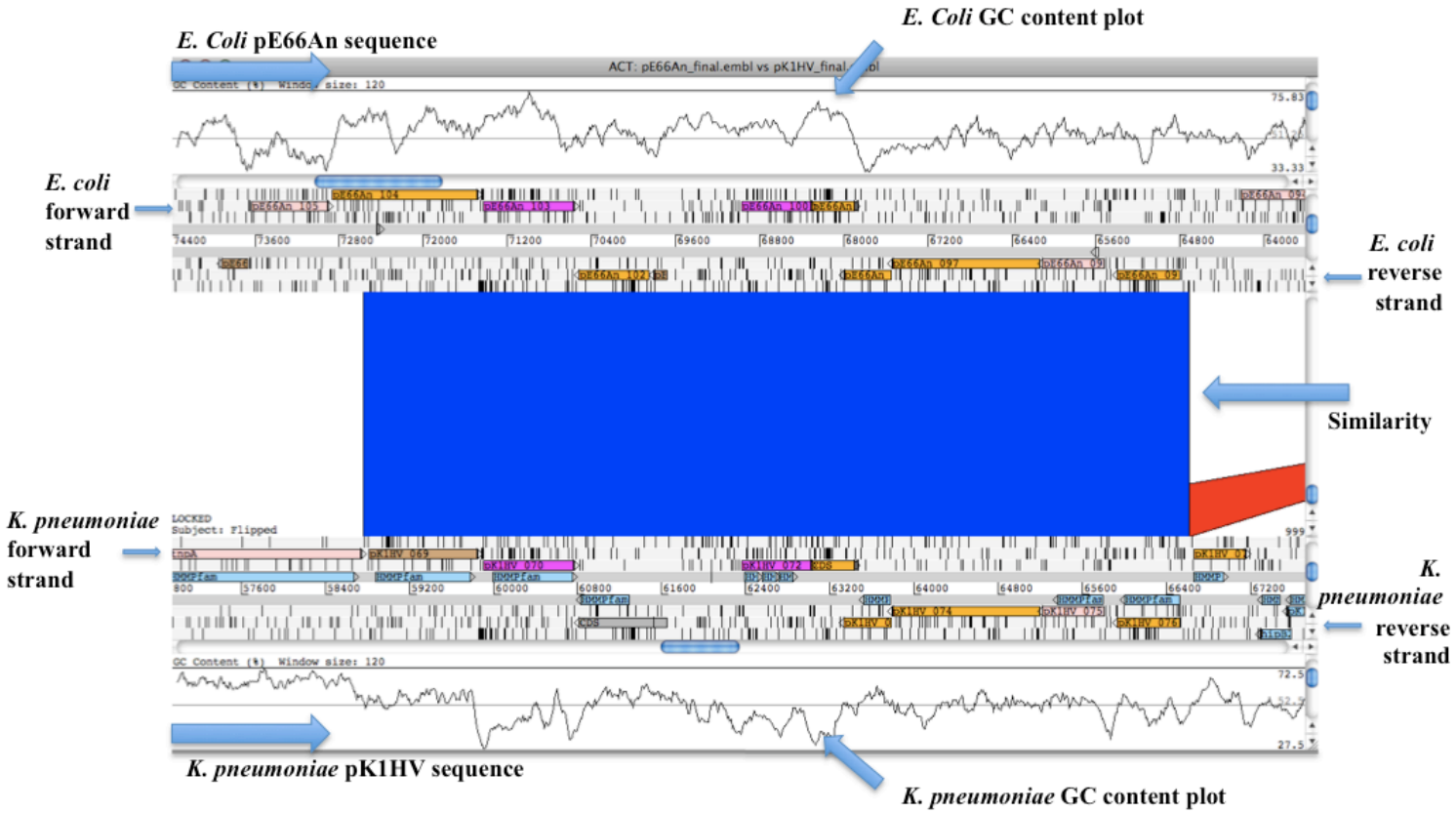


Figure 2.5 Comparing two genome sequences using Artemis Comparison Tool (ACT).

Displayed here is the DNA sequence of the pE66An aligned with pK1HV at the *qnrS1*-containing region. The representation of the two DNA sequences displays regions of high sequence similarity with a red bar connection the two regions, or blue if sequences in converse directions, regions with little or no similarity have no shading.

2.5. Identification of *qnrS1*-containing transposon circulating in the Enterobacteriaceae in Ho Chi Minh City

2.5.1. PCR detecting known *qnrS1*-containing transposon

In this study, the Expand Long Template PCR System was used (Roche, Germany) to amplify target sequences containing the *qnrS1*-containing mobile elements with unknown size. With the thermostable Taq DNA polymerase and Tgo DNA polymerase, this system is optimized to amplify large genomic DNA fragments up to 20 kb long.

The reactions were prepared on ice in 0.2 ml thin wall PCR tubes with the primers Trans-qnrS-F and Trans-qnrS-R (Table 2.6), following the manufacturer's instructions in a total volume of 50 μl, using the Expand Long Template PCR System. The quantities used in the PCR reaction mixture are outlined in Table 2.5.

DNA amplifications were performed using the following programme:

i. 1 cycle of 94^{o}C for 2 minutes.
ii. 20 cycles of 94^{o}C for 10 seconds, 55^{o}C for 30 seconds and 68^{o}C for 5 minutes and 30 seconds.
iii. 15 cycles of 94^{o}C for 10 seconds, 55^{o}C for 30 seconds and 68^{o}C for 5 minutes with 20 second increments per cycle.
iv. 1 cycle of 68^{o}C for 7 minutes.
v. Held at 12^{o}C.

Table 2.5 PCR mixture for PCR for detecting *qnrS*-containing transposon.

Reagents	Volume	Final concentration
10X buffer	5 µl	1X
50 mM $MgCl_2$	1 µl	1 mM
25 mM dNTPs	0.5 µl	0.25 mM
10 µM primer forward	1 µl	0.2 µM
10 µM primer reverse	1 µl	0.2 µM
5 U/µl Taq polymerase	0.15 µl	0.75 U
DNA template	5 µl	10 ng genomic DNA
H_2O	36.35 µl	-
	50 µl	

Table 2.6 Oligonucleotide primers used in PCR reactions for detecting *qnrS*-containing transposon.

Target genes	Primers	Predicted product size (bp)	Annealing temperature	Primer sequence (5' → 3')	Sources of reference
***qnrS1*-containing transposon**	Trans-qnrS-F Trans-qnrS-R	NA	55°C	CAGGAAGAGGCATTGTCAAAGG GGTGCTTGTCAGCGTAAA	This study

2.5.2. *qnrS1*-containing transposon Restriction Fragment Length Polymorphism (RFLP) typing

qnrS1-containing PCR amplicons were typed by RFLP with 3 different restriction endonucleases: *Eco*RV, *Hind*III and *Pvu*II (New England Biolabs). The restriction digested PCR amplicons were carried out with 100 ng of PCR amplicons which was digested at 37^{o}C overnight with 20U of *Eco*RV or *Hind*III or *Pvu*II in a 20 µl reaction, including of 2 µl of 10X buffer, 0.2 µl of 100X BSA, 1 µl of enzyme, 100 ng of PCR amplicons and distilled water up to 20 µl. The digested products were analyzed by electrophoresis at 80V for 2 hours with a 1% agarose gel and visualized in UV light after staining with 2% Ethidium Bromide for 10 minutes and examined on UV visualization system.

RFLP patterns were clustered and interpreted by combining the resulting digestion patterns of all three enzymes and analyzed using BioNumerics software. *qnrS1*-containing transposons were considered in a group when they had RFLP patterns that were identical in all 3 patterns digesting with the 3 restriction endonucleases *Eco*RV, *Hind*III and *Pvu*II.

2.5.3. Sequence of *qnrS1*-containing transposon using primer walking sequence method

The sequence of *qnrS1*-containing transposon from K34N strain was sequenced using primer walking sequence method with the primers are listed in Table 2.8. Each sequenced fragment was repeated 4 times for the reliability using ABI 3130xl machine (Applied Biosystems, US). The sequences were then assembled using Vector NTI software (InforMax, Inc.)

DNA amplifications for sequencing were performed using the following programme:

i. 1 cycle of 96°C for 1 minute.

ii. 26 cycles of 95°C for 5 seconds, 50°C for 10 seconds and 60°C for 2 minutes.

iii. Held at 12°C.

Table 2.7 PCR mixture for PCR for sequencing *qnrS*-containing transposon of K34N strain.

Reagents	Volume	Final concentration
Big dye Terminator	4 μl	1X
Buffer	2 μl	1X
DNA template	2 μl	20 ng genomic DNA
H_2O	12 μl	-
	20 μl	

Table 2.8 Oligonucleotide primers used in primer walking sequence of *qnrS1*-containing transposon of K34N strain.

Primers	Annealing temperature	Primer sequence (5' → 3')	Sources of reference
Trans-qnrS-F	55°C	CAGGAAGAGGCATTGTCAAAGG	This study
Trans-qnrS-R		GGTGCTTGTCAGCGTAAA	
Trans-qnrS-K34N-1-F	55°C	CGGTGCCGTTGTGAAGAGAG	
Trans-qnrS-K34N-1-R		GCGAGCCGTCCTGAAGTG	
Trans-qnrS-K34N-2-F	55°C	CAGGGAATGCAGGCCAACC	
Trans-qnrS-K34N-2-R		CCACTAACCAGTCAGCACATTG	
Trans-qnrS-K34N-3-F	55°C	TCAGTACGCTATGCCAGGAAC	
Trans-qnrS-K34N-3-R		CTGTCCGGTTTCGGCATGAT	
Trans-qnrS-K34N-4-F	55°C	CAGCGATTTTCAAACAACTC	
Trans-qnrS-K34N-4-R		TTCGTCGTTCCCAGGCAA	

2.5.4. Detection of known *qnrS1*-containing transposon subfamily

Based on sequences of the 3 sequenced-plasmids, it was found that in the *qnrS1*-containing transposon, the *qnrS1* gene was next to bla_{LAP-2} gene, a gene that confers resistance to β-lactam antimicrobials. As a result, a Southern blotting assay was designed to detect a subfamily of known *qnrS1*-containing transposon, which contains *qnrS1* and bla_{LAP-2} genes, in the isolates negative with PCR of detecting known *qnrS1*-containing transposon.

Plasmids containing subfamily of known *qnrS1*-containing transposon was detected by Southern blotting with 2 different probes: qnrS1 and bla_{LAP-2} (primer bla_{LAP-2}_F: 5' - TAC TCC AGC ATT ATC TAC ACC – 3'; blaLAP-2_R: 5' - CCG CCG ACT TTA TCT TCC – 3'), using Amersham ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare), according to the manufacturer's instructions.

Briefly, *qnrS1*-containing plasmid DNA from isolates negative with PCR of *qnrS1*-containing transposon was extracted using the QIAGEN Midi kit prep plasmid DNA extraction kit, as per manufacturer's recommendations (Qiagen, USA). These *qnrS1*-containing plasmids were then digested with *Eco*RI restriction endonuclease and run duplicated on a gel.

2.5.4.1. Treatment of gel

The gel was first soaked in depurination solution (0.25M HCl) twice for 10 minutes. The gel was then soaked in a denaturing solution (0.5M NaOH, 1.5M NaCl) for 2 x 20 minutes. Once denatured, the gel was neutralized by soaking twice for 20 minutes in a neutralizing solution (0.5M Tris HCl, 1.5M NaCl pH7.5). Finally, the gel was soaked in 20X SSC (175.3 g/l NaCl, 88.2 g/l sodium citrate) solution for 20 minutes.

2.5.4.2. DNA transfer onto nitrocellulose membrane

The DNA was transferred to a Hybond-N nitrocellulose membrane (Amersham). A platform was placed in 6X SSC and a filter paper wick was placed in the SSC and spanned the platform. The gel was positioned on the wick and three layers of wet (6X SSC) filter paper and three layers of dry filter paper were placed on the gel. To absorb the solution, 10 cm of paper towels were positioned on the stack. A perspex board was placed on top of the stack, and was weighted with approximately 5 kg. The DNA was then left to transfer under capillary action overnight at ambient temperature. After transfer, DNA was then linked to the membrane by placing the membrane in saran wrap and UV cross-linked using an automated cycle.

2.5.4.3. Labeling of Southern probes

The Southern probes (qnrS1 and bla_{LAP-2}) were amplified using PCR. Once quantified, 50-100 ng of the selected PCR products were placed in ultra pure water up to a final volume of 34 μl. DNA was denatured by boiling for 5 minutes and snap-cooled by placing immediately on ice. Once cooled, the following was added to give a total volume of 50 μl; 10 μl of nucleic acid mix, 5 μl of random primer mix and 1 μl of klenow enzyme (4U) (Amersham). The reaction mix was incubated at 37^{o}C for 1 hour. The reaction was stopped by the addition of 2 μl of 0.5M EDTA. Probes were stored at -20^{o}C until required.

2.5.4.4. Hybridisation

The nitrocellulose membrane was cut into 2 pieces and hybridized individually with 2 different probes, qnrS1 or bla_{LAP-2}. The membrane was placed in a hybridisation tube with 50 ml of hybridisation buffer (5X SSC, 5% dextran sulphate, 0.1% SDS,

5% (w/v) blocking agent), which was pre-heated to 42°C. The membrane was pre-hybridised for 1 hour at 42°C with rotation. Twenty microlitres of the labeled probe was boiled for 5 minutes and then snap cooled on ice. The probe was briefly centrifuged in a benchtop microcentrifuge, placed in the hybridisation buffer and incubated overnight at 42°C with rotation. The membrane was then washed 2 times x 20 minutes in 50 ml of pre-heated primary wash buffer (0.5X SSC, 0.4% (w/v) SDS, 6M urea) at 42°C with rotation. The membrane was then washed 2 times x 5 minutes in 50 ml of secondary wash buffer (2X SSC) at room temperature with rotation. Once washed, the membrane was removed, drained and placed DNA side down onto a piece of saran wrap containing 5 ml of detection reagent, and incubated for 1 minute at room temperature. The membrane was then removed, drained, covered in saran wrap and placed in a light proof cassette. The blot was exposed for 30 seconds and developed. If plasmid DNA of an isolate had a signal with both probes qnrS1 and bla_{LAP-2} at the same fragment, it was then assigned as carrying the subfamily of *qnrS1*-containing transposon.

2.6. Characterization of *qnrS1*-harbouring plasmids isolated from Enterobacteriaceae in Ho Chi Minh City

2.6.1. Electro-transformation

2.6.1.1. Electrocompetent cell preparation

The method used for electro-transformation was adapted from Bjorkman et al. (Bjorkman 1996). A colony of lab strain *E. coli* DH5α was grown in 5 ml of LB overnight. Two microlitres of the *E. coli* DH5α cultured broth was transferred to a 100 ml 37°C pre-warmed-LB flask and incubated with shaking at 37°C for 3 hours (or when OD600 = 0.3 - 0.4 and < 0.6). The culture was incubated on ice and

aliquoted into two 50 ml tubes. The cells were then harvested by centrifuging at 4,000 rpm for 15 minutes at 4°C. The bacterial pellet was subsequently washed with 10 ml of ice-cold glycerol 10% then centrifuged at 4,000 rpm for 10 minutes at 4°C. The bacterial pellet was washed another 5 times with 5 ml of ice-cold glycerol 10% with centrifugation as above. The tubes were always kept on ice and centrifugation was performed at 4°C. Once washed, the pellet was resuspended in 100 µl of glycerol 10% and aliquoted in 2 microfuge tubes, each contained 50 µl of competent cells. The competent cells were subsequently stored at -80°C until required.

2.6.1.2. Electro-transformation

The electro-transformation procedure used here was adapted from Dower et al. (Dower *et al.,* 1988). Aliquots (50 µl) of competent cells were thawed on ice and transferred to a cold 1.5 ml microfuge tube. One microlitre of plasmid DNA was added to the competent cells. The suspension was mixed carefully by tapping the side of the microfuge tube and was then transferred to a chilled 1-mm electroporation cuvette (Invitrogen, USA) and kept on ice. Competent cells alone with no plasmid DNA served as a control. Electro-competent bacteria were electroporated using a Bio-Rad Gene Pulser with the following conditions: 1.8 kV, 25 µF and 200 Ωhms. After the electrical pulse, the cells were immediately re-suspended in 1 ml fresh of electroporation recovery media; S.O.C solution (LB broth, 10mM MgCl2, 10mM MgSO4, 20mM glucose) (Invitrogen, USA) and incubated at 37°C for one hour. Transformants were selected by plating 100 µl of the culture on LB agar plates supplemented with 0.03 µg/ml of ciprofloxacin. All isolated organisms were sub-cultured and subjected to PCR amplification for the *qnrS* (as described in 2.3.8) to ensure transformation of the appropriate plasmid.

2.6.2. Plasmid sizing

2.6.2.1. Plasmid extraction using Kado-Liu method

Plasmid sizes were initially estimated using the alkaline lysis procedure, as described by Kado and Liu (Kado and Liu 1981). The method utilises the molecular characteristics of covalently closed circular DNA that is released from cells on digestion under conditions that denatures chromosomal DNA by using alkaline sodium dodecyl sulfate (pH 12.6) at elevated temperatures. Proteins and cell debris are removed by extraction with phenol-chloroform. Under these conditions, chromosomal DNA concentrations were reduced or eliminated. The extracted plasmid DNA was only used for electrophoresis analysis but not purified enough to do further molecular experiments such as running PCR or digesting with restriction enzymes.

Bacteria were grown overnight in 5 ml of LB broth. The cells were harvested by centrifuging at 4,000 rpm for 15 minutes at 4^{o}C. The bacterial pellet was subsequently resuspended in 150 μl of E buffer and 300 μl of lysis buffer (3 g SDS, 0.6055 g Tris base, 3.2 ml 2M NaOH, dissolved in 100 ml distilled water) and mixed gently. Once mixed, the mixture was heated at 55^{o}C for 1 hour. After incubation, the mixture was added with 600 μl of Phenol:Chloroform (24:1) and mixed gently until the solutions emulsified. The mixture was then centrifuged for 30 minutes at 15^{o}C to separate layers.

2.6.2.2. Plasmid sizing analysis

Thirty-two microlitre aliquots from the middle of the top layer were removed and separated by electrophoresis in 0.7% agarose gels made with 1X E buffer. Agarose

gels were run at 90 V for 3 h, stained with ethidium bromide, and photographed. *E. coli* 39R861 was used for plasmid sizing on agarose gels which contains plasmids of 7, 36, 63, and 147 kb. Estimated plasmid sizes (circular replicons) were subsequently adjusted according BioNumerics software, version 5.1 (Applied Maths, Belgium).

2.6.3. Plasmid typing

Plasmid DNA extracted from the *qnrS1*-tranformants was typed after endonuclease restriction mapping with *Eco*RI (GAATTC) (New England Biolabs, USA). One hundred nanograms of plasmid DNA was digested at 37^{o}C overnight with 20U of *Eco*RI in a 20 µl reaction, including of 2 µl of 10X buffer, 0.2 µl of 100X BSA, 1 µl of enzyme, plasmid DNA and distilled water up to 20 µl, prior to electrophoresis at 80V for two hours with a 1% agarose gel and visualized in UV light after staining with ethidium bromide. One kilobase pair ladder (Invitrogen, USA) was used as a fragment size marker.

2.6.4. Plasmid incompatibility grouping

Plasmids from *qnrS1*-transformants were assigned to incompatibility groups by PCR-based replicon typing performed on total DNA using previously described primers and conditions (Carattoli *et al.,* 2005; Garcia-Fernandez *et al.,* 2009). The methodology utilizes 21 pairs of primers targeting the replication genes of the incompatibility groups; FIA, FIB, FIC, HI1, HI2, I1-Igamma, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, R, U, colE and FIIA (Table 2.10). Positive controls strains for all incompatibility groups were provided by Carattoli.

The reactions were prepared on ice in 0.2 ml thin wall PCR, in a total volume of 50 µl, using Bioline Taq polymerase. The quantities used in the PCR reaction mixture

are outlined in Table 2.9. DNA amplifications were performed using the following programme:

i. 1 cycle of 94°C for 5 minutes.

ii. 35 cycles of 94°C for 30 seconds, 55°C* for 30 seconds, and 72°C for 30 seconds.

iii. 1 cycle of 72°C for 5 minutes.

iv. Held at 12°C.

*Annealing temperature depends on Tm of primers; 55°C is an estimated average. The precise annealing temperature of each target is listed on Table 2.10.

Table 2.9 PCR mixture for PCR for determining Inc plasmid groups.

Reagents	**Volume**	**Final concentration**
10X buffer	5 μl	1X
50 mM $MgCl_2$	2.5 μl	25 mM
25 mM dNTPs	0.5 μl	0.25 mM
10 μM primer forward	1 μl	0.2 μM
10 μM primer reverse	1 μl	0.2 μM
5 U/μl Taq polymerase	0.15 μl	0.75 U
DNA template	5 μl	10 ng genomic DNA
H_2O	34.85 μl	-
	50 μl	

Table 2.10 Oligonucleotide primers used in PCR reactions for determining Inc plasmid groups.

Target genes	Primers	Predicted product size (bp)	Annealing temperature	Primer sequence (5' → 3')	Sources of reference
parA-parB	HI1 FW	471	60°C	GGAGCGATGGATTACTTCAGTAC	(Carattoli *et al.,* 2005)
	HI1 RV			TGCCGTTTCACCTCGTGAGTA	
iterons	HI2 FW	644	60°C	TTTCTCCTGAGTCACCTGTTAACAC	
	HI2 RV			GGCTCACTACCGTTGTCATCCT	
RNAI	I1 FW	139	60°C	CGAAAGCCGGACGGCAGAA	
	I1 RV			TCGTCGTTCCGCCAAGTTCGT	
ori γ	X FW	376	60°C	AACCTTAGAGGCTATTTAAGTTGCTGAT	
	X RV			TGAGAGTCAATTTTTATCTCATGTTTTAGC	
repA,B,C	L/M FW	785	60°C	GGATGAAAACTATCAGCATCTGAAG	
	L/M RV			CTGCAGGGGCGATTCTTTAGG	
repA	N FW	559	60°C	GTCTAACGAGCTTACCGAAG	
	N RV			GTTTCAACTCTGCCAAGTTC	
iterons	FIA FW	462	60°C	CCATGCTGGTTCTAGAGAAGGTG	
	FIA RV			GTATATCCTTACTGGCTTCCGCAG	
repA	FIB FW	702	60°C	GGAGTTCTGACACACGATTTTCTG	
	FIB RV			CTCCCGTCGCTTCAGGGCATT	
repA	W FW	242	60°C	CCTAAGAACAACAAAGCCCCCG	
	W RV			GGTGCGCGGCATAGAACCGT	
repA	Y FW	765	60°C	AATTCAAACAACACTGTGCAGCCTG	
	Y RV			GCGAGAATGGACGATTACAAAACTTT	
iterons	P FW	534	60°C	CTATGGCCCTGCAAACGCGCCAGAAA	
	P RV			TCACGCGCCAGGGCGCAGCC	
repA2	FIC FW	262	60°C	GTGAACTGGCAGATGAGGAAGG	
	FIC RV			TTCTCCTCGTCGCCAAACTAGAT	
repA	A/C FW	465	60°C	GAGAACCAAAGACAAAGACCTGGA	
	A/C RV			ACGACAAACCTGAATTGCCTCCTT	
repA	T FW	750	60°C	TTGGCCTGTTTGTGCCTAAACCAT	
	T RV			CGTTGATTACACTTAGCTTTGGAC	
repA	FII_S FW	270	60°C	CTGTCGTAAGCTGATGGC	
	FII_S RV			CTCTGCCACAAACTTCAGC	
RNAI/repA	F_{repB} FW	270	60°C	TGATCGTTTAAGGAATTTTG	
	F_{repB} RV			GAAGATCAGTCACACCATCC	
RNAI	K/B FW	160	60°C	GCGGTCCGGAAAGCCAGAAAAC	
	K RV			TCTTTCACGAGCCCGCCAAA	
RNAI	K/B FW	159	60°C	GCGGTCCGGAAAGCCAGAAAAC	
	B/O RV			TCTGCGTTCCGCCAAGTTCGA	
colE	oricolE FW	187	55°C	GTT CGT GCA TAC AGT CCA	(Garcia-Fernandez *et al.,* 2009)
	oricolE RV			GGC GAA ACC CGA CAG GAC T	
IncR	IncR FW	251	55°C	TCG CTT CAT TCC TGC TTC AGC	
	IncR RV			GTG TGC TGT GGT TAT GCC TCA	
IncU	IncU FW	843	55°C	TCA CGA CAC AAG CGC AAG GG	
	IncU RV			TCA TGG TAC ATC TGG GCG C	

2.6.5. Antimicrobial resistance gene miniaturized DNA microarray

Genomic DNA was isolated from 33 *qnrS1* plasmid containing transformants and the original recipient strain using the wizard genomic DNA preparation Kit (Promega, USA) after bacterial cells were cultured overnight at 37^{o}C in LB broth. Genomic DNA was hybridized to a miniaturized DNA microarray containing antimicrobial resistance genes. The miniaturized DNA microarray contains oligonucleotide probes for 54 antimicrobial resistance genes considered to be of clinical importance, including Beta-lactams, tetracycline, aminoglycosides, sulphonamides, chloramphenicol, trimethoprim, quinolones and two integrase genes associated with class 1 and class 2 integrons (Kauko *et al.,* 2010). All hybridizations to the antimicrobial resistance gene miniaturized DNA microarray were conducted by AbuOun and Morrison at the Veterinary Laboratories Agency, Weybridge, Surrey in the United Kingdom, using the following method.

Two micrograms of genomic DNA from each strain were linearly amplified using a set of 89 antisense primers and simultaneously biotin labelled (Batchelor *et al.,* 2008). Single-stranded labeled amplified products were then hybridized to the arrays (Anjum *et al.,* 2007). The signal intensity was determined for each spot on the array by calculating the quantitative staining value using IconoClust software (Figure 2.6). Based on previous data analysis and validation, probes with intensity values ≥ 0.4 were considered positive whilst those < 0.3 were considered negative (Kauko *et al.,* 2010). Probes with intensity values between 0.3 and <0 .4 were considered ambiguous.

The result of miniaturized DNA microarray with antimicrobial probes was imported to BioNumerics in the type of “presence” (with positive probe) or “absence” (with

negative probe). The Pearson correlation algorithm was used to group these transformants based on the combination of presence or absence of the tested antimicrobial probes in each isolate.

2.6.6. Microarray phenotyping (Biolog)

Phenotypic characteristics were assayed using Biolog's phenotype microarray $(PM)^3$ system (Hayward, CA), allowing high-through-put screening of bacterial respiration response against a range of stimuli including carbon, nitrogen, phosphorus and sulphur sources and a range of metabolic effectors including antimicrobials (AbuOun *et al.,* 2009; Kauko *et al.,* 2010). All hybridizations to the microarray biolog were conducted by AbuOun and Morrison at the Veterinary Laboratories Agency, Weybridge, Surrey in the United Kingdom**.**

The micro-plates used for phenotypic analysis were carbon sources (manufacturers codes; PM01 and 02A), nitrogen sources (03B), phosphorus and sulphur (04A) and chemicals/antimicrobials (13B, 14A, 16A and 18C). All fluids, reagents and PM panels were used according to the manufacturer's instructions. Briefly, bacterial strains were cultured for 16 hours on LB-G agar plates at 37^oC, aerobically. Cells were harvested from the agar surface and adjusted to 85% transmittance (T) in inoculating fluid (IF-0a). Prior to addition to PM microtitre plates, bacterial suspensions were further diluted into 12 ml of IF-0a (per plate) in the relevant inoculating fluid. For PM03B and PM04A that measure nitrogen, phosphorus and sulphur utilization, IF-0a was supplemented with a final concentration of 0.02 M sodium succinate/0.002 mM ferric citrate and 25 μM L-leucine. Substrate utilization was measured via cellular respiration using a tetrazolium dye A, supplied by Biolog Inc (Hayward, USA). All microplates were incubated in the Omnilog instrument at

37^{o}C and monitored for colour change at 15-minute intervals for 26 hours. Kinetic data was analysed with OmniLog-PM software (Biolog). All experiments were performed in duplicate and comparisons between strains were based on the average of the area under the curve values at 26 hours (Figure 2.7) (Kauko *et al.,* 2010).

For each chemical test, Chi-squared test was used to analyse differences of area under the curve values at 26 hours of all isolates. If the Chi-squared test showed any difference among them with p value less than 0.05, the growth curves of all isolates in the presence of the tested chemical were drawn.

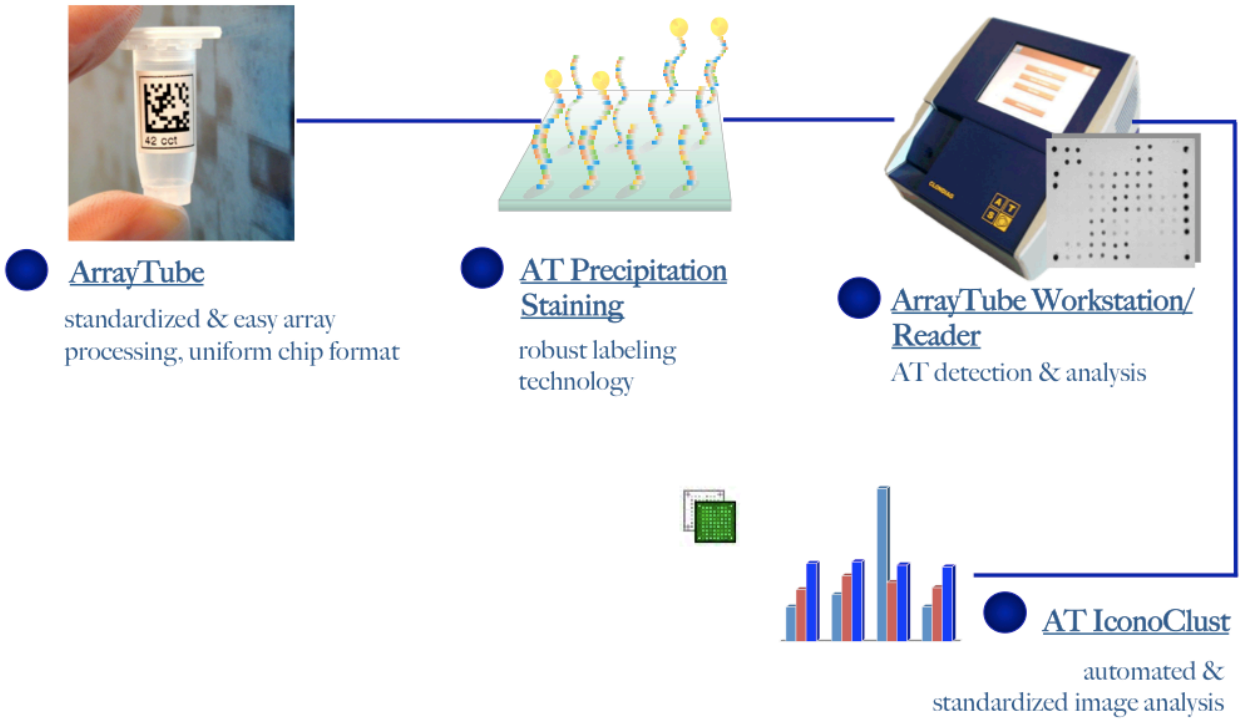


Figure 2.6 Scheme of procedure of miniaturized DNA microarray.

A primer elongation reaction was used before hybridization with the DNA array. An aliquot of this labeled sample was then transferred into the ArrayTube and incubated for 60 minutes at 50°C. The staining reaction was performed according to the manufacturer's instructions. The ArrayTube was then placed into the ATR 04 reading device. The AT IconoClust software was used to record and analyzed the arrays.

Figure 2.7 Scheme of procedure of microarray phenotyping (Biolog).

Bacterial suspensions were added into the plates containing tetrazolium dye. If the phenotype is strongly "positive" in a well, the cells form a strong colour and if the phenotype is weakly negative, no colour is formed in the well (a). The OmniLog instrument used to incubate plates and capture digital image of the microarray several times each hour and stores the quantitative color change values into computer files (b). The computer files are displayed in the form of kinetic graphs. To compare the phenotype of 2 cell lines, one is recorded as a red tracing and one as green tracing. The graphs were then overlaid by the software to detect differences. Areas of overlap are coloured yellow, whereas differences are highlighted as patches of red or green (c).

2.7. Real-time PCR to quantify PMQR determinants in children with ARIs

2.7.1. Introduction of samples

The inclusion criteria for this study were children less than 16 years of age, symptoms of an ARI, not admitted to a hospital, informed consent given by parents or legal guardians, living in Ho Chi Minh City and agreeing to return for follow up visit after 1 week. Age, demographics, and prescribed antimicrobials on enrolment were recorded on the first day. Six hundred rectal swabs were taken from 300 children with ARIs admitted to the Outpatient Department in Children's Hospital Number 1 in Ho Chi Minh City and were collected at enrolment and day 7. Rectal swabs were then submerged in 1 ml of sterile saline and stored at -80 °C until analysis.

2.7.2. Colony counting

To calculate the number of bacteria in the rectal swabs collected from children with ARIs, a set of ten-fold serial dilutions from 10^{-1} to 10^{-8} of samples in saline were made. Fifty microlitres of each was spread on MacConkey agar and incubated at 37^{o}C overnight. The colony number was counted and the dilution factor of the plate containing 20-200 colonies was used for calculations. The estimated number of bacteria in 50 μl of specimen was calculated by the number of colonies counted on the plate multiplied by the dilution factor.

2.7.3. Total DNA extraction from rectal swabs

Total DNA from rectal swabs was extracted from 200 μl of solution of rectal swab in 1 ml of saline with 20 μl of DNA of Phocine herpesvirus (PhHV) (van Doornum *et al.,* 2003), which was used as an internal control, and eluted in 50 μl of elution buffer

using the automated nucliSENS EasyMag system (bioMérieux, Mercy l'Etoile, France) according to the manufacturer's instructions. To monitor the efficiency of DNA extraction, and real-time PCR reaction later, a known quantity of PhHV DNA was included with the rectal samples before DNA extraction. We added the PhHV DNA in each rectal sample with the amount that the cycle threshold (Ct) value of PhHV in real-time PCR was from 33 to 35 cycles. After running real-time PCR, the Ct value of PhHV was used as signal to calculate the efficiency of DNA extraction and the real-time PCR.

The automated nucliSENS EasyMag system automated extract DNA based on the property of nucleic acid binding to silica. In brief, the procedure includes 4 main steps: lysis and magnetic silica binding; washing to purify nucleic acid bound to silica; elution mixing of magnetic silica in final buffer combined with heating results in increasing efficient elution; and the last step is final purification to remove magnetic silica and elute with pure, concentrated nucleic acid. Eluted DNA was then stored at -20^{o}C until used.

2.7.4. Primers and probes design for Real-time PCR

Nucleotide sequences of the required full-length alleles of target genes (*qnrA*, *qnrB* and *qnrS*) were retrieved from NCBI and were aligned using Vector NTI (Invitrogen). Primers and probes were designed using Primer Express version 2.0 (Applied Biosystems Inc.). These primers and probes were then checked using BLAST against the NCBI database and were synthetized by Sigma-Proligo, Singapore. The primers and probes used for real-time PCR are shown in Table 2.11.

Table 2.11 Oligonucleotide primers used in real-time PCR reactions for PMQR determinants.

Target genes	Primers	Predicted product size (bp)	Annealing temperature	Primer sequence (5' → 3')	Sources of reference
qnrA	qnrA_RT_F	148	52°C	CAGTTTCGAGGATTGCAGTT	This study
	qnrA_RT_R			CCTGAACTCTATGCCAAAGC	
	qnrA_probe			**Fam - AAGGGTGYCACTTCAGCTATGCC - Tamra**	
qnrB	qnrB_RT_F	104	54°C	CAGATTTYCGCGGCGCAAG	This study
	qnrB_RT_R			TTCCCACAGCTCRCAYTTTTC	
	qnrB_probe			**Fam - CGCACCTGGTTTTGYAGYGCMTATATCAC - Tamra**	
qnrS	qnrS_RT_F	157	55°C	TCAAGTGAGTAATCGTATGTA	This study
	qnrS_RT_R			GTCTGACTCTTTCAGTGAT	
	qnrS_probe			**Fam – CCAGCGATTTTCAAACAACTCAC - Tamra**	
PhHV	PhHVforw	89	52-55°C	GGGCGAATCACAGATTGAATC	(van Doornum *et al.,* 2003)
	PhHVrev			GCGGTTCCAAACGTACCAA	
	PhHVprobe			**Cy5 – TTTTTATGTGTCCGCCACCATCTGGATC - BHQ3**	

2.7.5. Cloning of target gene to plasmids

We chose 3 *E. coli* isolates that contained *qnrA1*, *qnrB1* and *qnrS1*, which had been confirmed by PCR and sequencing, the *qnrA1*, *qnrB1* and *qnrS1* PCR fragments were cloned into plasmid pCR2.1-TOPO, and transferred into competent *E. coli* TOP10 cells using TOPO TA Cloning (Invitrogen), according to the manufacturer's instructions.

The transformation mix included 2 μl of PCR product, 0.5 μl of salt solution and 0.5 μl of TOPO vector; the solution was mixed gently and incubated at room temperature for 15 minutes. Once incubated, 15 μl of pre-thawed One Shot *E. coli* was added to the mix and incubated again on ice for 15 minutes. The cells were heat shocked by incubating the mix at 42^{o}C for 30 seconds and the tubes were transferred directly to ice. One hundred microlitres of S.O.C medium was added to each tube and incubated at 37^{o}C for 1 hour. Once incubated, the mixture was spread onto LB plates supplemented with 40 μg/ml of X-gal and 50 μg/ml of Ampicillin and incubated at 37^{o}C for overnight.

On the following day, 8 white colonies were selected and cultured on LB agar supplemented with X-gal and Ampicillin. After subculture, a colony was re suspended in 5 μl of LB broth and was used as DNA template for colony PCR with M13 primers (Table 2.13) to detect successfully cloned genes. The quantities used in the PCR reaction mixture are outlined in Table 2.12. DNA amplifications were performed using the following programme:

i. 1 cycle of 95^{o}C for 5 minutes.

ii. 35 cycles of 95^{o}C for 15 seconds, 55^{o}C for 30 seconds, and 72^{o}C for 1

minute.

iii. 1 cycle of 72^{o}C for 5 minutes.

iv. Held at 12^{o}C.

PCR amplification was analyzed on electrophoresis and any transformants produced the PCR amplification more than 200 bp were further extracted plasmid DNA from these transformants using the QIAprep Miniprep kit (QIAGEN) and carried out PCR amplification for the inserted gene. Once the inserted gene had been confirmed, the transformants were stored in BHI broth plus 5% glycerol at -80^{o}C until required.

Table 2.12 PCR mixture used in PCR with primer M13 for detecting successful transformants.

Reagents	Volume	Final concentration
10X buffer	5 μl	1X
50 mM $MgCl_2$	2.5 μl	25 mM
25 mM dNTPs	0.5 μl	0.25 mM
10 μM primer M13 forward	1 μl	0.2 μM
10 μM primer M13 reverse	1 μl	0.2 μM
5 U/μl Taq polymerase	0.15 μl	0.75 U
DNA template	7 μl	-
H_2O	32.85 μl	-
	50 μl	

Table 2.13 Oligonucleotide of M13 primer used in PCR for detecting successful transformants.

Primers	Predicted product size (bp)	Annealing temperature	Primer sequence (5' → 3')	Sources of reference
M13-F	NA	55°C	GTAAAACGACGGCCAG	(commercial)
M13-R			CAGGAAACAGCTATGAC	

2.7.6. Standard curve

The plasmid DNA from the transformants was digested with Xho enzyme (which has no restriction sites within the *qnrA*, *qnrB* and *qnrS* genes) to cut the supercoiled plasmids to form a linear structure, DNA was purified using QIAquick PCR Purification Kit (QIAGEN) and quantified using the formula (created by Andrew Staroscik, the University of Rhode Island Genomics & Sequencing Center, http://www.uri.edu/research/gsc/resources/cndna.html):

number of copies = (amount x $6.022x10^{23}$) / (length x $1x10^{9}$ x 650)

Where amount is calculated in ng; length is calculated in bp; $6.022x10^{23}$ is Avogadro's number (number of molecules per mole) and $1x10^{9}$x 650 is the number of nanograms per mole of DNA (per bp). Based on copy number results, serial dilutions of plasmid DNA were used to produce a standard curve and a linear regression was calculated based on the standard curve.

2.7.7. Quantitative real-time PCR

Real-time PCR was performed to quantify *qnr* genes copy number in rectal swabs collected from children with ARIs, using a Chromo 4 real-time PCR machine (Bio-Rad). The scheme of real-time PCR is shown in Figure 2.8. Reactions were prepared on ice in 0.2 ml thin wall PCR tubes, using a total volume of 25 μl with HotStart Taq polymerase (QIAGEN). The quantities in PCR reactions are outlined in Table 2.14 and amplifications were performed using the following programme:

i. 1 cycle of 95^{o}C for 15 minutes.

ii. 45 cycles of 95^{o}C for 30 seconds, 55^{o}C* for 30 seconds, and 72^{o}C for 30 seconds.

*Annealing temperature is dependent on the Tm of primers where 55^{o}C is an average. The precise annealing amplification temperatures are listed in Table 2.11.

Table 2.14 PCR mixture used in real-time PCR for quantifying PMQR determinants.

Reagents	**Volume**	**Final concentration**
10X buffer	2.5 µl	1X
25 mM $MgCl_2$	3.5 µl	3.5 mM + 1.5 mM in 10X buffer
25 mM dNTPs	0.5 µl	0.25 mM
10 µM primer forward	1 µl	0.4 µM
10 µM primer reverse	1 µl	0.4 µM
10 µM probe	0.5 µM	0.2 µM
10 µM primer PhHV forward	1 µM	0.4 µM
10 µM primer PhHV reverse	1 µM	0.4 µM
10 µM probe PhHV	0.5 µM	0.2 µM
5 U/µl Taq polymerase	0.15 µl	0.75 U
DNA template	5 µl	-
H_2O	33.35 µl	-
	50 µl	

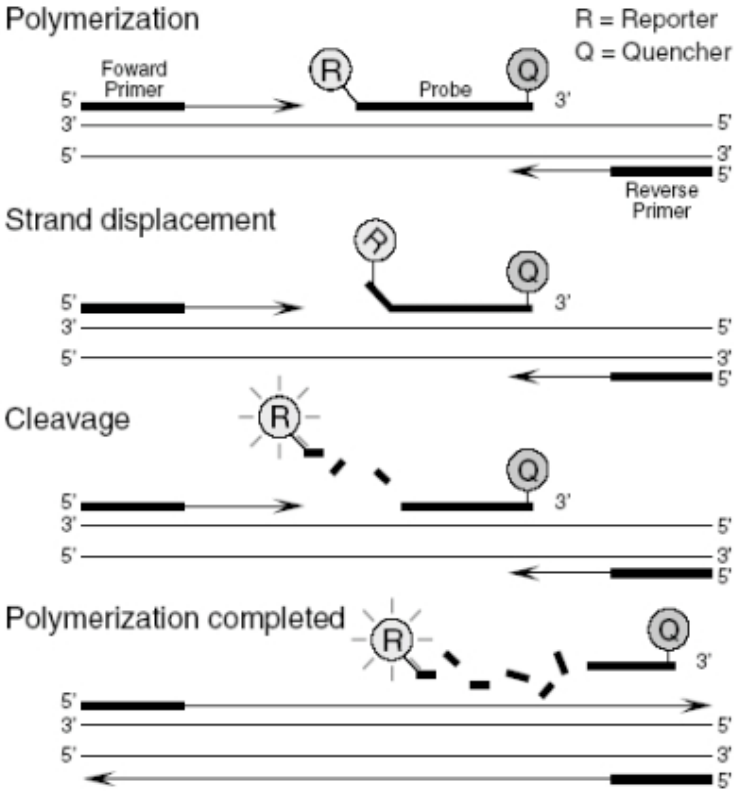


Figure 2.8 Schematic diagram of real-time PCR amplification.

A probe is designed to anneal to the target sequence between the forward and reverse PCR primers. A fluorescent reporter and a quencher are attached to the 5' and 3' ends of the probe. When the probe is intact, the reporter dye emission is quenched. During each PCR extension cycle, the DNA polymerase has a 5' to 3' nuclease activity and cleaves the reporter dye from the probe. Once separated from the quencher, the reporter dye emits a fluorescent signal.

2.7.8. Analysis of real-time PCR data

For each real-time PCR replicate, positive controls were amplified along with the samples to create a standard curve. *qnr* genes were then quantified using the standard curve as described above. The Paired Wilcoxon rank-sum and Pearson's Chi-squared test were used to compare non-normal distribution data between groups. Statistical analysis was performed using R 2.12.0 (*www.r-project.org).* A p value of ≤ 0.05 was used to infer significance for all statistical analyses.

3. Prevalence of plasmid-mediated quinolone resistance determinants in commensal members of the Enterobacteriaceae in Ho Chi Minh City, Vietnam

3.1. Abstract

Antimicrobial-resistant pathogenic members of the Enterobacteriaceae are a well-defined global problem. It was hypothesized that one of the main reservoirs of dissemination of antimicrobial resistance genes in Vietnam is non-pathogenic intestinal flora, and this study sought to isolate antimicrobial-resistant organisms from hospitalized patients and non-hospitalized healthy individuals in Ho Chi Minh City. The results identified substantial faecal carriage of gentamicin, ceftazidime and nalidixic acid resistant members of the Enterobacteriaceae in both hospitalized patients and non-hospitalized healthy individuals. A high prevalence of quinolone resistance determinants was identified, particularly the *qnrS* gene, in both community-associated and hospital-associated strains. Furthermore, the results demonstrate that a combination of quinolone resistance determinants can confer an increased resistance to nalidixic acid and ciprofloxacin, even in the apparent absence of additional chromosomal resistance mutations in wild-type strains and laboratory strains with transferred plasmids. These data suggest that intestinal commensal organisms are a significant reservoir for the dissemination of plasmid-mediated quinolone resistance in Ho Chi Minh City.

3.2. Introduction

Pathogenic enteric bacteria that exhibit antimicrobial resistance are widespread phenomenon and arguably constitute a global epidemic (Denton 2007). Whilst the depth of knowledge is broad regarding antimicrobial resistant organisms isolated from infected patients or circulating in the hospital environment, less is known about antimicrobial resistant organisms that are disseminated in the community. Furthermore, little is known about the antimicrobial resistance patterns of community-acquired organisms that circulate in developing countries where antimicrobials are available without prior consultation of a physician, such as Vietnam.

Fluoroquinolone resistance is an emerging problem, particularly in the treatment of Enterobacteriaceae infections, such as urinary tract infections, pneumonia or meningitis (Murray *et al.,* 1998). Many studies have highlighted that resistance to fluoroquinolones has increased globally over the last ten or more years (Figure 3.1), particularly in the Enterobacteriaceae including *E. coli*, *Salmonella* and *Klebsiella* species (Chau *et al.,* 2007; Denton 2007; Strahilevitz *et al.,* 2007; Wang, A. *et al.,* 2008a; Warburg *et al.,* 2008). Resistance to fluoroquinolones in the Enterobacteriaceae is mainly associated with mutations in the *gyrA* or *parC* genes, which are transmitted vertically (Weigel *et al.,* 1998). However, with the recent detection of plasmid-mediated quinolone resistance determinants, it has been shown that a fluoroquinolone resistance phenotype can also be transferred horizontally between organisms. These data suggest that the fluoroquinolone resistance may be able to spread rapidly between organisms that share the same environment (Warburg *et al.,* 2008). As shown in Figure 3.1, the percentage of organisms that are non-

susceptible to fluoroquinolones increases with the presence of *aac(6')-Ib-cr*, a type of PMQR determinant (Warburg *et al.,* 2008).

There are several reports from multiple continents regarding the prevalence of PMQR determinants in Enterobacteriaceae showing the prevalence of PMQR genes has increased over time (Strahilevitz *et al.,* 2009). The diversity of PMQR determinants has also changed over time. For example, in Asia, the majority of isolates collected before 2005 were only positive with *qnrA* gene. However, in isolates collected after 2005, almost all of the studies showed positive results with four PMQR types (*qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*) (Strahilevitz *et al.,* 2009).

In this chapter, an investigation was conducted on the frequency of carriage of nalidixic, ciprofloxacin, gentamicin and amikacin resistant Enterobacteriaceae isolated from patients and healthy individuals living in Ho Chi Minh City, Vietnam. Additionally the prevalence of 5 PMQR genes (*qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr* and *qepA*) was determined in the isolated strains and their effect on the MIC with and without additional resistance associated chromosomal mutations.

The samples used here were isolated from two individual populations: hospital strains and community strains. The hospital strains were comprised of the Enterobacteriaceae isolated from 194 patients admitted to the Tetanus ward of the Hospital for Tropical Diseases between April to November 2004 and May to December 2005. The community strains were comprised of Enterobacteriaceae isolated from 27 healthy adults and 77 healthy children who participated in a typhoid vaccine trial and from 100 healthy neonates from an obstetric department from Hung Vuong hospital in Ho Chi Minh City from 29th May to 29th June 2006 (methods section 2.1).

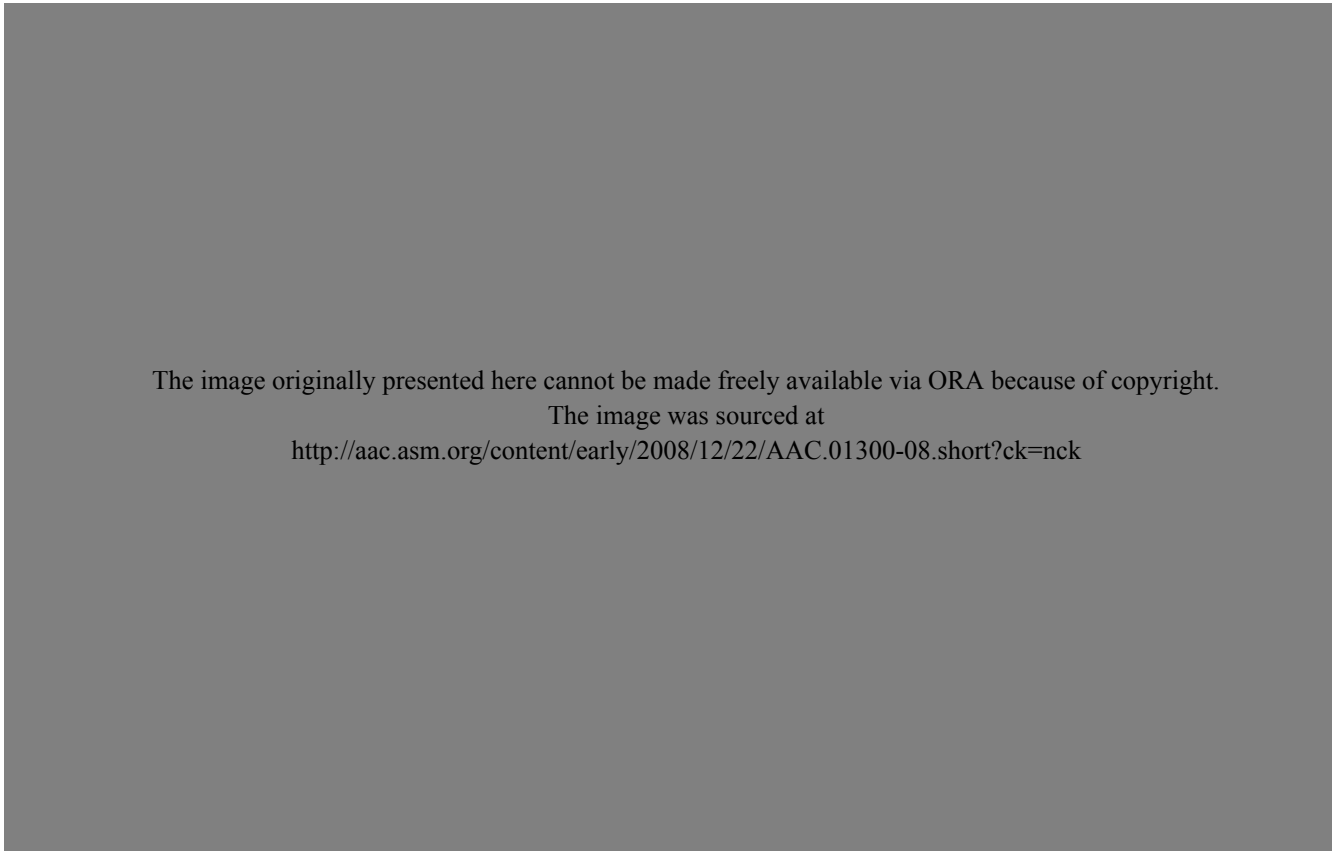


Figure 3.1 Graph showing the percentage of clinical *E. coli* isolates non-susceptible to ciprofloxacin in a hospital in Israel.

The figure shows the percentage of clinical *E. coli* isolates (n = 718) harbouring resistance to ciprofloxacin (MIC ≥ 2 μg/ml) and harbouring *aac(6')-Ib-cr* from 1991 to 2005 in a hospital in Israel. The figure demonstrates a gradual increase of ciprofloxacin resistance from 0% in 1991 to 35% in 2005, adapted from Warburg and colleagues (Warburg *et al.,* 2008).

3.3. Results

3.3.1. Isolation of antimicrobial-resistant commensal Enterobacteriaceae from hospital group

Three thousand one hundred and twenty three swabs from the axilla, nose, sputum and anus were collected from 194 hospitalized patients admitted to the tetanus ward of the Hospital for Tropical Diseases, Ho Chi Minh City, between April to November 2004 and May to December 2005. These swabs were taken on admission and twice weekly to discharge day. The collected swabs were cultured on MacConkey agar supplemented with gentamicin (8 μg/ml) or ceftazidime (2 μg/ml). Only the first isolated *K. pneumoniae* grew on MacConkey supplemented with gentamicin and the first *E. coli* or other Enterobacteriaceae (except for *K. pneumoniae*) grew on MacConkey supplemented with ceftazidime and confirmed ESBL positive were included in the study. The detail of differences between hospital isolates selection is shown in the flowchart in Figure 3.2.

In total, 70 *E. coli*, 123 *K. pneumoniae* and 29 other members of Enterobacteriaceae were isolated, including *Citrobacter* species, *Enterobacter cloacae*, *Proteus mirabilis*, *Klebsiella orrithinolytica*, *Pantoea* species and *Vibrio fluvialis*. All isolated organisms were resistant to gentamicin, ceftazidime or a combination of these antimicrobials.

RAPD was used to distinguish in all isolated bacteria and to determine the proportion of unique isolates. From the RAPD results, 53 unique *E. coli* strains, 62 unique *K. pneumoniae* strains and 16 other unique Enterobacteriaceae were identified (Figure 3.2, Figure 3.3) (Table 9.4, Appendices) during this sampling regime.

In summary, from the 194 tetanus-hospitalized patients, 45 (23.2%) patients carried antimicrobial resistant Enterobacteriaceae on admission the hospital, 104 (53.6%) patients acquired antimicrobial resistant Enterobacteriaceae after admission to hospital and in 45 (23.2%) patients it was not possible to isolate antimicrobial resistant Enterobacteriaceae on admission or during the period of stay at the hospital.

Based on the combination of RAPD patterns with 3 primers ERIC1, ERIC2 and TT3, a phylogenetic tree was constructed using BioNumerics software. Two isolates were designated to be the same type if the patterns for all 3 primers were identical.

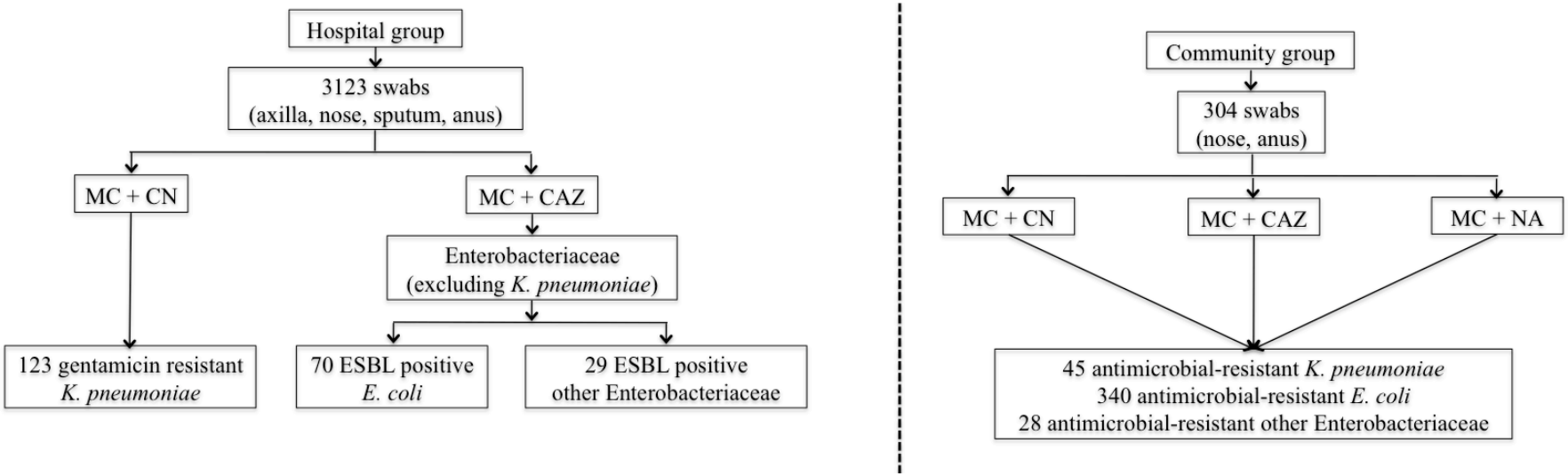


Figure 3.2. Flowchart isolate selection within the hospital and community group.

The figure shows how isolates from the hospital and community groups were selected. In the hospital group, only gentamicin-resistant *K. pneumoniae* and Enterobacteriaceae that were ESBL positive were analyzed. In the community group, all Enterobacteriaceae that were resistant to gentamicin and/or ceftazidime and/or nalidixic acid were examined.

* MC: MacConkey media; CN: gentamicin (8 μg/ml); CAZ: ceftazidime (2 μg/ml); NA: nalidixic acid (16 μg/ml).

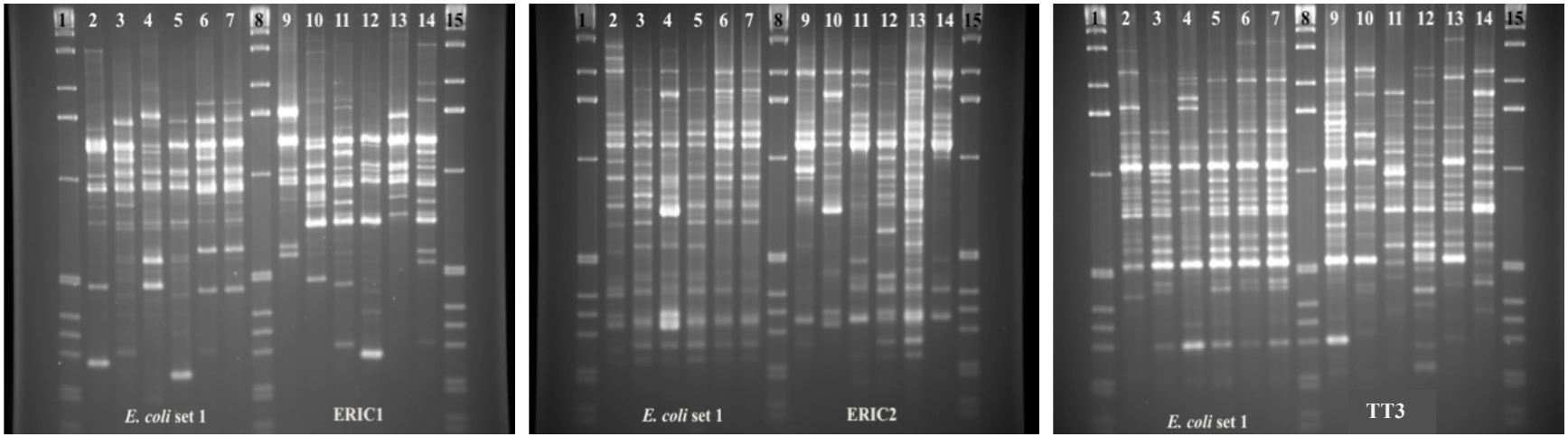


Figure 3.3 RAPD of *E. coli* isolates using the PCR primers ERIC1, ERIC2 and TT3.

Example of an RAPD profile from a set of *E. coli* isolates using the PCR primers ERIC1, ERIC2 and TT3. Lanes: 1; 1 Kb plus ladder (Invitrogen), 2; E18An, 3; E20An, 4; E23An, 5; E27An, 6; E29An, 7; E31An, 8; 1 Kb plus ladder (Invitrogen), 9; E32An, 10; E34An, 11; E35An, 12; E38An, 13; E44An, 14; E45An, 15; 1 Kb plus ladder (Invitrogen).

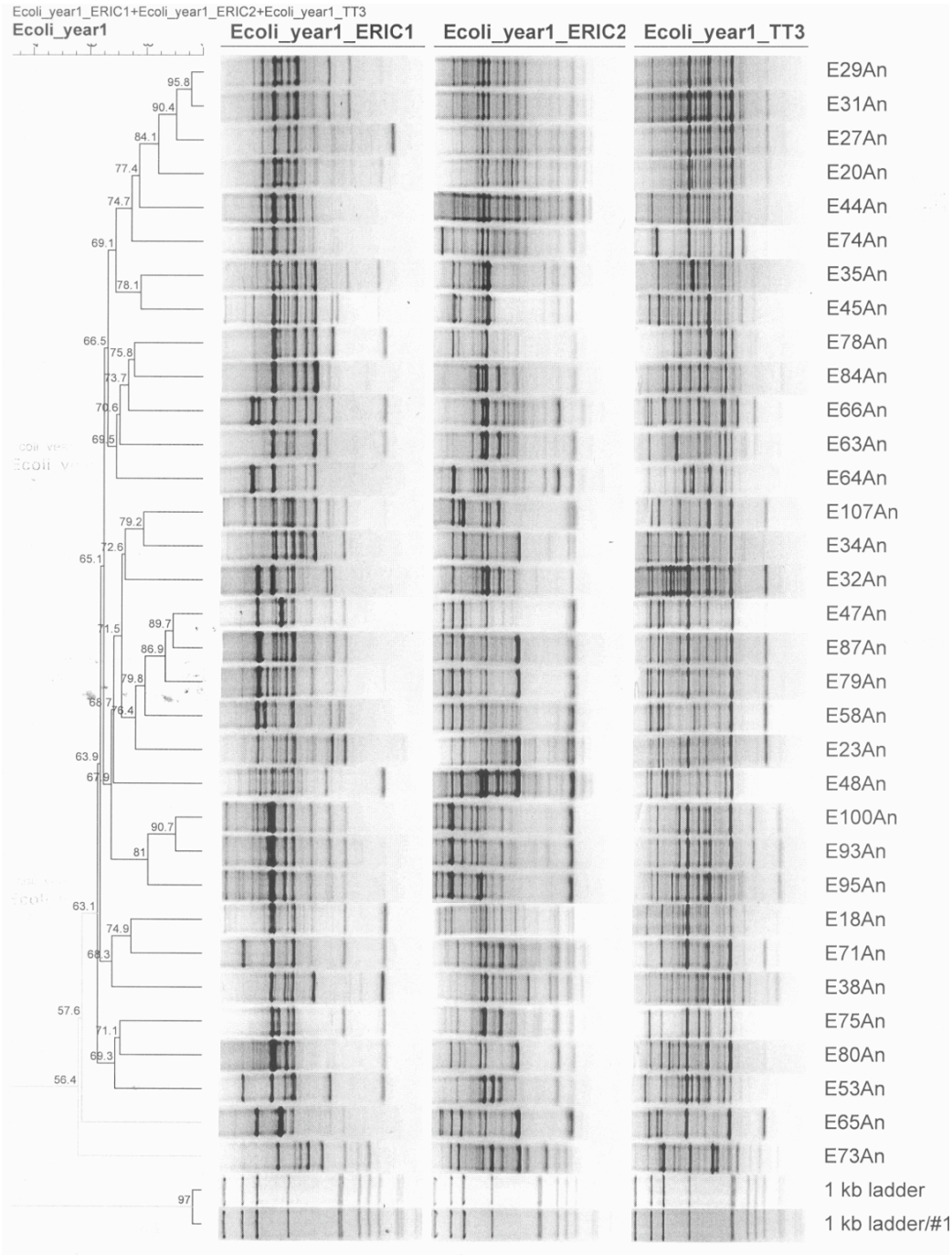


Figure 3.4 An example of phylogenetic tree of *E. coli* strains isolated from hospitalized patients with RAPD.

The figure presents an example of phylogenetic tree, drawn by BioNumerics, of 33 *E. coli* strains isolated from 26th April to 18th November from hospitalized patients. In the 33 strains, E29An is identical with E31An and E93An is identical with E100An.

3.3.2. Isolation of antimicrobial-resistant commensal Enterobacteriaceae from the community group

One hundred and four anal swabs were collected from 27 healthy adults and 77 healthy children and 100 nasal swabs and 100 anal swabs were collected from 100 healthy neonates. On the basis of culture on selective media containing gentamicin (8 μg/ml), ceftazidime (2 μg/ml) or nalidixic acid (16 μg/ml), 340 resistant *E. coli* strains, 45 resistant *K. pneumoniae* and 28 other resistant members of Enterobacteriaceae were isolated, including *Citrobacter* species, *Enterobacter* species, *Escherichia fergusoni*, *Pantoea* spp., *Kluyvera* spp., *Leclercia adecarboxylata*, *Morganella morganii*, *Proteus* species and *Klebsiella terrigena* (Figure 3.2). Again, to identify individual strains RAPD was performed as before. Analysis of the RAPD patterns showed that all strains isolated from the different individuals were all unique, i.e. it was not possible to find two strains with identical RAPD patterns in any of the bacterial isolates (Table 9.4, Appendices).

From the 204 healthy volunteers, organisms were isolated from 25/27 (93%) of the healthy adults, 71/77 (92%) of the healthy children and 64/100 (64%) of the healthy neonates that were resistant to gentamicin, ceftazidime, or nalidixic acid, or a combination of these antimicrobials.

3.3.3. Amplification of plasmid-mediated quinolone resistance genes

All bacterial isolates were subjected to PCR to individually amplify the *qnrA*, *qnrB*, *qnrS*, *qepA* and *aac(6')-Ib* genes according to the methods described in section 2.3.8. The majority of strains that generated a PMQR amplicon were positive for a single PMQR determinant only (Table 3.1).

All PCR amplicons of *qnrS* were sequenced and these data demonstrated that the sequences for all the amplicons of the *qnrS* fragment were indistinguishable and demonstrated 100% sequence identity with *qnrS1* from *K. pneumoniae* strain 052250 (GenBank accession no. EF683584). The sequences of all *qnrA* and *qnrB* amplicons and the single *qepA* PCR amplicon demonstrated 100% identity to *qnrA1* and *qnrB1* from *K. pneumoniae* plasmid pMG252 (GenBank accession no. DQ831140) and *K. pneumoniae* plasmid pMG298 (GenBank accession no. DQ351241) and *E. coli* plasmid pHPA (GenBank accession no AB263754), respectively.

Variability in PMQR determinant content was observed for a number of strains with identical RAPD patterns among the hospital strains. The number of unique strains was adjusted by combining the RAPD pattern and PMQR content. Therefore, from the hospital isolates, 55 unique *E. coli* strains, 66 *K. pneumoniae* strains and 18 other members of Enterobacteriaceae in this group were identified (Table 3.2).

Table 3.1 The number of unique bacterial isolates from the hospital and community groups carrying fluoroquinolone resistance determinants.

Source of bacterial isolate	Bacterial species (n)	PCR positive strains [n (%)]					
		qnrA	***qnrB***	***qnrS***	***aac(6')-Ib-cr***	***qepA***	**> 1 PMQR***
Hospital group	*E. coli* (55)	5 (9.0)	1 (1.8)	5 (9.0)	9 (16.4)	1 (1.8)	2 (3.6)
	K. pneumoniae (66)	4 (6.1)	8 (12.1)	52 (78.8)	11 (16.7)	0 (0)	11 (16.7)
	Other† (18)	6 (33.3)	8 (44.4)	6 (33.3)	0 (0)	0 (0)	4 (22.2)
Community group	*E. coli* (340)	2 (0.6)	0 (0)	32 (9.4)	9 (2.6)	0 (0)	1 (0.3)
	K. pneumoniae (45)	0 (0)	0 (0)	15 (33.3)	1 (2.2)	0 (0)	1 (2.2)
	Other‡ (28)	1 (3.6)	2 (7.1)	2 (7.1)	1 (3.6)	0 (0)	1 (3.6)

The table presents the prevalence of Enterobacteriaceae isolates carrying fluoroquinolone resistance determinants in Ho Chi Minh City from 2 different groups; hospital and community population.

* PCR positive for more than one PMQR gene inclusive of data in the rest of the table.

† Other bacterial species isolated from patients included *Enterobacter cloacae*, *Citrobacter youngae*, *Citrobacter freundii*, *Proteus mirabilis*, *Citrobacter* species and *Citrobacter koseri*.

‡ Other bacterial species isolated from the community included *Citrobacter* species, *Enterobacter cloacae* and *Klebsiella terrigena*.

Table 3.2 The designation of isolates with the same RAPD pattern but differing PMQR gene content.

Species	Isolates	RAPD patterns	PMQR – content					Genotype results
			qnrA	*qnrB*	*qnrS*	*qepA*	*aac(6')-Ib-cr*	
E. coli	E210An	E2-16	-	-	-	-	-	E2-16a
E. coli	E228An	E2-16	+	-	-	-	-	E2-16b
E. coli	E230An	E2-18	-	-	-	-	-	E2-18a
E. coli	E237An	E2-18	-	-	-	-	+	E2-18b
K. pneumoniae	K84N	K1-18	-	-	-	-	-	K1-18a
K. pneumoniae	K86N	K1-18	-	+	-	-	+	K1-18b
K. pneumoniae	K222N	K2-3	-	-	+	-	-	K2-3a
K. pneumoniae	K222Ca	K2-3	+	-	+	-	-	K2-3b
K. pneumoniae	K226Ax	K2-29	-	-	+	-	+	K2-29a
K. pneumoniae	K279N	K2-29	+	-	+	-	+	K2-29b
K. pneumoniae	K286N	K2-24	-	+	-	-	+	K2-24a
K. pneumoniae	K288An	K2-24	-	-	-	-	-	K2-24b
Proteus mirabilis	CV209Ax	II-P m.1	+	-	-	-	-	II-P m.1a
Proteus mirabilis	CV269Ax	II-P m.1	+	-	+	-	-	II-P m.1b
Proteus mirabilis	CV279An	II-P m.1	-	-	+	-	-	II-P m.1c

This table shows the designation of isolates based on RAPD pattern and the re-designation after combining the result of RAPD and PMQR content. For example, 2 isolates (E210An and E228An) have the same RAPD pattern and are designated as E2-16. However, since the 2 isolates contain different type of PMQR determinants, they were treated as 2 unique isolates and re-designated as E2-16a and E2-16b.

3.3.3.1. The prevalence of PMQR determinants in hospital strains

From the 66 *K. pneumoniae* isolated in the hospital group, 6.1% (4/66), 12.1% (8/66), 78.8% (52/66) and 16.7% (11/66) isolates produced amplicons for the *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr* gene, respectively. The proportions of *E. coli* strains carrying these PMQR genes from the same group were 5/55 (9%), 1/55 (1.8%), 5/55 (9%) and 9/55 (16.4%), respectively. In the remaining 18 Enterobacteriaceae, an amplicon for *qnrA* was produced by 6/18 (33.3%) isolates (3 *Proteus mirabilis*, 1 *Citrobacter youngae*, 1 *Enterobacter cloacae* and 1 *Citrobacter koseri*); an amplicon for *qnrB* in 8/18 (44.4%) isolates (3 *Citrobacter youngae*, 3 *Citrobacter freundii*, 1 *Enterobacter cloacae* and 1 *Citrobacter* spp.); an amplicon for *qnrS* in 6 (33.3%) isolates (3 *Proteus mirabilis*, 1 *Vibrio fluvialis*, 1 *Citrobacter koseri* and 1 *Enterobacter cloacae*) and no positive amplification of *aac(6')-Ib-cr*. From all antimicrobial resistant Enterobacteriaceae isolated from the hospital and the community, only one *E. coli* strain isolated from hospital group produced an amplicon for the *qepA* gene (Table 3.1).

3.3.3.2. Prevalence of PMQR determinants in community strains

The overall number of PMQR genes identified was less in the community strains than the hospital strains; nevertheless, 9.4% (32/340) of the *E. coli*, 33.3% (15/45) of the *K. pneumoniae* and 7.1% (2/28) other members of Enterobacteriaceae in community strains were PCR positive for the *qnrS* gene. Additionally, *aac(6')-Ib-cr* positive *E. coli*, *K. pneumoniae* and other members of Enterobacteriaceae from community strains were detected, albeit at a comparatively low frequency. The detailed results are shown in Table 3.1.

3.3.4. Prevalence of antimicrobial resistance in Enterobacteriaceae in Ho Chi Minh City

The MIC to nalidixic acid and ciprofloxacin was tested for all isolates in the hospital group and for PMQR-carrying isolates in the community group. In the hospital group, the MIC50 and MIC90 for the 66 *K. pneumoniae* strains were 16 μg/ml and >256 μg/ml, respectively, for nalidixic acid and 1 μg/ml and >32 μg/ml for ciprofloxacin, respectively. Likewise, the MIC50 and MIC90 for the 55 *E. coli* strains were >256 μg/ml and >256 μg/ml, respectively for nalidixic acid and >32 μg/ml and >32 μg/ml for ciprofloxacin, respectively. Similarly, in 18 other members of Enterobacteriaceae, the MIC50 and MIC90 to nalidixic acid were >256 μg/ml and >256 μg/ml, respectively and to ciprofloxacin were 8 μg/ml and >32 μg/ml, respectively (Figure 3.4 and Figure 3.5). A total of 139 Enterobacteriaceae strains isolated from the hospitalized-patients admitted to the Tetanus ward of the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam, the resistance prevalence to nalidixic acid and ciprofloxacin was 60.4% (84/139) and 48.9% (68/139), respectively. Similarly, in 92 PMQR-carrying Enterobacteriaceae strains isolated from hospitalized-patients, the resistance prevalence to nalidixic acid and ciprofloxacin was 48.9% (45/92) and 43.5% (40/92), respectively.

In PMQR-carrying community strains, the MIC50 and MIC90 for the 16 *K. pneumoniae* strains were 12 μg/ml and >256 μg/ml for nalidixic acid and 0.75 μg/ml and >32 for ciprofloxacin. Likewise, the MIC50 and MIC90 for the 43 *E. coli* strains were 64 μg/ml and>256 μg/ml for nalidixic acid, respectively and 1 μg/ml and 32 μg/ml for ciprofloxacin, respectively. Similarly, in 5 other members of Enterobacteriaceae the MIC50 and MIC90 to nalidixic acid were 24 μg/ml and>256

μg/ml, respectively, and to ciprofloxacin were 0.75 μg/ml and 3 μg/ml, respectively (Figure 3.3). In total of 64 PMQR-carrying Enterobacteriaceae strains isolated from the healthy volunteers living in Ho Chi Minh City, Vietnam, the resistance prevalence to nalidixic acid and ciprofloxacin were 42.2% (27/64) and 28.1% (18/64), respectively.

All community isolates were tested for susceptibility to a number of antimicrobials, including of gentamicin, amikacin, imipenem and piperacillin-tazobactam. The detailed results of susceptibility testing to the tested antimicrobials in the community isolates are shown in Figure 3.6. In addition, the prevalence of ESBL positive isolates in community group was also determined using the double disc diffusion method. The prevalence of ESBL positivity was 21% (70/340) in *E. coli*, 40% (18/45) in *K. pneumoniae* and 29% (8/28) in other members of Enterobacteriaceae. The prevalence of ESBL positive in *K. pneumoniae* in hospital group was determined to be 83% (55/66).

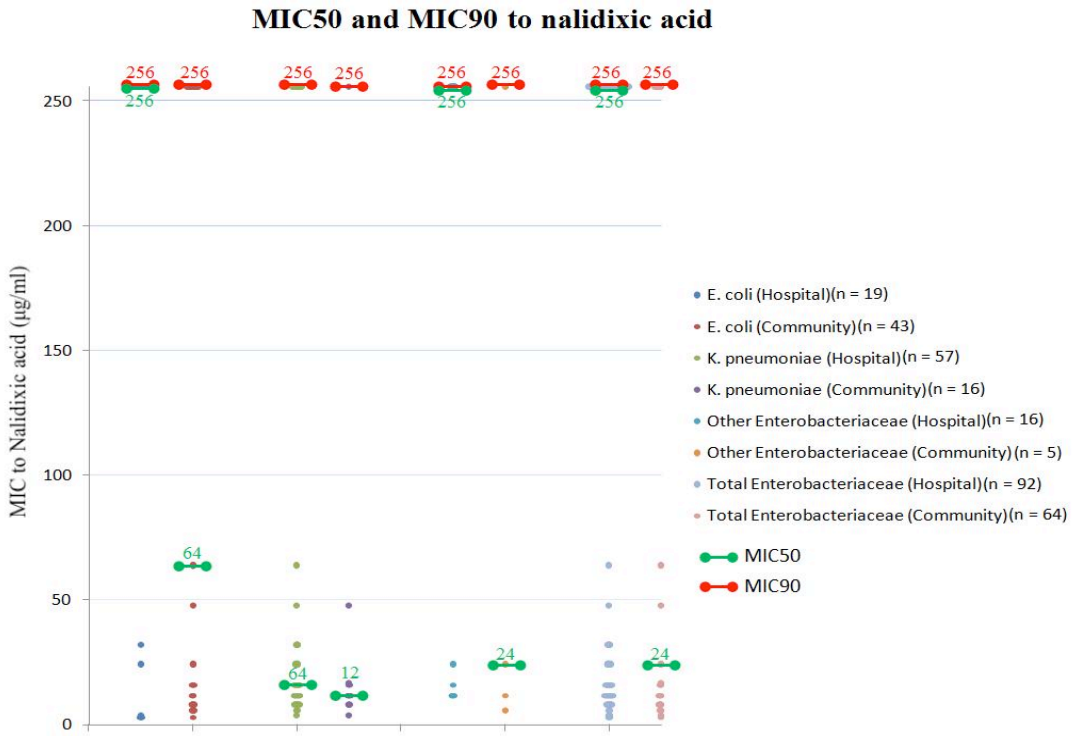


Figure 3.5 The MIC50 and MIC90 to nalidixic acid of PMQR-carrying Enterobacteriaceae isolates from the hospital and community groups.

Of all 156 Enterobacteriaceae strains isolated from Ho Chi Minh City, the MIC90 to nalidixic acid is 256 µg/ml while the MIC50 to nalidixic acid varies from 12 - 256 µg/ml. The MIC50 to nalidixic acid in *E. coli* is higher than that in *K. pneumoniae* ($p < 0.01$; for hospital strains, $p = 0.03$, for community strains, Wilcoxon rank sum test). Although the figure shows the MIC50 to nalidixic acid in hospital strains is higher than that in community strains, it is not statistically significant ($p = 0.12$, Wilcoxon rank sum test).

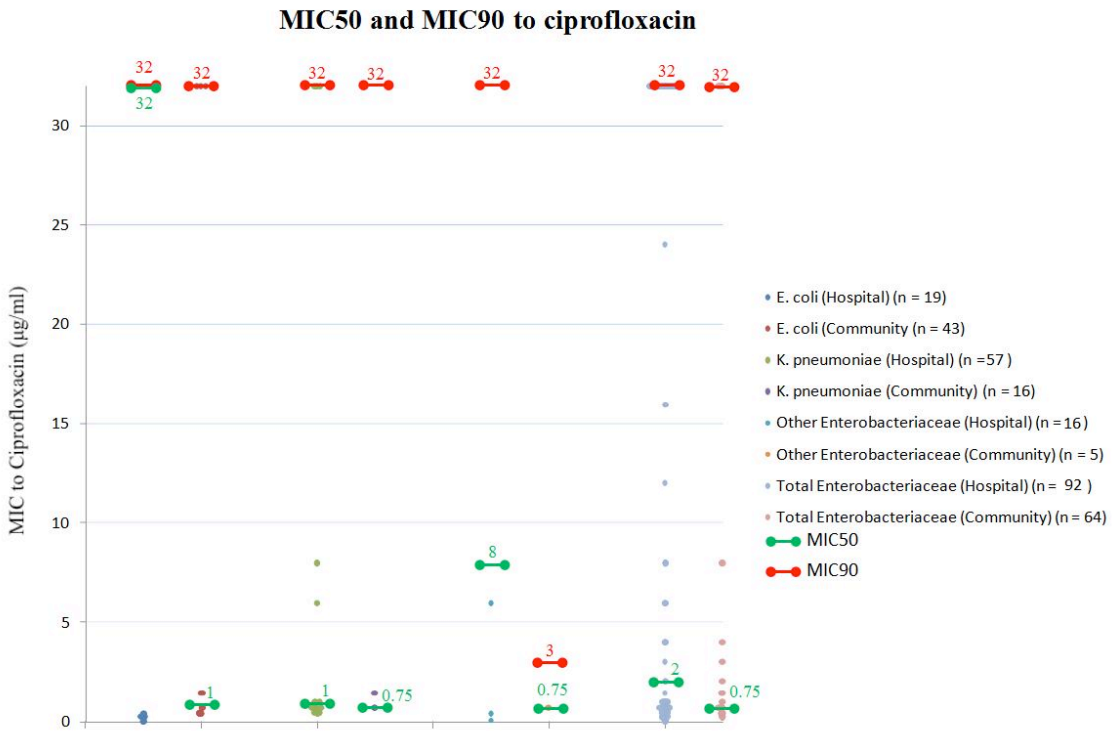


Figure 3.6 The MIC50 and MIC90 to ciprofloxacin of PMQR-carrying Enterobacteriaceae isolates from the hospital and community groups.

Of all 156 Enterobacteriaceae strains isolated from Ho Chi Minh City, the MIC90 to ciprofloxacin is 32 µg/ml while the MIC50 to ciprofloxacin varies from 0.75 - 32 µg/ml. The MIC50 to ciprofloxacin in *E. coli* is higher than that in *K. pneumoniae* strains in hospital group ($p < 0.01$, Wilcoxon rank sum test), but not in the community group ($p = 0.79$).

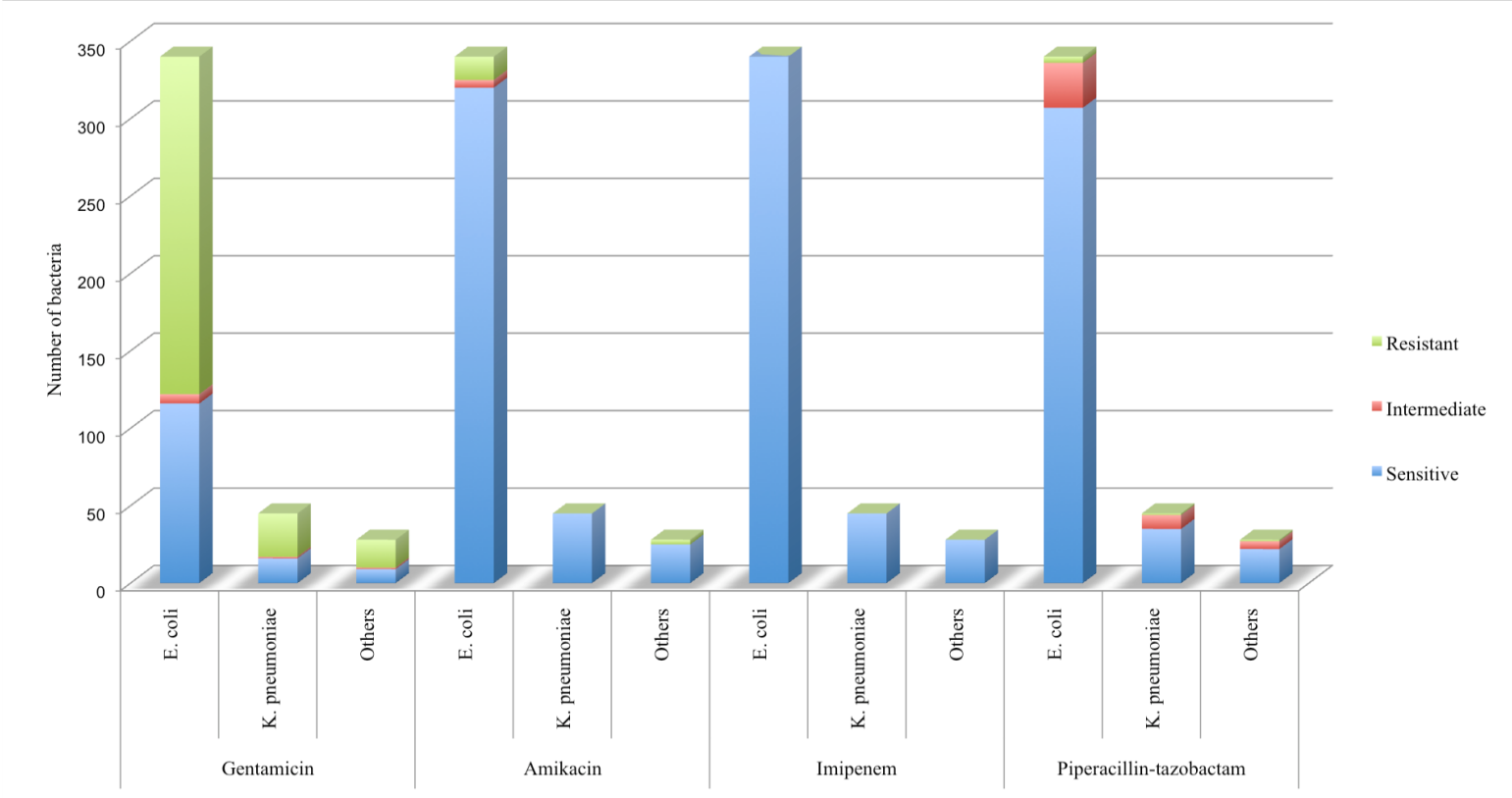


Figure 3.7 Graph demonstrating the susceptibility to gentamicin, amikacin, imipenem and piperacillin-tazobactam of community strains

The majority of tested isolates were sensitive to amikacin, imipenem and piperacillin-tazobactam. The prevalence of gentamicin resistance in *E. coli*, *K. pneumoniae* and other members of Enterobacteriaceae was 64%, 62.2% and 64.2%, respectively.

3.3.5. The association of ESBL production and PMQR determinants

One hundred and twenty eight strains isolated from hospital patients were ESBL positive, from these 128 strains 15 (11.7%) additionally carried the *qnrA* gene, 15 (11.7%) additionally carried the *qnrB* gene, 55 (43%) additionally carried the *qnrS* gene, 1 (0.8%) additionally carried the *qepA* gene and 18 (14.1%) additionally carried the *aac(6')-Ib-cr* gene. In 96 ESBL strains identified from the community isolates, 2 (2.1%) strains carried the *qnrA* gene, 1 (1%) strain carried *qnrB* gene, 5 (5.2%) strains carried *qnrS* gene, 8 (8.3%) carried the *aac(6')-Ib-cr* gene, no strains were found to carry the *qepA* gene. The prevalence of PMQR determinants in ESBL positive *E. coli*, *K. pneumoniae* and other members of Enterobacteriaceae strains in both hospital and community populations are shown in Table 3.3.

Table 3.3 The prevalence of PMQR determinants in ESBL producing isolates.

Source of bacterial isolate	Bacterial species (n)	PCR positive strains [n (%)]					
		qnrA	***qnrB***	***qnrS***	***aac(6')-Ib-cr***	***qepA***	**> 1 PMQR***
Hospital group	*E. coli* (55)	5 (9.0)	1 (1.8)	5 (9.0)	9 (16.4)	1 (1.8)	2 (3.6)
	K. pneumoniae (55)	4 (7.3)	6 (10.9)	44 (80)	9 (16.4)	0 (0)	11 (1.8)
	Other† (18)	6 (33.3)	8 (44.4)	6 (33.3)	0 (0)	0 (0)	4 (22.2)
Community group	*E. coli* (70)	1 (1.4)	0 (0)	2 (2.9)	6 (8.6)	0 (0)	1 (1.4)
	K. pneumoniae (18)	0 (0)	0 (0)	2 (11.1)	1 (5.6)	0 (0)	1 (5.6)
	Other‡ (8)	1 (12.5)	1 (12.5)	1 (12.5)	1 (12.5)	0 (0)	1 (12.5)

The table presents the prevalence of ESBL-producing Enterobacteriaceae isolates carrying fluoroquinolone resistance determinants in Ho Chi Minh City from 2 different groups; hospital and community population.

* PCR positive for more than one PMQR gene inclusive of data in the rest of the table.

† Other bacterial species isolated from patients included *Enterobacter cloacae*, *Citrobacter youngae*, *Citrobacter freundii*, *Proteus mirabilis*, *Citrobacter* species and *Citrobacter koseri*.

‡ Other bacterial species isolated from the community included *Citrobacter* species, *Enterobacter cloacae* and *Klebsiella terrigena*.

3.3.6. The prevalence of *gyrA* and *parC* mutations in *E. coli* and *K. pneumoniae* isolated from hospital and community populations

To assess the combinatorial effect of MIC to nalidixic acid and ciprofloxacin of PMQR gene and mutations in the *gyrA* and *parC* genes, *gyrA* and *parC* were sequenced in all *E. coli* and *K. pneumoniae* (from both settings) that contained a PMQR gene. From 55 unique hospital *E. coli* strains, 10 (18.2%) had a single mutation at codon 83 (Serine→ Leucine), none had a single mutation at codon 87, and 38 (69.1%) had a double mutation in the *gyrA* gene at codon 83 (Serine→ Leucine) and codon 87 (Aspartic acid→ Asparagine). In the 66 unique hospital *K. pneumoniae* strains, 11 (16.7%) had a single mutation at codon 83, in which Serine was substituted to Tyrosine or Isoleucine in 4 (6%) and 7 (10.6%) strains, respectively; none had a single mutation at codon 87; and 5 strains had a double mutation in the *gyrA* gene at codon 83 (Serine → Tyrosine in 3 strains and Serine → Phenylalanine in 2 strains) and codon 87 (Aspartic acid → Alanine).

In the 42 community *E. coli* strains, 6 (14.3%) strains had a single mutation at codon 83 (Serine→Alanine in 2 strains and Serine → Leucine in 4 strains), none had a single mutation at codon 87 and 14 (33.3%) had a double mutation in *gyrA* gene at codon 83 (Serine→ Leucine) and codon 87(Aspartic acid→ Asparagine). Of the 15 community *K. pneumoniae* strains, only one (6%) isolate had a double mutation. In this double mutant, Serine was substituted to Tyrosine at codon 83 and Aspartic acid to Alanine at codon 87. None of the strains with *parC* sequenced contained a significant mutation (codon 80 and 84), which was related to fluoroquinolone susceptibility. The proportions of mutation types of each group (community or hospital) are shown on Figure 3.7.

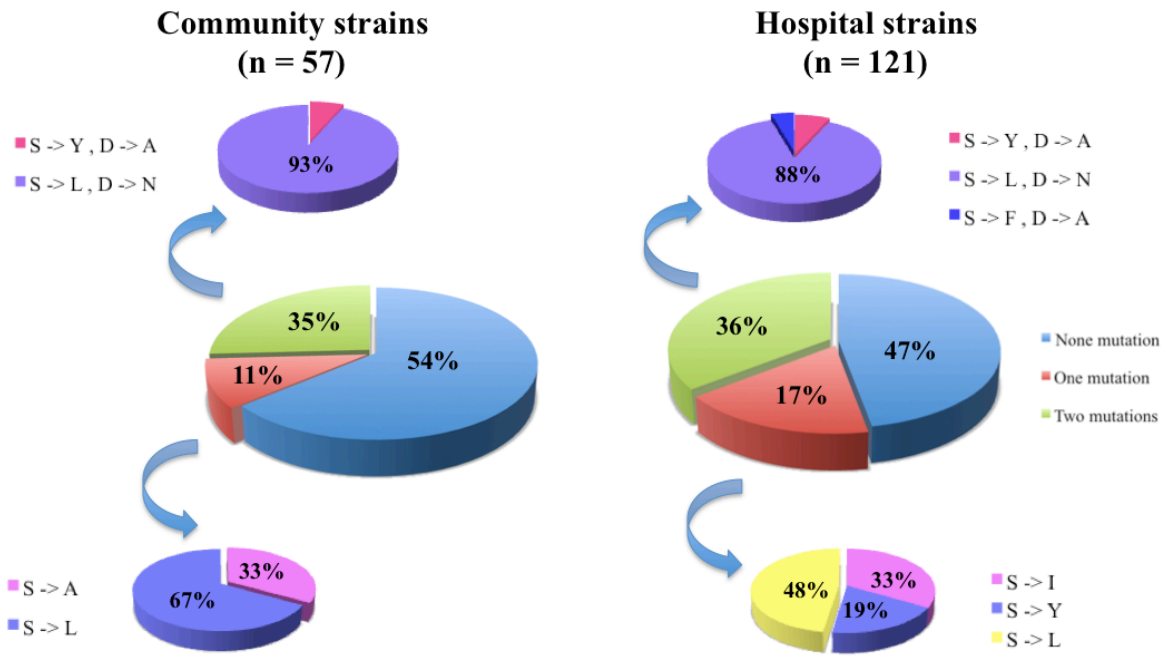


Figure 3.8 The proportion and types of mutations in the *gyrA* gene in *E. coli* and *K. pneumoniae* isolates from the community and the hospital.

This figure shows the proportion of the various *gyrA* mutations in *E. coli* and *K. pneumoniae* isolated from hospital (*E. coli*, n = 55; *K. pneumoniae*, n = 66) and community (*E. coli*, n = 42; *K. pneumoniae*, n =15). The predominant mutations in these two groups are a single mutation inducing the change from a Serine to a Leucine at codon 83 and mutations changing Serine to Leucine and Aspartic acid to Asparagine at codons 83 and 87, respectively.

* One mutation: mutation at codon 83 of GyrA; Two mutations: mutations at codon 83 and 87 of GyrA.

3.3.7. Effect of *gyrA* and *parC* mutations and PMQR genes on susceptibility to nalidixic acid and ciprofloxacin

All the 21 hospital *E. coli* and *K. pneumoniae* isolates with a mutation at position 83 have an MIC to nalidixic acid of >256 µg/ml whilst the MIC to ciprofloxacin of these isolates varied from 0.19 – 0.25 µg/ml in *E. coli* and 4 - >32 in *K. pneumoniae*. In the 6 community *E. coli* strains with a mutation at position 83, the MIC to nalidixic acid is also >256 µg/ml and the MIC to ciprofloxacin varied from 1.5 to >32 µg/ml. The MIC to nalidixic acid and ciprofloxacin in the 43 strains from hospital group and 15 strains from community group with a double mutation at position 83 and 87 are >256 µg/ml and >32 µg/ml, respectively, with the exception of one community *E. coli* strain with an MIC to ciprofloxacin of 8 µg/ml. The combinatorial effect of harbouring one or more of the PMQR genes in isolates with or without mutations on the *gyrA* gene on susceptibility to nalidixic acid and ciprofloxacin was assessed in all unique hospital strains (Table 3.4) and all PMQR positive community strains (Table 3.5).

Table 3.4 The MIC range (µg/ml) of *E. coli* and *K. pneumoniae* strains isolated from hospitalized patients.

PCR positive gene	Strains (n)	Number of mutations in gyrA								
		0			1			2		
		n	NAL	CIP	n	NAL	CIP	n	NAL	CIP
None	*E. coli* (36)	5	3 - 6	0.012 – 0.016	10	>256	0.19 – 0.38	21	>256	>32
	K. pneumoniae (9)	3	4 - 32	0.06 – 1	4	>256	6 - >32	2	>256	>32
qnrA	*E. coli* (5)	0			0			0	>256	>32
	K. pneumoniae (1)	1	8	0.25	0			0		
qnrB	*E. coli* (1)	0			0			1	>256	>32
	K. pneumoniae (1)	1	12	0.38	0			0		
qnrS	*E. coli* (4)	2	24, 32	0.38	0			2	>256	>32
	K. pneumoniae (42)	38*	6 – 64	0.38 – 4	3	>256	3 – 8	1	>256	>32
aac(6')-Ib-cr	*E. coli* (8)†	0			0			8	>256	>32
qnrA + qnrS	*K. pneumoniae* (2)	2	24, 32	0.75, 1	0			0		
qnrB + aac(6')-Ib-cr	*K. pneumoniae* (3)	0			1	>256	>32	2	>256	>32
qnrS + aac(6')-Ib-cr	*E. coli* (1)	0			0			1	>256	>32
	K. pneumoniae (3)	2	16, 32	2	1	>256	>32	0		
qnrA + qnrS + aac(6')-Ib-cr	*K. pneumoniae* (1)	1	16	3	0			0		
qnrB + qnrS + aac(6')-Ib-cr	*K. pneumoniae* (4)	2	>256	>32	0			2	>256	>32

The table presents the MIC range (µg/ml) of *E. coli* and *K. pneumoniae* strains isolated from hospitalized patients, associated with a variety of combinations of PMQR genes and mutations in the *gyrA* gene.

None of the strains contained mutations at codon 80 and 84 of the *parC* gene. NAL, nalidixic acid; CIP, ciprofloxacin.

* NAL: MIC_{50} = 16, MIC_{90} = 32; CIP: MIC_{50} = 0.75, MIC_{90} = 1.5

† Included one *qepA* positive strain.

Table 3.5 The MIC range (µg/ml) of *E. coli* and *K. pneumoniae* strains isolated from healthy individuals.

PCR positive gene	Strains (n)	Number of mutations in *gyrA*								
		0			1			2		
		n	NAL	CIP	n	NAL	CIP	N	NAL	CIP
qnrA	*E. coli* (1)	0			0			1	>256	8
qnrS	*E. coli* (31)	22*	3 – 64	0.19 – 1.5	5	>256	1.5 - >32	4	>256	>32
	K. pneumoniae (14)	14†	8 – 48	0.5 – 1.5	0			0		
aac(6')-Ib-cr	*E. coli* (9)	0			1	>256	2	8	>256	>32
qnrA + qnrS	*E. coli* (1)	1	6	0.5	0			0		
qnrS + aac(6')-Ib-cr	*K. pneumoniae* (1)	0			0			1	>256	>32

The table presents the MIC range (µg/ml) of *E. coli* and *K. pneumoniae* strains isolated from healthy individuals, associated with a variety of combinations of PMQR genes and mutations in the *gyrA* gene.

None of the strains contained mutations at codon 80 and 84 of the *parC* gene. NAL, nalidixic acid; CIP, ciprofloxacin.

* NAL: MIC_{50} = 16, MIC_{90} = 32; CIP: MIC_{50} = 0.75, MIC_{90} = 1.5

† Included one *qepA* positive strain

None of the strains contained mutations at codon 80 and 84 of the *parC* gene.

NAL, nalidixic acid; CIP, ciprofloxacin.

* NAL: MIC50 = 12, MIC90 = 48; CIP: MIC50 = 0.38, MIC90 = 0.75

† NAL: MIC50 = 12, MIC90 = 16; CIP: MIC50 = 0.75, MIC90 = 1.5

3.3.8. Conjugation of *qnrS*-harbouring plasmids

Three *E. coli* strains and 20 *K. pneumoniae* strains were randomly chosen from the hospital group, which were positive for only the *qnrS* determinant, to conduct a series of conjugation experiments to test for the ability of the plasmids to transfer between strains. Only 1 of the 3 *E. coli* strains demonstrated the ability to transfer nalidixic acid resistance (and the corresponding *qnrS* plasmid) to a J53 *E. coli* recipient. However, it was not possible to transfer any of the 20 *qnrS*-containing plasmids in *K. pneumoniae* strains to a J53 *E. coli* recipient.

3.3.9. The effect of *qnrS* on the MIC to nalidixic acid and ciprofloxacin

Three *qnrS1* PCR positive strains were selected (*E. coli* E66An (hospital), *K. pneumoniae* K1HV (community) and *K. pneumoniae* K18An (hospital)) for further analysis of the *qnrS1* (Table 3.6). It was not possible to transfer nalidixic acid resistance by conjugation from either of the 2 *K. pneumoniae* strains into the *E. coli* J53 recipient. However, it was possible to transform an *E. coli* strain with purified *qnrS*-harbouring plasmid DNA and also to transfer nalidixic acid resistance via conjugation and transformation from *E. coli* E66An into another *E. coli* strain.

The MIC to nalidixic acid and ciprofloxacin of transformants and transconjugants was assessed (Table 3.6). The *qnrS* plasmid containing transformants demonstrated an MIC increase from 1.5 μg/ml to 4 μg/ml (~ 3-fold) and 0.006 μg/ml to 0.094 μg/ml or 0.125 μg/ml (~ 15- to 20-fold) for nalidixic acid and ciprofloxacin respectively. The results were similar for the *E. coli* transconjugant, after gaining *qnrS*-harbouring plasmid, the MIC increased from 4 μg/ml to 16 μg/ml (4-fold) to nalidixic acid and from 0.008 μg/ml to 0.75 μg/ml (~ 93-fold) to ciprofloxacin (Table 3.6).

Table 3.6 The susceptibility patterns of wild-type, transconjugants and electro-transformants of *E. coli* and *K. pneumoniae* strains.

Strain	NAL MIC (µg/ml)	CIP MIC (µg/ml)	ESBL*	*qnrS* PCR
E. coli TOP10	1.5	0.006	-	-
E. coli J53-Azi	4	0.008	-	-
K. pneumoniae K1HV	8	0.75	-	+
E. coli transformant 1 K1HV	4	0.125	-	+
E. coli transformant 2 K1HV	4	0.125	-	+
K. pneumoniae K18An	>256	>32	+	+
E. coli transformant 1 K18An	4	0.125	-	+
E. coli transformant 2 K18An	4	0.094	-	+
E. coli E66An	>256	>32	+	+
E. coli transformant E66An	4	0.125	+	+
E. coli transconjugant E66An	16	0.75	+	+

The table presents the MIC to nalidixic acid, ciprofloxacin as well as ESBL producing property of wild-type, transconjugants and electro-transformants from 3 *E. coli* and *K. pneumoniae* isolates. Transformants and transconjugant were composed from *qnrS*-carrying plasmids from wild-type strains and *E. coli* TOP10 or *E. coli* J53-Azi, respectively. From the 3 *qnrS*-carrying plasmids, only the plasmid from *E. coli* E66An strain expresses the ESBL producing feature.

NAL, nalidixic acid; CIP, ciprofloxacin.

* ESBL production was identified by double-disc method.

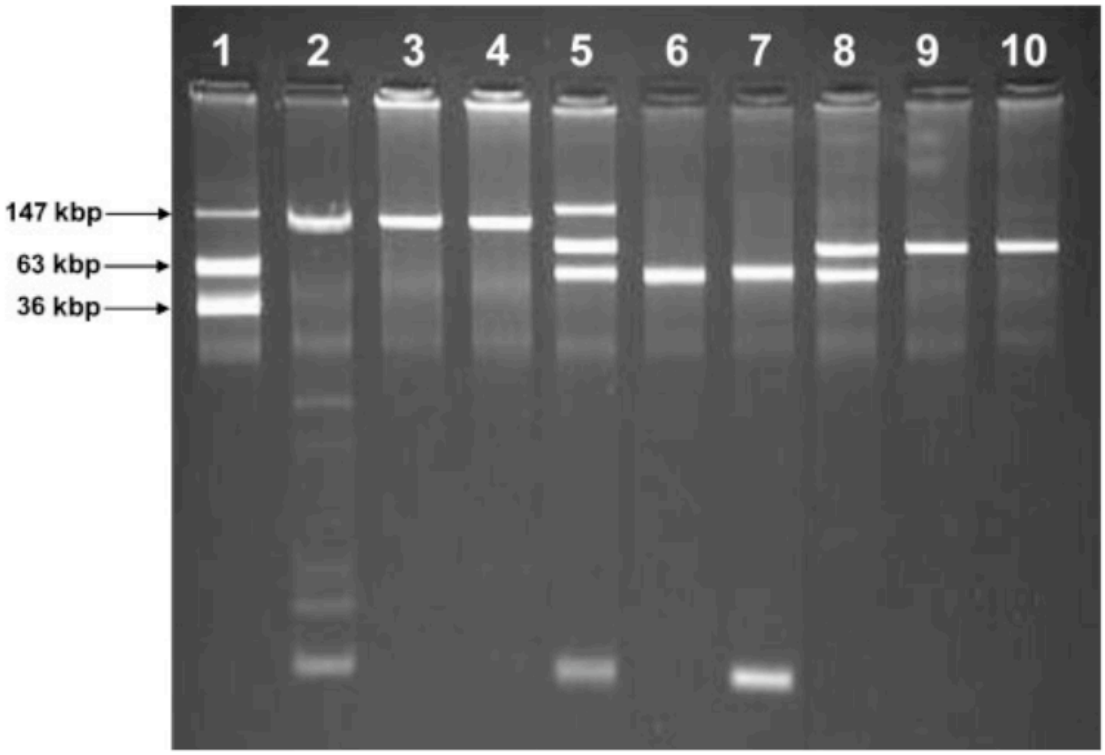


Figure 3.9 Agarose gel electrophoresis of plasmid DNA from clinical isolate, transconjugant and electrotransformant *E. coli* and *K. pneumoniae* strains.

The figure presents the agarose gel electrophoresis of alkaline plasmid lysis preparation from wild-type, transconjugant and electrotransformant of the 3 aforementioned *E. coli* and *K. pneumoniae* strains. In addition of carrying *qnrS*-embedded plasmid, the wild-type strains also carry other plasmids. The *qnrS*-carrying plasmids vary in size from around 50 kb to approximately 140 kb.

Lanes: 1, *E. coli* 39R861; 2, *K. pneumoniae* K1HV; 3, *E. coli* transformant 1 K1HV; 4, *E. coli* transformant 2 K1HV; 5, *K. pneumoniae* K18An; 6, *E. coli* transformant 1 K18An; 7, *E. coli* transformant 2 K18An; 8, *E. coli* E66An; 9, *E. coli* transformant E66An; 10, *E. coli* transconjugant E66An.

3.4. Discussion

These data regarding the prevalence of antimicrobial resistant Enterobacteriaceae show that the dissemination of antimicrobial resistant enteric bacteria is widespread in the hospital and the community in Ho Chi Minh City. Antimicrobials are available without prescription in Vietnam; therefore, it is tempting to suggest that this is the primary source of selection for resistant organisms. However, as part of the enrollment criteria, none of the healthy individuals had received any antimicrobial therapy for at least 4 weeks prior to sample collection. An alternative hypothesis that the use of antimicrobials in the production of meat and vegetables is another potential major source of the ongoing selection of resistant organisms, a view which is shared by others (Stobberingh and van den Bogaard 2000; Teuber 2001). In recent reports, investigators have shown intestinal colonization with fluoroquinolone resistant and ESBL producing *E. coli* in healthy schoolchildren in Latin America and vegetarians in the USA (Pallecchi *et al.,* 2007a; Pallecchi *et al.,* 2007b; Sannes *et al.,* 2008). These data suggest that daily exposure to particular antimicrobials is sufficient as to maintain resistant organisms in the intestinal flora, and/or transmission of resistant strains from individuals exposed to these agents followed by persistent carriage.

The data indicates a very high prevalence of *qnr* genes, in particular the *qnrS* gene, in commensal organisms in Ho Chi Minh City. Recent reports showing *qnr* genes originating from waterborne bacteria may explain dissemination of *qnr* genes in commensal bacteria (Cattoir *et al.,* 2007a; Cattoir *et al.,* 2008a). Faecal-oral transmission may facilitate exchange of *qnr* genes between water-borne and intestinal bacteria in the human host.

Chromosomal quinolone resistance in *E. coli* and *K. pneumoniae* is predominantly determined by mutations at codon 83 and codon 87 in the *gyrA* gene and codon 80 and 84 in *parC* gene (Vila *et al.,* 1996; Weigel *et al.,* 1998; Lee *et al.,* 2005; Hu *et al.,* 2007). Here, a high prevalence of mutation in the *gyrA* gene was found, both single and double mutations, but no isolates with mutation in *parC* gene were identified. This result again supports the hypothesis that mutation on *parC* gene is the complementary factor for the higher resistance to quinolones in Enterobacteriaceae (Chen *et al.,* 2003).

No isolates with a single mutation at codon 87 were identified, but the prevalence of double mutations at codon 83 and 87 was high, and corresponds with a high MIC to nalidixic acid and ciprofloxacin in these isolates, which suggests sequential stepwise mutations in *gyrA* is occurring to drive quinolone resistance in commensal Enterobacteriaceae in Ho Chi Minh City. The potential association between type of substitution and the level of MIC to nalidixic acid and ciprofloxacin in the strains with one mutation at codon 83 was investigated; however, no significant relationship between MIC and mutation at this location was found.

The combinatorial effects of the various PMQR genes on the MIC to nalidixic acid and ciprofloxacin were assessed. The biggest MIC increases associated with a single gene were with either *qnrS* or *aac(6')-Ib-cr*. Two strains carrying the *qnrS*, *qnrB* and *aac(6')-Ib-cr* genes were obtained which demonstrated complete resistance to nalidixic acid and ciprofloxacin, in the absence of known selective mutation in the *gyrA* gene. The effect of PMQR genes on the resulting MIC to fluoroquinolones thus appears to be reliant on the number and type of PMQR genes carried by the bacteria. The co-existence of *qnrB* and *qnrS* genes on 2 different plasmids within a strain has

been shown previously. However, unlike the data demonstrates here, this combination did not have any additional effect on the resistance to nalidixic acid (Hu *et al.,* 2008). Therefore, a combination effect is suggested, due to the proteins working in an independent manner; as QnrB and QnrS protect the DNA gyrase and Aac(6')-Ib-cr modifies the fluoroquinolone. However, it is not possible to rule out any possible additional effects of other quinolone resistance mechanisms, such as unspecific efflux pump activity. Furthermore, none of strains examined demonstrated any mutation at position 80 and 84 of the *parC* gene, which have been shown to decrease the susceptibility of *E. coli* and *K. pneumoniae* to both nalidixic acid and ciprofloxacin (Vila *et al.,* 1996; Brisse and Verhoef 2001).

Some primary analysis of the *qnrS* containing plasmids on the transformants derived from plasmid DNA isolated from both *K. pneumoniae* strains and the transformant and transconjugant derived from *E. coli* E66An indicate that the sizes and resistance profiles of *qnrS* containing plasmids demonstrates significant variability. These data formed the basis of further work, which is presented later in the thesis. The transconjugant strain derived from *E. coli* E66An, but not the transformants derived from the *K. pneumoniae* strains, additionally showed that ESBL production could be transferred alongside the *qnrS* gene (Table 3.6). The correlation between *qnrA* or *qnrB* and ESBL genes is well known (Iabadene *et al.,* 2008; Jiang *et al.,* 2008; Oktem *et al.,* 2008; Szabo *et al.,* 2008; Wang, A. *et al.,* 2008a), whilst the relationship between *qnrS* and other resistance genes is less well described. ESBL production was observed in only 52.7% of the *qnrS* positive strains in our study, however, it was not possible to confirm the number of strains, which harbour a *qnrS* plasmid that also harbor an ESBL gene.

3.5. Conclusion

In conclusion, this chapter indicates that commensal organisms represent a significant reservoir and source of dissemination of plasmid mediated antimicrobial resistance genes, such as *qnrS,* in Vietnam.

4. A common *qnrS1*-containing transposon circulating in Enterobacteriaceae in Ho Chi Minh City, Vietnam and the characterization of 3 multi-drug resistance *qnrS1* plasmids

4.1. Abstract

If there is a common genetic environment of PMQR genes such as *qnrS1* is a question that has yet to be definitively answered. It is known that PMQR genes are not commonly located on *sul1*-type class 1 integrons, yet *qnrA* and *qnrB*, *qnrS* are still widely distributed throughout the Enterobacteriaceae. The aim of this chapter is to characterize the *qnrS*-containing mobile element found in Enterobacteriaceae in Ho Chi Minh City, Vietnam. Furthermore, I selected 3 *qnrS1*-carrying plasmids to sequence and annotate to understand the structure of such *qnrS1*-embedded plasmids and also the association of *qnrS1* to other antimicrobial resistance genes. I previously found that 71 out of 112 *qnrS1*-carrying Enterobacteriaceae isolates (63.4%) were positive by PCR for a known *qnrS1*-containing transposon. Of the 71 strains, 63 strains (88.7%) carried a *qnrS1* containing transposon, which was named type A. Annotation of the 3 *qnrS1*-containing plasmids revealed evidences of genetic transfer by means of mobile elements. Plasmid pE66An contains both *qnrS1* and $bla_{CTX\text{-}M\text{-}14}$, with an appropriate transposase to facilitate the spread of $bla_{CTX\text{-}M\text{-}14}$. In addition, these plasmids carry specific genes for adaptation within variety of hosts and environments.

4.2. Introduction

Since the first report on the identification of plasmid-mediated quinolone resistance (PMQR) determinant in *K. pneumoniae* in 1998 (Martinez-Martinez *et al.,* 1998), there has been a dramatic spread of these *qnr* genes throughout the Enterobacteriaceae in many countries (Strahilevitz *et al.,* 2009). Because of the high dissemination of the *qnr* genes, there have been several studies investigating the mobile elements that carry the *qnr* genes. In contrast to the *qnrA* and *qnrB* genes, which are normally a part of complex *sul1*-type class 1 integrons (Garnier *et al.,* 2006; Poirel *et al.,* 2006a), the *qnrS* gene has been found to be located in a cluster of genes which is flanked by 2 IS26 transposase elements at 2 either terminals (Chen, Y. *et al.,* 2006; Hu *et al.,* 2008). However, it remains unknown if *qnrS* is consistently associated with this element or if the gene can be transferred/harboured by other transposable elements.

Currently, there are two completely sequenced *qnrS1*-carrying plasmids that are available in public databases. Both plasmids were isolated from patients admitted to hospital. The first is a 10kb plasmid, named pTPqnrS-1a and was obtained from a multiresistant *S. enterica* Typhimurium DT193 in the UK (Kehrenberg *et al.,* 2007). The other is pK245, a 98 kb plasmid which identified in a clinical *Klebsiella pneumoniae* from Taiwan (Chen, Y. *et al.,* 2006). With a size of 10,066 bp, plasmid pTPqnrS-1a is generally smaller than other reported *qnrS*-containing plasmids. The *qnrS1* gene of this plasmid encodes a 218 amino acid protein indistinguishable from the QnrS1 protein previously detected in the *S. enterica* serovar Infantis (accession no. CAJ84117). A detailed sequence analysis of this plasmid revealed that the surrounding of the *qnrS1* of this plasmid is similar with the surrounding of the *qnrS1*

gene in plasmids pAH0376 from *S. flexneri* (Hata *et al.,* 2005) and pINF5 from *Salmonella* Infantis (Kehrenberg *et al.,* 2006).

In contrast to plasmid pTPqnrS-1a, pK245 is a large plasmid of 98,264 bp. After transferring the *qnrS1*-containing plasmid pK245 to a laboratory strain, the electrotransformant pK245-km demonstrated an increase in MIC to multiple classes of antimicrobials, including aminoglycosides, ß-lactams, (fluoro)quinolones and also had an ESBL phenotype (Chen, Y. *et al.,* 2006). Similar to plasmid pTPqnrS-1a, the genetic environment surrounding *qnrS1* in plasmid pK245 is related to genetic environment of the *qnrS1* gene in plasmids pAH0376 and pINF5 (Figure 4.1).

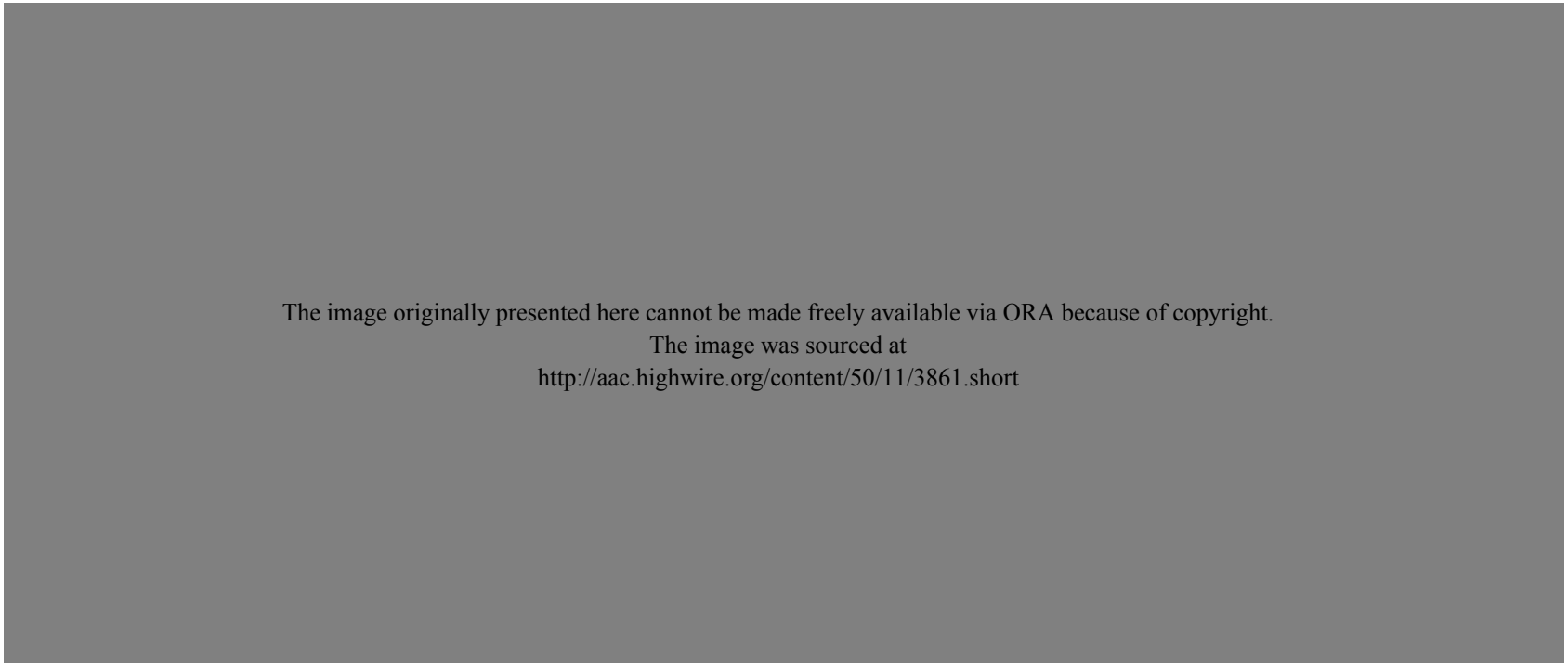


Figure 4.1 Schematic diagram comparing the *qnrS*-containing region of pK245 with those of pAH0376 from *S. flexneri* and pINF5 from *S. enterica* serovar Infantis.

This figure adapted from Chen et al. (Chen, Y. *et al.,* 2006)

The ORFs are shown as arrows, with the arrowhead indicating the direction of transcription. A 12.4-kb region of pK245, which is flanked by two copies of IS26, is illustrated on the top. The terminal inverted repeats on both sides of IS26 are shown in triangles.

A high prevalence of *qnrS1* gene in both hospital (63/139, 45%) and community strains (49/413, 12%) was shown in Chapter 3. Hence, I hypothesized that the *qnrS1* gene was embedded on a related mobile element, which contributed to the spread of the *qnrS1* gene throughout the Enterobacteriaceae in Ho Chi Minh City. As a result, in this chapter, the goal was to characterize the dominant *qnrS1*-containing mobile elements circulating in Enterobacteriaceae isolated from both hospital and community groups in Ho Chi Minh City. Furthermore, three *qnrS1*-containing plasmids were selected from 2 different groups, hospital and community for DNA sequencing and analysis. The annotation and investigation of the comparative structure of the mobile element that carries *qnrS1* will broaden the knowledge regarding the genetic background of *qnrS1* and also the relationship between this gene and additional resistance genes within different populations.

The three plasmids that were sequenced and annotated are the three plasmids that were used to examine the influence of the *qnrS* on the MIC to fluoroquinolones in Chapter 3, which were pE66An, pK18An (from hospital strains) and pK1HV (from community strain) (Table 3.6, Chapter 3). The sequences of the three plasmids were produced in collaboration with the Sanger Institute, UK. The common *qnrS1*-containing transposons circulating in Enterobacteriaceae were investigated in 112 *qnrS*-positive Enterobacteriaceae strains, which were also analyzed in Chapter 3. The PCR amplification and RFLP for *qnrS1*-containing transposon are detailed in 2.5.1 and 2.5.2, and isolates containing subfamily of known *qnrS1*-containing transposon were detected by Southern Blotting, which is detailed in 2.5.3.

4.3. Results

4.3.1. Characterization of *qnrS1*-containing transposons

Based on the alignments of the 3 plasmid sequences (pE66An, pK18An and pK1HV), the initial focus was placed on the *qnrS1*-containing fragment. All 3 *qnrS1*-containing fragments were identical, except for one, which contained a 980-bp insertion within the *qnrS1*-containing fragment (Figure 4.2). Unlike the *qnrA* gene, which has been shown to be embedded on integron, the *qnrS1* gene is located within a transposon, which has 2 identical IS26 elements at either terminal portion of the transposon. In addition to containing the *qnrS1* gene, these transposons also carry the bla_{LAP-2} gene, a gene which induces resistance to β-lactam antimicrobials. The annotation of the 3 sequenced *qnrS1*-containing transposons in this study and an alignment with other reported *qnrS*-containing fragments are shown in Figure 4.3.

4.3.2. PCR amplification for *qnrS1*-containing transposons

Based on the sequences of the *qnrS1*-containing transposons from the three sequenced plasmids (Figure 4.2), PCR primers were designed that would, theoretically, amplify similar *qnrS1*-containing transposons. The primer-binding site is shown on Figure 4.3 and the sizes of PCR amplicons are 6,859 bp (for the transposon identical with pE66An and pK1HV) or 8,059 bp (for the transposon identical with pK18An). PCR was performed on DNA extracted from all the 112 *qnrS1*-positive strains. Seventy-one out of 112 isolates (63.4%) were positive by PCR for the known *qnrS1*-containing transposon that was related to the three sequenced plasmids.

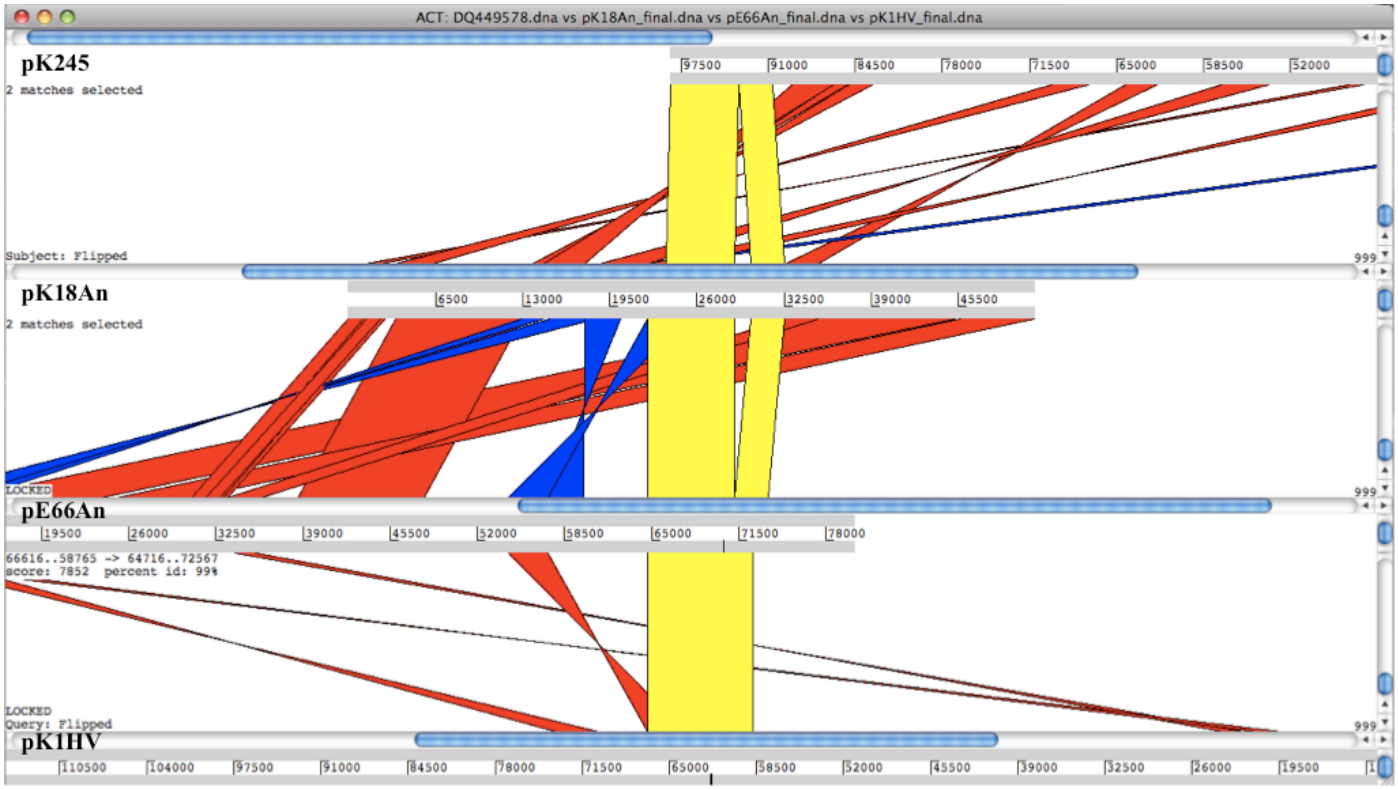


Figure 4.2 Artemis comparison tool alignment of the three sequenced plasmids containing the *qnrS1* carrying transposon with the previously sequenced pK245 plasmid.

This figure shows the similarity of sequence amongst 4 plasmids. The red, blue or yellow fragment shows the identity with the cut off 99% of each pair of sequences. The yellow fragment is the region containing *qnrS1*, which are identical in all 4 plasmids except for a 980 bp insert of pK18An.

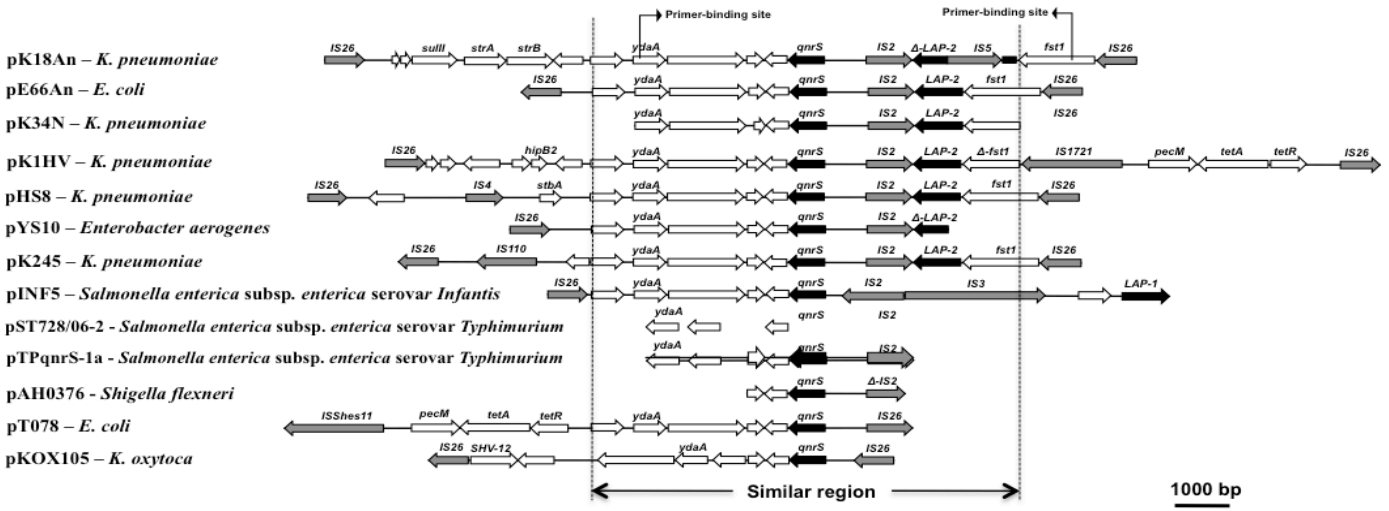


Figure 4.3 A schematic diagram of the *qnrS*-containing transposon and alignment with other sequenced *qnrS*-containing fragments.

This figure shows the similarity of the *qnrS*-containing fragment between our 3 plasmids (pK18An, pE66An and pK1HV) and other sequenced fragment. The homologous region was includes of putative IS2 element, a putative resolvase protein YdaA, whose predicted product is distantly related to a family of universal stress proteins (Beliaev *et al.,* 2002), and 3 other hypothetical uncharacterized proteins.

The figure also includes the plasmid names and the strains from which these plasmids were isolated and the primer-binding site of the PCR for detecting *qnrS*-containing transposon. Genes are colour coded as followed: black; *qnrS*, bla_{LAP-2}; grey, IS elements; white without the name above, hypothetical uncharacterized protein; white with the name above, genes with the name indicated.

4.3.3. Typing of *qnrS*-containing transposons

The 71 positive PCR amplicons with the known *qnrS1*-containing transposons were digested individually with *Eco*RV, *Hind*III and *Pvu*II restriction enzymes. In addition to the 2 known *qnrS1*-containing transposons that were identified from the 3 sequenced plasmids, the restriction fragment polymorphism mapping (RFLP) patterns from these 71 amplicons also revealed a transposon with a third structure (K34N strain). The DNA sequence of the *qnrS1*-containing fragment from the K34N strain revealed that the *qnrS1*-containing transposon in K34N strain is 6,652 bp and identical with the *qnrS1*-containing transposon from E66An strain, except for a deletion of a 200 bp fragment in a hypothetical gene (Figure 4.3). Therefore, 3 main types of *qnrS1*-containing transposons were identified to be circulating in these Enterobacteriaceae isolated in Ho Chi Minh City. The size of these transposons varies between 6,859 bp and 8,059 bp. The RFLP patterns of these transposons digested by 3 enzymes (*Eco*RV, *Hind*III and *Pvu*II) are shown in Figure 4.4.

The proportion of the 3 transposon types is shown in Table 4.1. In the 63 hospital strains, 45 (71.4%) carry the *qnrS1*-containing transposon identical with pE66An (type A), 1 isolate (1.6%) carries the *qnrS1*-containing transposon identical with pK18An (type B) and 3 isolates (4.8%) carry the *qnrS1*-containing transposon identical with K34N (type C). Similarly, in the 49 community strains, there were 18 (36.7%), 1 (2%) and 3 (6.1%) isolates carrying type A, B and C *qnrS1* transposon, respectively. However, 14 isolates (22.2%) from hospital strains and 27 isolates (55.1%) from the community strains were negative by PCR targeting the *qnrS1*-containing transposon, suggesting that the *qnrS1* in these strains is embedded on a different genetic element which was undetectable by the described methods.

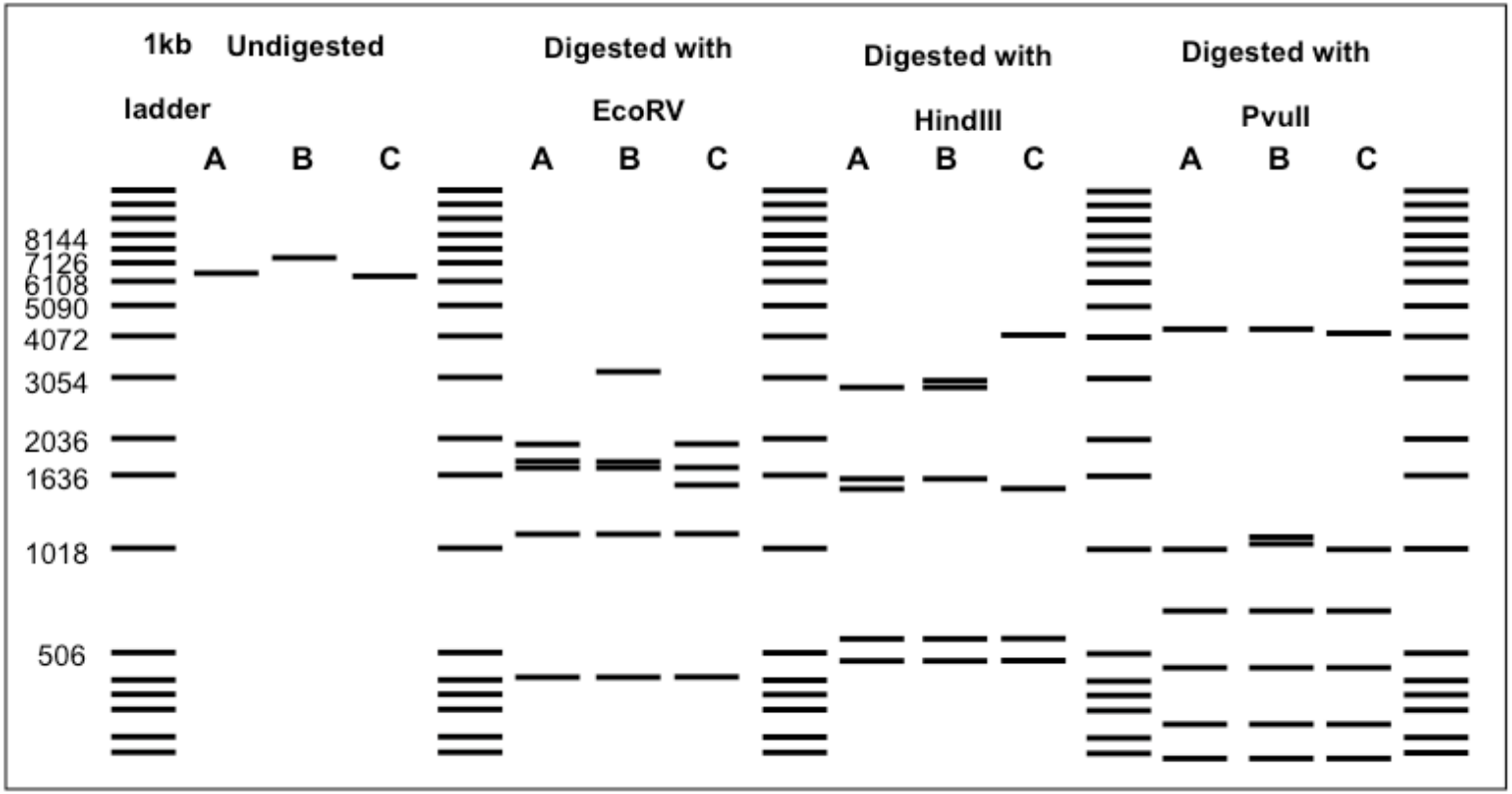


Figure 4.4 Illustration of 3 different *qnrS1*-containing transposon structures by RFLP.

This figure shows the undigested and RFLP patterns of 3 types of *qnrS1*-containing transposon after digesting with *Eco*RV, *Hind*III and *Pvu*II.

A, B and C are type A, type B and type C which are *qnrS1*-containing transposons of pE66An, pK18An and pK34N strains, respectively.

4.3.4. Subfamily of known *qnrS1*-containing transposon

Forty-one isolates with undescribed (by PCR) *qnrS1*-containing mobile elements were further investigated for the presence of subfamily of the known transposon. *E. coli* TOP10 was successfully transformed with plasmid DNA from 27 out of the 41 isolates with undefined *qnrS1* plasmid structure. Plasmid DNA from 27 transformants were digested with *Eco*RI and probed by Southern blotting with 2 probes: qnrS1 and bla_{LAP-2}. In the resulting hybridization of the plasmid DNA extracted from the 27 isolates, 2 isolates produced a detectable signal with both the qnrS1 and bla_{LAP-2} probes on the same digestion fragment, implying these 2 isolates contain *qnrS1*-containing transposon of the same subfamily as described in the sequenced plasmids (Figure 4.5). The 39 *qnrS1*-positive isolates, which were not detected on the mobile elements that the *qnrS1* embedded on, were stored in BHI at -20^{o}C.

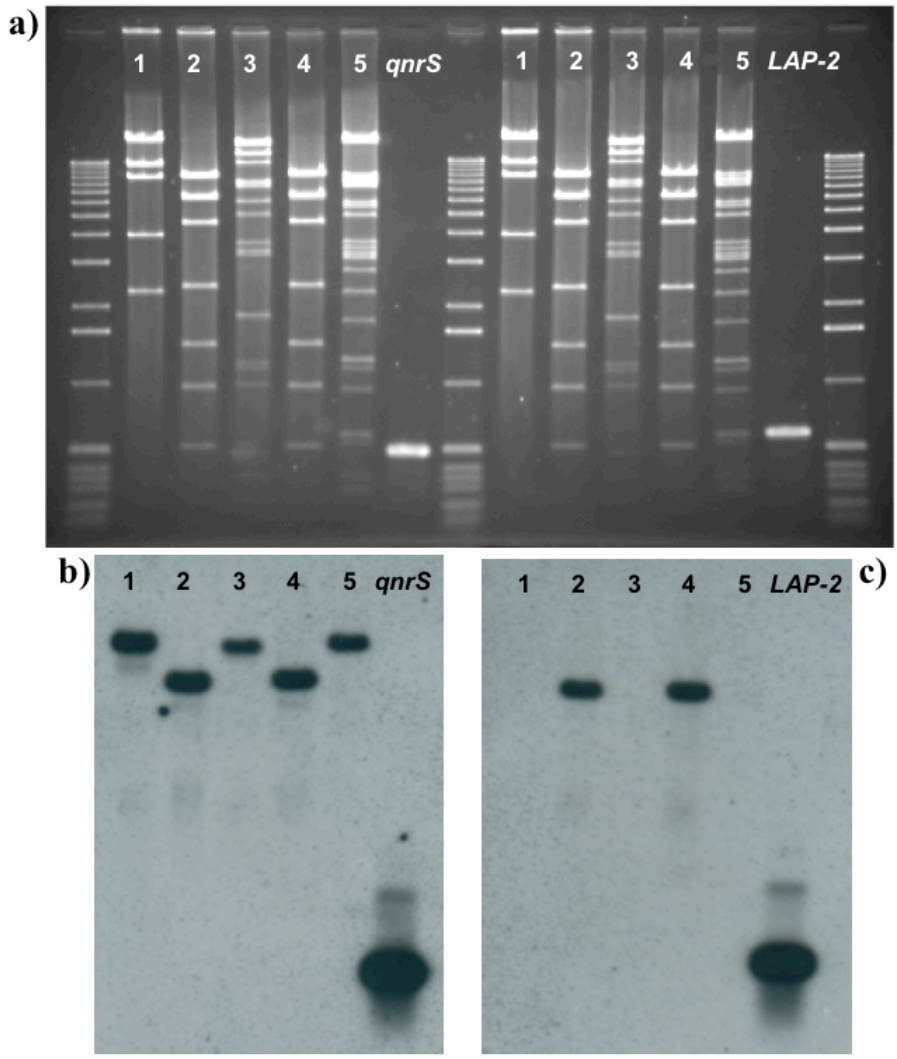


Figure 4.5 Agarose gel electrophoresis of digested plasmids with *Eco*RI and Southern hybridization with qnrS and bla_{LAP-2} probes.

a) Agarose gel electrophoresis showing the RFLP patterns of 5 plasmids (1, isolate LTMV18; 2, isolate LTMV33; 3, isolate LTMV6; 4, isolate LTMV30; 5, isolate LTMV1) after digestion with *Eco*RI and run in duplicate. PCR amplicons of qnrS and bla_{LAP-2} are included as a positive control.

b) Hybridisation with qnrS1 probe after Southern blotting of 5 mentioned plasmids. All 5 plasmids contain fragments corresponding with probe qnrS1.

c) Hybridisation with probe bla_{LAP-2} after Southern blotting of 5 mentioned isolates. In these 5 isolates, isolate 2 and 4 contain fragment hybridized with probe bla_{LAP-2}, which is precisely the fragment hybridized with probe qnrS1.

Table 4.1 The distribution of the various *qnrS*-containing transposons.

		Identical with pE66An (type A)	**Identical with pK18An (type B)**	**Identical with pK34N (type C)**	**Sub-type of known transposon**	**Unknown mobile element**
Hospital strains (n = 63)	*E. coli* (n = 5)	3 (60%)	0	0		2 (40%)
	K. pneumoniae (n = 52)	40 (77%)	1 (2%)	3 (5.7%)		8 (15.3%)
	Others (n = 6)	2 (33.3%)	0	0		4 (66.7%)
Community strains (n = 49)	*E. coli* (n = 32)	13 (40.6%)	0	0	1 (3.1%)	18 (56.3%)
	K. pneumoniae (n = 15)	5 (33.3%)	1 (6.7%)	3 (20%)	1 (6.7%)	5 (33.3%)
	Others (n = 2)	0	0	0		2 (100%)

Table showing the distribution of the various *qnrS*-containing transposon types from Enterobacteriaceae in Ho Chi Minh City, Vietnam. Based on RFLP results of all PCR amplicon positive replicons for *qnrS*-containing transposon, we can identify 3 main types of *qnrS*-containing transposon which are identical to pE66An (type A), pK18An (type B) and pK34N (type C). The "Sub-type of known transposon" column shows the number of isolates that were positive after hybridisation with both qnrS1 and bla_{LAP-2} probes.

4.3.5. General features from the DNA sequencing of three *qnrS1*-containing plasmids

Plasmid pE66An was extracted from an *E. coli* strain isolated from a patient admitted to tetanus ward of the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam. Plasmid pE66An was determined to be an 80,105 bp circular replicon with a G+C content of 51.52%. From the annotated sequence, 109 potential coding sequences were identified, of which 7 genes were predicted to be involved in resistance to a variety of classes of antimicrobials. Plasmid pE66An has two large fragments demonstrating significant homology with plasmid pKOX105 (Carattoli *et al.,* 2010) and 3 small regions demonstrating homology to plasmid pIP843 (Cao *et al.,* 2002), pRAx (Fricke *et al.,* 2009) and pKF3-94 (Zhao *et al.,* 2010) (Figure 4.6).

Similar to plasmid pE66An, plasmid pK18An was also extracted from a strain that was isolated from a patient in the tetanus ward of the hospital for tropical diseases, but in this case it was isolated from a *K. pneumoniae* strain. Plasmid pK18An is also a circular replicon; it is 51,160 bp in size and has a G+C content of 51.32%. The annotation of this pK18An identified 72 genes coding sequences, of which, 5 genes are potentially responsible for resistance to antimicrobials. Plasmid pK18An has 2 large regions demonstrating homology to plasmid pKOX105 (Carattoli *et al.,* 2010) (Figure 4.7).

Plasmid pK1HV was extracted from a *K. pneumoniae* strain from a healthy neonate living in Ho Chi Minh City. Plasmid pK1HV is a 133,191 bp circular plasmid with a G+C content of 52.5%. The plasmid contains 167 predicted coding sequences, of which, the majority encode conserved hypothetical uncharacterized proteins. However, pK1HV also carries 11 genes, which may induce resistance to various

antimicrobial groups. Plasmid pK1HV has a very large fragment (~84 kb), which demonstrates significant homology with pKOX105 (Carattoli *et al.,* 2010) (Figure 4.8). Plasmid pKOX105 was extracted from a *Klebsiella oxytoca* strain from the gut flora of a long-term-care-facility resident in Bolzano, Italy in 2005. This plasmid belongs to IncN group, and subsequently was confirmed to contain a very well-conserved IncN backbone (Carattoli *et al.,* 2010). All of genes on the common fragment to pK1HV are generally conserved hypothetical uncharacterized genes or genes encoding the components required for conjugal transfer. The remaining region of pK1HV contains genes responsible for resistance to antimicrobials or IS elements (Figure 4.6c).

The general characteristics of the 3 sequenced plasmids are shown in Table 4.2. The annotations of these 3 plasmids are described in Table 9.1, Table 9.2 and Table 9.3, Appendices.

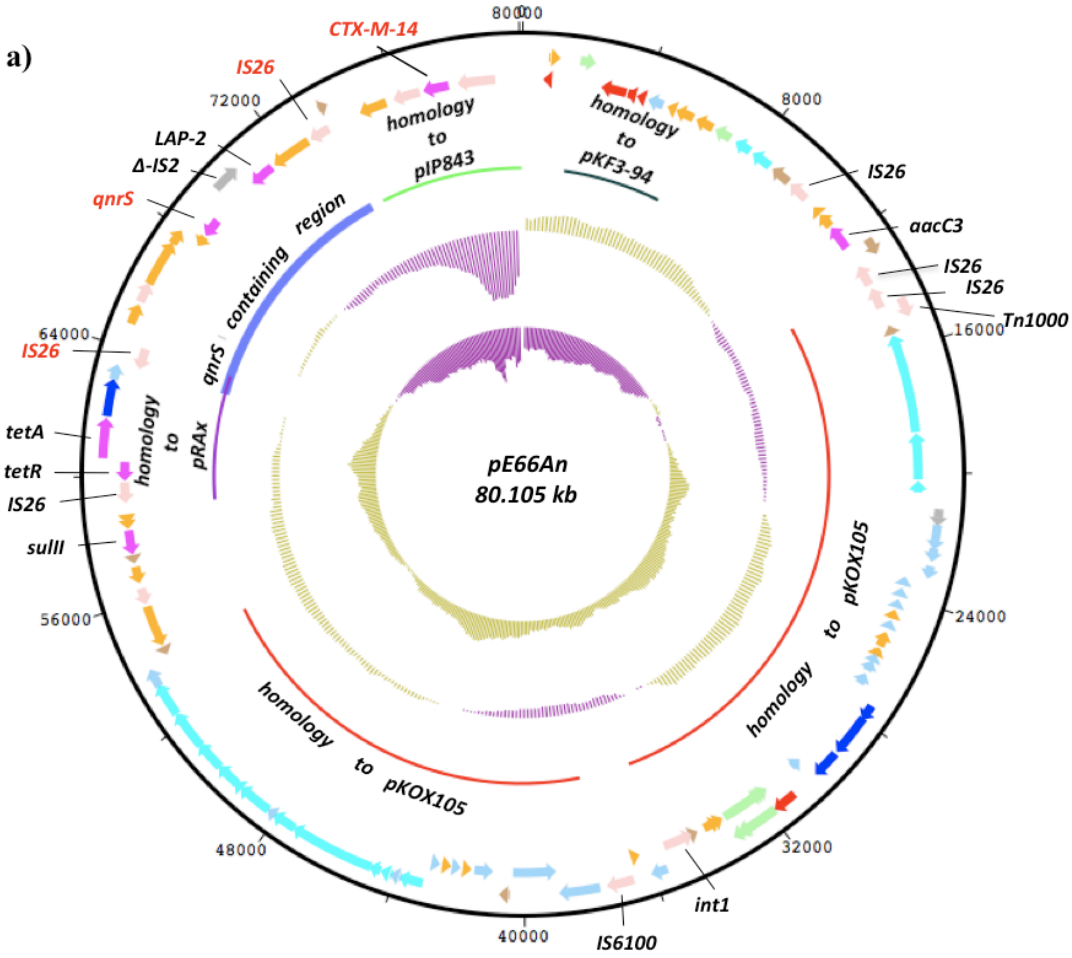


Figure 4.6 Circular map of plasmid pE66An.

Open Reading Frames (ORFs), which is defined as the region of the nucleotide sequences from the start codon (ATG) to the stop codon, are colour coded as followed: red, plasmid replication; dark blue, inorganic/metal/UV resistance; sky blue, conjugal transfer; dark pink, antimicrobial resistance; light green, unknown; light blue, regulators; orange, conserved hypothetical; brown, pseudogenes or partial genes; light pink, IS elements. Arrows show the direction of transcription. The most inner circle shows the GC skew ([G-C]/[G+C]) of that plasmid. The next circle shows %GC plot of that plasmid. This figure also shows the fragments that share significant homology with other plasmids.

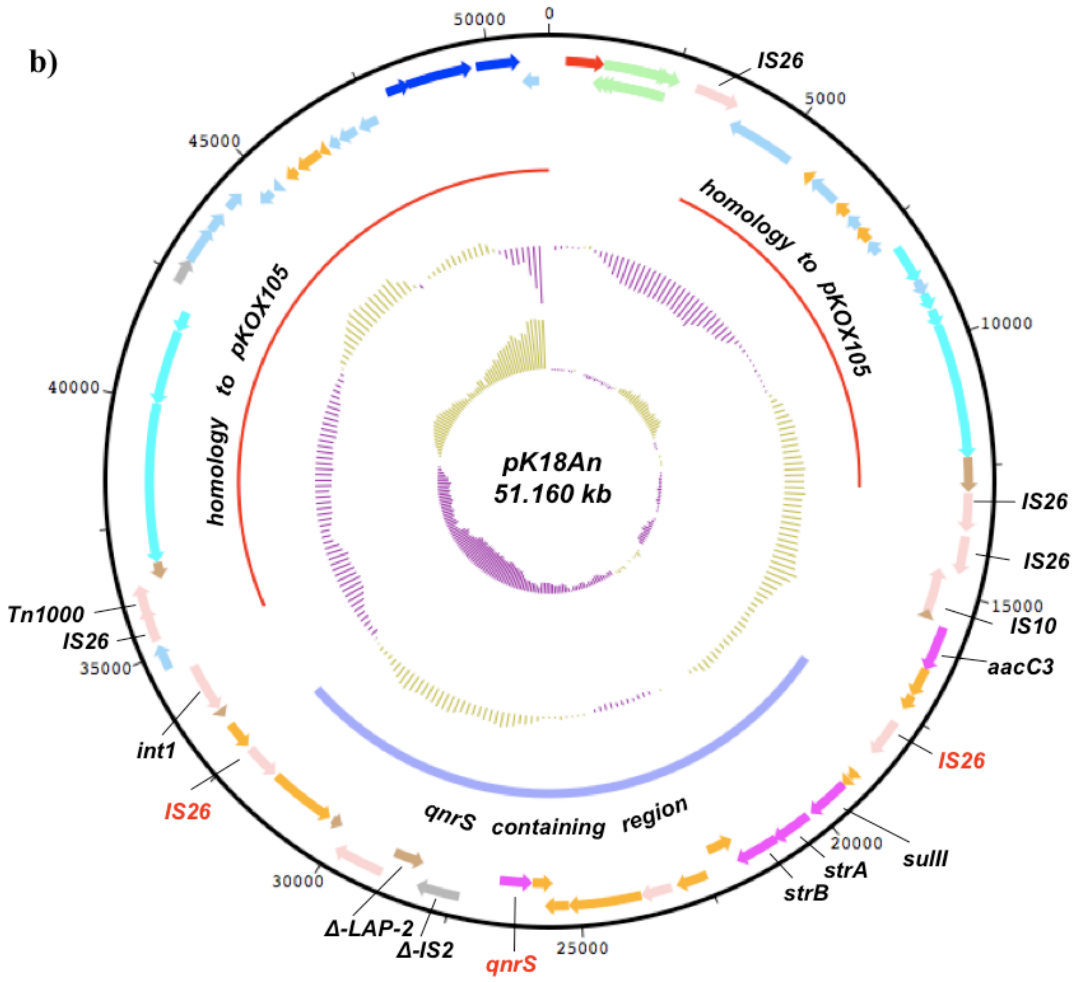


Figure 4.7 Circular map of plasmid pK18An.

ORFs are colour coded as followed: red, plasmid replication; dark blue, inorganic/metal/UV resistance; sky blue, conjugal transfer; dark pink, antibiotic resistance; light green, unknown; light blue, regulators; orange, conserved hypothetical; brown, pseudogenes or partial genes; light pink, IS elements. The arrows show the direction of transcription. The most inner circle shows the GC skew ([G-C]/[G+C]) of that plasmid. The next circle shows %GC plot of that plasmid. This figure also shows the fragments homology with other plasmids.

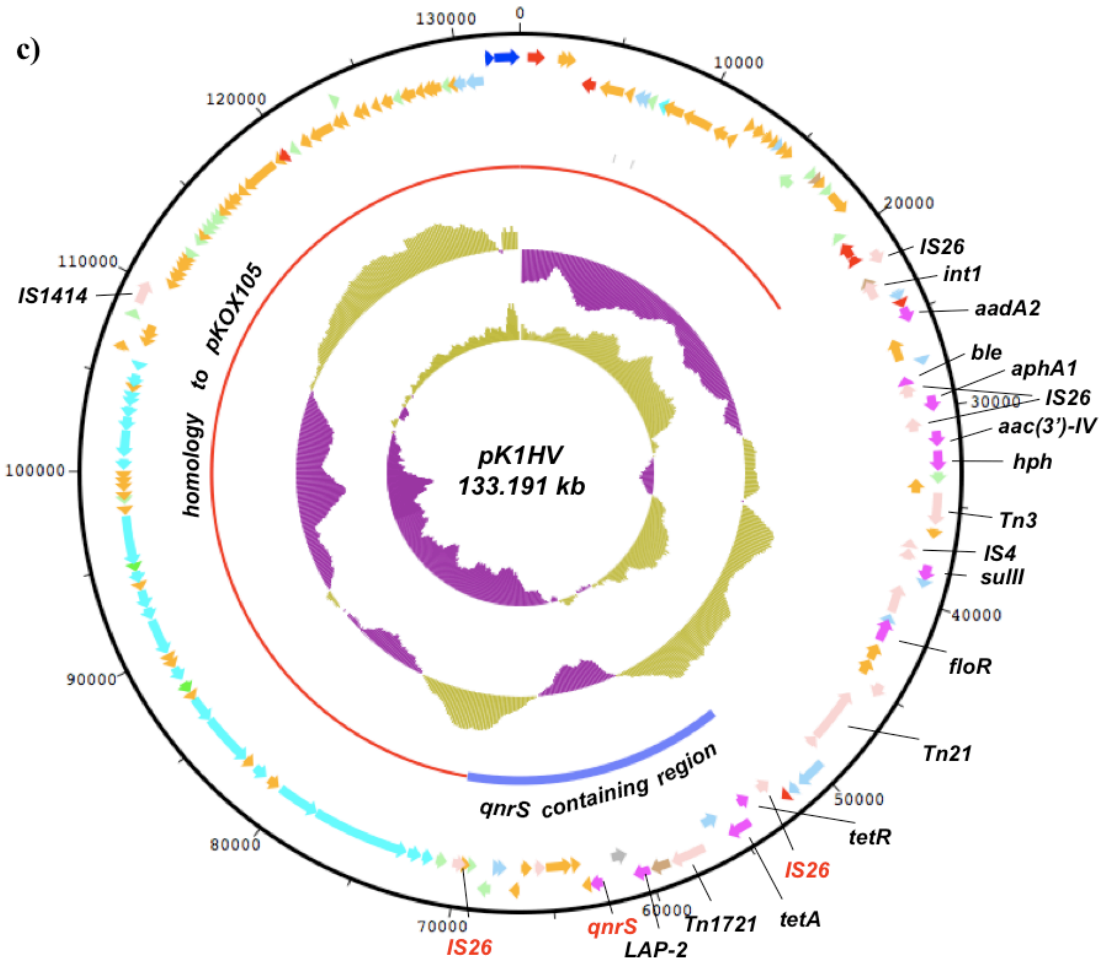


Figure 4.8 Circular map of plasmid pK1HV.

ORFs are colour coded as followed: red, plasmid replication; dark blue, inorganic/metal/UV resistance; sky blue, conjugal transfer; dark pink, antibiotic resistance; light green, unknown; light blue, regulators; orange, conserved hypothetical; brown, pseudogenes or partial genes; light pink, IS elements. The arrows show the direction of transcription. The most inner circle shows the GC skew ([G-C]/[G+C]) of that plasmid. The next circle shows %GC plot of that plasmid. This figure also shows the fragments homology with other plasmids.

Table 4.2 Characterization of 3 *qnrS*-containing plasmid sequences.

	pE66An	**pK18An**	**pK1HV**
Original strain	*E. coli*	*K. pneumoniae*	*K. pneumoniae*
Source	Hospital	Hospital	Community
Size (kb)	80.105	51.160	133.191
G + C (%)	51.25	51.32	52.5
Inc group	IncN	IncN	IncFII
Predicted genes (number of genes)	109	72	167
Essential function genes (number of genes)	22	15	14
Conjugative system (number of genes)	16	6	21
Resistance genes (number of genes)	7	5	11
IS elements	IS26, IS6100, IS903D, ISEcp1	IS26, IS10, IS5	IS26, IS4, IS1414
Integron	Integron 1	Integron 1	Integron 1

Table showing the general characteristics of 3 sequenced *qnrS1*-containing plasmids from *E. coli* and *K. pneumoniae*. The numbers in the predicted genes, essential function genes, conjugative system and resistance genes rows are the number of genes of each correlative plasmids. The IS elements and the integron rows show the type of IS transposase and integron present in each plasmids**.**

4.4. Discussion

Data describing the *qnrS1* transposon demonstrated that there is a dominant *qnrS1*-containing transposon (type A) circulating in *qnrS1* positive Enterobacteriaceae in Ho Chi Minh City, Vietnam, which we found to present in 71.4% of *qnrS1* positive isolates in hospital group and 36.7% of *qnrS1* positive isolates in community group. The other 2 types of *qnrS1*-containing transposon (type B and type C) differ from type A based on endonuclease digestion pattern with a deletion of 200 bp in a hypothetical gene (type C) or a 980 bp insert fragment of IS5 in type B with comparison to type A. Beside the *qnrS1* gene, type A and type C also contain the bla_{LAP-2} gene which confers resistance to β-lactam antimicrobials (Huang *et al.,* 2008; Wang, C. X. *et al.,* 2009). In contrast to type A and type C, the type B transposon has an insert of 980 bp of IS5 at the middle of bla_{LAP-2} gene, which presumably inactivates this gene. Moreover, these 3 transposons additionally carry a putative *fst1* gene. The *fst1* gene contains a penicillin-binding protein transpeptidase domain, which may additionally stimulate increased resistance to penicillin (Perna *et al.,* 2001).

By comparing the sequences of these 3 *qnrS1*-containing transposons with other previously sequenced *qnrS*-containing fragments, it was possible to identify a region of significant similarity among these sequences. This region of DNA homology was comprised of an IS2 element, a putative resolvase protein YdaA, whose predicted product is distantly related to a family of universal stress proteins (Beliaev *et al.,* 2002), and 3 other hypothetical uncharacterized proteins (Hata *et al.,* 2005; Chen, Y. *et al.,* 2006; Kehrenberg *et al.,* 2006; Kehrenberg *et al.,* 2007; Hu *et al.,* 2008; Wu *et al.,* 2008a; Park *et al.,* 2009; Carattoli *et al.,* 2010) (Figure 4.3). To date, there is no

report as to the function of the aforementioned proteins in the *qnrS1*-containing transposons yet, but it seems that these genes are probably part of the same mobilisable unit. Moreover, the G+C percentage of the 2 transposons (type A and type B) is 50.3%, which is lower than the mean GC percentage of all 3 plasmids, which suggests that the *qnrS1* encoding transposons have been inserted into these three sequenced plasmids by horizontal gene transfer.

Out of 112 *qnrS1*-positive strains, it was not possible to generate a PCR amplicon for the known *qnrS1*-containing transposon in 41 strains. An attempt was made to detect the mobile elements carrying the *qnrS1* in these isolates. There have been multiple reports regarding the association between *qnrS1* and bla_{LAP-2} genes (Poirel *et al.,* 2006a; Poirel *et al.,* 2007; Huang *et al.,* 2008; Cano *et al.,* 2009; Dahmen *et al.,* 2009; Park *et al.,* 2009). Yet, due to a lack of a PCR amplicon, it was hypothesized that some isolates contain the *qnrS1* and bla_{LAP-2} sequence, but the adjacent regions demonstrate a different structure. Therefore, the primers used to detect the *qnrS1*-containing transposons may not have been able to define the *qnrS1* transposon in some strains (Figure 4.3). Consequently, plasmid DNA from the 41 undetectable *qnrS1*-containing mobile element isolates were digested with *Eco*RI, which was selected because there was not an *Eco*RI restriction site inside the *qnrS1*-containing transposons type A, type B and type C, and hybridized with qnrS1 and bla_{LAP-2} probes. If the same restriction fragment demonstrates hybridisation for qnrS1 and bla_{LAP-2} probes, it may be assumed that the plasmid carries the so-called subfamily of known *qnrS1*-containing transposon. However, only two from 27 plasmids were positive by Southern blotting hybridisation. Therefore, there are still a number of *qnrS1*-containing plasmids (which are mainly from community) that obviously demonstrate significant diversity from the previously described *qnrS1* transposons;

such element require further investigation.

Figure 4.2 shows the similarity between the 3 sequenced plasmids and plasmid pK245 from *K. pneumoniae* isolated in Taiwan (Chen, Y. *et al.,* 2006), using ACT software. The comparison demonstrates that the two plasmids isolated from the hospital exhibit greater similarity than with the plasmid isolated in the community. Analysis and comparison of the coding sequences between plasmids pK18An and pE66An demonstrated that four out of five antimicrobial resistance genes are shared between them and are identical at the DNA level. These homologous genes were *aacC3* (resistance to aminoglycosides), *sulII* (resistance to sulfonamides), *strA* (resistance to aminoglycosides) and *qnrS1* (resistance to fluoroquinolones). All of these antimicrobial resistance genes are spanned by IS26 elements or other transposases, suggesting that common antimicrobial resistance genes are present and may be co-circulating throughout the Enterobacteriaceae on the ward of the hospital.

All 3 sequenced plasmids (pE66An, pK18An and pK1HV) contain regions that demonstrate significant homology with plasmid pKOX105 (Figure 4.6, 4.7 and 4.8). However, the region from pK1HV (from community), which is homologous with pKOX105, is different from the region of homology shared between pKOX105 and pE66An and pK18An (from hospital). The majority of the genes in the homology fragments between pE66An, pK18An and pKOX105 are predicted regulators or involved in conjugal transfer whilst the genes in the homologous fragment between pK1HV and pKOX105 are related conjugal transfer or are uncharacterized genes of unknown function.

Plasmid pE66An contains 2 regions of 10,280 bp and 5,183 bp respectively which include coding sequences that are predicted to be responsible for the conjugal

transfer. Similarly, plasmid pK18An also contains 2 regions containing coding sequences that are predicted to be responsible for the conjugal transfer, but these genes are disrupted by several IS26 elements. These data explain why plasmid pE66An was successfully conjugated in the laboratory, whilst it was not possible to conjugate pK18An into a recipient *E. coli* (results are shown on Chapter 3). Although pK18An is not conjugative, this plasmid sequence is littered with multiple IS elements, particularly surrounding resistance genes, suggests that such elements within the plasmid are highly promiscuous and may facilitate the transfer of these genes to other plasmids.

There are several features of the hospital-isolated plasmid pE66An that suggest it may be able to replicate and adapt in a variety of host and environments. For example, plasmid pE66An contains an *frmA* gene with 99.5% similarity to the alcohol dehydrogenase class III of *Pasteurella piscicida* (Kim and Aoki 1994) and 98.1% similarity to alcohol dehydrogenase class III of *E. coli* (Chen, S. L. *et al.,* 2006; Hochhut *et al.,* 2006). The function of this enzyme is related to the metabolism of formaldehyde dehydrogenase, and presence of *frmA* which may provide an added survival advantage to the host in the hospital environment where disinfectants containing formaldehyde are routinely used (Chen, Y. *et al.,* 2006). Furthermore, a *mucAB* repair system operon containing the genes *mucA* and *mucB* was identified in both hospital plasmids, pE66An and pK18An. These genes demonstrate 100% DNA identity to *mucA* and *mucB* genes from the IncN plasmid pKM101 which was isolated from an *E. coli* strain (Perry *et al.,* 1985). The *mucA* and *mucB* genes, also known as *umuD* and *umuC* genes, have long been used in the Ames test to increase the rate of mutagenesis (Kitagawa *et al.,* 1985). Therefore, the existence of the *mucAB* operon may facilitate mutation within genes that are targeted by

antimicrobials, which may enhance the resistance to antimicrobials with isolates that harbor the *mucAB* operon.

Plasmid pK1HV does not contain the *mucAB* operon like in pE66An and pK18An, but does contains the *imp* operon, which was 85% homologous to *impA* and *impB* extracted encoded on the IncI1 plasmid TP110 from *Salmonella* Typhimurium (Lodwick *et al.,* 1990). The DNA sequence of the *imp* operon demonstrates a close relationship to the two UV protection/mutation operons, *muc* and *umu*. The high degree of translated amino acid conservation amongst the predicted proteins of these 3 operons indicates these proteins have the similar and essential function (Lodwick *et al.,* 1990). As a result, the existence of the *imp* operon in plasmid pK1HV may also facilitate mutation within antimicrobial target genes.

Plasmids pK18An and pE66An both contain restriction modification systems; pK18An contains the *ecoRIIM* gene and pE66An contains the *ecoRIIR* and *ecoRIIM* genes, which exhibit an opposite orientation between 2 IS elements. The *ecoRIIR* and *ecoRIIM* genes are 98.8% identical at the DNA level with the Endonuclease *ecoRII* gene (Bhagwat *et al.,* 1990) and 100% identity with the modification methylase gene, *ecoRII* (Som *et al.,* 1987) from *E. coli*. In addition to assisting with defense against bacteriophage, this restriction modification system has also been reported to contribute to the spread and maintenance of plasmid which contains this system (Kobayashi 2001).

Plasmid pK18An carries the *aacC3*, *sulII*, *strA*, *strB* and *qnrS1* genes, which encode proteins that catalyze resistance to kanamycin (*aacC3*), gentamicin (*aacC3*), tobramycin (*aacC3*), neomycin (*aacC3*), apramycin (*aacC3*), sulfonamide (*sulII*), streptomycin (*strA* and *strB*) and quinolones (*qnrS1*). Plasmid pK18An also carries

bla$_{LAP-2}$ gene, which is in close proximity to *qnrS1*, but it is presumably inactivated by the insertion of IS5 element. The combination *sulII-strA-strB* is also present in plasmid pK18An and is adjacent to an IS26 element. In bacterial isolates found in humans and animals, *strA-strB* is often linked with the *sulII* sulfonamide-resistance gene and is encoded on broad-host-range non-conjugative plasmids. Although the usage of streptomycin in clinical and animal medicine has diminished, the persistence of *sulII-strA-strB* in bacterial populations implies that factors other than direct antimicrobial selection are involved in maintenance of these genes (Sundin and Bender 1996).

Plasmid pE66An contains the *aacC3*, *sulII*, *tetR*, *tetA*, *qnrS1*, *bla*$_{LAP-2}$, *bla*$_{CTX-M-14}$ gene which induce resistance to kanamycin (*aacC3*), gentamicin (*aacC3*), tobramycin (*aacC3*), neomycin (*aacC3*), apramycin (*aacC3*), sulfonamide (*sulII*), tetracycline (*tetR* and *tetA*), quinolone (*qnrS1*), β-lactam antimicrobials (*bla*$_{LAP-2}$) and third generation cephalosporins (*bla*$_{CTX-M-14}$). The association between the *qnrA* gene and ESBL encoding genes has been reported previously (Lavigne *et al.,* 2006; Castanheira *et al.,* 2007; Hamouda *et al.,* 2008). However, there are limited reports regarding the association between *qnrS* and ESBL producing genes (Strahilevitz *et al.,* 2009). Plasmid pE66An was identified to contain a *qnrS1* gene and a *bla*$_{CTX-M-14}$. In plasmid pE66An, *bla*$_{CTX-M-14}$ is adjacent to ISEcp1, an insertion element, which has been described as a mechanism of spreading of *bla*$_{CTX-M-14}$ (Bou *et al.,* 2002). As the *bla*$_{CTX-M-14}$ gene is in association with the insertion element ISEcp1, the dissemination of this gene to other plasmids or transposable elements is significantly enhanced (Snyder and Champness 2003). In a study regarding the genetic background of the *qnrB* and *qnrS* genes, Hu *et al.* also reported a non-conjugative plasmid (pHS8) from a *K. pneumoniae* which was identified to contain both *qnrS* and

$bla_{CTX\text{-}M\text{-}14}$ (Hu *et al.,* 2008) but the authors did not describe the genetic surrounding of $bla_{CTX\text{-}M\text{-}14}$.

The majority of the predicted coding sequences on plasmid pK1HV are hypothetical and uncharacterized. However, plasmid pK1HV also contains multiple genes related to antimicrobial resistance including *aadA2*, *ble*, *aphA1*, *aac(3')-IV*, *hph*, *sulII*, *floR*, *tetR*, *tetA*, $bla_{LAP\text{-}2}$ and *qnrS1*. These genes are related to reducing susceptibility to streptomycin (*aadA2*), bleomycin (*ble*), kanamycin (*aphA1*), amikacin (*aphA1*), gentamicin (*aac(3')-IV*), tobramycin (*aac(3')-IV*), neomycin (*aac(3')-IV*), apramycin (*aac(3')-IV*), hygromycin (*hph*), sulfonamide (*sulII*), chloramphenicol (*floR*), tetracycline (*tetR* and *tetA*), β-lactam ($bla_{LAP\text{-}2}$) and quinolones (*qnrS1*). Similar to plasmid pE66An, pK1HV also carries an IS26-*tetR*-*tetA* complex, which is a common means of the transfer of tetracycline resistance. Plasmid pK1HV also carries a type 1 integron containing the *dfrA12*-*orfF*-*aadA2* cassette, which was present in 2 of the earliest-known plasmids and remains very common in modern-day isolates (Gestal *et al.,* 2005). The presence of *dfrA12*-*orf*-*aadA2* in plasmid pK1HV (in community) implied that streptomycin is still present in community. Both plasmids pK18An and pE66An (hospital plasmids) carry an *int1* that implies the presence of an integron type 1 and they are both inserted with IS26 right after *dhfrV* gene, implying of a transmission of transposon containing integron in hospital strains.

4.5. Conclusion

Here I have characterized the dominant *qnrS1*-containing transposons circulating in the Enterobacteriaceae in Ho Chi Minh City, Vietnam. Moreover, the complete nucleotide sequences of the plasmids pE66An, pK18An and pK1HV identified the

molecular mechanisms contributing to multidrug resistance in 3 isolates from hospital and community and also provided insight into the means for adaptation of these plasmids within a variety of host and environments. The DNA sequence data demonstrated a high degree similarity between the 2 plasmids isolated from the hospital, presenting evidence of genetic transfer among isolates from the nature of the mobile genetic elements. In addition, the annotation of plasmid pE66An provides evidence for the association of plasmid-mediated quinolone resistance gene *qnrS* and the ESBL gene $bla_{CTX\text{-}M\text{-}14}$. This is also the first time that the complete sequencing of *qnrS1*-containing plasmid isolated from commensal enteric bacteria has been performed.

5. Differential phenotypic and genotypic characteristics of *qnrS1* harbouring plasmids carried by hospital and community commensal Enterobacteriaceae

5.1. Abstract

The *qnrS1* gene induces reduced susceptibility to fluoroquinolones in the Enterobacteriaceae. The prospect that there is co-selection of additional antimicrobial resistance genes with *qnrS1* was explored in addition to whether the spectrum of co-transferred genes is dependent on the environment where such plasmids originate. A naive *E. coli* was transformed with plasmid DNA extracted from *E. coli* (n = 15) and *K. pneumoniae* (n = 17) that were isolated in the hospital (n = 11) or the community (n = 21), and were PCR amplification positive for *qnrS1* and exhibited reduced susceptibility to ciprofloxacin. These and other findings show that whilst the diversity in these plasmids is substantial, the antimicrobial resistance patterns and antimicrobial resistance gene microarray combined with fragment mapping differentiates the plasmids into 2 groups. The two groups correspond to strains, which originate in the community or in the hospital ($p < 0.001$). All plasmids that conferred an ESBL phenotype (n = 6) were identified exclusively from strains isolated in the hospital. These data demonstrate that the *qnrS1* gene is extremely widespread and is stable in a number of highly variable replicons. Furthermore, differential genotypic and phenotypic characteristics of *qnrS1* containing plasmids isolated in the hospital and the community was found, indicative of distinct selective pressures in the two environments.

5.2. Introduction

The *qnrS1* gene is an example of a PMQR gene and encodes an amino acid pentapeptide repeat protein which is capable of protecting the DNA gyrase from the inhibitory activity of quinolones (Tran and Jacoby 2002). The transfer of a *qnrS1* gene into a naive laboratory recipient strain generally confers an increase in the MIC to fluoroquinolones, such as ciprofloxacin (Martinez-Martinez *et al.,* 1998) and the magnitude of the MIC increase is dependent on the background of the strain and the quinolone (Martinez-Martinez *et al.,* 1998; Wang *et al.,* 2003; Wang *et al.,* 2004b). In addition to increasing the MIC to quinolones, many reports, including the data presented in Chapter 4, show that the recipient strains are also resistant to other antimicrobials (Chen, Y. *et al.,* 2006). A study of the *qnrS1* in *Salmonella enterica* strains in the UK found that *qnrS1*-containing plasmids isolated from *Salmonella* also contain additional resistance genes, stimulating resistance to ampicillin, chloramphenicol, gentamicin, cefuroxime, ceftriaxone, cefotaxime, amikacin, kanamycin, streptomycin, sulphonamide, tetracycline and trimethoprim (Hopkins *et al.,* 2007).

In Chapter 3, I demonstrated that in commensal Enterobacteriaceae isolated from people resident in Ho Chi Minh City, 79% and 33% of *K. pneumoniae* isolated in the hospital and the community, respectively, were PCR amplification positive for *qnrS1* sequences. Furthermore, it was demonstrated that when *qnrS1* was combined with other PMQRs or mutations in DNA gyrase gene, *gyrA*, there was a substantial increase (up to 5 fold) in the MIC to ciprofloxacin and an MIC of >256 μg/ml to nalidixic acid. Based on the sequences of the 3 *qnrS1*-containing plasmids in Chapter 4, additional antimicrobial resistance genes were identified on the same plasmid with

the *qnrS1* gene, which suggests that the co-selection of additional antimicrobial resistance genes along with *qnrS1* gene is common. It was surmised that Gram-negative commensal organisms (such as *K. pneumoniae* and *E. coli*) in the community and the hospital in Ho Chi Minh City represent a substantial reservoir and source of dissemination of PMQR genes including the most frequently identified *qnrS1*.

The majority of work on PMQR plasmids has been performed on clinical strains isolated from infection sites (Iabadene *et al.,* 2008; Jiang *et al.,* 2008; Oktem *et al.,* 2008; Szabo *et al.,* 2008; Wang, A. *et al.,* 2008a). Consequently, little is known about PMQR encoding plasmids in commensal Enterobacteriaceae and their roles in disseminating resistance genes in areas where antimicrobial usage is unrestricted. In urban Vietnam, fluoroquinolones and other antimicrobials are available at low cost and without prescription in an array of outlets. It was hypothesized that *qnrS1*-containing plasmids that originate in the hospital environment may have similarities in both architecture and antimicrobial resistance gene content whilst *qnrS1* plasmids in the community have, in comparison, greater variability. Furthermore, an investigation was conducted on the association of the *qnrS1* gene with other antimicrobial resistance genes, which may be co-transferred and add be maintained via additional selective pressure.

Here, *qnrS1* plasmids from 32 unique strains (Table 5.1) that were isolated in the hospital or the community in Ho Chi Minh City are studied and some of their phenotypic and genotypic characteristics are examined. These 32 plasmids include of 26 *qnrS1*-containing plasmids with unknown *qnrS1*-containing transposons (Chapter 4), 3 *qnrS1*-containing plasmids with known *qnrS1*-containing transposons (Chapter

4) and the 3 sequenced plasmids (pE66An, pK18An and pK1HV). These 32 plasmids were electrotransferred into *E. coli* TOP10; therefore, *E. coli* TOP10 was used as a control in all experiments. As a result, any differences among these 32 transformants when compared to the control *E. coli* strain are the outcomes of *qnrS1*-containing plasmids.

The 32 transformants were compared using antimicrobial susceptibility testing (2.3.3, Chapter 2) and a DNA nanoarray containing probes for antimicrobial resistance genes (2.6.5, Chapter 2). Individual plasmids were assigned to incompatibility groups by PCR replicon typing (2.6.4, Chapter 2) and were compared using endonuclease digestion with *Eco*RI (2.6.3, Chapter 2). Sizes of these *qnrS1*-containing plasmids were also assigned by analyzing the restriction fragments after agarose gel electrophoresis using BioNumerics (2.6.2, Chapter 2). To investigate if there were any additional phenotypic characteristics of a subset of the *qnrS1* plasmid, transformants were assayed using a Biolog phenotype microarray (PM) (2.6.6, Chapter 2).

Table 5.1 List of the organisms used in this Chapter.

Nr	Strain ID	Plasmid name	Bacterial species	Year	Source
1	LTMV1	pEW-62R-MAN	*K. pneumoniae*	2006	Community
2	LTMV2	p033-CAZ-2	*E. coli*	2006	Community
3	LTMV3	p036-CN-2	*E. coli*	2006	Community
4	LTMV4	pA-003-I-a-1	*K. pneumoniae*	2005	Community
5	LTMV5	pK261An	*K. pneumoniae*	2005	Hospital
6	LTMV6	p023-CN-2	*E. coli*	2006	Community
7	LTMV7	p038-CN-2	*K. pneumoniae*	2006	Community
8	LTMV8	p008-CN-1	*E. coli*	2006	Community
9	LTMV9	p001-CN-2	*E. coli*	2006	Community
10	LTMV11	p024-CAZ-2	*E. coli*	2006	Community
11	LTMV12	pD-022-I-b-1	*K. pneumoniae*	2005	Community
12	LTMV13	p045-CN-2	*E. coli*	2006	Community
13	LTMV14	pK233Ca	*K. pneumoniae*	2005	Hospital
14	LTMV15	p025-CN-1	*E. coli*	2006	Community
15	LTMV16	p065-CN-2	*K. pneumoniae*	2006	Community
16	LTMV17	p048-CN-2	*E. coli*	2006	Community
17	LTMV18	pE18An	*E. coli*	2004	Hospital
18	LTMV19	pD-025-I-a-1	*E. coli*	2005	Community
19	LTMV20	p051-CN	*E. coli*	2006	Community
20	LTMV21	pK300N	*K. pneumoniae*	2005	Hospital
21	LTMV23	p039-CN-2	*E. coli*	2006	Community
22	LTMV24	p009-Na-2	*E. coli*	2006	Community
23	LTMV25	pA-001-I-a-1	*E. coli*	2005	Community
24	LTMV26	pB-011-I-a-1	*E. coli*	2005	Community
25	LTMV27	pEW-20N-MAG	*K. pneumoniae*	2006	Community
26	LTMV28	pK18An	*K. pneumoniae*	2004	Hospital
27	LTMV29	pE66An	*E. coli*	2004	Hospital
28	LTMV30	pK255An	*K. pneumoniae*	2005	Hospital
29	LTMV31	pK218Ca	*K. pneumoniae*	2005	Hospital
30	LTMV32	pK79N	*K. pneumoniae*	2004	Hospital
31	LTMV33	pK263Ax	*K. pneumoniae*	2005	Hospital
32	LTMV34	K279N	*K. pneumoniae*	2005	Hospital

5.3. Results

5.3.1. Characterization *qnrS1*-containing plasmids

Total plasmid DNA extracted from 32 commensal Enterobacteriaceae isolated from hospital inpatients (n = 11, *K. pneumoniae*; 9, *E. coli*; 2) or healthy volunteers (n = 21, *K. pneumoniae*; 6, *E. coli*; 15) were PCR amplicon positive for *qnrS1* (Table 5.2). A common *E. coli* strain (TOP10) was transformed with total plasmid DNA. Thirty-two novel strains that were isogenic, except for the transferred plasmid, were constructed. All strains were confirmed to be PCR amplification positive for the *qnrS1* gene and were confirmed to contain only one plasmid by alkaline lysis comparison on gel. All the newly constructed strains had an elevated MIC to nalidixic acid and ciprofloxacin, in comparison to the naive *E. coli* background strain (Table 5.2). However, no strains were categorized as resistant to ciprofloxacin (median; 0.25, range; 0.12 - 0.38) and all but one strain still demonstrated susceptibility to nalidixic acid (median; 4, range; 4 - 12), according to current CLSI susceptibility breakpoints (Table 2.1, Chapter 2) (CLSI 2007).

Table 5.2 Bacterial strains and the characteristics of *qnrS1* encoding plasmids.

Strain ID	Plasmid name	Source[a]	Bacterial Species[b]	Incompatibility group[c]	Size (Kbp)	ESBL	MIC (µg/ml)		Resistance pattern[d]
							NAL	CIP	
Top10	-	-	*E. coli*	-	-	-	1.5	0.006	-
LTMV1	pEW-62R-MAN	Community	*K. pneumoniae*	A/C	9	-	6	0.38	CIP_L
LTMV2	p033-CAZ-2	Community	*E. coli*	FIA; A/C	101	-	4	0.25	CHL, TET, SXT, CIP_L
LTMV3	p036-CN-2	Community	*E. coli*	repF; HI1; FIA; A/C	80	-	12	0.38	CHL, GEN, CIP_L
LTMV4	pA-003-I-a-1	Community	*K. pneumoniae*	FIA; A/C; R	132	-	4	0.25	CHL, GEN, TET, SXT, CIP_L
LTMV5	pK261An	Hospital	*K. pneumoniae*	R	70	-	4	0.25	AMP, CHL, GEN, TET, SXT , CIP_L
LTMV6	p023-CN-2	Community	*E. coli*	repF; FIB	140	-	4	0.25	AMP, CHL, CIP_L
LTMV7	p038-CN-2	Community	*K. pneumoniae*	R	64	-	4	0.25	AMP, CHL, GEN, TET, SXT , CIP_L
LTMV8	p008-CN-1	Community	*E. coli*	repF; FIB	140	-	8	0.38	CHL, GEN, CIP_L
LTMV9	p001-CN-2	Community	*E. coli*	unknown	96	-	4	0.38	AMP, CHL, GEN, TET, CIP_L
LTMV11	p024-CAZ-2	Community	*E. coli*	FIA	44	-	6	0.38	AMP, TET, SXT, CIP_L
LTMV12	pD-022-I-b-1	Community	*K. pneumoniae*	unknown	98	-	4	0.25	AMP, TET, SXT, CIP_L
LTMV13	p045-CN-2	Community	*E. coli*	repF	107	-	6	0.25	AMP, CHL, CIP_L
LTMV14	pK233Ca	Hospital	*K. pneumoniae*	unknown	51	+	4	0.12	AMP, GEN, CRO, CIP_L
LTMV15	p025-CN-1	Community	*E. coli*	unknown	135	-	6	0.38	AMP, CHL, TET, SXT, CIP_L
LTMV16	p065-CN-2	Community	*K. pneumoniae*	unknown	71	-	4	0.25	AMP, CHL, GEN, TET, CIP_L
LTMV17	p048-CN-2	Community	*E. coli*	R	26	-	6	0.38	CHL, CIP_L
LTMV18	pE18An	Hospital	*E. coli*	unknown	58	-	4	0.25	AMP, CIP_L
LTMV19	pD-025-I-a-1	Community	*E. coli*	unknown	130	-	6	0.38	AMP, CHL, GEN, TET, SXT, CIP_L
LTMV20	p051-CN	Community	*E. coli*	R; colE	108	-	4	0.25	AMP, CHL, GEN, TET, SXT , KAN, CIP_L
LTMV21	pK300N	Hospital	*K. pneumoniae*	R	57	+	6	0.25	AMP, FEP, GEN, TIC, CRO, CIP_L
LTMV23	p039-CN-2	Community	*E. coli*	unknown	109	-	4	0.25	AMP, CHL, GEN, TET, TIC, SXT , KAN, CIP_L
LTMV24	p009-Na-2	Community	*E. coli*	unknown	43	-	6	0.12	AMP, CIP_L
LTMV25	pA-001-I-a-1	Community	*E. coli*	unknown	118	-	3	0.25	AMP, CHL, GEN, SXT , CIP_L
LTMV26	pB-011-I-a-1	Community	*E. coli*	Y	103	-	3	0.25	AMP, CHL, GEN, CIP_L
LTMV27	pEW-20N-MAG	Community	*K. pneumoniae*	unknown	132	-	4	0.25	AMP, GEN, TET, SXT, KAN, CIP_L
LTMV28	pK18An	Hospital	*K. pneumoniae*	N	58	-	4	0.25	GEN, CIP_L
LTMV29	pE66An	Hospital	*E. coli*	N	87	+	4	0.25	AMP, FEP, GEN, TET, SXT, CRO, CIP_L
LTMV30	pK255An	Hospital	*K. pneumoniae*	A/C	48	-	4	0.12	GEN, CIP_L
LTMV31	pK218Ca	Hospital	*K. pneumoniae*	N	60	+	6	0.38	AMP, FEP, GEN, CRO, CIP_L
LTMV32	pK79N	Hospital	*K. pneumoniae*	N	74	+	6	0.38	AMP, FEP, GEN, TET, SXT, CRO, CIP_L
LTMV33	pK263Ax	Hospital	*K. pneumoniae*	unknown	49	-	6	0.19	GEN, CIP_L
LTMV34	pK279N	Hospital	*K. pneumoniae*	unknown	105	+	4	0.25	AMP, FEP, GEN, CRO, CIP_L

a) Source of isolation of the original bacterial isolates containing *qnrS1*encoding plasmid.
b) Original bacterial species from which the *qnrS1*-encoding plasmid was isolated.
c) Incompatibility group determined by PCR (Carattoli *et al.,* 2005; Garcia-Fernandez *et al.,* 2009).
d) NAL: Nalidixic acid; CIP_L: reduced susceptibility to Ciprofloxacin; CHL: Chloramphenicol; TET: Tetracycline; SXT: Trimethoprim/Sulphamethazole; GEN: Gentamicin; AMP: Ampicillin; CRO: Ceftriaxone; KAN: Kanamycin; FEP: Cefepime; TIC: Ticarcillin

Plasmid sizes were variable, as were the incompatibility groups. The plasmid sizes ranged from 9 to 140 Kbp, with a median of 84 Kbp (Table 5.2). The *qnrS1* encoding plasmids that originated from the hospital ranged from 48 to 105 Kbp (median; 58 Kbp) and those originating from the community ranged from 9 to 140 Kbp (median; 103 Kbp). The difference in plasmid size between these two groups was statistically significant (p = 0.024, calculated by 2 tailed t-test). All strains were subjected to PCR for 21 different incompatibility replicons. I identified 11 different incompatibility group combinations, of which incN (n = 4) and incR (n = 4) were the most common (Table 5.2). I was unable to PCR amplify any replicon genes in 13 strains and 6 strains demonstrated various amplicons, signifying multiple replication origins.

All 32 *qnrS1* plasmids were digested using *Eco*R1. An example of the variability in restriction patterns is shown in Figure 5.1. Plasmid restriction patterns were compared using a Jacquard correlation to identify common banding patterns (Figure 5.2). There were only plasmids from two strains (LTMV30 and LTMV33) that demonstrated an identical restriction pattern. Thirty strains demonstrated less than 67% banding identity. The digestion patterns were subdivided into two main groups (1 and 2) on the basis of having less than 20% commonality (Figure 5.2). The *qnrS1* plasmids originating in the community were generally associated with group 1 and the hospital plasmids were correspondingly associated with group 2 (p = 0.0017, two tailed Fishers exact test), suggesting some commonality in *Eco*R1 restriction patterns.

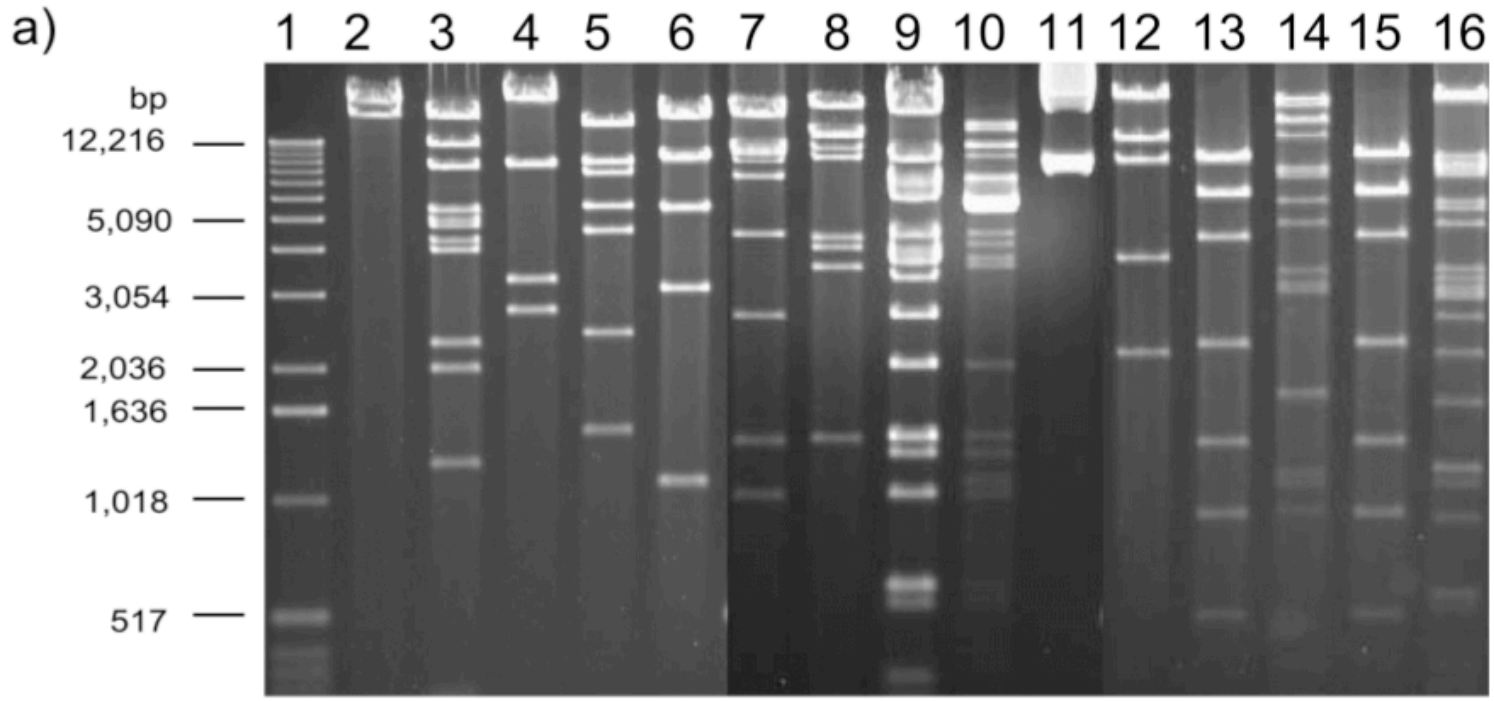


Figure 5.1 *Eco*RI restriction digest analysis of *qnrS1*-encoding plasmids.

Thirty-two *qnrS1*-harbouring plasmids were isolated from commensal Enterobacteriaceae and were transferred into naive *E. coli* TOP10 cell. Plasmid DNA was extracted from the 32 transformants and digested with *Eco*RI and compared by agarose electrophoresis.

Example of *Eco*RI digestion of 15 *qnrS1*-encoding plasmids, Lanes: 1; 1 Kb ladder (Invitrogen), 2; LTMV21, 3; LTMV16, 4; LTMV28, 5; LTMV14, 6; LTMV11, 7; LTMV25, 8; LTMV5, 9; LTMV2, 10; LTMV20, 11; LTMV1, 12; LTMV18, 13; LTMV33, 14;LTMV6, 15; LTMV30 and 16; LTMV19.

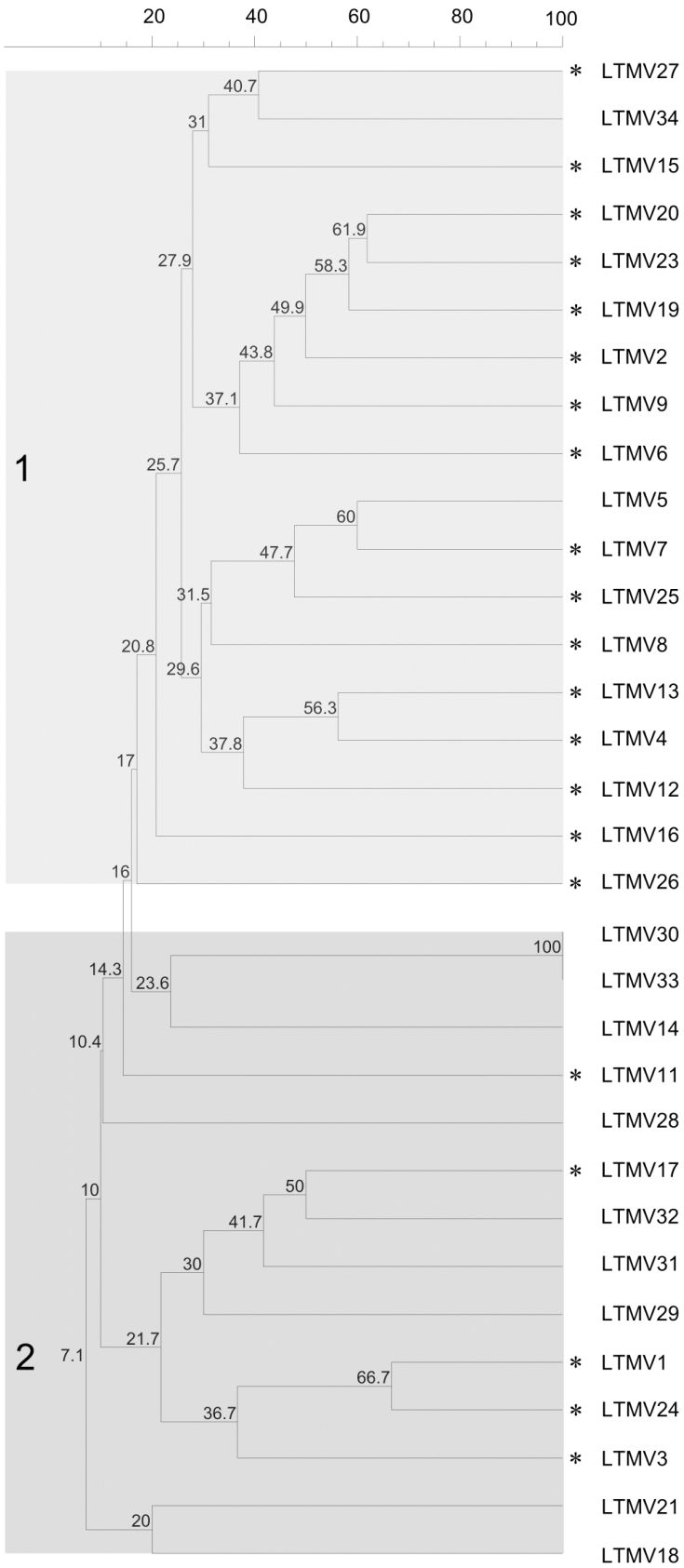


Figure 5.2 Neighbour joining tree of 32 *qnrS1*-encoding plasmids.

Neighbour joining tree of 32 *qnrS1*-encoding plasmids derived from digestion with *Eco*RI (names of the strains are shown). Plasmids isolated from strains originating in the community are highlighted by the asterisk. Numbers within the shaded boxes correspond with the percentage relationship between the individual nodes. The two shaded boxes (1 and 2) correspond with the two major groups of digested plasmid.

5.3.2. Antimicrobial susceptibility testing

The 32 strains were subjected to susceptibility testing with 9 (supplementary to nalidixic acid and ciprofloxacin) antimicrobials (Table 5.2). As all plasmids were harboured by an isogenic *E. coli*, any additional resistance determinants were, consequently, co-transferred with the *qnrS1* encoding region. Ampicillin resistance was the most common additional phenotype (23/32, 71.9%) and resistance to ticarcillin was the least common (2/32, 6.3%).

The resistance profiles were subdivided in groups according to the origin of the plasmids; firstly *E. coli* and *K. pneumoniae* and additionally into those from community and hospital (Table 5.3). There was no significant disparity (with the exception of chloramphenicol resistance) between the resistance profiles of plasmids isolated from *E. coli* and *K. pneumoniae.* However, the resistance profiles between plasmids isolated in the community and hospital demonstrated several differences. The *qnrS1* plasmids isolated in the hospital were more frequently resistant to gentamicin, ceftriaxone (corresponding with ESBL production, 6/11, 55.4%) and cefepime. Whilst only chloramphenicol resistance was associated with plasmids isolated from the community (Table 5.3).

Table 5.3 The proportion of *qnrS1* plasmid containing strains exhibiting a co-resistance with nine antimicrobials.

	Antimicrobial tested								
	AMP	**GEN**	**CHL**	**TET**	**SXT**	**CRO[c]**	**FEP[c]**	**KAN**	**TIC**
Number of resistant organisms (from 32) [n (%)]	23 (71.9)	21 (65.6)	18 (56.3)	15 (46.9)	15 (46.9)	6 (18.8)	5 (15.6)	2 (6.3)	2 (6.3)
Resistant *E. coli* (from 17) [n (%)]	13 (76.5)	9 (52.9)	13 (76.5)	8 (47.1)	8 (47.1)	1 (5.9)	1 (5.9)	2 (11.8)	1 (5.9)
Resistant *K. pneumoniae* (from 15) [n (%)]	10 (66.7)	13 (86.7)	4 (26.7)	7 (46.7)	6 (40.0)	5 (33.3)	4 (26.7)	1 (6.7)	1 (6.7)
***p* value [a]**	0.6989	0.0605	0.0118*	1	0.7345	0.0755	0.1609	1	1
Resistant organisms community (from 21) [n (%)]	15 (68.2)	11 (50.0)	17 (77.3)	12 (54.5)	11 (50.0)	0 (0.0)	0 (0.0)	2 (9.1)	1 (4.5)
Resistant organisms hospital (from 11) [n (%)]	8 (72.7)	10 (90.9)	1 (9.1)	3 (27.3)	4 (36.4)	6 (54.5)	5 (45.5)	0 (0.0)	1 (9.1)
***p* value [b]**	1	0.0273*	0.0005*	0.2659	0.712	0.0004*	0.0019*	0.5417	1

a) *p* value calculated on the proportion of sensitive/resistant strains after plasmid transformation with plasmid DNA isolated from either *E. coli* and *K. pneumoniae* using two tailed Fishers exact test.

b) *p* value calculated on the proportion of sensitive/resistant strains after plasmid transformation with plasmid DNA prepared from either community and hospital isolates using two tailed Fishers exact test.

c) All strains that were resistant to cefepime and ceftriaxone were ESBL positive

*Indicates statistical significance ($p < 0.05$)

5.3.3. Antimicrobial resistance gene content

Genomic DNA was extracted from the 32 strains and hybridised to a miniaturized antimicrobial resistance gene DNA microarray to assess the antimicrobial resistance gene content. The hybridisation patterns to specific antimicrobial resistance gene loci are shown in Figure 5.3 and are subdivided into genes responsible for resistance (or reduced susceptibility) to quinolones, aminoglycosides, chloramphenicol, trimethoprim, sulphonamide, ß-lactams and tetracycline. As predicted, DNA extracted from all strains demonstrated hybridisation to *qnrS1*. Twenty-five strains demonstrated hybridisation to probes for genes encoding ß-lactam resistance, with 19/32 (59.4%) and 4/32 (12.5%) producing a positive signal for *tem1* and *ctxM9*, respectively (Figure 5.3). Overall, 17 strains demonstrated positive hybridisation to the class one integrase probe (Figure 5.3).

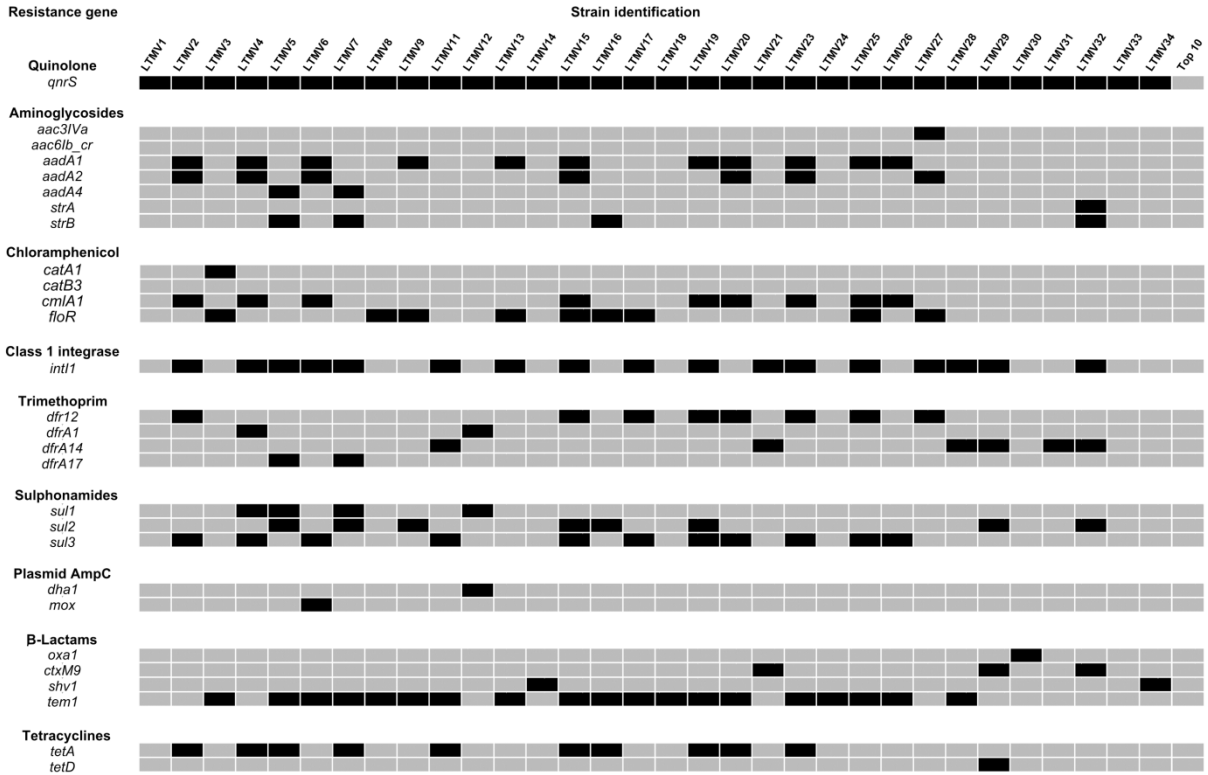


Figure 5.3 Antimicrobial resistance gene content of 32 *qnrS1* encoding plasmids assessed by hybridization to antimicrobial resistance gene DNA nanoarray.

Genomic DNA from 32 strains carrying *qnrS1* encoding plasmids was hybridized to a miniaturized antimicrobial resistance gene DNA microarray containing 54 antimicrobial resistance gene probes, representing 10 different groups of antimicrobial agents. Map shows the pattern of 27 antimicrobial resistance genes and one class one integrase gene (*intI1*) that demonstrated hybridization (black) or no hybridization (grey) according to signal intensities of ≥ 0.4 (positive) and < 0.3 (negative).

The hybridisation patterns were subdivided, as before, into groups according to the origin of the plasmids; *E. coli* or *K. pneumoniae* (Figure 5.4) and community or hospital (Figure 5.5). There was a clear variability in hybridisation patterns between plasmids that originated in the hospital and in the community (Figure 5.5). None of the plasmids originating from the hospital carried any of the four chloramphenicol resistance determinants, confirming phenotypic differences (Table 5.2). Hybridisation to the *oxa1*, *ctxM9* or *shv1* was only detected in the hospital plasmids, whereas, hybridisation to *tem1* was more common in community plasmids. Correspondingly, only the hospital plasmids had a transferable ESBL phenotype. However, two community plasmids were found to contain plasmid AmpC genes. The average number of genes detected by the assay in the community (6.3 per plasmid) and the hospital plasmids (3.7 per plasmid) was also significantly different (p = 0.013 calculated by 2 tailed t-test).

The hybridisation data were subjected to a Pearson correlation analysis on the basis of the presence or absence of hybridisation to the antimicrobial resistance gene probes, the resulting neighbour joining tree is shown in Figure 5.6. The hybridisation patterns divided the plasmids into two main groups (A and B), the partitioning corresponded with the community and the hospital plasmids ($p < 0.001$, by Fishers exact test). This grouping was also concurrent with the plasmid digestion patterns, with 14 and 2 community and hospital group 1A plasmids, respectively, and 8 and 1 hospital and community group 2B plasmids, respectively ($p < 0.001$, by Fishers exact test).

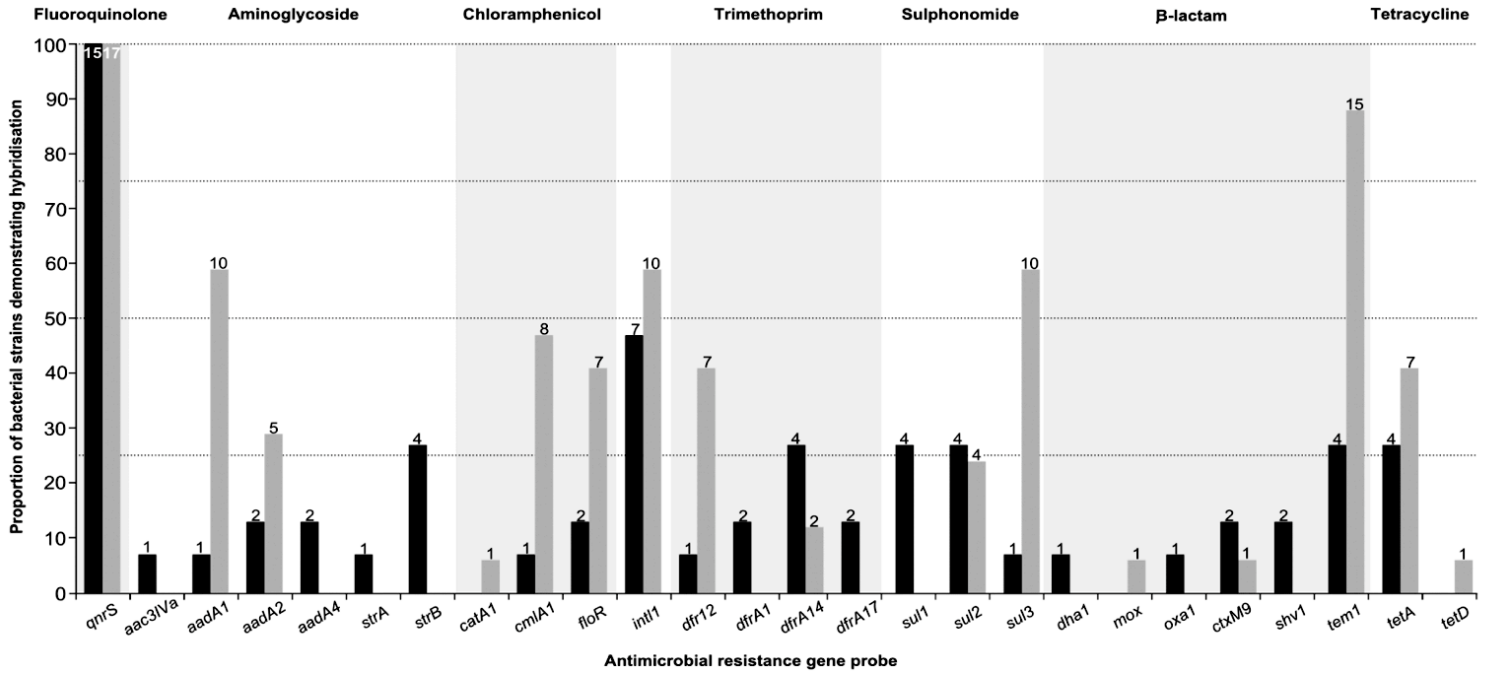


Figure 5.4 The antimicrobial resistance gene content of 32 *qnrS1* encoding plasmids according to the bacterial species of plasmid isolation.

Graph presents the proportion of transformants containing *qnrS1* plasmids that demonstrated hybridization to 25 specific antimicrobial resistance genes and a class one integrase gene (*intI1*). Graph is subdivided into those plasmids which were originally isolated from the *K. pneumoniae* (black) (n = 15) and the *E. coli* (grey) (n = 17). The labels at the head of the diagram highlight the groups to which the antimicrobial resistance gene confers resistance.

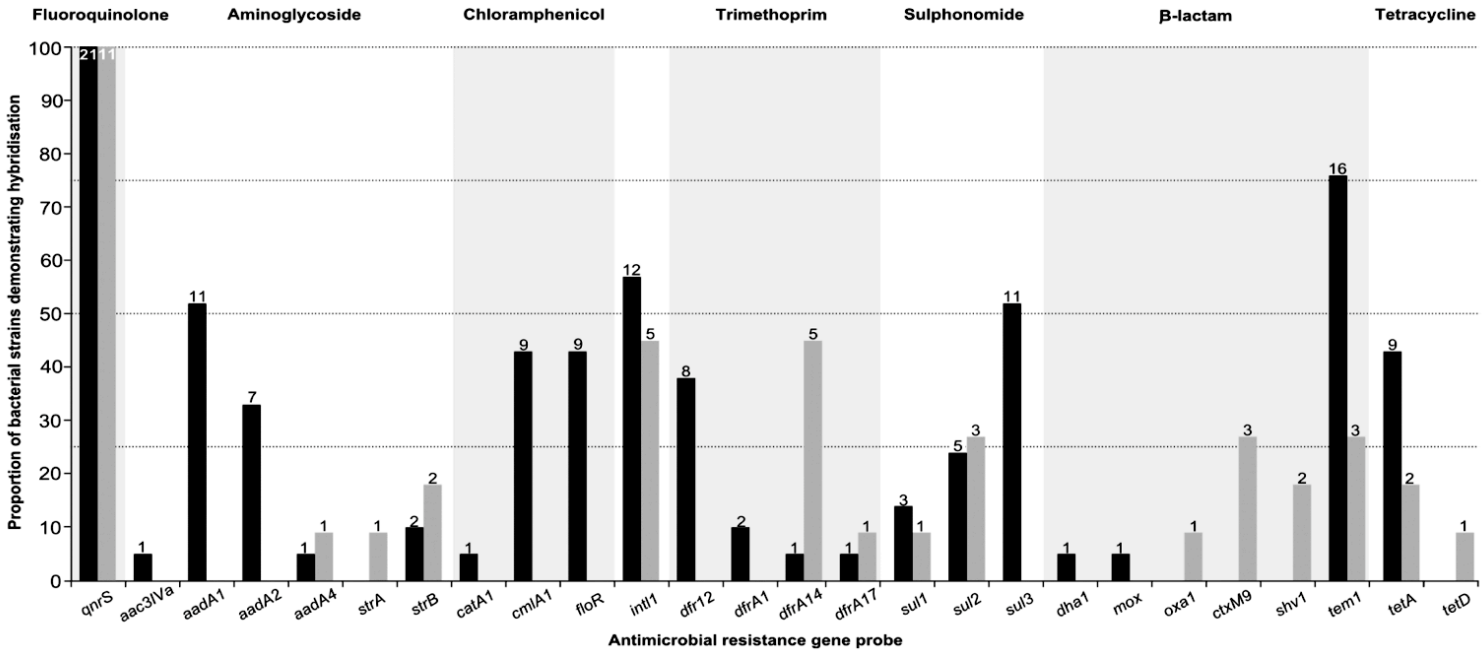


Figure 5.5 The antimicrobial resistance gene content of 32 *qnrS1* encoding plasmids according to the original setting of plasmid isolation.

Graph as Figure 5.4 presents the proportion of transformants containing *qnrS1* plasmids that demonstrated hybridization according to the originally isolated in the community (black) (n = 21) and the hospital (grey) (n = 11).

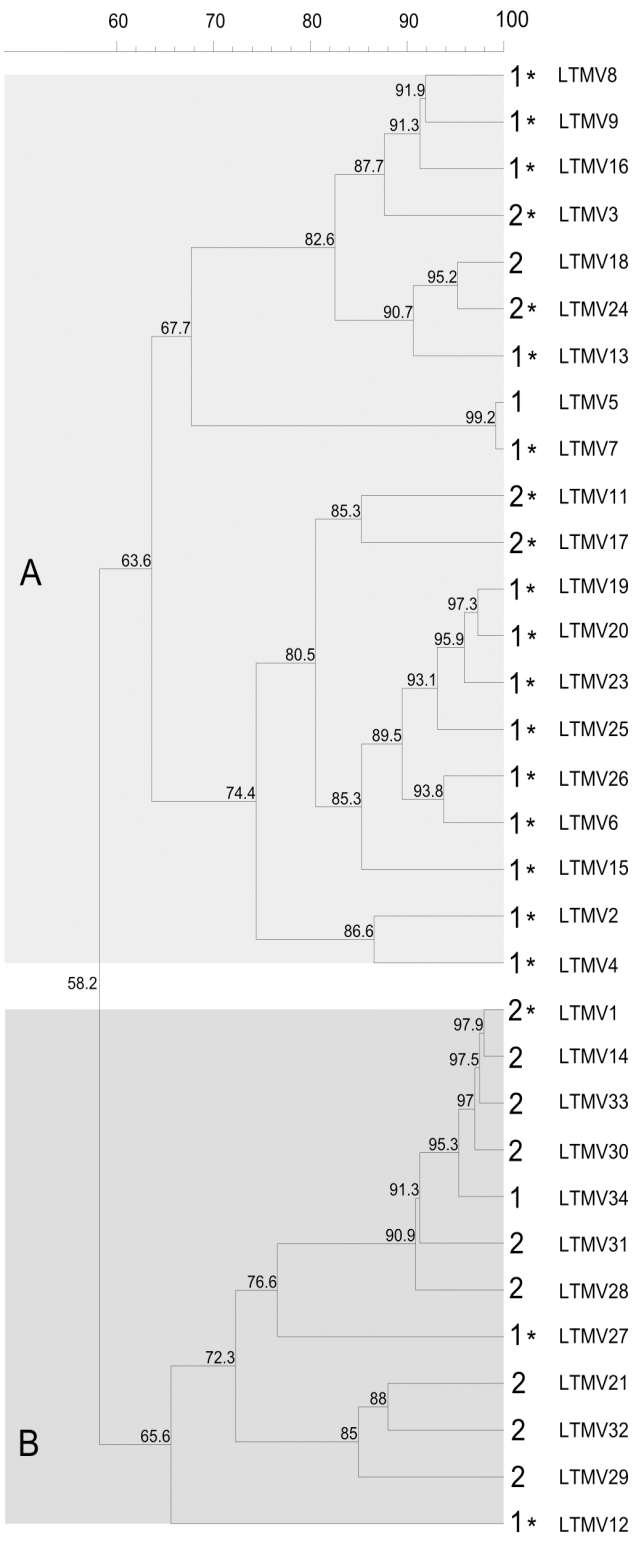


Figure 5.6 Neighbour joining tree derived from antimicrobial resistance gene content of 32 *qnrS1* encoding plasmids assessed by hybridization to DNA nanoarray.

This neighbour joining tree is comprised of data produced from hybridization to miniaturized DNA microarray (presence or absence), by Pearson correlation. The 32 strains fall into two main clusters (A and B), which are distinguished by grey shading. The strain names are shown on the right and correspond with Table 1. The asterisk identifies those strains containing community plasmids; the numbers adjacent to the strain names correspond with the major groups according to *Eco*R1 restriction digestion (Figure 5.2). Plasmids originating in the community were significantly associated with group 1 and plasmids originating in the hospital were correspondingly associated with group B (p = 0.0021, using two tailed Fishers exact test). The numbers on each branch correspond to percentage identity between individual branches.

5.3.4. High throughput phenotyping

To investigate if the transferred plasmids could stimulate any additional phenotypic characteristics, 6 strains were subjected to high throughput phenotyping analysis. Six strains were selected on the basis of plasmid, nanoarray and antimicrobial resistance diversity, which were: LTMV1, LTMV5, LTMV14, LTMV18, LTMV20 and LTMV29 (Table 5.2). These 6 strains contained plasmids from the hospital and the community belonging to groups that we defined as 1A, 2A and 2B (Figure 5.6). The naive *E. coli* strain was used as a control and the output data from the test strains was compared to baseline data from this strain.

In total, the respiration of the six strains was compared for 427 chemicals, with the control strain as a comparator. The resulting data was compared by ANOVA to identify those agents, which stimulated either an increase or a decrease in cellular respiration over 26 hours. Four hundred and three chemical assays demonstrated no significant change over the experimental period. However, we were able to identify a reproducible, differential change in cellular respiration in at least 1 strain out of 7 strains in 24 chemical assays. Figure 5.7, shows the data from the 24 assays, which demonstrated a differential pattern in the 7 strains. The respiration response in Figure 5.7 is subdivided into those demonstrating strong respiration (black), intermediate respiration (grey) and no respiration (white). The group demonstrating the most differences were those that demonstrated respiration in the presence of ß-lactams, these data concur with the susceptibility patterns, resistance gene content and ESBL production. The exception being LTMV29, which was able to respire in the presence of third generation cephalosporins (Figure 5.7), yet was considered as sensitive to

these compounds when compared to CLSI guidelines (Table 5.1). Respiration in presence of ß-lactams was more common in plasmids isolated in hospital strains.

All 6 experimental strains were capable of respiration in norfloxacin; however, only LTMV1, which had the highest MIC to nalidixic acid from the six strains (6µg/ml), was capable of respiration in the presence of a range of quinolones. Another notable difference in the presence of antimicrobials was strain LTMV20, which contains a community isolated plasmid and demonstrated respiration in the macrolide, tylosin. Atypically, LTMV1 demonstrated a number of inverse phenotypes, with respect to the control strain and the other 5 experimental strains. LTMV1 was unable to respire in the presence of Glucose-1-phosphate and the antiseptic agents 9 aminoacridine and acriflavine. Additionally, strain LTMV5 was resistant to the tyrosine phosphatase inhibitor, sodium orthovanadate, and strain LTMV29 was unable to respire in the presence of potassium telluride (Figure 5.7).

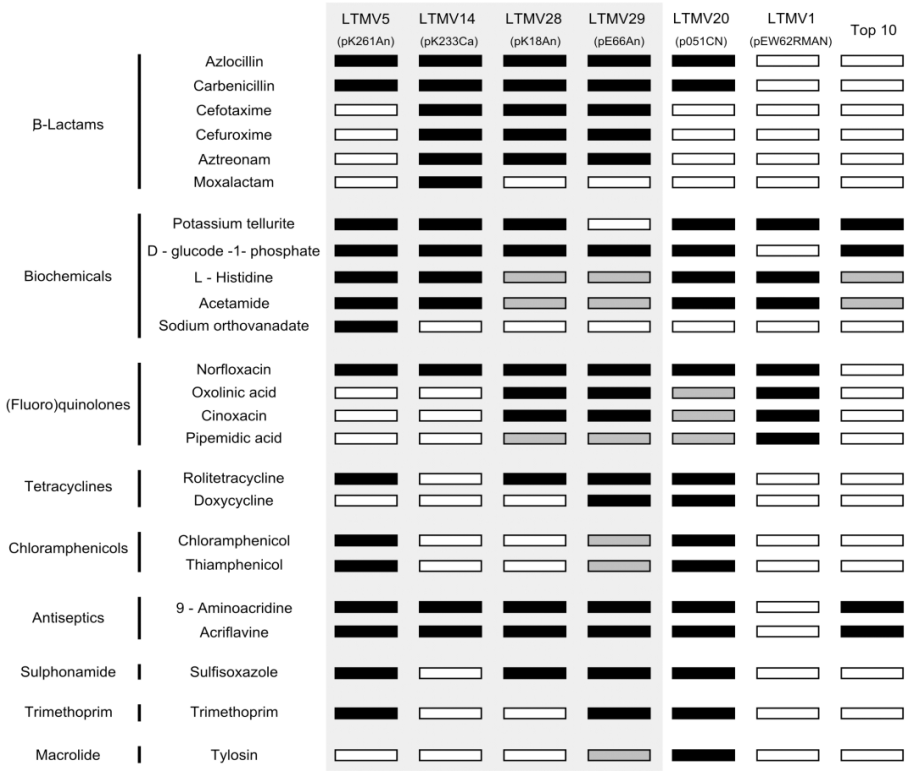


Figure 5.7 High throughput phenotyping data from six selected *qnrS1* plasmid transformants.

Diagram showing data from the 24 phenotypic assays from which a differential respiration characteristic between *qnrS1* plasmid transformants and a control was observed. The six selected strains were: LTMV1, LTMV5, LTMV14, LTMV20, LTMV28, LTMV29 and a control (TOP10). Strains containing hospital plasmids are highlighted by the grey box. Any differential respiration in the seven strains was assessed statically by ANOVA (all of the 24 assays had a *p* value of >0.01). The 24 chemicals and the nine groups to which they were assigned are shown on the left of the figure. Box colours represent: Black; respiration with no inhibition, grey; weak respiration with inhibition and white; no respiration/total inhibition.

5.4. Discussion

There has been some prior characterization of plasmids which can carry and transfer PMQR genes (Poirel *et al.,* 2005c; Chen, Y. *et al.,* 2006; Garcia-Fernandez *et al.,* 2009). In chapter 4, the variability in the size of three *qnrS1*-encoding plasmids was described. It was suggested that the genetic elements carrying the *qnrS1* genes are highly promiscuous and the transfer and sustained maintenance of such genes is not purely dependent on the structure of plasmids on which the *qnr* genes are located. Here, these preliminary findings are advanced and have demonstrated that the size, incompatibility group, resistance gene content and phenotypes of *qnrS1*-encoding plasmids are highly variable. Yet I also show that the characteristics of the plasmids are related to the location of their isolation. It has been shown that the carriage of resistant isolates can vary between the community and hospital cohorts in Asia (Duerink *et al.,* 2007). It can now be concluded that *qnrS1* carrying plasmids carry additional antimicrobial resistance genes, which are dependent on location of isolation. Gentamicin, cefepime and ceftriaxone are all administered intravenously and are, therefore, not commonly used in the community. Additionally, despite the use of oral cephalosporins in the community, only the hospital-associated plasmids were capable of expressing ESBLs.

The 32 plasmids described here with the *qnrS1* gene belonged to multiple incompatibility groups, of which IncN and IncR were the most common and all four IncN *qnrS1* positive plasmids were isolated from hospital patients. An association between *qnr* genes and IncN plasmids has been previously identified and is likely due to the integration of a *qnr* gene into a particularly prevalent plasmid group (Garcia-Fernandez *et al.,* 2009). The majority of *qnrS1* plasmids from the community isolates

were positive for more than one incompatibility group amplicon (which can be common in diverse plasmids) (Carattoli *et al.,* 2005), or did not generate any PCR amplicon. Plasmid variability between the two groups (hospital and community) was further defined by substantial differences in sizes. The range and the median size of the plasmids from the community were considerably greater than those from the hospital. Despite a large range of plasmid size, there were some groups with *Eco*R1 digestion, indicative of some common restriction fragments. The restriction fragment groups were also related to the original location of plasmid isolation. The concept that *qnr* genes circulate on a range of potentially unrelated plasmids is supported by work which characterized plasmids harbouring *qnrS1*, *qnrB2* and *qnrB19* genes in non-typhoid *Salmonella* strains isolated from patients and poultry meat in The Netherlands (Garcia-Fernandez *et al.,* 2009). The authors demonstrated that 5 distinct plasmids carrying *qnrS1*, *qnrB2* and *qnrB19* were IncN replicons. Additionally, *qnrS1* was also located on three other plasmid replicons, belonging to the ColA, IncR and the IncHI2 groups. Work conducted in Asia investigating the genetic properties of two plasmids containing *qnrS1* and *qnrB4* isolated from an infectious *K. pneumoniae* strain in China. Hu *et al.* found that the *qnrB4* and *qnrS1* genes were located on two plasmids which were genetically distinct (Hu *et al.,* 2008).

Hybridisation data produced from the resistance gene nanoarray established that plasmids originating in the community contained a higher mean number of the assayed genes, compared to those originating in the hospital. Variation in gene content corresponded to plasmid sizes in these strains and the spectrum of co-transferred resistance genes was also variable between the two groups (hospital and community). Chloramphenicol resistance was the most common phenotype associated with community plasmids and it was possible to detect hybridisation to only

chloramphenicol resistance determinants in this plasmid group. It remains uncertain as to why chloramphenicol resistance remains in this group, as it is known that chloramphenicol usage in both the hospital and the community has all but stopped in urban Vietnam. The potential for aminoglycoside resistance was most frequently encoded by *aadA4*, *strA* and *strB* in hospital plasmids, whilst among community plasmids, *aac3Iva*, *aadA1* and *aadA2* were more abundant. The inclusion of an integron type 1 probe on the nanoarray also identified an association between *intI1* and *dfrA14* and *sul2* (trimethoprim and sulphonamide resistance respectively), which was associated with community plasmids.

Using high-throughput phenotyping, we were able to identify traits which may be specific to the natural environment of the plasmids. Namely, it was confirmed the relationship of resistance to ß-lactams with hospital plasmids. We also found that 2 community plasmids had the individual phenotypic traits of being unable to respire with glucose-1-phosphate as a carbon source and the other was resistant to tylosin. Tylosin is an antimicrobial agent commonly used in veterinary medicine, suggesting a difference source of origin of this plasmid. Furthermore, the unusual apparent toxicity of glucose-1-phosphate in a strain harbouring the smallest plasmid (pEW-62R-MAN) again suggests a differing origin of this replicon, with respect to the hospital plasmids.

The most common probe demonstrating hybridisation (after *qnrS1*) was *tem1*, which encodes a ß-lactamase. Due to DNA sequence homology between some TEM genes, cross hybridisation must be considered. Therefore, whilst these data are highly indicative, it cannot be categorically concluded that *tem1* is the most common co-transferred gene along with *qnrS1*. This co-transfer is, however, an observation which has been alluded to before (Jiang *et al.,* 2008). The potential linkage and co-transfer

of *qnrS1* and TEM class ß-lactamases on the same genetic element requires further investigation as resistance to ampicillin was the most commonly associated phenotype from the 32 isolates.

Fluoroquinolones are amongst the most common groups of antimicrobial agents to treat infections caused by the Enterobacteriaceae. In locations such as Vietnam, antimicrobial practice is poorly regulated, consequently, fluoroquinolone usage is common in both hospital and community settings. It is currently not possible to estimate the magnitude of the selective pressure maintaining PMQR genes, yet these data suggest that the association of other resistance genes with *qnrS1* is common, particularly in *qnrS1* plasmids circulating in the community. Indeed, the selective pressure induced by other antimicrobials may be as great (if not greater) as that of the fluoroquinolones. Alternative or additional selective pressure is inferred here as ESBL production was only detected in hospital plasmids (6/11, 54%). DNA microarray hybridisation identified that *oxa1*, *ctxM9* and *shv1* (or related sequences) were the most likely source of ESBL production. In addition, 2 plasmids encoded the AmpC gene for ß-lactamase production. A sentinel study investigating *E. coli* and *K. pneumoniae* in China found that 8% the investigated ESBL producing strains were also PCR amplicon positive for a *qnr* gene. The $bla_{CTX\text{-}M}$ gene was the most commonly identified in 27 of the 29 *qnr* positive isolates and TEM-1 type ß-lactamase was detected in 16 *qnr*-positive isolates (Jiang *et al.,* 2008).

5.5. Conclusions

The spread of antimicrobial resistance genes is important globally; yet particularly pertinent to many developing countries with improving economic prospects and unregulated using of antimicrobials. A recent report of a novel resistance mechanism

to carbapenems conferred by a metallo ß-lactamase in India, highlights the origins and potential spread of such determinants (Kumarasamy *et al.,* 2010). The data presented here demonstrate that the *qnrS1* gene is extremely widespread and capable of transfer into a number of highly variable replicons, which may be under differential selection or co-selection. Furthermore, different genotypic and phenotypic characteristics of *qnrS1*-containing plasmids isolated in the hospital and the community were found, which is indicative of the selection of such replicons with the use of specific antimicrobial agents in these differing settings.

6. Using quantitative real-time PCR to detect and quantify *qnr* genes in rectal swabs collected from children with acute respiratory infections

6.1. Abstract

It is understood that consumption of antimicrobials may be a contributing factor of antimicrobial resistance in bacteria through the positive selection of resistant bacteria due to the antimicrobial pressure. In this chapter, a study is presented regarding the association of antimicrobial usage and the *qnr* gene copy number in rectal swabs collected from children with acute respiratory infections on enrollment and follow up at day 7. Of these children, 99% were prescribed antimicrobials by their physician, even though in the absence of aetiological diagnosis. The study showed an increase in both prevalence of *qnr* genes and *qnr* genes copy number at day 7 when compared with the day of enrolment ($p < 0.05$). In addition, it also suggested an increase in transmission of *qnrB* in the gut flora after using of antimicrobials ($p < 0.05$). These data suggested that the use of antimicrobials *per se* (but not necessarily fluoroquinolones) might play an important role in the absolute quantity of *qnr* genes in the gut flora.

6.2. Introduction

The appropriate use of antimicrobials in the treatment of infectious diseases is an important issue, not only for clinicians but also for patients. This issue is particularly important in countries where antimicrobial using is unrestricted (Okeke *et al.,* 2005). Inappropriate use of antimicrobials will, over time, reduce the effect of antimicrobials on pathogenic bacteria and may select the resistant commensal bacterial populations in patients receiving them (Goossens *et al.,* 2005; Lautenbach *et al.,* 2005; Bergman *et al.,* 2008). Antimicrobial resistance in pathogenic bacteria increases treatment duration and cost, as second line drugs are often more expensive, or patients may require a longer treatment (Travers and Barza 2002). In addition, resistant commensal bacteria may be shed and transmitted to other people, especially if the bacteria are in the gut flora (Bailey *et al.,* 2010).

Some groups have studied the effect in structure and number of bacteria in gut flora after using of antimicrobials (Kirjavainen and Gibson 1999; Levy 2000); however, there is no evidence of overall selection within the gut flora. In this chapter, I investigated the association between the use of antimicrobials and the *qnr* gene copy number in gut flora of children with acute respiratory infections (ARIs) before and after antimicrobial therapy.

ARIs are the most common diseases in children in both developed and developing countries and are the leading cause of death in children less than 5 years old. A recent meta-analysis study demonstrated that 1.9 million (95% CI 1.6-2.2 million) children died globally from ARIs in 2000, 70% of them in Africa and Southeast Asia (WHO 2002a). Viruses are known to be the dominant etiologic agents of ARIs, responsible for approximately 60% of all ARIs (WHO 2002a). However, treatment of ARIs is

usually focused on antimicrobials directed at bacterial pathogens. One of the main reasons for this high rate of antimicrobial use is the difficult nature of accurately discriminating viral from bacterial infections using readily available diagnostic testing, such as clinical presentation and chest x-rays. More specific (microbiologic) diagnosis is time consuming or not available; therefore, clinicians choose to address all possible treatable infections, including those of bacterial etiology.

The consumption of antimicrobials correlates with increasing levels of resistance to not only the antimicrobial used but also to other antimicrobials because of the genetic relationship of the resistance genes (e.g. multidrug resistance plasmids) (Peterson 2005; Bergman *et al.,* 2008). There have been many reports demonstrating the linkage of antimicrobial resistance genes, since they are often carried on the same mobile elements, such as plasmids, transposons or integrons (van der Veen *et al.,* 2009). Here, real-time PCR was used to detect the presence and quantities of *qnr* (*qnrA*, *qnrB* and *qnrS*) genes between two samples taken on the day of enrolment (prior to antimicrobials) and on day 7 from children with ARIs presenting at an outpatient clinic of a hospital in Ho Chi Minh City, Vietnam. The association between the type and/or duration of antimicrobial usage and the presence of *qnr* genes was examined.

Three hundred children less than 16 years of age presenting to the Outpatient Department in Children's Hospital Number 1 with ARIs from April to November 2009 were enrolled in this study. Rectal swabs from these children were collected at enrolment day and day 7 (2.7, Chapter 2). Total DNA from these samples were extracted and used as templates for real-time PCR targeting 3 different genes; *qnrA*, *qnrB* and *qnrS*. The relative *qnr* genes copy number for each sample was calculated

using quantitative real-time PCR (2.7.7, Chapter 2). The class and the duration of antimicrobials were also collected and combined in the analysis.

6.3. Results

6.3.1. Validation of quantitative real-time PCR for *qnr* genes

6.3.1.1. Primers and probes designing

Nucleotide sequences of all available full length of all *qnr* alleles were retrieved from NCBI (Jacoby *et al.,* 2008) and were aligned using Vector NTI (Invitrogen) (Figure 6.1, Figure 6.2 and Figure 6.3). Primers and probes were designed using Primer Express version 2.0 (Applied Biosystems Inc.). These primers and probes were then confirmed specificity by BLAST on NCBI database.

6.3.1.2. Sensitivity of quantitative real-time PCR for *qnr* (*qnrA*, *qnrB* and *qnrS*) genes

Ten-fold dilutions of plasmid cloned with target DNA fragment for each gene were used to create standard curves to determine the relative copy number of *qnr* genes in the rectal samples (Figure 6.4). The dynamic range of the assays was from 50 to 5 x 10^7 copies per PCR. The coefficient of variance of intra-assay and inter-assay of real-time PCR assay for each *qnr* gene are shown on Table 6.1.

6.3.1.3. Specificity of quantitative real-time PCR for *qnr* (*qnrA*, *qnrB* and *qnrS*) genes

Because of the high degree of genetic similarity between *qnrA*, *qnrB* and *qnrS* genes, the initial goal was to demonstrate that individual real-time PCR assays were specific to each target gene. Each individual real-time PCR assay for each target gene was

performed with positive controls for *qnrA*, *qnrB* and *qnrS* genes. These experiments were repeated in duplicate, each replicate produced the same result. Therefore, the assay for each gene only detected the target gene but not the other two genes. For example, the assay for detecting *qnrA* gene did not generate amplicons from samples containing *qnrB* or *qnrS* genes.

Table 6.1 The validation of quantitative real-time PCR for *qnr* genes.

Target	Unit	Gene copy number						
		$5x10^7$	$5x10^6$	$5x10^5$	$5x10^4$	$5x10^3$	$5x10^2$	50
qnrA	Ct value *	12.32	16.05	20.58	23.44	27.53	31.3	34.58
	Intra-assay CV%	0.75	0.91	0.79	0.74	1.46	2.00	2.72
	Ct value **	12.41	16.37	20.40	23.42	27.35	31.3	34.39
	Inter-assay CV%	1.43	1.57	1.45	2.55	2.90	2.86	2.96
qnrB	Ct value *	12.41	16.40	20.60	23.17	27.44	31.35	34.52
	Intra-assay CV%	0.65	0.76	0.56	1.22	1.28	1.47	1.91
	Ct value **	12.41	16.35	20.47	23.46	27.20	31.45	34.37
	Inter-assay CV%	1.83	2.19	2.46	1.86	2.12	2.45	2.83
qnrS	Ct value *	13.09	16.86	20.12	23.69	27.77	30.72	34.12
	Intra-assay CV%	1.38	0.65	1.43	2.02	1.62	1.13	1.59
	Ct value **	13.28	16.99	20.77	24.18	27.30	31.40	34.56
	Inter-assay CV%	2.67	1.27	2.18	2.12	2.09	1.70	2.45

The table presents the result of validation of quantitative real-time PCR for *qnr* (*qnrA*, *qnrB* and *qnrS*) genes. All of the intra-assay CV and inter-assay CV value were less than 3%, implying that the 3 quantitative real-time PCR assays for quantify *qnrA*, *qnrB* and *qnrS* genes were productive.

* Ct value: the mean Ct value of the intra assay experiment. The result based on 5 different amplification times.

** Ct value: the mean Ct value of the inter assay experiment. The experiment was repeated 5 times on 5 different days.

CV: Coefficient of variance

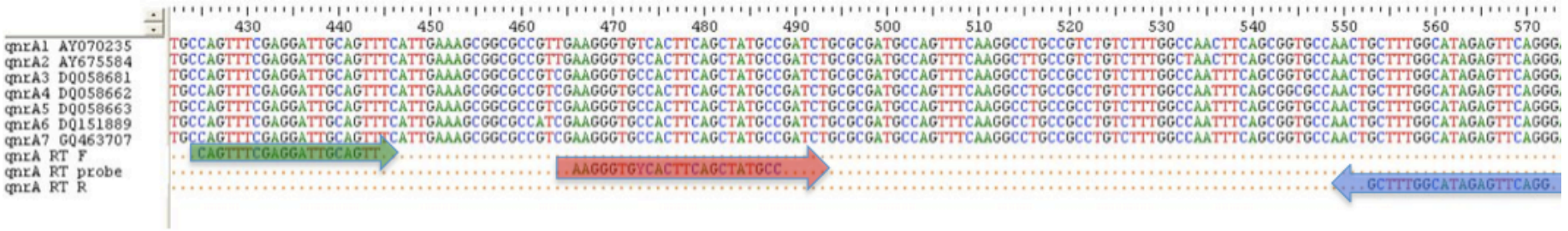


Figure 6.1 Alignment of all available *qnrA* allele sequences, from which the real-time *qnrA* primers and probe were designed.

The forward primer sequence is highlighted in green, the probe is highlighted in red and the reverse primer is highlighted in blue.

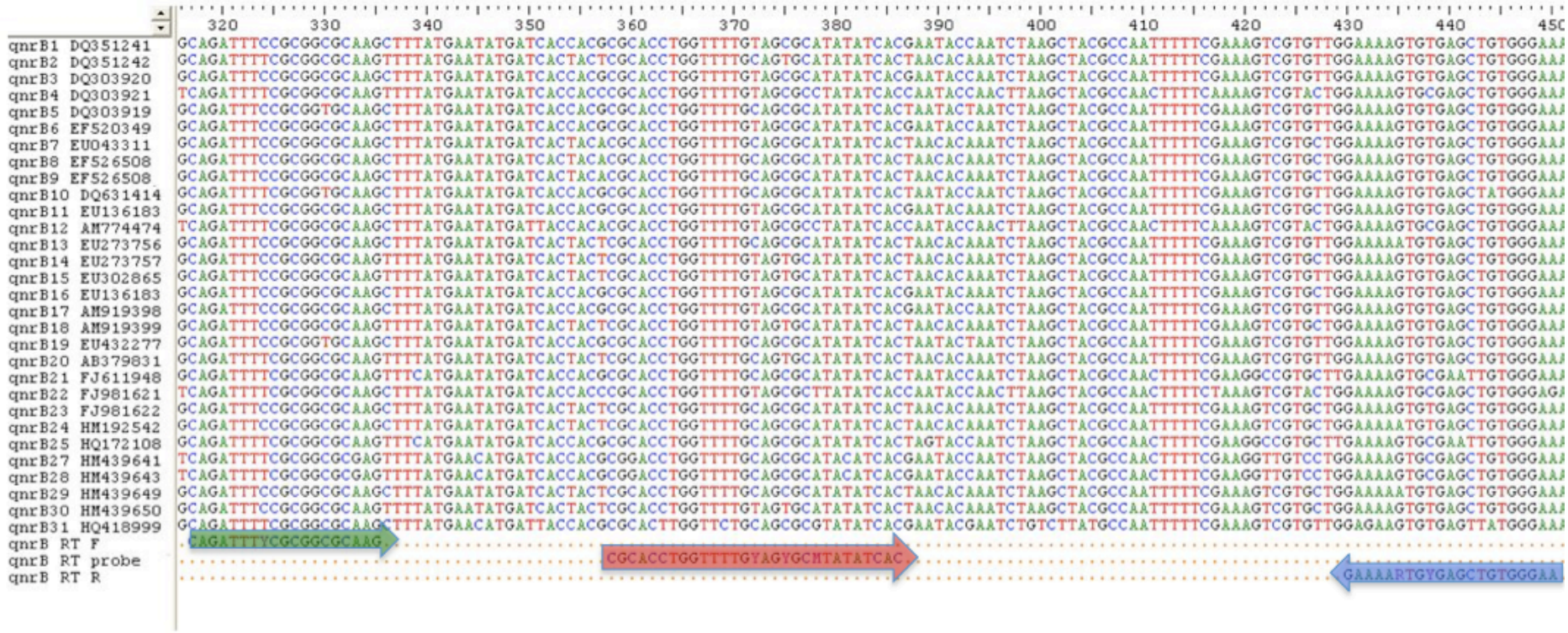


Figure 6.2 Alignment of all *qnrB* alleles sequences, from which the real-time *qnrB* primers and probe were designed.

The forward primer sequence is highlighted in green, the probe is highlighted in red and the reverse primer is highlighted in blue.

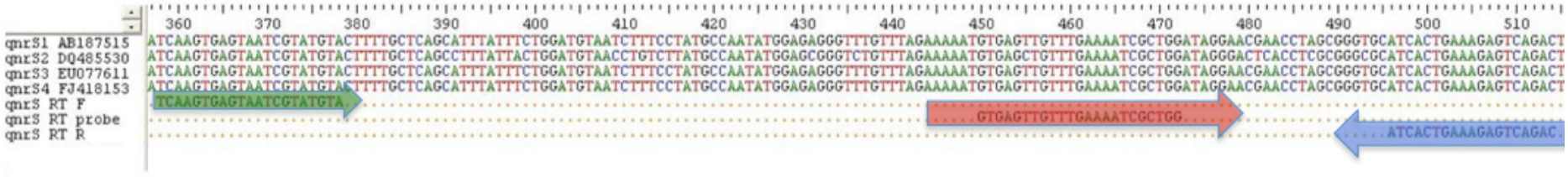
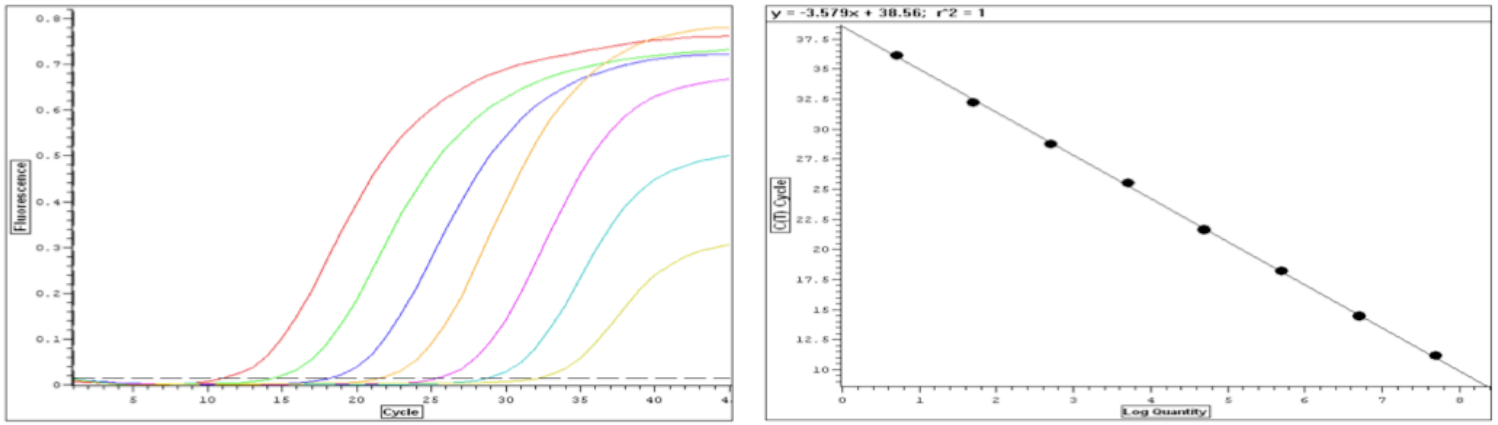


Figure 6.3 Alignment of all *qnrS* alleles sequences, from which the real-time *qnrS* primers and probe were designed.

The forward primer sequence is highlighted in green, the probe is highlighted in red and the reverse primer is highlighted in blue.



a) b)

Figure 6.4 Standard curve created from a serial dilution of the *qnrA*-cloned plasmid.

The standard curve was generated using a 10-fold dilution from $5x10^7$ to 50 copies of a *qnrA*-cloned plasmid template. a) Amplification curves of the dilution series. b) Standard curve with the Ct plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the r value are shown above the graph.

6.3.2. Characteristics of the study population

Children's Hospital Number 1 is one of the 2 children's hospitals in Ho Chi Minh City and contains an out patient department with the capacity to assess over 5,000 children per day. From the samples from 300 children initially enrolled in this study, 37 were rejected from the study (19 had a negative internal control in PCR of one of the rectal swabs, 16 had less than 20 colonies cultured from one of the rectal or both swabs and 2 children were not prescribed antimicrobials). Therefore, in total, samples from 263 children subjected to statistical analysis.

The 263 children enrolled in the study were resident in 18 different districts in Ho Chi Minh City (Table 6.2). The age of the participants was between 2 months and 12 years (median: 12 months). The male to female ratio of the participants was 1:0.83.

Table 6.2 Association of demographic of participants to prevalence of *qnr* genes.

Features	*qnrA*		*qnrB*		*qnrS*	
	Day 0	**Day 7**	**Day 0**	**Day 7**	**Day 0**	**Day 7**
Sex						
Male (n = 144)	6 (4.2%)	14 (9.7%)	67 (46.5%)	101 (70.1%)	111 (77.1%)	111 (77.1%)
Female (n = 119)	6 (5%)	9 (7.6%)	49 (41.2%)	78 (65.5%)	85 (71.4%)	96 (80.7%)
p value	0.73	0.53	0.38	0.42	0.29	0.47
Age						
<1 (n = 66)	3 (4.5%)	4 (6.1%)	37 (56.1%)	50 (75.8%)	57 (86.4%)	53 (80.3%)
1 (n = 75)	5 (6.7%)	11 (14.7%)	34 (45.3%)	53 (70.7%)	55 (73.3%)	63 (82.7%)
2 (n = 51)	1 (2%)	2 (3.9%)	20 (39.2%)	32 (62.7%)	34 (66.7%)	40 (78.4%)
3 (n = 43)	2 (4.7%)	4 (9.3%)	19 (44.2%)	29 (67.4%)	31 (72.1%)	35 (81.4%)
4 (n = 20)	0 (0%)	1 (5%)	4 (20%)	11 (55%)	15 (75%)	12 (60%)
5 (n = 4)	1 (25%)	1 (25%)	0 (0%)	2 (50%)	1 (25%)	2 (50%)
6 (n = 1)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1 (100%)	1 (100%)
7 (n = 2)	0 (0%)	0 (0%)	1 (50%)	1 (70%)	1 (50%)	1 (50%)
12 (n = 1)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	1 (100%)	1 (100%)
p value	0.587	0.488	0.073	0.456	0.105	0.372
Place of enrollment						
District 3 (n = 4)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	3 (75%)	3 (75%)
District 4 (n = 3)	0 (0%)	0 (0%)	2 (66.7%)	1 (33.3%)	2 (66.7%)	2 (66.7%)
District 5 (n = 7)	0 (0%)	0 (0%)	4 (57.1%)	4 (57.1%)	6 (85.7%)	6 (85.7%)
District 6 (n = 8)	2 (25%)	1 (12.5%)	4 (50%)	5 (62.5%)	4 (50%)	5 (62.5%)
District 7 (n = 4)	1 (25%)	1 (25%)	3 (75%)	4 (100%)	4 (100%)	4 (100%)
District 8 (n = 18)	0 (0%)	1 (5.6%)	9 (50%)	10 (55.6%)	13 (72.2%)	13 (72.2%)
District 9 (n = 5)	0 (0%)	1 (20%)	1 (20%)	4 (80%)	3 (60%)	4 (80%)
District 10 (n = 18)	1 (5.6%)	1 (5.6%)	7 (38.9%)	15 (83.3%)	13 (72.2%)	14 (77.8%)
District 11 (n = 12)	1 (8.3%)	0 (0%)	7 (58.3%)	8 (66.7%)	10 (83.3%)	9 (75%)
District 12 (n = 16)	0 (0%)	0 (0%)	11 (68.8%)	13 (81.2%)	11 (68.8%)	14 (87.5%)
Binh Chanh (n = 38)	1 (2.6%)	3 (7.9%)	13 (34.2%)	26 (68.4%)	30 (78.9%)	32 (84.2%)
Binh Tan (n = 39)	2 (5.1%)	6 (15.4%)	15 (38.5%)	29 (74.4%)	30 (76.9%)	35 (89.7%)
Cu Chi (n = 7)	0 (0%)	0 (0%)	4 (57.1%)	6 (85.7%)	4 (57.1%)	5 (71.4%)
Go Vap (n = 5)	0 (0%)	0 (0%)	2 (40%)	2 (40%)	4 (80%)	5 (100%)
Hoc Mon (n = 25)	3 (12%)	3 (12%)	12 (48%)	14 (56%)	20 (80%)	14 (56%)
Tan Binh (n = 28)	0 (0%)	3 (10.7%)	10 (35.7%)	18 (64.3%)	19 (67.9%)	22 (78.6%)
Tan Phu (n = 23)	1 (4.3%)	3 (13%)	10 (43.5%)	17 (73.9%)	17 (73.9%)	18 (78.3%)
Thu Duc (n = 3)	0 (0%)	0 (0%)	2 (66.7%)	2 (66.7%)	3 (100%)	2 (66.7%)
p value	0.271	0.837	0.483	0.386	0.917	0.442

This table shows the demographics of participants and the prevalence of *qnr* genes. There is no correlation between sex, age and location to the *qnr* gene prevalence ($p > 0.05$, calculated by two-sided Fisher's exact test).

6.3.3. Prevalence of *qnr* genes in rectal swabs collected from children with ARI in Ho Chi Minh City

Using real-time PCR for the 3 *qnr* genes, the prevalence of these 3 *qnr* genes in rectal swabs from children with ARI infection was determined. The prevalence of *qnrA* in the first sample set (admission date) and second sample set (day 7) was 4.5% (12/263) and 8.7% (23/263), respectively. The difference of these proportions was a statistically significant increase ($p < 0.001$, calculated by McNemar's test). Similarly, the prevalence of *qnrB* and *qnrS* in the first and second sample sets were also increase with 44.1% (116/263) to 68.1% (179/263) (for *qnrB*) and 74.5% (196/263) to 78.7% (207/263) (for *qnrS*). However, only *qnrS* demonstrates a statistically significant increase ($p < 0.001$) but not with *qnrB* ($p = 0.086$) (McNemar's test). A detailed description of the results of the prevalence and statistical analyses are shown in Table 6.3. No significant association between age, sex and or district of residency of the participants and the prevalence of *qnr* genes was found (Table 6.2).

Table 6.3 Comparison of two collected sample sets, at the day of enrolment and at the day 7.

Features	Day 0	Day 7	*p* value
Prevalence of *qnr* genes (%), (CI 95%)			
qnrA	12/263 (4.5%)	23/263 (8.7%)	< 0.001*
qnrB	116/263 (44.1%)	179/263 (68.1%)	0.086
qnrS	196/263 (74.5%)	207/263 (78.7%)	<0.001*
CFU per 1 ml specimen, median (range)			
	$1.26x10^7$	$3.26x10^7$	< 0.001*
	$(800 – 9x10^8)$	$(780– 5x10^{11})$	
***qnr* genes copy number per 1 ml specimen, median (range)**			
qnrA	0	0	0.1018
	$(0 – 5.2x10^7)$	$(0 – 2.8x10^7)$	
qnrB	0	28800	< 0.001*
	$(0 – 7.82x10^7)$	$(0 – 4.26x10^{10})$	
qnrS	86400	334000	< 0.001*
	$(0 – 7.88x10^8)$	$(0 – 2.36x10^9)$	
Average *qnr* genes copy number per CFU, median (range)			
qnrA	0	0	0.1018
	(0 – 4.13)	(0 – 0.723)	
qnrB	0	0.0012	< 0.001*
	$(0 – 1.2x10^3)$	$(0 – 5.2x10^4)$	
qnrS	0.00841	0.0195	0.2257
	(0 – 2660)	(0 – 6990)	

This table presents the comparison of prevalence of *qnr* genes, number of colony forming unit (CFU), *qnr* genes copy number per 1 ml specimen and average *qnr* genes copy number per CFU between samples collected at day of enrolment (Day 0) and after 1 week (Day 7). It shows that there has been an increasing of prevalence of all 3 types of *qnr* genes with the significant statistic difference. Besides, the number of CFU and *qnrB* and *qnrS* copy also expressed the difference with significant statistic.

p value in the prevalence of *qnr* genes were calculated by McNemar's test, the others were calculated by paired Wilcoxon rank-sum test.

* significant statistic difference ($p < 0.05$)

6.3.4. Quantification of colony forming unit (CFU) from stool swabs

The quantitative data produced for each of the three *qnr* genes was based upon amplification from the rectal swab; these data, therefore, represent the crude copy numbers of each gene. To investigate whether the increase of *qnr* genes copy number between day 0 and day 7 was the result of an increase in *qnr*-containing plasmids or the outcome of the increase in total gut flora, the average *qnr* genes copy number per CFU was examined. The number of bacterial colonies grew on MacConkey agar plate was counted and multiplied to the base of dilution to calculate the CFU per 1 ml of stool specimen. The median CFU number of the enrolment sample set was $1.26x10^7$ (range 800 – $9x10^8$) and of the day 7 sample set was $3.26x10^7$ (range 780 – $5x10^{11}$). The difference between these 2 groups was statistically significant ($p < 0.001$, paired Wilcoxon rank-sum) (Table 6.3), demonstrating that the CFU per 1 ml of specimen increased between the sample sets collected at day 0 and day 7.

6.3.5. Quantification of *qnr* genes copy numbers in total DNA

The *qnr* genes copy numbers per 1 ml of specimen were quantified in a total of 526 rectal swabs collected from 263 children. The median copy numbers of the *qnrA*, *qnrB* and *qnrS* genes on the day of enrolment were 0 (range 0 – $5.2x10^7$), 0 (range 0 – $7.8x10^7$) and 4,320 (range 0 – $7.8x10^8$), respectively. On day 7, these numbers were 0 (range 0 – $2.8x10^7$), 1,440 (range 0 – $4.2x10^{10}$) and 16,700 (range 0 – $2.2x10^9$), respectively. The difference between these 2 groups was calculated and *qnrB* and *qnrS* showed a statistically significant increase ($p < 0.001$). However, there was no significant variation in the quantity of *qnrA* between the two sampling point ($p = 0.1018$, paired Wilcoxon rank-sum) (Table 6.3, Figure 6.2).

The copy numbers of each gene were calculated using the CFU values calculated by culturing specimens on MacConkey plates. The CFU number per 1 ml of specimen was calculated and based on the real-time PCR result the gene copy number per 1 ml of specimen was determined; from the 2 results it was possible to calculate the *qnr* gene copy number per CFU of each specimen. The median *qnrA*, *qnrB* and *qnrS* copy number per CFU after adjusting were 0 (range 0 – 4.13), 0 (range 0 – 0.00017) and 0.0084 (range 0 – 2660) on day 0 and 0 (range 0 – 0.723), 0.012 (range 0 – $5.2x10^4$) and 0.0195 (range 0 – 6990) on day 7, respectively. Only *qnrB* showed a statistically significant increase between day 0 and day 7 ($p < 0.001$, paired Wilcoxon rank-sum) (Table 6.3).

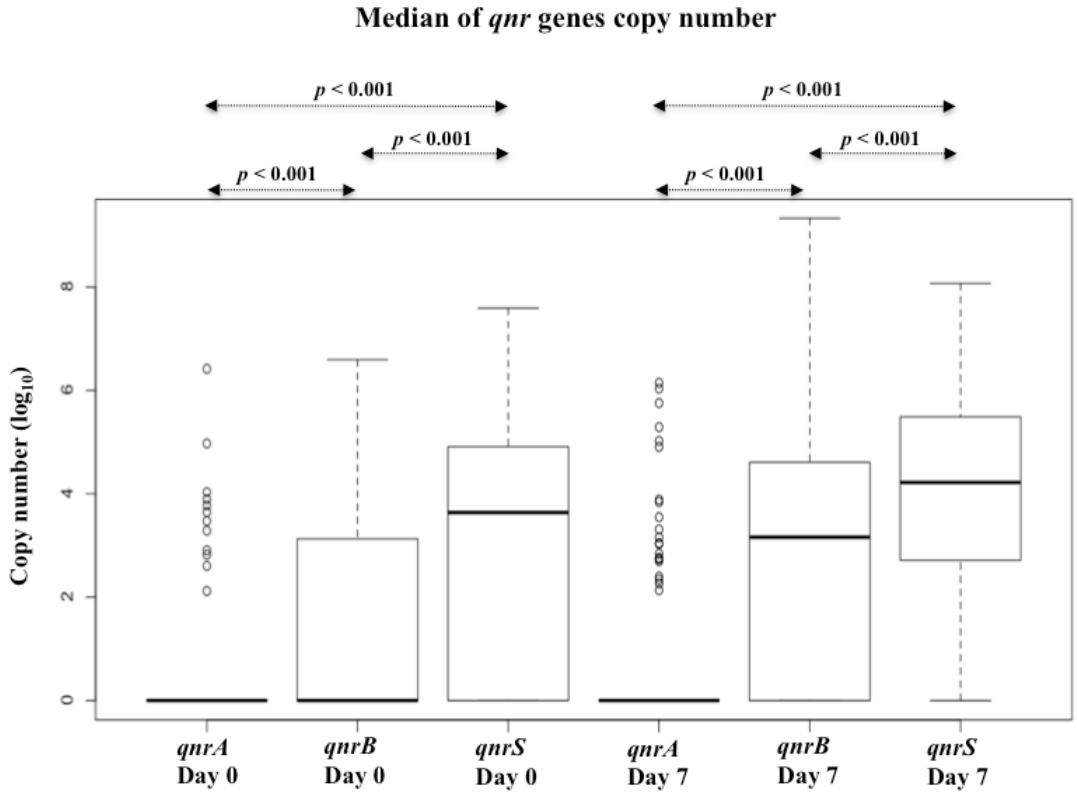


Figure 6.5 The median *qnr* gene copy number in 2 sample sets, day of enrolment (Day 0) and after antimicrobial treatment (Day 7).

This figure presents the *qnr* gene copy number in rectal swabs collected from children with ARIs at day of enrolment (Day 0) and after using antimicrobials (Day 7). This figure shows that in the 3 *qnr* genes, the copy number of *qnrS* gene is greatest and then the copy number of *qnrB* gene and the copy number of *qnrA* gene is the smallest. The *p* value was calculated using Wilcoxon rank sum test.

6.3.6. Assessing the association of the type of antimicrobial using to the change in *qnr* genes copy number

Thirteen antimicrobials belonging to 4 different classes (β-lactam, macrolide, cephalosporins and sulphonamide) were used to treat participants in this study. The antimicrobials were used at the discretion of the treating clinicians and were used separately or in combination. In total, there were 29 regimens prescribed, consisting of a single antimicrobial, or a combination of 2 or 3 antimicrobials. Four of them demonstrated a statistically significant increase in relative copy number from day 0 to day 7 for *qnrA* and/or *qnrB* genes but not with the *qnrS* gene (Table 6.4).

Similarly, when the difference in *qnr* gene copy number between day 0 and day 7 was calculated with respect to the antimicrobial combination used, there were 3 antimicrobial classes that showed a significant statistical difference, these were ß-lactam, ß-lactam + acid clavulanic and ß-lactam + acid clavulanic + cephalosporin (Table 6.5).

Table 6.4 Antimicrobial use and the effect on the copy number of *qnr* genes (based on antimicrobial regimes).

Type of antimicrobials	Day 0	Day 7	*p* value (CI 95%)
Group 1: Amoxicillin (n = 6)			
qnrA	0 (0 – 0.044)	0 (0 – 0.723)	1
qnrB	0 (0 – 0.0152)	0.002625 (0.0000483 – 52000)	0.03*
qnrS	0.02667 (0 – 0.276)	0.055 (0.01 – 0.512)	0.5625
Group 2: Amoxicillin + Acid clavulanic (n = 115)			
qnrA	0 (0 – 0.01562)	0 (0 – 0.35)	0.01577*
qnrB	0 (0 – 265)	0.0025 (0 – 5350)	< 0.001*
qnrS	0.0083 (0 – 2660)	0.025 (0 – 76.7)	0.391
Group 3: Amoxicillin + Acid clavulanic + Azithromycin (n = 3)			
qnrA	0 (0 – 0)	0 (0 – 0)	n/a
qnrB	0.000291 (0 – 0.00697)	0 (0 – 3.76)	0.4142
qnrS	0.106 (0.00227 – 0.373)	0 (0 – 0)	0.25
Group 4: Amoxicillin + Acid clavulanic + Cefaclor (n = 11)			
qnrA	0 (0 – 0.2145)	0 (0 – 0.015)	1
qnrB	0 (0 – 0.238)	0.00279 (0 – 22)	0.01782*
qnrS	0.00966 (0 – 83.9)	0.0673 (0.000486 – 15.4)	0.70
Group 5: Amoxicillin + Acid clavulanic + Cefixime (n = 4)			
qnrA	0 (0 – 0)	0 (0 – 0)	n/a
qnrB	0 (0 – 0.0003367)	0.40 (0 – 1.22)	0.1814
qnrS	0.107 (0 – 1.99)	0.25 (0 – 38.5)	0.7893
Group 6: Amoxicillin + Acid clavulanic + Cefuroxime (n = 13)			
qnrA	0 (0 - 0.004617)	0 (0 – 0.413)	0.3613
qnrB	0.0000233 (0 – 0.193)	0.011 (0 – 6)	0.041*
qnrS	0.00345 (0 – 11)	0.208 (0 – 1290)	0.1909
Group 7: Amoxicillin + Acid clavulanic + Cefpodoxime (n = 5)			
qnrA	0 (0 – 0.0045)	0 (0 – 0)	0.3711
qnrB	0.00084 (0 – 2.7)	0.0052 (0.00048 – 20.1)	0.8125
qnrS	0.0153 (0 – 17.9)	0.0053 (0.00057 – 175)	1
Group 8: Cefaclor (n = 14)			
qnrA	0 (0 – 0)	0 (0 – 0)	n/a
qnrB	0.00032 (0 – 0.0264)	0.00015 (0 – 0.0485)	0.4148
qnrS	0.00383 (0 - 2.7)	0.062 (0 – 3.01)	0.1842
Group 9: Cefaclor + Cefuroxime (n = 3)			
qnrA	0 (0 – 0)	0 (0 – 0)	n/a
qnrB	0.00037 (0 – 0.0287)	0 (0 – 0.000741)	0.1736
qnrS	0.307 (0 – 3.96)	0 (0 – 0.0662)	0.3711
Group 10: Cefixime (n = 17)			
qnrA	0 (0 – 0.0099)	0 (0 – 0.00008772)	0.5839
qnrB	0 (0 – 2.07)	0.000711 (0 – 34.8)	0.1261
qnrS	0.0081 (0 – 0.0459)	0.0111 (0 – 2.54)	0.2585
Group 11: Cefuroxime (n = 42)			
qnrA	0 (0 – 4.13)	0 (0 – 0.0023)	1
qnrB	0 (0 – 3.8)	0.00019 (0 – 4.65)	0.1945
qnrS	0.00321 (0 – 16.7)	0.0062 (0 – 89.5)	0.5916
Group 12: Cefpodoxime (n = 4)			
qnrA	0 (0 – 0)	0 (0 – 0)	n/a
qnrB	0 (0 – 0.00076)	0.0000545 (0 – 0.0031)	0.3711
qnrS	0.0268 (0 – 0.0682)	0 (0 – 0.0263)	0.375
Group 13: Erythromycin (n = 7)			
qnrA	0 (0 – 0)	0 (0 – 0)	n/a
qnrB	0 (0 – 1210)	0.000648 (0 – 49400)	0.2945
qnrS	0.00254 (0 – 2530)	0 (0 – 6990)	1

This table presents the correlation of antimicrobials using to the changing of *qnr* genes copy number. In these 13 groups, 4 of them showed the difference with the significant statistic (asterisk). Interestingly, in group 2, Amoxicillin + Acid clavulanic, both *qnrA* and *qnrB* showed the difference between 2 sample sets, before and after using antimicrobials.

Table 6.5 Antimicrobial use and the effect on the copy number of *qnr* genes (based on class antimicrobials).

Type of antimicrobial	Day 0	Day 7	*p* value (CI 95%)
Group 1: ß-lactam (n = 6)			
qnrA	0 (0 – 0.044)	0 (0 – 0.723)	1
qnrB	0 (0 – 0.0152)	0.002625 (0.0000483– 52000)	0.03*
qnrS	0.02667 (0 – 0.276)	0.055 (0.01 – 0.512)	0.5625
Group 2: ß-lactam + Acid clavulanic (n = 115)			
qnrA	0 (0 – 0.01562)	0 (0 – 0.35)	0.01577*
qnrB	0 (0 – 265)	0.0025 (0 – 5350)	< 0.001*
qnrS	0.0083 (0 – 2660)	0.025 (0 – 76.7)	0.391
Group 3: Cephalosporin (n = 86)			
qnrA	0 (0 – 4.13)	0 (0 – 0.0023)	0.5294
qnrB	0.00015 (0 – 7.62)	0.00016 (0 – 34.8)	0.1960
qnrS	0.0078 (0 – 178)	0.0077 (0 – 386)	0.5125
Group 4: ß-lactam + Acid clavulanic + Cephalosporin (n = 36)			
qnrA	0 (0 – 0.21)	0 (0 – 0.41)	0.9326
qnrB	0.000011 (0 – 2.7)	0.01 (0 – 22)	< 0.001*
qnrS	0.008 (0 – 83.9)	0.089 (0 – 1294)	0.1873
Group 5: ß-lactam + Acid clavulanic + Macrolide (n = 3)			
qnrA	0 (0 – 0)	0 (0 – 0)	n/a
qnrB	0.000291 (0 – 0.00697)	0 (0 – 3.76)	0.4142
qnrS	0.106 (0.00227 – 0.373)	0 (0 – 0)	0.25
Group 6: Macrolide (n = 10)			
qnrA	0 (0 – 0)	0 (0 – 0)	n/a
qnrB	0 (0 – 1210)	0 (0 – 49400)	0.1834
qnrS	0.0024 (0 – 2530)	0 (0 – 6992)	0.7263

This table presents the correlation of antimicrobials class using to the changing of *qnr* genes copy number. In these 6 groups, 3 of them showed a statistically significant difference (asterisk).

6.4. Discussion

Real-time PCR for *qnrA*, *qnrB* and *qnrS* on paired stool samples from children with ARIs demonstrated a difference in the prevalence of all 2 alleles *qnrA* and *qnrS* between the first sample set (collected on the day of enrolment) and the second sample set (collected on day 7) (($p < 0.05$). The results showed that 7.2% (19/263), 33% (87/263) and 17.9% (47/263) children were negative for PCR amplification of the *qnrA*, *qnrB* and *qnrS* genes, respectively, in samples collected on the day of enrolment but positive in samples collected on day 7 (after using antimicrobials). In contrast, 3% (8/263), 9.1% (24/263) and 13.7% (36/263) children were positive in PCR amplification for *qnrA*, *qnrB* and *qnrS*, respectively, in samples collected on day 0 but negative in samples collected at day 7. The resulting detection of PMQR genes in stool samples taken on day 0 yet not on day 7 implied that the antimicrobial regime reduced the proportion of bacteria carrying *qnr* genes to below a detectable level. However, children who were negative for PMQR determinants on day 0 and positive with PMQR determinants in the day 7 might be explained by the fact that the *qnr* gene copy number in stool samples collected on the day of enrolment of these children was less than the detection limitation of real-time PCR. Yet, 7 days from the post-enrolment, the copy number of the PMQR genes in these samples was large enough to be detected, and may be due to several possibilities, including the uptake of the PMQR genes from the environment through food. There have been many studies conducted revealing the presence of *qnr* genes in meat or food (Yue *et al.,* 2008; Huang *et al.,* 2009; Ma *et al.,* 2009).

The influence of temporal location on the prevalence of *qnr* genes carriage from the 263 participants was also determined. However, no relationship was found between the prevalence of children carrying *qnr* genes and their residence.

Table 6.3 shows the median CFU per 1 ml of the specimens and also a comparison of CFU per 1 ml of samples between the samples collected from day 0 and day 7. The difference between the values on day 0 and 7 was statistically significant ($p < 0.05$). It has been suggested that the number and structure of the bacteria population in the human gut depends on the health status, the diet and also the use of medicine, particularly with antimicrobials (Clarke 1974; Kirjavainen and Gibson 1999; Levy 2000). In this study, MacConkey agar was used for bacterial culture, which is a selective media for the Enterobacteriaceae (Blood and Curtis 1995). However, there are still many other bacteria in the intestinal tract which cannot grow in MacConkey agar, such as *Bacteroides*, *Clostridium*, *Fusobacterium* and *Peptostreptococcus* (Madigan *et al.,* 2003), where species from the genus *Bacteroides* alone constitute about 30% of all bacteria in the gut population (Moore and Holdeman 1974). Furthermore, assessing the antimicrobial susceptibility of these bacteria is difficult. When antimicrobials are used, the content of the gut flora changes because of the potential selection induced by the antimicrobial. Here, an increase of the number of colonies growing in MacConkey agar in the second sample was observed, implying that the number of sensitive bacteria, most of which were probably not Enterobacteriaceae, decreased which encouraged the development of the resistant or intermediately resistant bacteria due to the loss of competition. These results are supported by a concurrent study regarding the increase of antimicrobial resistance bacteria population in the gut flora after using antimicrobials. The study revealed that the number of colonies grown in MacConkey plates supplemented with

antimicrobials (amoxicillin, amoxicillin + acid clavulanic, ciprofloxacin, gentamicin and ceftazidime) increased significantly after using antimicrobials (unpublished data).

The *qnr* gene copy number in total DNA extracted from rectal swabs of children with ARIs at the day of enrolment (day 0) and after using antimicrobials (day 7) were calculated and compared. Both *qnrB* and *qnrS* showed a significant increase in copy number between day 0 and day 7. There are a number of reasons that may be responsible for this increase in *qnr* gene copy number. Firstly, there may be an effect of the type of antimicrobial agent used for treatment. In this study the clinicians did not use fluoroquinolones to treat participants, they commonly used β-lactams, macrolides, cephalosporins and sulphonamides. Previous data in Chapter 4 and Chapter 5 demonstrated that resistance genes to β-lactams, macrolides, cephalosporins and sulphonamides are commonly located on the same plasmids as *qnr* genes. In Chapter 4, a predominant *qnrS1*-containing transposon type carrying the bla_{LAP-2} gene, which encodes resistance to ß-lactam antimicrobials, was shown to be circulating in Enterobacteriaceae in Ho Chi Minh City. Furthermore, other reports have shown a close association between *qnrA* and *qnrB* gene with ESBL producing genes (Chen, S. L. *et al.,* 2006; Garnier *et al.,* 2006; Poirel *et al.,* 2006b; Kehrenberg *et al.,* 2007; Hu *et al.,* 2008). There has also been a report showing that after antimicrobial use, the number of bacteria containing the genes confer to resistance to those antimicrobials might be increased (Alali *et al.,* 2009). Here, the increase of *qnr* gene copy number, implying the increase of *qnr*-containing plasmids, is not due to fluoroquinolone use but due to an increase of plasmids containing the resistance gene carrying the *qnr* genes. Additionally, the difference in *qnr* gene copy number between day 0 and day 7 could be the result of the uptake of *qnr* gene-containing

bacteria from the environment through food or drink in the intermediate period. Surveillance have also revealed the presence of *qnr* genes in meat and other foodstuffs (Yue *et al.,* 2008; Huang *et al.,* 2009; Ma *et al.,* 2009).

To investigate whether the increase of *qnr* gene copy number between day 0 and day 7 is the result of an increase in *qnr*-containing plasmids or the outcome of the increase in total gut flora, the average *qnr* genes copy number per CFU was calculated. Table 6.3 shows a comparison of the average *qnr* gene copy number per CFU between the first and second sample set population. The *qnrB* gene showed a significant increase ($p < 0.001$) in the average gene copy number per CFU between these 2 sets, day 0 and day 7, which implied that an increase in *qnrB* gene quantity is not due to an increase in the total gut flora but partly a result of possible positive selection. There have been a number of studies showing the correlation of increasing resistance genes with antimicrobial usage, emphasizing the effect of selection pressure (van der Veen *et al.,* 2009). Here, the data shows an increase in *qnrB* gene copy number within the gut flora from a group of children undergoing antimicrobial therapy.

Thirteen antimicrobials belonging to 4 different antimicrobial groups (β-lactam, macrolide, cephalosporins and sulphonamides) were used to treat the enrolled children with ARIs in this study. Based on the antimicrobial regimes, the antimicrobial therapies were divided into 29 groups. Table 6.4 shows the detailed results comparing antimicrobial usage and the change in average *qnr* gene copy number per CFU between day 0 and day 7. Four out of 29 groups of antimicrobial therapies demonstrated a significant increase in the *qnrA* or *qnrB* gene copy number. All of the 4 antimicrobial therapies (Amoxicillin, Amoxicillin + Acid clavulanic,

Amoxicillin + Acid clavulanic + Cefaclor, Amoxicillin + Acid clavulanic + Cefuroxime) showed the increase in *qnrB* or *qnrA* gene copy numbers between the sample sets collected at day of enrolment (day 0) and day 7 (after using antimicrobials). Similarly, Table 6.5 details the comparison of *qnr* gene copy number between day 0 and day 7 based on the classification of antimicrobial classes. From this table, 3 types of antimicrobial class showed a significant increase in the *qnr* gene copy number between day 0 and day 7: ß-lactams, ß-lactams + clavulanic acid and ß-lactams + acid clavulanic + cephalosporins. These results were in agreement with the previous comparison results based on antimicrobials, which showed that ß-lactams and clavulanic acid play an important role in the increase of *qnr* gene copy number. The *qnrA* showed a significant increase in copy number between day 0 and day 7 in group of children using Amoxicillin + Clavulanic acid (n = 114) (p = 0.01). The *qnrA* and *qnrB* genes have been reported on the same plasmids as ESBL producing genes (Lavigne *et al.,* 2006; Oktem *et al.,* 2008; Pomba *et al.,* 2009). The results presented here again support the association of *qnrA*, *qnrB* genes and ESBL producing genes. With the use of cephalosporin, the number of bacteria sensitive to cephalosporin in the gut flora decreased, which stimulated an increase in bacteria resistant to cephalosporin through the reduction of competition in gut ecology. However, the underlying reason for a large increase in *qnr* gene copy number in the group of children using Amoxicillin + Clavulanic acid remains unclear. A comparison of demographic information as well as copy number of CFU to *qnr* gene of participants among these 3 antimicrobial usage groups (β-lactam, β-lactam + Clavulanic acid and β-lactam + Clavulanic acid + Cephalosporin) was also conducted (data not shown) with the results indicating that neither the residence location or the number of CFU had any effect on the average *qnr* gene copy number

per CFU between 2 sample sets on enrolment day and day 7. This might imply that the antimicrobials used could partly play a role in the observed increase. However, the uptake of *qnr* genes from the environment through food, drink and others during 7 days between the first and second samples collecting still remains a viable theory as well.

6.5. Conclusions

In this chapter, the prevalence and copy number of *qnr* genes from total DNA extracted from rectal swabs collected from children with acute respiratory infections before and after the use of antimicrobials was assessed. The results showed an increasing prevalence of *qnrA* and *qnrS* genes in the 2 sample sets collected on enrollment day and after 7 days. In addition, an increasing quantity of the *qnrB* gene in the gut flora after 7 days of using antimicrobials was shown. Further analysis of the data resulting from the real-time PCR amplification showed antimicrobials such as β-lactam, β-lactam + Clavulanic acid and β-lactam + Clavulanic acid + Cephalosporin, might play an important role in the increase of *qnr* gene copy numbers in gut flora, suggesting common co-selection. This work is the first such research related to the association between the use of non-quinolone antimicrobials and the increasing relative prevalence in *qnr* gene copy number in the gut flora.

7. General discussion

There have been many studies conducted regarding the mechanisms of fluoroquinolone resistance in bacteria; however, most of them have been focused on the clinical bacteria isolates from hospitalized patients, rarely have other studies focused on commensal bacterial flora isolated from healthy members of the community. Commensal flora appear to play an important role in the spread of antimicrobial resistance genes and can also be a source of infection, crossing the boundary between commensal and pathogen (Tancrede 1992). The results from this study have contributed to our knowledge of fluoroquinolone resistance mechanisms found and potentially disseminated by commensal Enterobacteriaceae, particularly in Ho Chi Minh City, Vietnam.

The prevalence of the PMQR determinants (*qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr* and *qepA*) in Enterobacteriaceae isolated from two groups (hospital and community populations) was determined. The results showed that the prevalence of *qnrS* was substantial in both hospital and community groups when compared to the prevalence of other PMQR determinants. All *qnrS* amplicons were sequenced and all were determined to be *qnrS1*. We do not currently understand why the prevalence of *qnrS1* in Ho Chi Minh City is so high. One possible contributing factor to the maintenance and spread of the *qnrS1* gene circulating in Ho Chi Minh City is that the *qnrS1* is embedded on a number of highly promiscuous mobile genetic elements which may be transferred from the original host to a recipient with high frequency hosts. In addition, the *qnrS1* gene in Enterobacteriaceae circulating in Ho Chi Minh City may also be co-transferred with additional antimicrobial resistance genes, as the data presented here demonstrates that such genes are commonly located on the same

transposons or integron. Vietnam is currently a country where pharmacies are abundant and individual can purchase antimicrobials without a clinician prescription. Because of this uncontrolled use of antimicrobials, outpatients can buy antimicrobials and may not follow the advice of the prescribing pharmacists (Quagliarello *et al.,* 2003). Current guidelines recommend that antimicrobials are used for 5 or 7 days, in the case of most Gram negative infections. However, after 3 days of antimicrobial, patients may begin to feel better (depending on the infection) and stop using antimicrobials (Quagliarello *et al.,* 2003). As a result, Enterobacteriaceae with antimicrobial resistance genes remain and colonize.

In this study, we found a high prevalence of a *qnrS1*-containing transposon circulating in Enterobacteriaceae isolated from both hospital and community populations. Analyzing the sequence of this transposon revealed that in addition to carrying the *qnrS1*, the transposon also carries a bla_{LAP-2} gene, a gene which confers the resistance to β-lactam antimicrobial group. This association may play an important role in the maintaining of *qnrS1* in Enterobacteriaceae in Ho Chi Minh City because of the common use of β-lactam antimicrobials in Vietnam. In addition, the analysis of the sequence of the *qnrS1*-containing fragment found that the *qnrS1*-containing fragment carried two IS26 elements at either ends of the element. The IS26 element is one of the most prevalent found within the Enterobacteriaceae (Iida *et al.,* 1984). As a result, these *qnrS1*-containing transposons may be easily transferred from this one location to another within the same plasmid or in different plasmids in the same organism. This element might also play an important role in the spreading of *qnrS1* in Enterobacteriaceae in Ho Chi Minh City.

Uncontrolled antimicrobial usage raises concerns about antimicrobial resistance in Enterobacteriaceae in Vietnam. With uninhibited antimicrobial use, the proportion of antimicrobial resistant bacteria will increase. In addition, the opportunity for novel antimicrobial resistance genes, which may induce resistance to multiple antimicrobials, may also increase. The appearance of NDM-1 gene is a prime and recent example. NDM-1 (New Delhi metallo-beta-lactamase 1) was first isolated in a *Klebsiella pneumoniae* strain from a patient in New Delhi, India (Yong *et al.,* 2009). What makes this enzyme so clinically important is that it can hydrolyze most known beta-lactam antimicrobials, including the carbapenems. A molecular examination revealed that the gene encoding the novel beta-lactamase is located on a large 180-kb multi drug resistance conferring genetic element that can be easily transferred to other Enterobacteriaceae. Thus, NDM-1 was transferred along with a gene encoding another broad-spectrum beta-lactamase (CMY-4) and genes which stimulate resistance to erythromycin, ciprofloxacin, rifampicin and chloramphenicol. The original organism from which NDM-1 was isolated was found to be resistant to all antimicrobial agents tested except colistin (Moellering 2010). Thus making the treatment for infections caused such NDM-1 carrying strains difficult. When NDM-1 or a similar novel enzyme with a similar property of inducing resistance to multiple antimicrobials is inevitably transferred to Vietnam, it will cause substantial difficulty to clinicians in treatment the infections.

Due to time and cost restrictions of this work, there are still many questions that need to be investigated further. It is hoped that in the future, it will be possible to sequence the *gyrA* genes in all community strains and also test the sensitivity test to new fluoroquinolones in all isolates to gain insight on the fluoroquinolone resistance situation in Enterobacteriaceae in Ho Chi Minh City. Moreover, characterizing all

qnrS1-containing plasmids would be valuable to obtain an accurate view on the different resistance patterns between plasmids extracted from hospital and community isolates. As genome sequencing becomes most cost effective through next generation methods, sequence of clinical isolates and resistance plasmids may become routine in a clinical setting.

The results of this thesis indicate a high prevalence of PMQR determinants, particularly *qnrS1* gene, in the Enterobacteriaceae isolated from both hospital and community populations from Ho Chi Minh City. Moreover, I demonstrated that the *qnrS1* gene have an intimate relationship with many other antimicrobial resistance genes. This selection was also demonstrated by the clinical use certain antimicrobials in children with ARI`s induced an increase in prevalence and quantity of PMQR genes. It is hoped that these results will lead to an impact on clinician prescribing behavior in order to avoid a further step-wise increase of fluoroquinolone resistance in Gram negative bacteria.

8. References

Abbott, J. C., Aanensen, D. M., Rutherford, K., Butcher, S. & Spratt, B. G. (2005). WebACT--an online companion for the Artemis Comparison Tool. *Bioinformatics* **21**(18), 3665-3666.

AbuOun, M., Suthers, P. F., Jones, G. I., Carter, B. R., Saunders, M. P., Maranas, C. D., Woodward, M. J. & Anjum, M. F. (2009). Genome scale reconstruction of a Salmonella metabolic model: comparison of similarity and differences with a commensal Escherichia coli strain. *J Biol Chem* **284**(43), 29480-29488.

Ahmed, A. M., Motoi, Y., Sato, M., Maruyama, A., Watanabe, H., Fukumoto, Y. & Shimamoto, T. (2007). Zoo animals as reservoirs of gram-negative bacteria harboring integrons and antimicrobial resistance genes. *Appl Environ Microbiol* **73**(20), 6686-6690.

Alali, W. Q., Scott, H. M., Norby, B., Gebreyes, W. & Loneragan, G. H. (2009). Quantification of the bla(CMY-2) in feces from beef feedlot cattle administered three different doses of ceftiofur in a longitudinal controlled field trial. *Foodborne Pathog Dis* **6**(8), 917-924.

Alkhayer, M., Jenkins, P. F. & Harrison, B. D. (1990). The outcome of community acquired pneumonia treated on the intensive care unit. *Respir Med* **84**(1), 13-16.

Almirall, J., Mesalles, E., Klamburg, J., Parra, O. & Agudo, A. (1995). Prognostic factors of pneumonia requiring admission to the intensive care unit. *Chest* **107**(2), 511-516.

Alvarez, C. A., Yomayusa, N., Leal, A. L., Moreno, J., Mendez-Alvarez, S., Ibanez, M. & Vanegas, N. (2010). Nosocomial infections caused by community-associated methicillin-resistant Staphylococcus aureus in Colombia. *American journal of infection control* **38**(4), 315-318.

Amabile-Cuevas, C. F. & Chicurel, M. E. (1992). Bacterial plasmids and gene flux. *Cell* **70**(2), 189-199.

Amin, A. K. & Wareham, D. W. (2009). Plasmid-mediated quinolone resistance genes in Enterobacteriaceae isolates associated with community and nosocomial urinary tract infection in East London, UK. *Int J Antimicrob Agents* **34**(5), 490-491.

Anderson, V. E. & Osheroff, N. (2001). Type II topoisomerases as targets for quinolone antibacterials: turning Dr. Jekyll into Mr. Hyde. *Curr Pharm Des* **7**(5), 337-353.

Anjum, M. F., Mafura, M., Slickers, P., Ballmer, K., Kuhnert, P., Woodward, M. J. & Ehricht, R. (2007). Pathotyping Escherichia coli by using miniaturized DNA microarrays. *Appl Environ Microbiol* **73**(17), 5692-5697.

Bailey, J. K., Pinyon, J. L., Anantham, S. & Hall, R. M. (2010). Commensal Escherichia coli of healthy humans: a reservoir for antibiotic-resistance determinants. *Journal of medical microbiology* **59**(Pt 11), 1331-1339.

Ball, P. (2000). Quinolone generations: natural history or natural selection? *J Antimicrob Chemother* **46 Suppl T1**, 17-24.

Baquirin, M. H. & Barlow, M. (2008). Evolution and recombination of the plasmidic qnr alleles. *J Mol Evol* **67**(1), 103-110.

Batchelor, M., Hopkins, K. L., Liebana, E., Slickers, P., Ehricht, R., Mafura, M., Aarestrup, F., Mevius, D., Clifton-Hadley, F. A., Woodward, M. J., Davies, R. H., Threlfall, E. J. & Anjum, M. F. (2008). Development of a miniaturised microarray-based assay for the rapid identification of antimicrobial resistance genes in Gram-negative bacteria. *Int J Antimicrob Agents* **31**(5), 440-451.

Bateman, A., Murzin, A. G. & Teichmann, S. A. (1998). Structure and distribution of pentapeptide repeats in bacteria. *Protein Sci* **7**(6), 1477-1480.

Beliaev, A. S., Thompson, D. K., Fields, M. W., Wu, L., Lies, D. P., Nealson, K. H. & Zhou, J. (2002). Microarray transcription profiling of a Shewanella oneidensis etrA mutant. *J Bacteriol* **184**(16), 4612-4616.

Bellido, F. & Pechere, J. C. (1989). Laboratory survey of fluoroquinolone activity. *Rev Infect Dis* **11 Suppl 5**, S917-924.

Benca, J., Ondrusova, A., Rudinsky, B., Bauer, F. & Kovac, M. (2007). Nosocomial meningitis caused by Enterobacteriaceae: risk factors and outcome in 18 cases in 1992-2007. *Neuro Endocrinol Lett* **28 Suppl 2**, 27-29.

Bergan, T., Dalhoff, A. & Rohwedder, R. (1988). Pharmacokinetics of ciprofloxacin. *Infection* **16 Suppl 1**, S3-13.

Berger, J. M. (1998). Structure of DNA topoisomerases. *Biochim Biophys Acta* **1400**(1-3), 3-18.

Bergman, M., Nyberg, S. T., Huovinen, P., Paakkari, P. & Hakanen, A. J. (2008). Association between Antimicrobial Consumption and Resistance in Escherichia coli. *Antimicrob Agents Chemother*.

Bergstrom, C. T., Lipsitch, M. & Levin, B. R. (2000). Natural selection, infectious transfer and the existence conditions for bacterial plasmids. *Genetics* **155**(4), 1505-1519.

Bhagwat, A. S., Johnson, B., Weule, K. & Roberts, R. J. (1990). Primary sequence of the EcoRII endonuclease and properties of its fusions with beta-galactosidase. *J Biol Chem* **265**(2), 767-773.

Bjorkman, P. (1996). Preparation of electrocompetent E. coli. California, USA, Howard Hughes Medical Institute.

Blood, R. M. & Curtis, G. D. (1995). Media for 'total' Enterobacteriaceae, coliforms and Escherichia coli. *Int J Food Microbiol* **26**(1), 93-115.

Bonemann, G., Stiens, M., Puhler, A. & Schluter, A. (2006). Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, qnrS2, isolated from the bacterial community of a wastewater treatment plant. *Antimicrob Agents Chemother* **50**(9), 3075-3080.

Bou, G., Cartelle, M., Tomas, M., Canle, D., Molina, F., Moure, R., Eiros, J. M. & Guerrero, A. (2002). Identification and broad dissemination of the CTX-M-14 beta-lactamase in different Escherichia coli strains in the northwest area of Spain. *J Clin Microbiol* **40**(11), 4030-4036.

Brenner, Don J., Krieg, Noel R. & Staley, James T. (2005). Bergey's manual of systematic bacteriology. New York, Springer.

Brisse, S. & Verhoef, J. (2001). Phylogenetic diversity of Klebsiella pneumoniae and Klebsiella oxytoca clinical isolates revealed by randomly amplified polymorphic DNA, gyrA and parC genes sequencing and automated ribotyping. *Int J Syst Evol Microbiol* **51**(Pt 3), 915-924.

British Thoracic Society (1992). The aetiology, management and outcome of severe community-acquired pneumonia on the intensive care unit. The British Thoracic Society Research Committee and The Public Health Laboratory Service. *Respir Med* **86**(1), 7-13.

Bryan, L. E. & Bedard, J. (1991). Impermeability to quinolones in gram-positive and gram-negative bacteria. *Eur J Clin Microbiol Infect Dis* **10**(4), 232-239.

Cambau, E., Lascols, C., Sougakoff, W., Bebear, C., Bonnet, R., Cavallo, J. D., Gutmann, L., Ploy, M. C., Jarlier, V., Soussy, C. J. & Robert, J. (2006). Occurrence of qnrA-positive clinical isolates in French teaching hospitals during 2002-2005. *Clin Microbiol Infect* **12**(10), 1013-1020.

Cano, M. E., Rodriguez-Martinez, J. M., Aguero, J., Pascual, A., Calvo, J., Garcia-Lobo, J. M., Velasco, C., Francia, M. V. & Martinez-Martinez, L. (2009). Detection of plasmid-mediated quinolone resistance genes in clinical isolates of Enterobacter spp. in Spain. *J Clin Microbiol* **47**(7), 2033-2039.

Cao, V., Lambert, T. & Courvalin, P. (2002). ColE1-like plasmid pIP843 of Klebsiella pneumoniae encoding extended-spectrum beta-lactamase CTX-M-17. *Antimicrob Agents Chemother* **46**(5), 1212-1217.

Carattoli, A., Aschbacher, R., March, A., Larcher, C., Livermore, D. M. & Woodford, N. (2010). Complete nucleotide sequence of the IncN plasmid pKOX105 encoding VIM-1, QnrS1 and SHV-12 proteins in Enterobacteriaceae from Bolzano, Italy compared with IncN plasmids encoding KPC enzymes in the USA. *J Antimicrob Chemother* **65**(10), 2070-2075.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L. & Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* **63**(3), 219-228.

Carver, T., Berriman, M., Tivey, A., Patel, C., Bohme, U., Barrell, B. G., Parkhill, J. & Rajandream, M. A. (2008). Artemis and ACT: viewing, annotating and comparing sequences stored in a relational database. *Bioinformatics* **24**(23), 2672-2676.

Castanheira, M., Pereira, A. S., Nicoletti, A. G., Pignatari, A. C., Barth, A. L. & Gales, A. C. (2007). First report of plasmid-mediated qnrA1 in a ciprofloxacin-resistant Escherichia coli strain in Latin America. *Antimicrob Agents Chemother* **51**(4), 1527-1529.

Cattoir, V., Poirel, L., Aubert, C., Soussy, C. J. & Nordmann, P. (2008a). Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental Aeromonas spp. *Emerg Infect Dis* **14**(2), 231-237.

Cattoir, V., Poirel, L., Mazel, D., Soussy, C. J. & Nordmann, P. (2007a). Vibrio splendidus as the source of plasmid-mediated QnrS-like quinolone resistance determinants. *Antimicrob Agents Chemother* **51**(7), 2650-2651.

Cattoir, V., Poirel, L. & Nordmann, P. (2007b). In-vitro mutagenesis of qnrA and qnrS genes and quinolone resistance in Escherichia coli. *Clin Microbiol Infect* **13**(9), 940-943.

Cattoir, V., Poirel, L. & Nordmann, P. (2008b). Plasmid-mediated quinolone resistance pump QepA2 in an Escherichia coli isolate from France. *Antimicrob Agents Chemother* **52**(10), 3801-3804.

Cattoir, V., Weill, F. X., Poirel, L., Fabre, L., Soussy, C. J. & Nordmann, P. (2007c). Prevalence of qnr genes in Salmonella in France. *J Antimicrob Chemother* **59**(4), 751-754.

Cavaco, L. M., Hasman, H., Xia, S. & Aarestrup, F. M. (2009). qnrD, a novel gene conferring transferable quinolone resistance in Salmonella enterica serovar Kentucky and Bovismorbificans strains of human origin. *Antimicrob Agents Chemother* **53**(2), 603-608.

Chau, T. T., Campbell, J. I., Galindo, C. M., Van Minh Hoang, N., Diep, T. S., Nga, T. T., Van Vinh Chau, N., Tuan, P. Q., Page, A. L., Ochiai, R. L., Schultsz, C., Wain, J., Bhutta, Z. A., Parry, C. M., Bhattacharya, S. K., Dutta, S., Agtini, M., Dong, B., Honghui, Y., Anh, D. D., Canh do, G., Naheed, A., Albert, M. J., Phetsouvanh, R., Newton, P. N., Basnyat, B., Arjyal, A., La, T. T., Rang, N. N., Phuong le, T., Van Be Bay, P., von Seidlein, L., Dougan, G., Clemens, J. D., Vinh, H., Hien, T. T., Chinh, N. T., Acosta, C. J., Farrar, J. & Dolecek, C. (2007). Antimicrobial drug resistance of Salmonella enterica serovar typhi in asia and molecular mechanism of reduced susceptibility to the fluoroquinolones. *Antimicrob Agents Chemother* **51**(12), 4315-4323.

Chen, F. J., Lauderdale, T. L., Ho, M. & Lo, H. J. (2003). The roles of mutations in gyrA, parC, and ompK35 in fluoroquinolone resistance in Klebsiella pneumoniae. *Microb Drug Resist* **9**(3), 265-271.

Chen, S. L., Hung, C. S., Xu, J., Reigstad, C. S., Magrini, V., Sabo, A., Blasiar, D., Bieri, T., Meyer, R. R., Ozersky, P., Armstrong, J. R., Fulton, R. S., Latreille, J. P., Spieth, J., Hooton, T. M., Mardis, E. R., Hultgren, S. J. & Gordon, J. I. (2006). Identification of genes subject to positive selection in uropathogenic strains of Escherichia coli: a comparative genomics approach. *Proc Natl Acad Sci U S A* **103**(15), 5977-5982.

Chen, Y., Shu, H. S, Li, L. H., Liao, T. L., Wu, K. M., Shiau, Y. R., Yan, J. J., Su, I. J., Tsai, S. H. & Lauderdale, T. L. (2006). Complete nucleotide sequence of pK245, a 98-kilobase plasmid conferring quinolone resistance and Extended-Spectrum-B-Lactamase activity in a clinical *Klebsiella pneumoniae* isolate. *Antimicrob Agents Chemother* **50**(11), 3861-3866.

Cheung, T. K., Chu, Y. W., Chu, M. Y., Ma, C. H., Yung, R. W. & Kam, K. M. (2005). Plasmid-mediated resistance to ciprofloxacin and cefotaxime in clinical isolates of Salmonella enterica serotype Enteritidis in Hong Kong. *J Antimicrob Chemother* **56**(3), 586-589.

Clarke, J. S. (1974). Bacteriology of the gut and its clinical implications. *West J Med* **121**(5), 390-403.

CLSI (2007). Performance Standards For Antimicrobial Susceptibility Testing. 17th Informational Supplement.

Coque, T. M., Novais, A., Carattoli, A., Poirel, L., Pitout, J. D., Peixe, L., Baquero, F., Canton, R. & Nordmann, P. (2008). Dissemination of Clonally Related *Escherichia coli* Strains Expressing Extended-Spectrum B-Lactamase CTX-M-15. *Emerg Infect Dis* **14**(2), 195-200.

Cordeiro, N. F., Robino, L., Medina, J., Seija, V., Bado, I., Garcia, V., Berro, M., Pontet, J., Lopez, L., Bazet, C., Rieppi, G., Gutkind, G., Ayala, J. A. & Vignoli, R. (2008). Ciprofloxacin-resistant enterobacteria harboring the aac(6')-Ib-cr variant isolated from feces of inpatients in an intensive care unit in Uruguay. *Antimicrob Agents Chemother* **52**(2), 806-807.

Craven, D. E. (2006). What is healthcare-associated pneumonia, and how should it be treated? *Curr Opin Infect Dis* **19**(2), 153-160.

Cui, S., Li, J., Sun, Z., Hu, C., Jin, S., Li, F., Guo, Y., Ran, L. & Ma, Y. (2009). Characterization of Salmonella enterica isolates from infants and toddlers in Wuhan, China. *J Antimicrob Chemother* **63**(1), 87-94.

Dahmen, S., Poirel, L., Mansour, W., Bouallegue, O. & Nordmann, P. (2009). Prevalence of plasmid-mediated quinolone resistance determinants in Enterobacteriaceae from Tunisia. *Clin Microbiol Infect*.

del Solar, G. & Espinosa, M. (2000). Plasmid copy number control: an ever-growing story. *Mol Microbiol* **37**(3), 492-500.

Denton, M. (2007). Enterobacteriaceae. *Int J Antimicrob Agents* **29 Suppl 3**, S9-S22.

Diver, J. M., Piddock, L. J. & Wise, R. (1990). The accumulation of five quinolone antibacterial agents by Escherichia coli. *J Antimicrob Chemother* **25**(3), 319-333.

Donskey, C. J. (2004). The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens. *Clin Infect Dis* **39**(2), 219-226.

Dower, W. J., Miller, J. F. & Ragsdale, C. W. (1988). High efficiency transformation of E. coli by high voltage electroporation. *Nucleic Acids Res* **16**(13), 6127-6145.

Drlica, K. (1999). Mechanism of fluoroquinolone action. *Curr Opin Microbiol* **2**(5), 504-508.

Dryden, M., Hand, K. & Davey, P. (2009). Antibiotics for community-acquired pneumonia. *J Antimicrob Chemother* **64**(6), 1123-1125.

Duerink, D. O., Lestari, E. S., Hadi, U., Nagelkerke, N. J., Severin, J. A., Verbrugh, H. A., Keuter, M., Gyssens, I. C. & van den Broek, P. J. (2007). Determinants of carriage of resistant Escherichia coli in the Indonesian population inside and outside hospitals. *J Antimicrob Chemother* **60**(2), 377-384.

Eliopoulos, G. M. (1988). New quinolones: pharmacology, pharmacokinetics, and dosing in patients with renal insufficiency. *Rev Infect Dis* **10 Suppl 1**, S102-105.

Fihman, V., Lartigue, M. F., Jacquier, H., Meunier, F., Schnepf, N., Raskine, L., Riahi, J., Sanson-le Pors, M. J. & Bercot, B. (2008). Appearance of aac(6')-Ib-cr gene among extended-spectrum beta-lactamase-producing Enterobacteriaceae in a French hospital. *J Infect* **56**(6), 454-459.

Franco, A. V. (2005). Recurrent urinary tract infections. *Best Pract Res Clin Obstet Gynaecol* **19**(6), 861-873.

Fricke, W. F., Welch, T. J., McDermott, P. F., Mammel, M. K., LeClerc, J. E., White, D. G., Cebula, T. A. & Ravel, J. (2009). Comparative genomics of the IncA/C multidrug resistance plasmid family. *J Bacteriol* **191**(15), 4750-4757.

Fung, H. B. & Monteagudo-Chu, M. O. (2010). Community-acquired pneumonia in the elderly. *The American journal of geriatric pharmacotherapy* **8**(1), 47-62.

Funnell, Barbara E. & Phillips, Gregory J.A.G. (2004). Plasmid biology. Wasington DC, ASM Press.

Garcia-Fernandez, A., Fortini, D., Veldman, K., Mevius, D. & Carattoli, A. (2009). Characterization of plasmids harbouring qnrS1, qnrB2 and qnrB19 genes in Salmonella. *J Antimicrob Chemother* **63**(2), 274-281.

Garnier, F., Raked, N., Gassama, A., Denis, F. & Ploy, M. C. (2006). Genetic environment of quinolone resistance gene qnrB2 in a complex sul1-type integron in the newly described Salmonella enterica serovar Keurmassar. *Antimicrob Agents Chemother* **50**(9), 3200-3202.

Gaynes, R. & Edwards, J. R. (2005). Overview of nosocomial infections caused by gram-negative bacilli. *Clin Infect Dis* **41**(6), 848-854.

Gerding, D. N. & Hitt, J. A. (1989). Tissue penetration of the new quinolones in humans. *Rev Infect Dis* **11 Suppl 5**, S1046-1057.

Gestal, A. M., Stokes, H. W., Partridge, S. R. & Hall, R. M. (2005). Recombination between the dfrA12-orfF-aadA2 cassette array and an aadA1 gene cassette creates a hybrid cassette, aadA8b. *Antimicrob Agents Chemother* **49**(11), 4771-4774.

Golet, E. M., Alder, A. C. & Giger, W. (2002). Environmental exposure and risk assessment of fluoroquinolone antibacterial agents in wastewater and river water of the Glatt Valley Watershed, Switzerland. *Environ Sci Technol* **36**(17), 3645-3651.

Goossens, H., Ferech, M., Vander Stichele, R. & Elseviers, M. (2005). Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* **365**(9459), 579-587.

Goossens, H. & Sprenger, M. J. (1998). Community acquired infections and bacterial resistance. *Bmj* **317**(7159), 654-657.

Hamouda, A., Vali, L., Alsultan, A., Dancer, S. & Amyes, S. G. (2008). First report of ciprofloxacin resistance among Klebsiella pneumoniae harbouring the qnrA1 gene and producing SHV-5 extended-spectrum beta-lactamase in Scotland. *J Chemother* **20**(6), 753-755.

Hansen, H. & Heisig, P. (2003). Topoisomerase IV mutations in quinolone-resistant salmonellae selected in vitro. *Microb Drug Resist* **9**(1), 25-32.

Hata, M., Suzuki, M., Matsumoto, M., Takahashi, M., Sato, K., Ibe, S. & Sakae, K. (2005). Cloning of a novel gene for quinolone resistance from a transferable plasmid in Shigella flexneri 2b. *Antimicrob Agents Chemother* **49**(2), 801-803.

Hentges, D. J., Maier, B. R., Burton, G. C., Flynn, M. A. & Tsutakawa, R. K. (1977). Effect of a high-beef diet on the fecal bacterial flora of humans. *Cancer Res* **37**(2), 568-571.

Hochhut, B., Wilde, C., Balling, G., Middendorf, B., Dobrindt, U., Brzuszkiewicz, E., Gottschalk, G., Carniel, E. & Hacker, J. (2006). Role of pathogenicity island-associated integrases in the genome plasticity of uropathogenic Escherichia coli strain 536. *Mol Microbiol* **61**(3), 584-595.

Holt, John G., Sneath, Peter H. & Krieg, Noel R. (1994). Bergey's manual of determinative bacteriology. Philadelphia, Lippincott Williams & Wilkins.

Hooper, D. C. (1999). Mechanisms of fluoroquinolone resistance. *Drug Resist Updat* **2**(1), 38-55.

Hopkins, K. L., Wootton, L., Day, M. R. & Threlfall, E. J. (2007). Plasmid-mediated quinolone resistance determinant qnrS1 found in Salmonella enterica strains isolated in the UK. *J Antimicrob Chemother* **59**(6), 1071-1075.

Hu, F. P., Xu, X. G., Zhu, D. M. & Wang, M. G. (2008). Coexistence of qnrB4 and qnrS1 in a clinical strain of Klebsiella pneumoniae. *Acta Pharmacol Sin* **29**(3), 320-324.

Hu, L. F., Li, J. B., Ye, Y. & Li, X. (2007). Mutations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV in clinical strains of fluoroquinolone-resistant Shigella in Anhui, China. *J Microbiol* **45**(2), 168-170.

Huang, S. Y., Dai, L., Xia, L. N., Du, X. D., Qi, Y. H., Liu, H. B., Wu, C. M. & Shen, J. Z. (2009). Increased prevalence of plasmid-mediated quinolone resistance determinants in chicken Escherichia coli isolates from 2001 to 2007. *Foodborne Pathog Dis* **6**(10), 1203-1209.

Huang, Z., Mi, Z. & Wang, C. (2008). A novel beta-lactamase gene, LAP-2, produced by an Enterobacter cloacae clinical isolate in China. *J Hosp Infect* **70**(1), 95-96.

Iabadene, H., Messai, Y., Ammari, H., Ramdani-Bouguessa, N., Lounes, S., Bakour, R. & Arlet, G. (2008). Dissemination of ESBL and Qnr determinants in Enterobacter cloacae in Algeria. *J Antimicrob Chemother*.

Iida, S., Mollet, B., Meyer, J. & Arber, W. (1984). Functional characterization of the prokaryotic mobile genetic element IS26. *Molecular & general genetics : MGG* **198**(1), 84-89.

Ikaheimo, R., Siitonen, A., Karkkainen, U. & Makela, P. H. (1993). Virulence characteristics of Escherichia coli in nosocomial urinary tract infection. *Clin Infect Dis* **16**(6), 785-791.

Jacoby, G. A., Chow, N. & Waites, K. B. (2003). Prevalence of plasmid-mediated quinolone resistance. *Antimicrob Agents Chemother* **47**(2), 559-562.

Jacoby, G. A., Walsh, K. E., Mills, D. M., Walker, V. J., Oh, H., Robicsek, A. & Hooper, D. C. (2006). qnrB, another plasmid-mediated gene for quinolone resistance. *Antimicrob Agents Chemother* **50**(4), 1178-1182.

Jacoby, G., Cattoir, V., Hooper, D., Martinez-Martinez, L., Nordmann, P., Pascual, A., Poirel, L. & Wang, M. (2008). qnr Gene Nomenclature. *Antimicrob Agents Chemother*.

Jeong, J. Y., Yoon, H. J., Kim, E. S., Lee, Y., Choi, S. H., Kim, N. J., Woo, J. H. & Kim, Y. S. (2005). Detection of qnr in clinical isolates of Escherichia coli from Korea. *Antimicrob Agents Chemother* **49**(6), 2522-2524.

Jiang, Y., Zhou, Z., Qian, Y., Wei, Z., Yu, Y., Hu, S. & Li, L. (2008). Plasmid-mediated quinolone resistance determinants qnr and aac(6')-Ib-cr in extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in China. *J Antimicrob Chemother* **61**(5), 1003-1006.

Kado, C. I. & Liu, S. T. (1981). Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* **145**(3), 1365-1373.

Kauko, T., Haukka, K., Abuoun, M., Anjum, M. F., Woodward, M. J. & Siitonen, A. (2010). Phenotype MicroArray in the metabolic characterisation of Salmonella serotypes Agona, Enteritidis, Give, Hvittingfoss, Infantis, Newport and Typhimurium. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **29**(3), 311-317.

Kehrenberg, C., Friederichs, S., de Jong, A., Michael, G. B. & Schwarz, S. (2006). Identification of the plasmid-borne quinolone resistance gene qnrS in Salmonella enterica serovar Infantis. *J Antimicrob Chemother* **58**(1), 18-22.

Kehrenberg, C., Hopkins, K. L., Threlfall, E. J. & Schwarz, S. (2007). Complete nucleotide sequence of a small qnrS1-carrying plasmid from Salmonella enterica subsp. enterica Typhimurium DT193. *J Antimicrob Chemother* **60**(4), 903-905.

Kim, E. H. & Aoki, T. (1994). The transposon-like structure of IS26-tetracycline, and kanamycin resistance determinant derived from transferable R plasmid of fish pathogen, Pasteurella piscicida. *Microbiol Immunol* **38**(1), 31-38.

Kim, H. B., Park, C. H., Kim, C. J., Kim, E. C., Jacoby, G. A. & Hooper, D. C. (2009). Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. *Antimicrob Agents Chemother* **53**(2), 639-645.

Kim, S. Y., Park, Y. J., Yu, J. K., Kim, Y. S. & Han, K. (2009). Prevalence and characteristics of aac(6')-Ib-cr in AmpC-producing Enterobacter cloacae, Citrobacter freundii, and Serratia marcescens: a multicenter study from Korea. *Diagn Microbiol Infect Dis* **63**(3), 314-318.

Kirjavainen, P. V. & Gibson, G. R. (1999). Healthy gut microflora and allergy: factors influencing development of the microbiota. *Ann Med* **31**(4), 288-292.

Kitagawa, Y., Akaboshi, E., Shinagawa, H., Horii, T., Ogawa, H. & Kato, T. (1985). Structural analysis of the umu operon required for inducible mutagenesis in Escherichia coli. *Proc Natl Acad Sci U S A* **82**(13), 4336-4340.

Kmet, V. & Piatnicova, E. (2010). Antibiotic resistance in commensal intestinal microflora. *Folia microbiologica* **55**(4), 332-335.

Ko, W. C., Paterson, D. L., Sagnimeni, A. J., Hansen, D. S., Von Gottberg, A., Mohapatra, S., Casellas, J. M., Goossens, H., Mulazimoglu, L., Trenholme, G., Klugman, K. P., McCormack, J. G. & Yu, V. L. (2002). Community-acquired Klebsiella pneumoniae bacteremia: global differences in clinical patterns. *Emerg Infect Dis* **8**(2), 160-166.

Kobayashi, I. (2001). Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution. *Nucleic Acids Res* **29**(18), 3742-3756.

Kumarasamy, K. K., Toleman, M. A., Walsh, T. R., Bagaria, J., Butt, F., Balakrishnan, R., Chaudhary, U., Doumith, M., Giske, C. G., Irfan, S., Krishnan, P., Kumar, A. V., Maharjan, S., Mushtaq, S., Noorie, T., Paterson, D. L., Pearson, A., Perry, C., Pike, R., Rao, B., Ray, U., Sarma, J. B., Sharma, M., Sheridan, E., Thirunarayan, M. A., Turton, J., Upadhyay, S., Warner, M., Welfare, W., Livermore, D. M. & Woodford, N. (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *The Lancet infectious diseases* **10**(9), 597-602.

Lautenbach, E., Metlay, J. P., Bilker, W. B., Edelstein, P. H. & Fishman, N. O. (2005). Association between fluoroquinolone resistance and mortality in Escherichia coli and Klebsiella pneumoniae infections: the role of inadequate empirical antimicrobial therapy. *Clin Infect Dis* **41**(7), 923-929.

Lavigne, J. P., Marchandin, H., Delmas, J., Bouziges, N., Lecaillon, E., Cavalie, L., Jean-Pierre, H., Bonnet, R. & Sotto, A. (2006). qnrA in CTX-M-producing Escherichia coli isolates from France. *Antimicrob Agents Chemother* **50**(12), 4224-4228.

Lavilla, S., Gonzalez-Lopez, J. J., Sabate, M., Garcia-Fernandez, A., Larrosa, M. N., Bartolome, R. M., Carattoli, A. & Prats, G. (2008). Prevalence of qnr genes among extended-spectrum beta-lactamase-producing enterobacterial isolates in Barcelona, Spain. *J Antimicrob Chemother* **61**(2), 291-295.

Lawley, Trevor, Wilkins, Brian M. & Frost, Laura S. (2004). Bacterial conjugation in Gram-Negative Bacteria. Plasmid Biology. B. E. Funnell and G. J. Phillips. Washington, D.C., ASM Press**:** 203-226.

Lee, Y. J., Cho, J. K., Kim, K. S., Tak, R. B., Kim, A. R., Kim, J. W., Im, S. K. & Kim, B. H. (2005). Fluoroquinolone resistance and gyrA and parC mutations of Escherichia coli isolated from chicken. *J Microbiol* **43**(5), 391-397.

Levine, C., Hiasa, H. & Marians, K. J. (1998). DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities. *Biochim Biophys Acta* **1400**(1-3), 29-43.

Levy, J. (2000). The effects of antibiotic use on gastrointestinal function. *Am J Gastroenterol* **95**(1 Suppl), S8-10.

Lim, W. S., Baudouin, S. V., George, R. C., Hill, A. T., Jamieson, C., Le Jeune, I., Macfarlane, J. T., Read, R. C., Roberts, H. J., Levy, M. L., Wani, M. & Woodhead, M. A. (2009). BTS guidelines for the management of community acquired pneumonia in adults: update 2009. *Thorax* **64 Suppl 3**, iii1-55.

Liu, J. H., Deng, Y. T., Zeng, Z. L., Gao, J. H., Chen, L., Arakawa, Y. & Chen, Z. L. (2008). Coprevalence of plasmid-mediated quinolone resistance determinants QepA, Qnr, and AAC(6')-Ib-cr among 16S rRNA methylase RmtB-producing Escherichia coli isolates from pigs. *Antimicrob Agents Chemother* **52**(8), 2992-2993.

Lodwick, D., Owen, D. & Strike, P. (1990). DNA sequence analysis of the imp UV protection and mutation operon of the plasmid TP110: identification of a third gene. *Nucleic Acids Res* **18**(17), 5045-5050.

Ma, J., Zeng, Z., Chen, Z., Xu, X., Wang, X., Deng, Y., Lu, D., Huang, L., Zhang, Y., Liu, J. & Wang, M. (2009). High prevalence of plasmid-mediated quinolone resistance determinants qnr, aac(6')-Ib-cr, and qepA among ceftiofur-resistant Enterobacteriaceae isolates from companion and food-producing animals. *Antimicrob Agents Chemother* **53**(2), 519-524.

Machado, E., Coque, T. M., Canton, R., Baquero, F., Sousa, J. C. & Peixe, L. (2006). Dissemination in Portugal of CTX-M-15-, OXA-1-, and TEM-1-producing Enterobacteriaceae strains containing the aac(6')-Ib-cr gene, which encodes an aminoglycoside- and fluoroquinolone-modifying enzyme. *Antimicrob Agents Chemother* **50**(9), 3220-3221.

Madigan, Michael T., Martinko, John M. & Parker, Jack (2003). Brock biology of microorganisms. New Jersey, Prentice Hall.

Martinez-Martinez, L., Pascual, A. & Jacoby, G. A. (1998). Quinolone resistance from a transferable plasmid. *Lancet* **351**(9105), 797-799.

Minarini, L. A., Poirel, L., Cattoir, V., Darini, A. L. & Nordmann, P. (2008). Plasmid-mediated quinolone resistance determinants among enterobacterial isolates from outpatients in Brazil. *J Antimicrob Chemother* **62**(3), 474-478.

Miyachi, H., Furuya, H., Umezawa, K., Itoh, Y., Ohshima, T., Miyamoto, M. & Asai, S. (2007). Controlling methicillin-resistant Staphylococcus aureus by stepwise implementation of preventive strategies in a university hospital: impact of a link-nurse system on the basis of multidisciplinary approaches. *Am J Infect Control* **35**(2), 115-121.

Moellering, R. C., Jr. (2010). NDM-1--a cause for worldwide concern. *The New England journal of medicine* **363**(25), 2377-2379.

Montero, C., Mateu, G., Rodriguez, R. & Takiff, H. (2001). Intrinsic resistance of Mycobacterium smegmatis to fluoroquinolones may be influenced by new pentapeptide protein MfpA. *Antimicrob Agents Chemother* **45**(12), 3387-3392.

Moore, W. E. & Holdeman, L. V. (1974). Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Appl Microbiol* **27**(5), 961-979.

Mori, N., Fujino, T., Uchida, H., Miyazawa, H., Minoura, S., Kawana, A., Kawahata, H., Gotoh, M., Yonamine, T., Yamanishi, F., Kuratsuji, T., Kudo, K., Kobori, O., Yazaki, Y. & Kirikae, T. (2001). Molecular epidemiology of methicillin-resistant Staphylococcus aureus infections in a newborn nursery, a neonatal intensive care unit, and a general pediatrics ward. *Jpn J Infect Dis* **54**(5), 189-190.

Mosby Inc. (2009). Mosby's medical dictionary. St. Louis, MO, Mosby.

Murray, Patrick R., Rosenthal, Ken S. , Kobayashi, George S. & Pfaller, Michael A. (1998). Medical Microbiology. Missouri, Mosby, Inc.

Nester, Eugene W., Anderson, Denise G., Roberts, C. Evans, Pearsall, Nancy N. & Nester, Martha T. (2004). Microbiology: A human perspective. Washington, D.C., McGraw-Hill.

Neu, H. C. (1992). Quinolone antimicrobial agents. *Annu Rev Med* **43**, 465-486.

NHS, Health Protection Agency (2010). National Standard Method. Identification of Enterobacteriaceae. Standard Unit, Department for Evaluations, Standards and Training. **16**.

Nikaido, H. (2009). Multidrug resistance in bacteria. *Annu Rev Biochem* **78**, 119-146.

Nishino, Y., Deguchi, T., Yasuda, M., Kawamura, T., Nakano, M., Kanematsu, E., Ozeki, S. & Kawada, Y. (1997). Mutations in the gyrA and parC genes associated with fluoroquinolone resistance in clinical isolates of Citrobacter freundii. *FEMS Microbiol Lett* **154**(2), 409-414.

Okeke, I. N., Laxminarayan, R., Bhutta, Z. A., Duse, A. G., Jenkins, P., O'Brien, T. F., Pablos-Mendez, A. & Klugman, K. P. (2005). Antimicrobial resistance in developing countries. Part I: recent trends and current status. *Lancet Infect Dis* **5**(8), 481-493.

Oktem, I. M., Gulay, Z., Bicmen, M. & Gur, D. (2008). qnrA prevalence in extended-spectrum beta-lactamase-positive Enterobacteriaceae isolates from Turkey. *Jpn J Infect Dis* **61**(1), 13-17.

Pallecchi, L., Bartoloni, A., Fiorelli, C., Mantella, A., Di Maggio, T., Gamboa, H., Gotuzzo, E., Kronvall, G., Paradisi, F. & Rossolini, G. M. (2007a). Rapid dissemination and diversity of CTX-M extended-spectrum beta-lactamase genes in commensal Escherichia coli isolates from healthy children from low-resource settings in Latin America. *Antimicrob Agents Chemother* **51**(8), 2720-2725.

Pallecchi, L., Lucchetti, C., Bartoloni, A., Bartalesi, F., Mantella, A., Gamboa, H., Carattoli, A., Paradisi, F. & Rossolini, G. M. (2007b). Population structure and resistance genes in antibiotic-resistant bacteria from a remote community with minimal antibiotic exposure. *Antimicrob Agents Chemother* **51**(4), 1179-1184.

Park, C. H., Robicsek, A., Jacoby, G. A., Sahm, D. & Hooper, D. C. (2006). Prevalence in the United States of aac(6')-Ib-cr encoding a ciprofloxacin-modifying enzyme. *Antimicrob Agents Chemother* **50**(11), 3953-3955.

Park, Y. J., Yu, J. K., Kim, S. I., Lee, K. & Arakawa, Y. (2009). Accumulation of plasmid-mediated fluoroquinolone resistance genes, qepA and qnrS1, in enterobacter aerogenes co-producing RmtB and class A beta-lactamase LAP-1. *Ann Clin Lab Sci* **39**(1), 55-59.

Park, Y. J., Yu, J. K., Lee, S., Oh, E. J. & Woo, G. J. (2007). Prevalence and diversity of qnr alleles in AmpC-producing Enterobacter cloacae, Enterobacter aerogenes, Citrobacter freundii and Serratia marcescens: a multicentre study from Korea. *J Antimicrob Chemother* **60**(4), 868-871.

Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T., Prentice, M. B., Sebaihia, M., James, K. D., Churcher, C., Mungall, K. L., Baker, S., Basham, D., Bentley, S. D., Brooks, K., Cerdeno-Tarraga, A. M., Chillingworth,

T., Cronin, A., Davies, R. M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Karlyshev, A. V., Leather, S., Moule, S., Oyston, P. C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S. & Barrell, B. G. (2001). Genome sequence of Yersinia pestis, the causative agent of plague. *Nature* **413**(6855), 523-527.

Perichon, B., Bogaerts, P., Lambert, T., Frangeul, L., Courvalin, P. & Galimand, M. (2008). Sequence of conjugative plasmid pIP1206 mediating resistance to aminoglycosides by 16S rRNA methylation and to hydrophilic fluoroquinolones by efflux. *Antimicrob Agents Chemother* **52**(7), 2581-2592.

Perichon, B., Courvalin, P. & Galimand, M. (2007). Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in Escherichia coli. *Antimicrob Agents Chemother* **51**(7), 2464-2469.

Perna, N. T., Plunkett, G., 3rd, Burland, V., Mau, B., Glasner, J. D., Rose, D. J., Mayhew, G. F., Evans, P. S., Gregor, J., Kirkpatrick, H. A., Posfai, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E. J., Davis, N. W., Lim, A., Dimalanta, E. T., Potamousis, K. D., Apodaca, J., Anantharaman, T. S., Lin, J., Yen, G., Schwartz, D. C., Welch, R. A. & Blattner, F. R. (2001). Genome sequence of enterohaemorrhagic Escherichia coli O157:H7. *Nature* **409**(6819), 529-533.

Perry, K. L., Elledge, S. J., Mitchell, B. B., Marsh, L. & Walker, G. C. (1985). umuDC and mucAB operons whose products are required for UV light- and chemical-induced mutagenesis: UmuD, MucA, and LexA proteins share homology. *Proc Natl Acad Sci U S A* **82**(13), 4331-4335.

Peterson, L. R. (2005). Squeezing the antibiotic balloon: the impact of antimicrobial classes on emerging resistance. *Clin Microbiol Infect* **11 Suppl 5**, 4-16.

Pham, H. N., Ohkusu, K., Mishima, N., Noda, M., Monir Shah, M., Sun, X., Hayashi, M. & Ezaki, T. (2007). Phylogeny and species identification of the family Enterobacteriaceae based on dnaJ sequences. *Diagn Microbiol Infect Dis* **58**(2), 153-161.

Picao, R. C., Poirel, L., Demarta, A., Silva, C. S., Corvaglia, A. R., Petrini, O. & Nordmann, P. (2008). Plasmid-mediated quinolone resistance in Aeromonas allosaccharophila recovered from a Swiss lake. *J Antimicrob Chemother* **62**(5), 948-950.

Pitout, J. D., Wei, Y., Church, D. L. & Gregson, D. B. (2008). Surveillance for plasmid-mediated quinolone resistance determinants in Enterobacteriaceae within the Calgary Health Region, Canada: the emergence of aac(6')-Ib-cr. *J Antimicrob Chemother* **61**(5), 999-1002.

Poirel, L., Cattoir, V., Soares, A., Soussy, C. J. & Nordmann, P. (2007). Novel Ambler class A beta-lactamase LAP-1 and its association with the plasmid-mediated quinolone resistance determinant QnrS1. *Antimicrob Agents Chemother* **51**(2), 631-637.

Poirel, L., Leviandier, C. & Nordmann, P. (2006a). Prevalence and genetic analysis of plasmid-mediated quinolone resistance determinants QnrA and QnrS in Enterobacteriaceae isolates from a French university hospital. *Antimicrob Agents Chemother* **50**(12), 3992-3997.

Poirel, L., Liard, A., Rodriguez-Martinez, J. M. & Nordmann, P. (2005a). Vibrionaceae as a possible source of Qnr-like quinolone resistance determinants. *J Antimicrob Chemother* **56**(6), 1118-1121.

Poirel, L., Nguyen, T. V., Weintraub, A., Leviandier, C. & Nordmann, P. (2006b). Plasmid-mediated quinolone resistance determinant qnrS in Enterobacter cloacae. *Clin Microbiol Infect* **12**(10), 1021-1023.

Poirel, L., Rodriguez-Martinez, J. M., Mammeri, H., Liard, A. & Nordmann, P. (2005b). Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob Agents Chemother* **49**(8), 3523-3525.

Poirel, L., Van De Loo, M., Mammeri, H. & Nordmann, P. (2005c). Association of plasmid-mediated quinolone resistance with extended-spectrum beta-lactamase VEB-1. *Antimicrob Agents Chemother* **49**(7), 3091-3094.

Pomba, C., da Fonseca, J. D., Baptista, B. C., Correia, J. D. & Martinez-Martinez, L. (2009). Detection of the pandemic O25-ST131 human virulent Escherichia coli CTX-M-15-producing clone harboring the qnrB2 and aac(6')-Ib-cr genes in a dog. *Antimicrob Agents Chemother* **53**(1), 327-328.

Quagliarello, AB., Parry, C. M., Hien, T. T. & Farrar, J. (2003). Factors associated with carriage of penicillin-resistant Streptococcus pneumoniae among Vietnamese children: a rural-urban divide. *J Health Popul Nutr* **21**(4), 316-324.

Quiroga, M. P., Andres, P., Petroni, A., Soler Bistue, A. J. C., GUerriero, L., Vargas, L. J., Zorreguieta, A., Tokumoto, M., Quiroga, C., Tolmasky, M. E., Galas, M. & Centron, D. (2007). Complex class 1 Integrons with diverse variable regions,

including aac(6')-Ib-cr and a novel allel qnrB10 associated with ISCR1 in clinical enterobacterial isolates from Argentina. *Antimicrob Agents Chemother* **51**(12), 4466-4470.

Rello, J., Quintana, E., Ausina, V., Net, A. & Prats, G. (1993). A three-year study of severe community-acquired pneumonia with emphasis on outcome. *Chest* **103**(1), 232-235.

Rello, J., Rodriguez, R., Jubert, P. & Alvarez, B. (1996). Severe community-acquired pneumonia in the elderly: epidemiology and prognosis. Study Group for Severe Community-Acquired Pneumonia. *Clin Infect Dis* **23**(4), 723-728.

Reznikoff, W. S. (1993). The Tn5 transposon. *Annu Rev Microbiol* **47**, 945-963.

Robicsek, A., Sahm, D. F., Strahilevitz, J., Jacoby, G. A. & Hooper, D. C. (2005). Broader distribution of plasmid-mediated quinolone resistance in the United States. *Antimicrob Agents Chemother* **49**(7), 3001-3003.

Robicsek, A., Strahilevitz, J., Jacoby, G. A., Macielag, M., Abbanat, D., Park, C. H., Bush, K. & Hooper, D. C. (2006a). Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat Med* **12**(1), 83-88.

Robicsek, A., Strahilevitz, J., Sahm, D. F., Jacoby, G. A. & Hooper, D. C. (2006b). qnr prevalence in ceftazidime-resistant Enterobacteriaceae isolates from the United States. *Antimicrob Agents Chemother* **50**(8), 2872-2874.

Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M. A. & Barrell, B. (2000). Artemis: sequence visualization and annotation. *Bioinformatics* **16**(10), 944-945.

Sabtcheva, S., Kaku, M., Saga, T., Ishii, Y. & Kantardjiev, T. (2009). High prevalence of the aac(6')-Ib-cr gene and its dissemination among Enterobacteriaceae isolates by CTX-M-15 plasmids in Bulgaria. *Antimicrob Agents Chemother* **53**(1), 335-336.

Saga, T., Kaku, M., Onodera, Y., Yamachika, S., Sato, K. & Takase, H. (2005). Vibrio parahaemolyticus chromosomal qnr homologue VPA0095: demonstration by transformation with a mutated gene of its potential to reduce quinolone susceptibility in Escherichia coli. *Antimicrob Agents Chemother* **49**(5), 2144-2145.

Sambrook, Joseph & Russell, David W. (2001). Molecular cloning : a laboratory manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press.

Sanchez, M. B., Hernandez, A., Rodriguez-Martinez, J. M., Martinez-Martinez, L. & Martinez, J. L. (2008). Predictive analysis of transmissible quinolone resistance

indicates Stenotrophomonas maltophilia as a potential source of a novel family of Qnr determinants. *BMC Microbiol* **8**, 148.

Sannes, M. R., Belongia, E. A., Kieke, B., Smith, K., Kieke, A., Vandermause, M., Bender, J., Clabots, C., Winokur, P. & Johnson, J. R. (2008). Predictors of antimicrobial-resistant Escherichia coli in the feces of vegetarians and newly hospitalized adults in Minnesota and Wisconsin. *J Infect Dis* **197**(3), 430-434.

Schultsz, C., Moussa, M., van Ketel, R., Tytgat, G. N. & Dankert, J. (1997). Frequency of pathogenic and enteroadherent Escherichia coli in patients with inflammatory bowel disease and controls. *J Clin Pathol* **50**(7), 573-579.

Shin, J. H., Jung, H. J., Lee, J. Y., Kim, H. R., Lee, J. N. & Chang, C. L. (2008). High rates of plasmid-mediated quinolone resistance QnrB variants among ciprofloxacin-resistant Escherichia coli and Klebsiella pneumoniae from urinary tract infections in Korea. *Microb Drug Resist* **14**(3), 221-226.

Snyder, Larry & Champness, Wendy (2003). Molecular genetics of bacteria. Washington, D.C., ASM Press.

Som, S., Bhagwat, A. S. & Friedman, S. (1987). Nucleotide sequence and expression of the gene encoding the EcoRII modification enzyme. *Nucleic Acids Res* **15**(1), 313-332.

Sorum, H. & Sunde, M. (2001). Resistance to antibiotics in the normal flora of animals. *Vet Res* **32**(3-4), 227-241.

Stiefel, U. & Donskey, C. J. (2004). The Role of the Intestinal Tract As a Source for Transmission of Nosocomial Pathogens. *Curr Infect Dis Rep* **6**(6), 420-425.

Stobberingh, E. E. & van den Bogaard, A. E. (2000). Spread of antibiotic resistance from food animals to man. *Acta Vet Scand Suppl* **93**, 47-50; discussion 51-42.

Strahilevitz, J., Engelstein, D., Adler, A., Temper, V., Moses, A. E., Block, C. & Robicsek, A. (2007). Changes in qnr prevalence and fluoroquinolone resistance in clinical isolates of Klebsiella pneumoniae and Enterobacter spp. collected from 1990 to 2005. *Antimicrob Agents Chemother* **51**(8), 3001-3003.

Strahilevitz, J., Jacoby, G. A., Hooper, D. C. & Robicsek, A. (2009). Plasmid-mediated quinolone resistance: a multifaceted threat. *Clin Microbiol Rev* **22**(4), 664-689.

Sundin, G. W. & Bender, C. L. (1996). Dissemination of the strA-strB streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. *Mol Ecol* **5**(1), 133-143.

Szabo, D., Kocsis, B., Rokusz, L., Szentandrassy, J., Katona, K., Kristof, K. & Nagy, K. (2008). First detection of plasmid-mediated, quinolone resistance determinants qnrA, qnrB, qnrS and aac(6')-Ib-cr in extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae in Budapest, Hungary. *J Antimicrob Chemother* **62**(3), 630-632.

Tancrede, C. (1992). Role of human microflora in health and disease. *Eur J Clin Microbiol Infect Dis* **11**(11), 1012-1015.

Taneja, J., Mishra, B., Thakur, A., Dogra, V. & Loomba, P. (2010). Nosocomial blood-stream infections from extended-spectrum-beta-lactamase-producing Escherichia coli and Klebsiella pneumonia from GB Pant Hospital, New Delhi. *Journal of infection in developing countries* **4**(8), 517-520.

Taylor, Diane E., Gibreel, Amera, Lawley, Trevor D. & Tracz, Dobryan M. (2004). Antibiotic resistance plasmids. Plasmid Biology. B. E. Funnell and G. J. Phillips. Washington, D.C., ASM Press**:** 473-491

Teuber, M. (2001). Veterinary use and antibiotic resistance. *Curr Opin Microbiol* **4**(5), 493-499.

Tran, J. H. & Jacoby, G. A. (2002). Mechanism of plasmid-mediated quinolone resistance. *Proc Natl Acad Sci U S A* **99**(8), 5638-5642.

Tran, J. H., Jacoby, G. A. & Hooper, D. C. (2005a). Interaction of the plasmid-encoded quinolone resistance protein Qnr with Escherichia coli DNA gyrase. *Antimicrob Agents Chemother* **49**(1), 118-125.

Tran, J. H., Jacoby, G. A. & Hooper, D. C. (2005b). Interaction of the plasmid-encoded quinolone resistance protein QnrA with Escherichia coli topoisomerase IV. *Antimicrob Agents Chemother* **49**(7), 3050-3052.

Tran, T. H., Nguyen, T. D., Nguyen, T. T., Ninh, T. T., Tran, N. B., Nguyen, V. M., Tran, T. T., Cao, T. T., Pham, V. M., Nguyen, T. C., Tran, T. D., Pham, V. T., To, S. D., Campbell, J. I., Stockwell, E., Schultsz, C., Simmons, C. P., Glover, C., Lam, W., Marques, F., May, J. P., Upton, A., Budhram, R., Dougan, G., Farrar, J., Nguyen, V. V. & Dolecek, C. (2010). A randomised trial evaluating the safety and immunogenicity of the novel single oral dose typhoid vaccine M01ZH09 in healthy Vietnamese children. *PLoS ONE* **5**(7), e11778.

Travers, K. & Barza, M. (2002). Morbidity of infections caused by antimicrobial-resistant bacteria. *Clin Infect Dis* **34 Suppl 3**, S131-134.

Van Bambeke, F., Michot, J. M., Van Eldere, J. & Tulkens, P. M. (2005). Quinolones in 2005: an update. *Clin Microbiol Infect* **11**(4), 256-280.

van der Veen, E. L., Schilder, A. G., Timmers, T. K., Rovers, M. M., Fluit, A. C., Bonten, M. J. & Leverstein-van Hall, M. A. (2009). Effect of long-term trimethoprim/sulfamethoxazole treatment on resistance and integron prevalence in the intestinal flora: a randomized, double-blind, placebo-controlled trial in children. *J Antimicrob Chemother* **63**(5), 1011-1016.

van Doornum, G. J., Guldemeester, J., Osterhaus, A. D. & Niesters, H. G. (2003). Diagnosing herpesvirus infections by real-time amplification and rapid culture. *J Clin Microbiol* **41**(2), 576-580.

Venter, J. C., Remington, K., Heidelberg, J. F., Halpern, A. L., Rusch, D., Eisen, J. A., Wu, D., Paulsen, I., Nelson, K. E., Nelson, W., Fouts, D. E., Levy, S., Knap, A. H., Lomas, M. W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, Y. H. & Smith, H. O. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**(5667), 66-74.

Versalovic, J., Koeuth, T. & Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**(24), 6823-6831.

Vetting, M. W., Hegde, S. S., Fajardo, J. E., Fiser, A., Roderick, S. L., Takiff, H. E. & Blanchard, J. S. (2006). Pentapeptide repeat proteins. *Biochemistry* **45**(1), 1-10.

Vetting, M. W., Park, C. H., Hegde, S. S., Jacoby, G. A., Hooper, D. C. & Blanchard, J. S. (2008). Mechanistic and structural analysis of aminoglycoside N-acetyltransferase AAC(6')-Ib and its bifunctional, fluoroquinolone-active AAC(6')-Ib-cr variant. *Biochemistry* **47**(37), 9825-9835.

Vila, J., Ruiz, J., Goni, P. & De Anta, M. T. J. (1996). Detection of mutation in *parC* in Quinolone-resistant clinical isolates of *Escherichia coli*. *Antimicrob Agents Chemother* **40**(2), 491-493.

Wang, A., Yang, Y., Lu, Q., Wang, Y., Chen, Y., Deng, L., Ding, H., Deng, Q., Wang, L. & Shen, X. (2008a). Occurrence of qnr-positive clinical isolates in Klebsiella pneumoniae producing ESBL or AmpC-type beta-lactamase from five pediatric hospitals in China. *FEMS Microbiol Lett* **283**(1), 112-116.

Wang, A., Yang, Y., Lu, Q., Wang, Y., Chen, Y., Deng, L., Ding, H., Deng, Q., Zhang, H., Wang, C., Liu, L., Xu, X., Wang, L. & Shen, X. (2008b). Presence of qnr gene

in Escherichia coli and Klebsiella pneumoniae resistant to ciprofloxacin isolated from pediatric patients in China. *BMC Infect Dis* **8**, 68.

Wang, C. X., Huang, Z. M., Mi, Z. H. & Chen, G. Q. (2009). A Klebsiella pneumoniae clinical isolate producing the LAP-2 beta-lactamase in China. *J Hosp Infect* **71**(2), 189-190.

Wang, F. D., Chen, Y. Y., Chen, T. L. & Liu, C. Y. (2008). Risk factors and mortality in patients with nosocomial Staphylococcus aureus bacteremia. *Am J Infect Control* **36**(2), 118-122.

Wang, M., Guo, Q., Xu, X., Wang, X., Ye, X., Wu, S. & Hooper, D. C. (2009). New plasmid-mediated quinolone resistance gene, qnrC, found in a clinical isolate of Proteus mirabilis. *Antimicrob Agents Chemother* **53**(5), 1892-1897.

Wang, M., Sahm, D. F., Jacoby, G. A. & Hooper, D. C. (2004a). Emerging plasmid-mediated quinolone resistance associated with the qnr gene in Klebsiella pneumoniae clinical isolates in the United States. *Antimicrob Agents Chemother* **48**(4), 1295-1299.

Wang, M., Sahm, D. F., Jacoby, G. A., Zhang, Y. & Hooper, D. C. (2004b). Activities of newer quinolones against Escherichia coli and Klebsiella pneumoniae containing the plasmid-mediated quinolone resistance determinant qnr. *Antimicrob Agents Chemother* **48**(4), 1400-1401.

Wang, M., Tran, J. H., Jacoby, G. A., Zhang, Y., Wang, F. & Hooper, D. C. (2003). Plasmid-mediated quinolone resistance in clinical isolates of Escherichia coli from Shanghai, China. *Antimicrob Agents Chemother* **47**(7), 2242-2248.

Warburg, G., Korem, M., Robicsek, A., Engelstein, D., Moses, A. E., Block, C. & Strahilevitz, J. (2008). Changes in aac(6')-Ib-cr prevalence and fluoroquinolone resistance in nosocomial isolates of E. coli.: 1991-2005. *Antimicrob Agents Chemother*.

Watanabe, T. (1963). Infective heredity of multiple drug resistance in bacteria. *Bacteriol Rev* **27**, 87-115.

Weigel, Linda M., Steward, Christine D. & Tenover, Fred C. (1998). *gyrA* Mutations Associated with Fluoroquinolone Resistance in Eight Species of *Enterobacteriaceae*. *Antimicrobial Agents and Chemotherapy* **42**(10), 2661 - 2667.

Whichard, J. M., Gay, K., Stevenson, J. E., Joyce, K. J., Cooper, K. L., Omondi, M., Medalla, F., Jacoby, G. A. & Barrett, T. J. (2007). Human Salmonella and

concurrent decreased susceptibility to quinolones and extended-spectrum cephalosporins. *Emerg Infect Dis* **13**(11), 1681-1688.

WHO (2002a). Acute respiratory infections (Updated 2002).

WHO (2002b). Prevetion of hospital-acquired infections - A practical guide (2nd edition).

Wolfson, J. S. & Hooper, D. C. (1991). Pharmacokinetics of quinolones: newer aspects. *Eur J Clin Microbiol Infect Dis* **10**(4), 267-274.

Wu, J. J., Ko, W. C., Chiou, C. S., Chen, H. M., Wang, L. R. & Yan, J. J. (2008a). Emergence of Qnr determinants in human Salmonella isolates in Taiwan. *J Antimicrob Chemother* **62**(6), 1269-1272.

Wu, J. J., Ko, W. C., Tsai, S. H. & Yan, J. J. (2007). Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, and QnrS among clinical isolates of Enterobacter cloacae in a Taiwanese hospital. *Antimicrob Agents Chemother* **51**(4), 1223-1227.

Wu, J. J., Ko, W. C., Wu, H. M. & Yan, J. J. (2008b). Prevalence of Qnr determinants among bloodstream isolates of Escherichia coli and Klebsiella pneumoniae in a Taiwanese Hospital, 1999-2005. *J Antimicrob Chemother* **61**(6), 1234-1239.

Xu, X., Wu, S., Ye, X., Liu, Y., Shi, W., Zhang, Y. & Wang, M. (2007). Prevalence and expression of the plasmid-mediated quinolone resistance determinant qnrA1. *Antimicrob Agents Chemother* **51**(11), 4105-4110.

Yamane, K., Wachino, J., Suzuki, S. & Arakawa, Y. (2008). Plasmid-mediated qepA gene among Escherichia coli clinical isolates from Japan. *Antimicrob Agents Chemother* **52**(4), 1564-1566.

Yamane, K., Wachino, J., Suzuki, S., Kimura, K., Shibata, N., Kato, H., Shibayama, K., Konda, T. & Arakawa, Y. (2007). New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an Escherichia coli clinical isolate. *Antimicrob Agents Chemother* **51**(9), 3354-3360.

Yang, H., Chen, H., Yang, Q., Chen, M. & Wang, H. (2008). High prevalence of plasmid-mediated quinolone resistance genes qnr and aac(6')-Ib-cr in clinical isolates of Enterobacteriaceae from nine teaching hospitals in China. *Antimicrob Agents Chemother* **52**(12), 4268-4273.

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., Lee, K. & Walsh, T. R. (2009). Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in

Klebsiella pneumoniae sequence type 14 from India. *Antimicrobial Agents and Chemotherapy* **53**(12), 5046-5054.

Yue, L., Jiang, H. X., Liao, X. P., Liu, J. H., Li, S. J., Chen, X. Y., Chen, C. X., Lu, D. H. & Liu, Y. H. (2008). Prevalence of plasmid-mediated quinolone resistance qnr genes in poultry and swine clinical isolates of Escherichia coli. *Vet Microbiol* **132**(3-4), 414-420.

Zhao, F., Bai, J., Wu, J., Liu, J., Zhou, M., Xia, S., Wang, S., Yao, X., Yi, H., Lin, M., Gao, S., Zhou, T., Xu, Z., Niu, Y. & Bao, Q. (2010). Sequencing and genetic variation of multidrug resistance plasmids in Klebsiella pneumoniae. *PLoS ONE* **5**(4), e10141.

9. Appendices

9.1. Annotation of the 3 plasmids

Table 9.1 Annotation of plasmid pE66An.

Gene name	Position	Function encoded	Amino acid identity, best hit	
			Ref (uniprot data)	Percentage
rop	compl. (652..843)	regulatory replication	Q939J9_KLEPN	100
pE66An_002	862..1149	hypothetical protein	B7UTN0_ECO27	81
pE66An_003	1813..2238	hypothetical protein	C1J8K7_ECOLX	85
repA	compl. (2544..3407)	replication initiation protein, Inc replicon	B5RKS4_KLEP3	99
tapA	compl. (3400..3474)	regulator of RepFIC repA1 expression	Q65AF5_YERPE	76
repA2	compl. (3717..3974)	replication regulatory protein	B2KAG0_YERPB	75
pE66An_007	compl. (4106..4657)	endonuclease	A6TI21_KLEP7	86
pE66An_008	compl. (4761..5069)	hypothetical protein	B5RKS0_KLEP3	99
pE66An_009	compl. (5066..5716)	hypothetical protein	B5RKR9_KLEP3	92
pE66An_010	compl. (5772..6416)	hypothetical protein	B5RKR8_KLEP3	88
pE66An_011	compl. (6466..7068)	hypothetical protein	B3HH93_ECOLX	43
finO	compl. (7229..7822)	fertility inhibition protein	B5RKR5_KLEP3	93
traX	compl. (7894..8619)	conjugal transfer protein	B5RKR4_KLEP3	95
traI	compl. (8699..9406)	conjugal transfer protein, truncated	A6TIJ2_KLEP7	99
tnpA	compl. (9461..10183)	transposase of IS26	B1LRU1_ECOSM	99
pE66An_016	compl. (10510..10785)	hypothetical protein	Q5QJL0_SALTY	99
pE66An_017	compl. (10821..11363)	hypothetical protein	C1KKB9_ENTCL	100
aacC3	compl. (11376..12236)	aminoglycoside N(3')-acetyltransferase III	A7LI78_ECOLX	100
tnpA	12379..12873	transposase of IS10, truncated	B6VP16_PROMI	99
tnpA	compl. (12907..13629)	transposase of IS26	A9QNW0_SALET	99
tnpA	compl. (13734..14456)	transposase of IS26	B1LRU1_ECOSM	99
tnpA	14429..15001	Transposase of Tn1000	B6UYN9_KLEPN	100
fipA	compl. (15032..15376)	fertility inhibition protein	B6UYN8_KLEPN	100
traI	compl. (15376..18612)	conjugal transfer protein	B6UYN7_KLEPN	100
traJ	compl. (18612..20141)	conjugal transfer protein	B6UYN6_KLEPN	100
traK	compl. (20143..20559)	conjugal transfer protein	B6RFQ7_KLEPN	100
stbA	21050..21520	stable plasmid inheritance	B6RFQ8_KLEPN	89
stdB	21529..22245	stable plasmid inheritance	B6RFQ9_KLEPN	100
stbC	22247..22615	stable plasmid inheritance	Q17U05_ECOLX	100
orfD	22797..23141	hypothetical protein	A8R738_SALDU	100
ccgAII	compl. (23252..23572)	CUP-controlled regulon	B6RFR2_KLEPN	100
ccgAI	compl. (23627..23806)	CUP-controlled regulon	A8R740_SALDU	100
ccgC	compl. (24014..24298)	growth arrest of plasmid free cells	B6RFR5_KLEPN	100
pE66An_034	compl. (24367..24708)	hypothetical protein	B6UZ15_KLEPN	99
ccgD	compl. (24723..25019)	hypothetical protein	B6RFR6_KLEPN	100
pE66An_036	compl. (25135..25677)	hypothetical protein	B6UYM7_KLEPN	100
pE66An_037	compl. (25677..25802)	hypothetical protein	B6UYM6_KLEPN	97
ccgEIII	compl. (25905..26144)	CUP-controlled regulon	B6RFR7_KLEPN	100
ardR	compl. (26154..26558)	type I antirestriction system	B6RFR8_KLEPN	100
ardB	compl. (26616..27041)	type I antirestriction system	Q17TZ9_ECOLX	100
mucA	27438..27896	mutagenesis enhancement	B6UYM2_KLEPN	99
mucB	27884..29149	mutagenesis enhancement	B6RFS1_KLEPN	100
mpr	29264..30091	zinc metalloproteinase	Q0ZKP8_ECOLX	99
ardK	compl. (30106..30507)	regulatory repressor	C2IBG0_VIBCH	99
repA	31007..31726	replicase protein, IncN replicon	B6RFL0_KLEPN	100
pE66An_046	compl. (31537..33000)	hypothetical protein	B8GCA4_CHLAD	34
pE66An_047	compl. (31659..33017)	hypothetical protein	B6VBW3_9PELO	33
pE66An_048	31755..32963	hypothetical protein	YPC2_ECOLX	86
pE66An_049	compl. (31769..33208)	hypothetical protein	B8GCA4_CHLAD	32
pE66An_050	31771..33033	hypothetical protein	Q0QCA4_KLEPN	66

pE66An_051	31814..33229	hypothetical protein	Q0QCA4_KLEPN	67
pE66An_052	compl. (33236..33388)	hypothetical protein	B6UYL7_KLEPN	100
pE66An_053	compl. (33385..33954)	hypothetical protein	B6RFL1_KLEPN	100
tnpA	compl. (34145..34294)	transposase of Tn21, truncated	Q2FCX9_ACIBA	98
intI1	compl. (34347..35360)	integrase of class 1 integron	INT2_SALTI	100
dhfrV	35516..35989	dihydrofolate reductase	B1LRU7_ECOSM	100
pE66An_057	compl. (36156..36440)	hypothetical protein	Q935L1_SALTI	100
tnpA	36589..37383	transposase of IS6100	Q2FD04_ACIBA	99
ecoRII	37644..38858	type II restriction enzyme	Q0QC82_KLEPN	100
ecoRIIM	compl. (38892..40325)	type II antirestriction enzyme	Q0ZKQ3_ECOLX	99
tnpA	40430..40696	transposase of IS1	B6UYV0_KLEPN	98
mrr	compl. (40918..41565)	restriction endonuclease	B6UYU8_KLEPN	99
pE66An_063	compl. (41615..41926)	hypothetical protein	B6RFN5_KLEPN	99
kikA	compl. (41962..42276)	killer protein of the TrbM family	B6RFN6_KLEPN	100
pE66An_065	compl. (42273..42617)	hypothetical protein	B6RFN7_KLEPN	100
korB	compl. (42633..42938)	DNA-binding protein	Q0ZKR0_ECOLX	100
traL	43092..43781	conjugal transfer protein	B6RFN9_KLEPN	100
korA	43790..44071	hypothetical protein	Q46695_ECOLX	100
traM	44081..44374	conjugal transfer protein	B6RFP1_KLEPN	100
traA	44424..44741	conjugal transfer protein	B6UYU0_KLEPN	100
traB	44741..47341	conjugal transfer protein	B6RFP2_KLEPN	100
traC	47359..48072	conjugal transfer protein	B6RFP3_KLEPN	100
eex	48080..48307	entry exclusion protein	B6RFP4_KLEPN	100
traD	48323..49363	conjugal transfer protein	B6RFP5_KLEPN	100
traN	49446..49592	conjugal transfer protein	B6UYS6_KLEPN	100
traE	49561..50280	conjugal transfer protein	B6UZ43_KLEPN	99
traO	50291..51175	conjugal transfer protein	B6RFP8_KLEPN	100
traF	51175..52335	conjugal transfer protein	B6RFP9_KLEPN	100
traG	52377..53372	conjugal transfer protein	B6RFQ0_KLEPN	100
nuc	53372..53905	phospholipase D family protein	C2IBT3_VIBCH	99
fipA	compl. (54079..54366)	fertility inhibition protein, truncated	A4IUR0_YERPE	100
pE66An_082	compl. (54401..55795)	hypothetical protein	B6DT21_ENTAE	100
ydaA	compl. (55829..56401)	putative resolvase protein	Q0QCB3_KLEPN	99
pE66An_084	compl. (56538..57128)	hypothetical protein	B6DT23_ENTAE	100
strA	compl. (57185..57430)	aminoglycoside resistance protein A, truncated	B1LRV3_ECOSM	88
sulII	compl. (57491..58306)	dihydropteroate synthase type-2	A6TIZ0_KLEP7	100
pE66An_087	compl. (58315..58497)	hypothetical protein	A0PAW9_PASPI	96
pE66An_088	compl. (58494..58637)	hypothetical protein	B6UYP4_KLEPN	100
tnpA	compl. (59149..59871)	transposase of IS26	B1LRU1_ECOSM	99
tetR	compl. (59865..60521)	tetracycline repressor	A0PAU7_PASPI	100
tetA	60617..61801	tetracycline repressor	Q84BR9_9ENTR	100
frmA	61896..63005	alcohol dehydrogenase	Q0QCD7_KLEPN	99
esd	63027..63461	S-formylglutathione hydrolase	A0PAV0_PASPI	100
tnpA	compl. (63495..64217)	transposase of IS26	B1LRU1_ECOSM	99
pE66An_095	64808..65398	hypothetical protein	B6DT23_ENTAE	100
ydaA	65535..66107	putative resolvase protein	Q0QCB3_KLEPN	99
pE66An_097	66141..67535	hypothetical protein	B6DT21_ENTAE	100
pE66An_098	67559..67996	hypothetical protein	B6DT20_ENTAE	100
pE66An_099	compl. (67895..68302)	hypothetical protein	Q0QC56_KLEPN	99
qnrS1	compl. (68315..68971)	quinolone resistance protein	Q17ZR5_SALIN	100
tnpA	69692..69805	transposase of IS2, truncated	A7WP56_SALTY	100
pE66An_102	69863..70519	hypothetical protein	Q0QC58_KLEPN	100
bla_{LAP-2}	compl. (70568..71425)	beta-lactamase	A8IDJ3_ENTCL	100
pE66An_104	compl. (71490..72866)	hypothetical protein	Q0QC60_KLEPN	99
tnpA	compl. (72914..73636)	transposase of IS26	B1LRU1_ECOSM	99
tetR	73677..73916	tetracycline repressor, truncated	TETR4_ECOLX	100
pE66An_107	compl. (74713..75663)	hypothetical protein	B6RFN1_KLEPN	100
tnpA	compl. (75867..76790)	transposase of IS903D	A4IUH9_YERRU	99

bla$_{CTX-M-14}$	compl. (76870..77745)	beta-lactamase CTX-M-14	Q2MJS3_PROST	100
tnpA	compl. (77995..79257)	transposase of ISEcp1	Q7WWK7_SALET	100

Table 9.2 Annotation of plasmid pK18An.

Gene name	Position	Function encoded	Amino acid identity, best hit	
			Ref (uniprot data)	Percentage
repA	355..1074	replication initiation protein, Inc replicon	B6RFL0_KLEPN	100
pK18An_002	compl. (888..2348)	hypothetical protein	Q0QCA4_KLEPN	66
pK18An_003	compl. (1010..2365)	hypothetical protein	Q0QCA4_KLEPN	67
pK18An_004	1103..2308	hypothetical protein	B8GCA4_CHLAD	32
pK18An_005	compl. (1117..2373)	hypothetical protein	YPC2_ECOLX	86
pK18An_006	1119..2378	hypothetical protein	B8GCA4_CHLAD	34
pK18An_007	1162..2574	hypothetical protein	B6VBW3_9PELO	33
tnpA	2942..3781	transposase of IS26	Q51938_ECOLX	99
ecoRIIM	compl. (3818..5248)	type II antirestriction enzyme, truncated	Q0ZKQ3_ECOLX	100
pK18An_010	compl. (5630..5836)	hypothetical protein	B6RFM5_KLEPN	100
mrr	compl. (5841..6488)	restriction endonuclease	B6UYU8_KLEPN	99
pK18An_012	compl. (6538..6849)	hypothetical protein	B6RFN5_KLEPN	99
kikA	compl. (6885..7199)	killer protein of the TrbM family	B6RFN6_KLEPN	100
pK18An_014	compl. (7196..7540)	hypothetical protein	B6RFN7_KLEPN	100
korB	compl. (7556..7861)	DNA-binding protein	Q0ZKR0_ECOLX	100
traL	7970..8704	conjugal transfer protein	B6RFN9_KLEPN	100
korA	8713..8994	hypothetical protein	B6RFP0_KLEPN	100
traM	9004..9297	conjugal transfer protein	B6RFP1_KLEPN	100
traA	9347..9664	conjugal transfer protein	Q0ZKR4_ECOLX	100
traB	9664..12264	conjugal transfer protein	B6RFP2_KLEPN	100
traC	12282..12926	conjugal transfer protein, truncated	B6RFP3_KLEPN	100
tnpA	12978..13694	transposase of IS26	B2YFH3_9ENTR	100
tnpA	13817..14521	transposase of IS26	C0JBB4_ACIBA	100
tnpA	compl. (14555..15475)	transposase of IS10	Q0QC68_KLEPN	100
env	compl. (15415..15579)	envelope glycoprotein gp160, truncated	Q6QLJ1_9HIV1	100
aacC3	15618..16478	aminoglycoside N(3')-acetyltransferase III	Q0QC65_KLEPN	100
pK18An_027	16491..17033	hypothetical protein	Q0QC64_KLEPN	100
pK18An_028	17069..17347	hypothetical protein	Q0QC63_KLEPN	100
tnpA	17671..18390	transposase of IS26	A9QNU3_SALET	99
pK18An_030	18905..19048	hypothetical protein	B6UYP4_KLEPN	100
pK18An_031	19045..19227	hypothetical protein	A0PAW9_PASPI	100
sulII	19236..20051	dihydropteroate synthase type-2	DHP2_SHIFL	100
strA	20157..20915	aminoglycoside resistance protein A	B5TTG3_9BACT	100
strB	20915..21751	streptomycin resistance protein	Q7WZH0_VIBCH	100
pK18An_035	compl. (21723..22262)	hypothetical protein	Q8VVM8_VIBCH	100
pK18An_036	22418..23008	hypothetical protein	B6DT23_ENTAE	100
ydaA	23145..23717	putative resolvase protein	Q0QCB3_KLEPN	99
pK18An_038	23751..25145	hypothetical protein	Q0QC54_KLEPN	100
pK18An_039	25169..25606	hypothetical protein	Q0QC55_KLEPN	100
qnrS1	compl. (25925..26581)	quinolone resistance protein	Q5H7L5_SHIFL	100
tnpA	27302..27415	transposase of IS2, truncated	A7WP56_SALTY	100
pK18An_042	27473..28129	hypothetical protein	Q0QC58_KLEPN	100
bla_{LAP-2}	compl. (28178..30235)	beta-lactamase, inserted	B6DT17_ENTAE	100
tnpA	28873..29853	transposase of IS5	A1KR19_PROMI	99
pK18An_045	compl. (30300..31676)	putative peptidoglycan synthetase	Q0QC60_KLEPN	99
tnpA	compl. (31727..32446)	transposase of IS26	A9QNU3_SALET	99
pK18An_047	compl. (32520..33089)	hypothetical protein	B6RFL1_KLEPN	100
tnpA	compl. (33280..33429)	transposase of Tn21, truncated	B7NGL3_ECOLU	100
intI1	compl. (33482..34495)	integrase of class 1 integron	Q701N0_SALCH	100
dhfrV	34666..35142	dihydrofolate reductase type 5	Q573P5_9BACT	100
tnpA	35265..35891	transposase of IS26	Q52625_PROVU	99
tnpA	35783..36355	transposase of Tn1000	B6UYN9_KLEPN	100
fipA	compl. (36386..36730)	fertility inhibition protein, truncated	B6UYN8_KLEPN	100
traI	compl. (36730..39966)	conjugal transfer protein	B6UYN7_KLEPN	100
traJ	compl. (39966..41495)	conjugal transfer protein	A8R733_SALDU	100
traK	compl. (41500..41913)	conjugal transfer protein	B6RFQ7_KLEPN	100
stbA	42404..42874	stable plasmid inheritance, inserted	B6RFQ8_KLEPN	89
stdB	42883..43599	stable plasmid inheritance	B6RFQ9_KLEPN	100

stbC	43601..43969	stable plasmid inheritance	B6RFR0_KLEPN	100
orfD	44151..44495	hypothetical protein	A8R738_SALDU	100
ccgAII	compl. (44606..44926)	CUP-controlled regulon	B6RFR2_KLEPN	100
ccgAI	compl. (44981..45160)	CUP-controlled regulon	Q79SD0_9ZZZZ	100
pK18An_063	compl. (45333..45599)	hypothetical protein	YPC2_ECOLX	81
pK18An_064	compl. (45640..46185)	hypothetical protein	B6UYM7_KLEPN	100
pK18An_065	compl. (46185..46310)	hypothetical protein	B6UYM6_KLEPN	97
ccgEIII	compl. (46413..46652)	CUP-controlled regulon	Q0ZKP3_ECOLX	100
ardR	compl. (46662..47066)	type I antirestriction system	B6RFR8_KLEPN	100
ardB	compl. (47127..47549)	type I antirestriction system	B6RFR9_KLEPN	100
mucA	47964..48404	mutagenesis enhancement	MUCA_SALTY	100
mucB	48392..49657	mutagenesis enhancement	B6RFS1_KLEPN	100
mpr	49772..50599	zinc metalloproteinase	Q0ZKP8_ECOLX	99
ardK	compl. (50614..50955)	regulatory repressor	B6RFS3_KLEPN	100

Table 9.3 Annotation of plasmid pK1HV.

Gene name	Position	Function encoded	Amino acid identity, best hit	
			Ref (uniprot data)	Percentage
repA	382..1275	replication initiation protein, Inc replicon	Q0QCA8_KLEPN	100
pK1HV_002	1940..2566	hypothetical protein	C1J8J6_ECOLX	97
pK1HV_003	2563..2865	hypothetical protein	Q3ZU34_ECOLX	83
resD	compl. (3316..4053)	plasmid resolvase	A6TJ26_KLEP7	96
pK1HV_005	compl. (4305..5615)	hypothetical protein	B5RKP5_KLEP3	65
pK1HV_006	compl. (5673..6146)	hypothetical protein	A6TIN4_KLEP7	87
ccdB	compl. (6237..6542)	cytotoxic protein	B5RKP4_KLEP3	99
ccdA	compl. (6544..6762)	antitoxin to CcdB	B5RKP3_KLEP3	95
pK1HV_009	compl. (6932..7354)	hypothetical protein	Q84A09_ECOLX	44
pK1HV_010	compl. (7580..7870)	hypothetical protein	B6IBZ7_ECOSE	78
pK1HV_011	compl. (7867..8994)	hypothetical protein	B6IBZ6_ECOSE	89
pK1HV_012	compl. (9028..10638)	hypothetical protein	B6IBZ5_ECOSE	91
pK1HV_013	compl. (10827..11606)	hypothetical protein	B6IBZ4_ECOSE	89
pK1HV_014	compl. (11619..12119)	hypothetical protein	Q5QJN8_SALTY	93
pK1HV_015	12394..12657	hypothetical protein	B6IC99_ECOSE	80
pK1HV_016	12663..13220	hypothetical protein	A6TI74_KLEP7	97
pK1HV_017	13251..13745	hypothetical protein	Q6U5Q6_KLEPN	97
pK1HV_018	13786..14157	hypothetical protein	B5RK66_KLEP3	88
ymoA	14191..14394	haemolysin expression modulating	B5RK65_KLEP3	86
pK1HV_020	14443..14700	hypothetical protein	A6TI71_KLEP7	96
pK1HV_021	14776..15030	hypothetical protein	A6TI70_KLEP7	89
pK1HV_022	compl. (15166..15945)	hypothetical protein	Q4ZSK9_PSEU2	25
pK1HV_023	16388..16603	hypothetical protein	C4DZ57_9FUSO	35
orf2	16877..16966	hypothetical protein, truncated	Q9ZEW3_KLEPN	95
pK1HV_025	16988..17257	hypothetical protein	B0ZDW7_ECOLX	74
pK1HV_026	17525..17752	hypothetical protein	Q07U38_RHOP5	58
pK1HV_027	17745..18926	hypothetical protein	Q6EVS8_YERPS	64
pK1HV_028	compl. (19434..19607)	hypothetical protein	Q8KQG1_ECOLX	41
repA	compl. (20118..20975)	replication initiation protein, Inc replicon	B5RKS4_KLEP3	99
tapA	compl. (20968..21045)	regulator of RepFIC repA1 expression	Q65AF5_YERPE	80
tnpA	21338..22060	transposase of IS26	B1LRU1_ECOSM	99
tnpA	compl. (22333..22596)	transposase of IS21, truncated	Q2FCX9_ACIBA	98
intI1	compl. (22535..23548)	integrase of class 1 integron	B0VCG5_ACIBY	100
dhfrXII	23693..24190	dihydrofolate reductase type XII	Q19NZ8_AERHY	100
orfF	24302..24592	hypothetical protein	A1YJJ6_SALTY	100
aadA2	24598..25389	aminoglycoside 3'-adenyltransferase	Q5UEP7_KLEPN	100
pK1HV_037	compl. (25951..27150)	hypothetical protein	Q83XH8_VIBAN	55
nimC/nimA	27317..27709	hypothetical protein	B4TM09_SALSV	99
ble	compl. (28029..28403)	bleomycin resistance	BLE_STAAR	99
tnpA	compl. (28443..29165)	transposase of IS26	A9QNW0_SALET	99
aphA1	29298..30113	aminoglycoside 3'-phosphotransferase	A8R6K9_SALET	100
tnpA	compl. (30303..31025)	transposase of IS26	B1LRU1_ECOSM	99
aac(3)-IV	31121..31897	aminoglycoside acetyltransferase	Q306W4_ECOLX	99
hph	32126..33151	hygromycin-B 4-O-kinase	KHYB_ECOLX	100
pK1HV_045	33226..33807	hypothetical protein	Q9RWR7_DEIRA	65
pK1HV_046	compl. (33573..34325)	hypothetical protein	B6D4X1_ECOLX	100
tnpA	34270..35910	transposase of Tn3	B4TM31_SALSV	99
pK1HV_048	36136..36621	hypothetical protein	B5TTN3_9BACT	100
tnpA	compl. (36818..37264)	transposase of IS4	B2Z3W5_ACIBA	99
tnpA	compl. (37339..37908)	transposase of IS4	B7I4U9_ACIB5	100
sulII	37998..38813	dihydropteroate synthase type-2	B3H353_ACTP7	97
glmM	38900..39199	phosphoglucosamine mutase	C1IV02_ENTCL	100
pK1HV_053	compl. (39378..40871)	putative transposase	Q7DFW6_VIBCH	99
lysR	compl. (40983..41288)	transcriptional regulator	B6ET83_PASMU	100
floR	compl. (41316..42530)	florfenicol export	B7U6G1_EDWIC	100
pK1HV_056	compl. (42747..43631)	hypothetical protein	Q8VVN1_VIBCH	100
pK1HV_057	compl. (43662..44498)	hypothetical protein	B5TTT3_9BACT	99
pK1HV_058	44547..45260	putative transposase	B5TTT2_9BACT	99

tnpA	compl. (45755..48706)	transposase of Tn21	B4F4G5_SALTY	99
tnpR	compl. (48709..49269)	recombinase resolvase	Q7BGA0_SHIFL	100
orfI	49529..51106	hypothetical protein	Q56321_ECOLX	100
pK1HV_062	51310..51789	phospholipase D protein	B5RKS1_KLEP3	93
repA2	51925..52275	replication regulatory protein	B5RKS2_KLEP3	96
tnpA	compl. (52221..52943)	transposase of IS26	B1LRU1_ECOSM	99
tetR	compl. (53576..54226)	tetracycline repressor	Q5W3B9_9BACT	100
tetA	54257..55531	tetracycline repressor	A9QNT5_SALET	100
pecM	compl. (55563..56447)	regulator protein	Q7X3B3_9BACT	100
tnpA	56912..58732	transposase of Tn1721	B5PQE9_SALHA	99
ftsI	58817..59842	peptidoglycan glycosyltransferase, truncated	Q0QC60_KLEPN	100
bla_{LAP-2}	59907..60764	beta-lactamase	A8IDJ3_ENTCL	100
tnpA	compl. (60813..61640)	transposase of IS2, truncated	B5PMB7_SALET	86
qnrS1	62361..63017	quinolone resistance protein	Q17ZR5_SALIN	100
pK1HV_073	compl. (63336..63773)	hypothetical protein	B6DT20_ENTAE	100
pK1HV_074	compl. (63797..65191)	hypothetical protein	B6DT21_ENTAE	100
ydaA	compl. (65225..65797)	putative resolvase protein	Q0QCB3_KLEPN	99
pK1HV_076	compl. (65934..66524)	hypothetical protein	B6DT23_ENTAE	100
pK1HV_077	66668..67150	hypothetical protein	A7FCE2_YERP3	70
hipB2	compl. (67297..67578)	predicted transcriptional regulator	Q74YR9_YERPE	100
pK1HV_079	compl. (67559..67888)	putative prophage protein gp49	B2CBG6_KLEPN	100
pK1HV_080	68103..68765	hypothetical protein	B1J8Z5_PSEPW	43
pK1HV_081	compl. (68905..69189)	hypothetical protein	B5RKL3_KLEP3	84
pK1HV_082	compl. (69261..69482)	hypothetical protein	B7T4W9_KLEPN	91
tnpA	compl. (69488..70210)	transposase of IS26	A9QNW0_SALET	99
pK1HV_084	compl. (70507..71109)	hypothetical protein	B3HH93_ECOLX	42
finO	compl. (71270..71863)	fertility inhibition protein	B5RKR5_KLEP3	92
traX	compl. (71935..72660)	conjugal transfer protein	A6TIJ3_KLEP7	97
traI	compl. (72741..78002)	conjugal transfer protein	A6TIJ2_KLEP7	98
traD	compl. (78002..80314)	conjugal transfer protein	A6TIJ1_KLEP7	97
pK1HV_089	compl. (80441..81130)	hypothetical protein	A6TIJ0_KLEP7	96
traT	compl. (81323..82093)	conjugal transfer protein	A6TII9_KLEP7	96
pK1HV_091	compl. (82240..82779)	hypothetical protein	Q7B3V4_ECOLX	31
traG	compl. (82776..85625)	conjugal transfer protein	A6TII7_KLEP7	84
traH	compl. (85625..87034)	conjugal transfer protein	A6TIV9_KLEP7	97
pK1HV_094	compl. (86982..87425)	hypothetical protein	A6TII5_KLEP7	98
trbB	compl. (87471..88058)	inner membrane protein	A6TII4_KLEP7	100
traF	compl. (88250..89002)	conjugal transfer protein	A6TII2_KLEP7	100
pK1HV_097	compl. (89023..89349)	hypothetical protein	A6TII1_KLEP7	100
pK1HV_098	compl. (89362..89610)	hypothetical protein	A6TII0_KLEP7	100
traN	compl. (89874..91829)	conjugal transfer protein	A6TIH8_KLEP7	99
trbC	compl. (91888..92526)	F pilus assembly protein	A6TIV0_KLEP7	100
traU	compl. (92539..93528)	conjugal transfer protein	A6TIU9_KLEP7	99
pK1HV_102	compl. (93525..93959)	hypothetical protein	A6TIU8_KLEP7	92
traW	compl. (93956..94582)	conjugal transfer protein	A6TIH5_KLEP7	99
trbI	compl. (94585..94974)	inner membrane protein	A6TIU6_KLEP7	97
traC	compl. (94971..97610)	conjugal transfer protein	A6TIU5_KLEP7	99
pK1HV_106	compl. (97682..98080)	hypothetical protein	A6TIU4_KLEP7	94
pK1HV_107	compl. (98231..98434)	hypothetical protein	A0LYL1_GRAFK	29
pK1HV_108	compl. (98456..98890)	hypothetical protein	A6TIU2_KLEP7	98
pK1HV_109	compl. (98927..99238)	hypothetical protein	A6TIH0_KLEP7	91
pK1HV_110	compl. (99239..99457)	hypothetical protein	A6TIU0_KLEP7	95
pK1HV_111	compl. (99563..99913)	hypothetical protein	A6TIG7_KLEP7	85
traV	compl. (100105..100689)	conjugal transfer protein	A6TIT8_KLEP7	99
traB	compl. (100803..102227)	conjugal transfer protein	A6TIG4_KLEP7	99
traK	compl. (102227..102967)	conjugal transfer protein	A6TIT6_KLEP7	99
traE	compl. (102954..103520)	conjugal transfer protein	A6TIT5_KLEP7	100
traL	compl. (103540..103845)	conjugal transfer protein	A6TIT4_KLEP7	100
traA	compl. (103859..104227)	conjugal transfer protein	A6TIT3_KLEP7	98
pK1HV_118	compl. (104296..104496)	hypothetical protein	Q0H074_ECOLX	52
traJ	compl. (104582..105283)	putative conjugal transfer protein	Q9X9C5_SALTY	32
traM	compl. (105513..105905)	putative conjugal transfer protein	A6TIT0_KLEP7	67
pK1HV_121	106337..106822	hypothetical protein	A6TIS9_KLEP7	100

pK1HV_122	compl. (106858..107184)	hypothetical protein	A6TIF5_KLEP7	100
pK1HV_123	compl. (107217..108053)	hypothetical protein	A6TIS7_KLEP7	98
pK1HV_124	108244..108525	hypothetical protein	A7ZH46_ECO24	45
tnpA	108846..110054	transposase of IS1414	Q1XCE1_EDWIC	93
pK1HV_126	compl. (110193..110603)	hypothetical protein	A6TIF3_KLEP7	93
pK1HV_127	compl. (110604..110882)	hypothetical protein	A6TIF2_KLEP7	100
pK1HV_128	compl. (110872..111192)	hypothetical protein	A6TIF1_KLEP7	100
pK1HV_129	compl. (111273..111497)	hypothetical protein	A6TIF0_KLEP7	100
pK1HV_130	compl. (111508..111720)	hypothetical protein	A6TIE9_KLEP7	97
pK1HV_131	compl. (111781..112137)	hypothetical protein	A6TIE8_KLEP7	99
pK1HV_132	compl. (112134..112259)	hypothetical protein	A6TIS7_KLEP7	79
pK1HV_133	compl. (112979..113368)	hypothetical protein	A7MNN4_ENTS8	47
pK1HV_134	compl. (113311..113580)	hypothetical protein	A6TIE7_KLEP7	95
pK1HV_135	compl. (113658..113951)	hypothetical protein	Q1XGH5_PSEPU	29
pK1HV_136	compl. (113978..114313)	hypothetical protein	A6TIS2_KLEP7	77
pK1HV_137	compl. (114390..114554)	hypothetical protein	B8HAJ2_ARTCA	47
pK1HV_138	compl. (114687..114839)	hypothetical protein	Q9ZGR6_ECO57	56
pK1HV_139	compl. (115009..115410)	hypothetical protein	A6TIE5_KLEP7	98
pK1HV_140	compl. (115439..115765)	hypothetical protein	A6TIE4_KLEP7	98
pK1HV_141	compl. (115762..116490)	putative plasmid SOS inhibition	A6TIE3_KLEP7	98
pK1HV_142	compl. (116487..116918)	putative plasmid SOS inhibition protein B	A6TIE2_KLEP7	98
pK1HV_143	compl. (116963..119020)	hypothetical protein	A6TIE1_KLEP7	98
pK1HV_144	compl. (119090..119320)	hypothetical protein	A6TIE0_KLEP7	97
ssb	compl. (119387..119929)	single-stranded DNA-binding	A6TID9_KLEP7	97
pK1HV_146	compl. (120759..121322)	hypothetical protein	A6TIR5_KLEP7	100
pK1HV_148	compl. (121370..122725)	hypothetical protein	A6TIX2_KLEP7	98
pK1HV_149	compl. (122777..123007)	hypothetical protein	A6TIR3_KLEP7	100
pK1HV_150	compl. (123099..123326)	hypothetical protein	A6TIX3_KLEP7	100
pK1HV_151	123439..123741	hypothetical protein	A7ZH46_ECO24	45
pK1HV_152	compl. (124006..124326)	hypothetical protein	A6TIX4_KLEP7	100
pK1HV_153	compl. (124361..124615)	hypothetical protein	A6TIR0_KLEP7	100
pK1HV_154	compl. (125037..125543)	hypothetical protein	A6TIX7_KLEP7	99
klcA	compl. (125586..126251)	hypothetical protein	A6TIQ7_KLEP7	98
pK1HV_156	compl. (126266..126661)	hypothetical protein	B5GAS7_9ACTO	32
pK1HV_157	compl. (126695..127519)	hypothetical protein	A6TIQ6_KLEP7	99
pK1HV_158	compl. (127519..127935)	hypothetical protein	A6TIQ5_KLEP7	100
pK1HV_159	compl. (127948..128166)	hypothetical protein	A6TIQ4_KLEP7	100
pK1HV_160	compl. (128166..128942)	hypothetical protein	A6TIQ3_KLEP7	99
pK1HV_161	compl. (128939..129130)	hypothetical protein	C1PMS3_9ACTN	37
pK1HV_162	compl. (129304..129534)	hypothetical protein	A6TIQ2_KLEP7	100
stbB	compl. (129598..130269)	stable plasmid inheritance	A6TIQ1_KLEP7	98
stbA	compl. (130272..131246)	stable plasmid inheritance	A6TJ34_KLEP7	99
impA	131477..131908	error-prone repair protein	B7L3X0_ESCF3	99
umuC	131908..133179	error-prone repair protein	Q0QCC9_KLEPN	97

9.2. List of strains used in this study

Table 9.4 List of strains used in this study.

Nr	Strain ID	Bacterial species	Year	Samples	Source	RAPD
1	K18An	*K. pneumoniae*	2004	Anus	Hospital	K1-1
2	K21Sp	*K. pneumoniae*	2004	Sputum	Hospital	K1-2
3	K23N	*K. pneumoniae*	2004	Nose	Hospital	K1-4
4	K31N	*K. pneumoniae*	2004	Nose	Hospital	K1-5
5	K34N	*K. pneumoniae*	2004	Nose	Hospital	K1-6
6	K35N	*K. pneumoniae*	2004	Nose	Hospital	K1-3
7	K38An	*K. pneumoniae*	2004	Anus	Hospital	K1-7
8	K39N	*K. pneumoniae*	2004	Nose	Hospital	K1-8
9	K62N	*K. pneumoniae*	2004	Nose	Hospital	K1-9
10	K65An	*K. pneumoniae*	2004	Anus	Hospital	K1-10
11	K66Sp	*K. pneumoniae*	2004	Sputum	Hospital	K1-11
12	K67An	*K. pneumoniae*	2004	Anus	Hospital	K1-12
13	K71N	*K. pneumoniae*	2004	Nose	Hospital	K1-13
14	K73Ax	*K. pneumoniae*	2004	Axilla	Hospital	K1-14
15	K75Ax	*K. pneumoniae*	2004	Axilla	Hospital	K1-16
16	K79N	*K. pneumoniae*	2004	Nose	Hospital	K1-17
17	K84N	*K. pneumoniae*	2004	Nose	Hospital	K1-18a
18	K86N	*K. pneumoniae*	2004	Nose	Hospital	K1-18b
19	K99Sp	*K. pneumoniae*	2004	Sputum	Hospital	K1-19
20	K101Sp	*K. pneumoniae*	2004	Sputum	Hospital	K1-20
21	K102An	*K. pneumoniae*	2004	Anus	Hospital	K1-24
22	K107N	*K. pneumoniae*	2004	Nose	Hospital	K1-21
23	K109Ax	*K. pneumoniae*	2004	Axilla	Hospital	K1-22
24	K112An	*K. pneumoniae*	2004	Anus	Hospital	K1-23
25	K218Ca	*K. pneumoniae*	2005	Canula	Hospital	K2-1
26	K219An	*K. pneumoniae*	2005	Anus	Hospital	K2-32
27	K222N	*K. pneumoniae*	2005	Nose	Hospital	K2-3a
28	K222Ca	*K. pneumoniae*	2005	Canula	Hospital	K2-3b
29	K223N	*K. pneumoniae*	2005	Nose	Hospital	K2-6
30	K226An	*K. pneumoniae*	2005	Anus	Hospital	K2-33
31	K228An	*K. pneumoniae*	2005	Anus	Hospital	K2-21
32	K228Ca	*K. pneumoniae*	2005	Canula	Hospital	K2-2
33	K231An	*K. pneumoniae*	2005	Anus	Hospital	K2-10
34	K232An	*K. pneumoniae*	2005	Anus	Hospital	K2-37
35	K233Ca	*K. pneumoniae*	2005	Canula	Hospital	K2-5
36	K235Ax	*K. pneumoniae*	2005	Axilla	Hospital	K2-31
37	K242Ax	*K. pneumoniae*	2005	Axilla	Hospital	K2-13
38	K251An	*K. pneumoniae*	2005	Anus	Hospital	K2-35
39	K255An	*K. pneumoniae*	2005	Anus	Hospital	K2-12
40	K259An	*K. pneumoniae*	2005	Anus	Hospital	K2-11
41	K261An	*K. pneumoniae*	2005	Anus	Hospital	K2-19
42	K262N	*K. pneumoniae*	2005	Nose	Hospital	K2-14
43	K263Ax	*K. pneumoniae*	2005	Axilla	Hospital	K2-30
44	K265Ax	*K. pneumoniae*	2005	Axilla	Hospital	K2-36
45	K266Ax	*K. pneumoniae*	2005	Axilla	Hospital	K2-29a
46	K268An	*K. pneumoniae*	2005	Anus	Hospital	K2-7
47	K269N	*K. pneumoniae*	2005	Nose	Hospital	K2-20
48	K277N	*K. pneumoniae*	2005	Nose	Hospital	K2-27
49	K279N	*K. pneumoniae*	2005	Nose	Hospital	K2-29b
50	K280N	*K. pneumoniae*	2005	Nose	Hospital	K2-23
51	K281An	*K. pneumoniae*	2005	Anus	Hospital	K2-38
52	K282Ax	*K. pneumoniae*	2005	Axilla	Hospital	K2-34
53	K283An	*K. pneumoniae*	2005	Anus	Hospital	K2-15
54	K286N	*K. pneumoniae*	2005	Nose	Hospital	K2-24a
55	K287N	*K. pneumoniae*	2005	Nose	Hospital	K2-17
56	K287Ca	*K. pneumoniae*	2005	Canula	Hospital	K2-39

57	K288An	*K. pneumoniae*	2005	Anus	Hospital	K2-24b
58	K289Ax	*K. pneumoniae*	2005	Axilla	Hospital	K2-28
59	K289An	*K. pneumoniae*	2005	Anus	Hospital	K2-18
60	K289Ca	*K. pneumoniae*	2005	Canula	Hospital	K2-26
61	K296N	*K. pneumoniae*	2005	Nose	Hospital	K2-8
62	K300N	*K. pneumoniae*	2005	Nose	Hospital	K2-4
63	K301An	*K. pneumoniae*	2005	Anus	Hospital	K2-25
64	K303Ax	*K. pneumoniae*	2005	Axilla	Hospital	K2-16
65	K306N	*K. pneumoniae*	2005	Nose	Hospital	K2-22
66	K307An	*K. pneumoniae*	2005	Anus	Hospital	K2-9
67	E18An	*E. coli*	2004	Anus	Hospital	E1-14
68	E20An	*E. coli*	2004	Anus	Hospital	E1-8
69	E23An	*E. coli*	2004	Anus	Hospital	E1-28
70	E27An	*E. coli*	2004	Anus	Hospital	E1-9
71	E29An	*E. coli*	2004	Anus	Hospital	E1-10
72	E32An	*E. coli*	2004	Anus	Hospital	E1-13
73	E34An	*E. coli*	2004	Anus	Hospital	E1-22
74	E35An	*E. coli*	2004	Anus	Hospital	E1-1
75	E38An	*E. coli*	2004	Anus	Hospital	E1-4
76	E44An	*E. coli*	2004	Anus	Hospital	E1-11
77	E45An	*E. coli*	2004	Anus	Hospital	E1-2
78	E47An	*E. coli*	2004	Anus	Hospital	E1-25
79	E48An	*E. coli*	2004	Anus	Hospital	E1-24
80	E53An	*E. coli*	2004	Anus	Hospital	E1-17
81	E58An	*E. coli*	2004	Anus	Hospital	E1-29
82	E63An	*E. coli*	2004	Anus	Hospital	E1-5
83	E64An	*E. coli*	2004	Anus	Hospital	E1-20
84	E65An	*E. coli*	2004	Anus	Hospital	E1-26
85	E66An	*E. coli*	2004	Anus	Hospital	E1-21
86	E71An	*E. coli*	2004	Anus	Hospital	E1-15
87	E74An	*E. coli*	2004	Anus	Hospital	E1-12
88	E75An	*E. coli*	2004	Anus	Hospital	E1-16
89	E78An	*E. coli*	2004	Anus	Hospital	E1-3
90	E79An	*E. coli*	2004	Anus	Hospital	E1-27
91	E80An	*E. coli*	2004	Anus	Hospital	E1-23
92	E84An	*E. coli*	2004	Anus	Hospital	E1-6
93	E87An	*E. coli*	2004	Anus	Hospital	E1-31
94	E93An	*E. coli*	2004	Anus	Hospital	E1-18
95	E95An	*E. coli*	2004	Anus	Hospital	E1-19
96	E107An	*E. coli*	2004	Anus	Hospital	E1-7
97	E210An	*E. coli*	2005	Anus	Hospital	E2-16a
98	E212An	*E. coli*	2005	Anus	Hospital	E2-10
99	E214An1	*E. coli*	2005	Anus	Hospital	E2-9
100	E215An	*E. coli*	2005	Anus	Hospital	E2-13
101	E216An	*E. coli*	2005	Anus	Hospital	E2-11
102	E222An	*E. coli*	2005	Anus	Hospital	E2-5
103	E223An	*E. coli*	2005	Anus	Hospital	E2-4
104	E228An	*E. coli*	2005	Anus	Hospital	E2-16b
105	E230An	*E. coli*	2005	Anus	Hospital	E2-18a
106	E233An	*E. coli*	2005	Anus	Hospital	E2-1
107	E234An	*E. coli*	2005	Anus	Hospital	E2-19
108	E237An	*E. coli*	2005	Anus	Hospital	E2-18b
109	E239An	*E. coli*	2005	Anus	Hospital	E2-2
110	E242An	*E. coli*	2005	Anus	Hospital	E2-17
111	E252An	*E. coli*	2005	Anus	Hospital	E2-3
112	E255An	*E. coli*	2005	Anus	Hospital	E2-7
113	E265An	*E. coli*	2005	Anus	Hospital	E2-12
114	E268An	*E. coli*	2005	Anus	Hospital	E2-20
115	E276An	*E. coli*	2005	Anus	Hospital	E2-8
116	E289N	*E. coli*	2005	Nose	Hospital	E2-14
117	E290An	*E. coli*	2005	Anus	Hospital	E2-15
118	E300An1	*E. coli*	2005	Anus	Hospital	E2-6
119	E300An2	*E. coli*	2005	Anus	Hospital	E2-21

120	E302An	*E. coli*	2005	Anus	Hospital	E2-23
121	E304An	*E. coli*	2005	Anus	Hospital	E2-22
122	CV28An	*Pantoea* spp.	2004	Anus	Hospital	I-P.s.1
123	CV37An	*Citrobacter* spp.	2004	Anus	Hospital	I-C.s.1
124	CV39An	*Citrobacter youngae*	2004	Anus	Hospital	I-C.y.1
125	CV62N	*Citrobacter freundii*	2004	Nose	Hospital	I-C f.1
126	CV67An	*Citrobacter youngae*	2004	Anus	Hospital	I-C.y.2
127	CV77N	*Vibrio fluvialis*	2004	Nose	Hospital	I-V.f.1
128	CV89An	*Klebsiella orrithinolytica*	2004	Anus	Hospital	I-K.o.1
129	CV109N	*Citrobacter freundii*	2004	Nose	Hospital	I-C f.2
130	CV209Ax	*Proteus mirabilis*	2005	Axilla	Hospital	II-P m.1a
131	CV217Ax	*Enterobacter cloacae*	2005	Axilla	Hospital	II-E.c.1
132	CV218An	*Citrobacter youngae*	2005	Anus	Hospital	II-C.y.1
133	CV227An	*Citrobacter koseri*	2005	Anus	Hospital	II-C k.2
134	CV240Ax	*Enterobacter cloacae*	2005	Axilla	Hospital	II-E.c.2
135	CV245Ax	*Enterobacter cloacae*	2005	Axilla	Hospital	II-E.c.3
136	CV248Ax	*Citrobacter freundii*	2005	Axilla	Hospital	II-C f.1
137	CV264Ax	*Proteus mirabilis*	2005	Axilla	Hospital	II-P m.2
138	CV269Ax	*Proteus mirabilis*	2005	Axilla	Hospital	II-P m.1b
139	CV279An	*Proteus mirabilis*	2005	Anus	Hospital	II-P m.1c
140	A-001-I-a-1	*E. coli*	2005	Stool	Community	E15
141	A-002-I-a-1	*E. coli*	2005	Stool	Community	
142	A-002-I-a-2	*E. coli*	2005	Stool	Community	
143	A-003-I-a-2	*E. coli*	2005	Stool	Community	
144	A-003-I-b-1	*E. coli*	2005	Stool	Community	
145	A-003-I-b-2	*E. coli*	2005	Stool	Community	
146	A-004-I-a-1	*E. coli*	2005	Stool	Community	
147	A-004-I-b-1	*E. coli*	2005	Stool	Community	E34
148	A-005-I-a-1	*E. coli*	2005	Stool	Community	E13
149	A-006-I-a-2	*E. coli*	2005	Stool	Community	
150	A-007-I-a-1	*E. coli*	2005	Stool	Community	
151	A-007-I-a-2	*E. coli*	2005	Stool	Community	
152	A-007-I-b-1	*E. coli*	2005	Stool	Community	
153	B-008-I-a-1	*E. coli*	2005	Stool	Community	
154	B-008-I-a-2	*E. coli*	2005	Stool	Community	
155	B-008-I-b-1	*E. coli*	2005	Stool	Community	
156	B-010-I-a-1	*E. coli*	2005	Stool	Community	
157	B-010-I-b-1	*E. coli*	2005	Stool	Community	
158	B-011-I-a-1	*E. coli*	2005	Stool	Community	E16
159	B-011-I-a-2	*E. coli*	2005	Stool	Community	
160	B-012-I-a-1	*E. coli*	2005	Stool	Community	
161	C-015-I-a-1	*E. coli*	2005	Stool	Community	
162	C-016-I-a-1	*E. coli*	2005	Stool	Community	
163	C-016-I-a-2	*E. coli*	2005	Stool	Community	E14
164	C-016-I-b-1	*E. coli*	2005	Stool	Community	
165	C-016-I-b-2	*E. coli*	2005	Stool	Community	
166	C-017-I-a-2	*E. coli*	2005	Stool	Community	
167	C-017-I-b-1	*E. coli*	2005	Stool	Community	
168	C-018-I-a-1	*E. coli*	2005	Stool	Community	
169	C-019-I-a-1	*E. coli*	2005	Stool	Community	
170	C-020-I-a-1	*E. coli*	2005	Stool	Community	
171	C-021-I-a-1	*E. coli*	2005	Stool	Community	
172	C-021-I-a-3	*E. coli*	2005	Stool	Community	
173	C-021-I-b-1	*E. coli*	2005	Stool	Community	
174	D-022-I-a-2	*E. coli*	2005	Stool	Community	
175	D-023-I-a-1	*E. coli*	2005	Stool	Community	
176	D-023-I-a-2	*E. coli*	2005	Stool	Community	
177	D-023-I-b-1	*E. coli*	2005	Stool	Community	E34
178	D-024-I-a-1	*E. coli*	2005	Stool	Community	
179	D-024-I-b-1	*E. coli*	2005	Stool	Community	
180	D-025-I-a-1	*E. coli*	2005	Stool	Community	E17
181	D-026-I-a-1	*E. coli*	2005	Stool	Community	
182	D-027-I-a-1	*E. coli*	2005	Stool	Community	

183	D-027-I-b-1	*E. coli*	2005	Stool	Community	
184	001-CN-1	*E. coli*	2007	Stool	Community	E22
185	001-CN-2	*E. coli*	2007	Stool	Community	E23
186	001-CN-3	*E. coli*	2007	Stool	Community	
187	001-Na-1	*E. coli*	2007	Stool	Community	
188	001-Na-2	*E. coli*	2007	Stool	Community	E40
189	001-Na-3	*E. coli*	2007	Stool	Community	
190	003-CN-1	*E. coli*	2007	Stool	Community	
191	003-Na-1	*E. coli*	2007	Stool	Community	
192	003-Na-2	*E. coli*	2007	Stool	Community	
193	004-CN-1	*E. coli*	2007	Stool	Community	E36
194	004-CN-2	*E. coli*	2007	Stool	Community	
195	004-Na-1	*E. coli*	2007	Stool	Community	
196	005-Na-1	*E. coli*	2007	Stool	Community	
197	007-CN-1	*E. coli*	2007	Stool	Community	
198	007-Na-2	*E. coli*	2007	Stool	Community	
199	008-CN-1	*E. coli*	2007	Stool	Community	E25
200	008-Na-1	*E. coli*	2007	Stool	Community	
201	008-Na-3	*E. coli*	2007	Stool	Community	
202	009-CN-1	*E. coli*	2007	Stool	Community	
203	009-Na-1	*E. coli*	2007	Stool	Community	
204	009-Na-2	*E. coli*	2007	Stool	Community	E20
205	010-CN-1	*E. coli*	2007	Stool	Community	
206	010-CN-2	*E. coli*	2007	Stool	Community	
207	010-Na-1	*E. coli*	2007	Stool	Community	
208	010-Na-2	*E. coli*	2007	Stool	Community	
209	011-Na-1	*E. coli*	2007	Stool	Community	
210	011-Na-2	*E. coli*	2007	Stool	Community	
211	012-CN-1	*E. coli*	2007	Stool	Community	
212	012-Na-1	*E. coli*	2007	Stool	Community	
213	013-CAZ-1	*E. coli*	2007	Stool	Community	E35
214	014-CN-1	*E. coli*	2007	Stool	Community	
215	014-CN-2	*E. coli*	2007	Stool	Community	E21
216	014-Na-1	*E. coli*	2007	Stool	Community	
217	014-Na-2	*E. coli*	2007	Stool	Community	E18
218	015-CN-1	*E. coli*	2007	Stool	Community	
219	015-Na-1	*E. coli*	2007	Stool	Community	
220	016-Na-1	*E. coli*	2007	Stool	Community	
221	016-Na-2	*E. coli*	2007	Stool	Community	
222	017-CN-1	*E. coli*	2007	Stool	Community	E19
223	017-Na-1	*E. coli*	2007	Stool	Community	
224	017-Na-2	*E. coli*	2007	Stool	Community	E26
225	017-Na-3	*E. coli*	2007	Stool	Community	
226	018-CN-1	*E. coli*	2007	Stool	Community	
227	018-CN-2	*E. coli*	2007	Stool	Community	
228	018-Na	*E. coli*	2007	Stool	Community	
229	019-Na	*E. coli*	2007	Stool	Community	
230	022-CN-1	*E. coli*	2007	Stool	Community	E24
231	022-CN-2	*E. coli*	2007	Stool	Community	E27
232	022-Na-1	*E. coli*	2007	Stool	Community	
233	022-Na-2	*E. coli*	2007	Stool	Community	
234	023-CAZ-2	*E. coli*	2007	Stool	Community	
235	023-CN-1	*E. coli*	2007	Stool	Community	
236	023-CN-2	*E. coli*	2007	Stool	Community	E9
237	023-Na-1	*E. coli*	2007	Stool	Community	
238	023-Na-2	*E. coli*	2007	Stool	Community	
239	024-CAZ-1	*E. coli*	2007	Stool	Community	
240	024-CAZ-2	*E. coli*	2007	Stool	Community	E4
241	024-CN-1	*E. coli*	2007	Stool	Community	E11
242	024-CN-2	*E. coli*	2007	Stool	Community	
243	024-CN-3	*E. coli*	2007	Stool	Community	
244	024-Na-1	*E. coli*	2007	Stool	Community	
245	024-Na-2	*E. coli*	2007	Stool	Community	

246	025-CN-1	*E. coli*	2007	Stool	Community	E5
247	025-Na	*E. coli*	2007	Stool	Community	
248	026-CAZ	*E. coli*	2007	Stool	Community	
249	026-CN	*E. coli*	2007	Stool	Community	
250	026-Na	*E. coli*	2007	Stool	Community	
251	027-CN	*E. coli*	2007	Stool	Community	
252	027-Na	*E. coli*	2007	Stool	Community	
253	028-CAZ	*E. coli*	2007	Stool	Community	
254	028-CN	*E. coli*	2007	Stool	Community	
255	028-Na	*E. coli*	2007	Stool	Community	
256	029-CAZ-1	*E. coli*	2007	Stool	Community	
257	029-CN-1	*E. coli*	2007	Stool	Community	
258	029-Na	*E. coli*	2007	Stool	Community	
259	030-Na	*E. coli*	2007	Stool	Community	
260	031-Na	*E. coli*	2007	Stool	Community	
261	032-CN	*E. coli*	2007	Stool	Community	
262	032-Na	*E. coli*	2007	Stool	Community	
263	033-CAZ-2	*E. coli*	2007	Stool	Community	E6
264	033-CN-1	*E. coli*	2007	Stool	Community	
265	033-CN-2	*E. coli*	2007	Stool	Community	
266	033-CN-3	*E. coli*	2007	Stool	Community	
267	033-Na-1	*E. coli*	2007	Stool	Community	
268	033-Na-3	*E. coli*	2007	Stool	Community	
269	034-CAZ-1	*E. coli*	2007	Stool	Community	E31
270	034-CAZ-2	*E. coli*	2007	Stool	Community	
271	034-CN-2	*E. coli*	2007	Stool	Community	
272	034-CN-3	*E. coli*	2007	Stool	Community	
273	034-Na	*E. coli*	2007	Stool	Community	
274	035-CAZ	*E. coli*	2007	Stool	Community	
275	035-CN-1	*E. coli*	2007	Stool	Community	E8
276	035-CN-2	*E. coli*	2007	Stool	Community	
277	035-Na-1	*E. coli*	2007	Stool	Community	
278	035-Na-2	*E. coli*	2007	Stool	Community	
279	036-CAZ-2	*E. coli*	2007	Stool	Community	
280	036-CN-1	*E. coli*	2007	Stool	Community	
281	036-CN-2	*E. coli*	2007	Stool	Community	E12
282	036-Na-1	*E. coli*	2007	Stool	Community	
283	036-Na-2	*E. coli*	2007	Stool	Community	
284	037-CN-1	*E. coli*	2007	Stool	Community	E7
285	037-CN-2	*E. coli*	2007	Stool	Community	
286	037-Na	*E. coli*	2007	Stool	Community	
287	038-CN-1	*E. coli*	2007	Stool	Community	
288	039-CAZ	*E. coli*	2007	Stool	Community	E32
289	039-CN-1	*E. coli*	2007	Stool	Community	
290	039-CN-2	*E. coli*	2007	Stool	Community	E1
291	039-Na	*E. coli*	2007	Stool	Community	
292	039-Na-1	*E. coli*	2007	Stool	Community	
293	040-CN	*E. coli*	2007	Stool	Community	
294	040-Na-1	*E. coli*	2007	Stool	Community	
295	040-Na-2	*E. coli*	2007	Stool	Community	
296	041-CN	*E. coli*	2007	Stool	Community	
297	041-Na-1	*E. coli*	2007	Stool	Community	
298	041-Na-2	*E. coli*	2007	Stool	Community	
299	043-CAZ-1	*E. coli*	2007	Stool	Community	E41
300	043-CAZ-2	*E. coli*	2007	Stool	Community	
301	043-CN	*E. coli*	2007	Stool	Community	
302	043-Na-1	*E. coli*	2007	Stool	Community	
303	043-Na-2	*E. coli*	2007	Stool	Community	
304	044-CN	*E. coli*	2007	Stool	Community	
305	044-Na	*E. coli*	2007	Stool	Community	
306	045-CN-1	*E. coli*	2007	Stool	Community	
307	045-CN-2	*E. coli*	2007	Stool	Community	E2
308	045-Na-1	*E. coli*	2007	Stool	Community	

309	045-Na-2	*E. coli*	2007	Stool	Community	
310	046-CAZ	*E. coli*	2007	Stool	Community	
311	046-CN	*E. coli*	2007	Stool	Community	E3
312	046-Na-1	*E. coli*	2007	Stool	Community	
313	046-Na-2	*E. coli*	2007	Stool	Community	
314	047-CN	*E. coli*	2007	Stool	Community	
315	048-CN-1	*E. coli*	2007	Stool	Community	
316	048-CN-2	*E. coli*	2007	Stool	Community	E39
317	048-Na	*E. coli*	2007	Stool	Community	
318	049-Na	*E. coli*	2007	Stool	Community	
319	050-CAZ	*E. coli*	2007	Stool	Community	
320	050-CN-1	*E. coli*	2007	Stool	Community	
321	050-CN-2	*E. coli*	2007	Stool	Community	
322	050-Na	*E. coli*	2007	Stool	Community	
323	051-CAZ	*E. coli*	2007	Stool	Community	
324	051-CN	*E. coli*	2007	Stool	Community	E37
325	051-Na-1	*E. coli*	2007	Stool	Community	
326	051-Na-2	*E. coli*	2007	Stool	Community	
327	052-Na	*E. coli*	2007	Stool	Community	
328	053-CAZ	*E. coli*	2007	Stool	Community	
329	053-CN-1	*E. coli*	2007	Stool	Community	
330	053-CN-2	*E. coli*	2007	Stool	Community	E10
331	053-Na-1	*E. coli*	2007	Stool	Community	
332	053-Na-2	*E. coli*	2007	Stool	Community	
333	053-Na-3	*E. coli*	2007	Stool	Community	
334	054-CN-1	*E. coli*	2007	Stool	Community	
335	054-CN-2	*E. coli*	2007	Stool	Community	
336	054-Na-1	*E. coli*	2007	Stool	Community	
337	054-Na-2	*E. coli*	2007	Stool	Community	
338	055-CN	*E. coli*	2007	Stool	Community	
339	055-Na-1	*E. coli*	2007	Stool	Community	
340	055-Na-2	*E. coli*	2007	Stool	Community	
341	055-Na-3	*E. coli*	2007	Stool	Community	
342	056-CAZ	*E. coli*	2007	Stool	Community	
343	056-CN	*E. coli*	2007	Stool	Community	
344	056-Na-1	*E. coli*	2007	Stool	Community	
345	056-Na-2	*E. coli*	2007	Stool	Community	
346	057-CAZ	*E. coli*	2007	Stool	Community	
347	057-CN	*E. coli*	2007	Stool	Community	
348	057-Na	*E. coli*	2007	Stool	Community	
349	058-CAZ	*E. coli*	2007	Stool	Community	
350	058-CN-1	*E. coli*	2007	Stool	Community	
351	058-CN-2	*E. coli*	2007	Stool	Community	
352	058-Na-1	*E. coli*	2007	Stool	Community	
353	058-Na-2	*E. coli*	2007	Stool	Community	
354	059-CN	*E. coli*	2007	Stool	Community	
355	059-Na-1	*E. coli*	2007	Stool	Community	
356	059-Na-2	*E. coli*	2007	Stool	Community	
357	060-CAZ-1	*E. coli*	2007	Stool	Community	
358	060-CAZ-2	*E. coli*	2007	Stool	Community	
359	060-CN-1	*E. coli*	2007	Stool	Community	
360	060-CN-2	*E. coli*	2007	Stool	Community	
361	060-Na	*E. coli*	2007	Stool	Community	
362	061-CAZ	*E. coli*	2007	Stool	Community	
363	061-CN	*E. coli*	2007	Stool	Community	
364	061-Na-1	*E. coli*	2007	Stool	Community	
365	061-Na-2	*E. coli*	2007	Stool	Community	
366	062-CN	*E. coli*	2007	Stool	Community	
367	062-Na	*E. coli*	2007	Stool	Community	
368	064-CN-1	*E. coli*	2007	Stool	Community	
369	064-CN-2	*E. coli*	2007	Stool	Community	
370	064-Na-2	*E. coli*	2007	Stool	Community	E42
371	064-Na-3	*E. coli*	2007	Stool	Community	

372	065-CN-1	*E. coli*	2007	Stool	Community	
373	065-Na-1	*E. coli*	2007	Stool	Community	
374	065-Na-2	*E. coli*	2007	Stool	Community	
375	067-CAZ-1	*E. coli*	2007	Stool	Community	E29
376	067-CN-1	*E. coli*	2007	Stool	Community	
377	067-CN-2	*E. coli*	2007	Stool	Community	
378	067-Na-1	*E. coli*	2007	Stool	Community	
379	067-Na-2	*E. coli*	2007	Stool	Community	
380	068-CN	*E. coli*	2007	Stool	Community	
381	068-Na-1	*E. coli*	2007	Stool	Community	
382	068-Na-2	*E. coli*	2007	Stool	Community	
383	069-CN	*E. coli*	2007	Stool	Community	
384	069-Na	*E. coli*	2007	Stool	Community	
385	070-CAZ-1	*E. coli*	2007	Stool	Community	E30
386	070-CN-1	*E. coli*	2007	Stool	Community	
387	070-CN-2	*E. coli*	2007	Stool	Community	
388	070-Na-1	*E. coli*	2007	Stool	Community	
389	070-Na-2	*E. coli*	2007	Stool	Community	
390	073-CAZ	*E. coli*	2007	Stool	Community	E33
391	074-CN-1	*E. coli*	2007	Stool	Community	
392	074-CN-2	*E. coli*	2007	Stool	Community	
393	074-Na-1	*E. coli*	2007	Stool	Community	
394	074-Na-2	*E. coli*	2007	Stool	Community	
395	075-CN	*E. coli*	2007	Stool	Community	
396	075-Na-1	*E. coli*	2007	Stool	Community	
397	075-Na-2	*E. coli*	2007	Stool	Community	
398	076-CN	*E. coli*	2007	Stool	Community	
399	076-Na-1	*E. coli*	2007	Stool	Community	
400	076-Na-2	*E. coli*	2007	Stool	Community	
401	077-CN	*E. coli*	2007	Stool	Community	
402	077-Na	*E. coli*	2007	Stool	Community	
403	EW-06-N-MAG-1	*E. coli*	2006	Nose	Community	
404	EW-06-N-MAG-2	*E. coli*	2006	Nose	Community	
405	EW-06-N-MAC-1	*E. coli*	2006	Nose	Community	
406	EW-06-N-MAC-3	*E. coli*	2006	Nose	Community	
407	EW-06-N-MAN	*E. coli*	2006	Nose	Community	
408	EW-06-R-MAG	*E. coli*	2006	Rectal	Community	
409	EW-10-R-MAG	*E. coli*	2006	Rectal	Community	
410	EW-14-R-MAN	*E. coli*	2006	Rectal	Community	
411	EW-20-N-MAN	*E. coli*	2006	Nose	Community	
412	EW-20-R-MAG-2	*E. coli*	2006	Rectal	Community	
413	EW-20-R-MAG-3	*E. coli*	2006	Rectal	Community	
414	EW-20-R-MAC-2	*E. coli*	2006	Rectal	Community	
415	EW-20-R-MAN-1	*E. coli*	2006	Rectal	Community	
416	EW-20-R-MAN-2	*E. coli*	2006	Rectal	Community	
417	EW-21-R-MAG-1	*E. coli*	2006	Rectal	Community	
418	EW-21-R-MAG-2	*E. coli*	2006	Rectal	Community	
419	EW-21-R-MAC	*E. coli*	2006	Rectal	Community	
420	EW-21-R-MAN	*E. coli*	2006	Rectal	Community	
421	EW-22-R-MAG-1	*E. coli*	2006	Rectal	Community	
422	EW-22-R-MAN-1	*E. coli*	2006	Rectal	Community	
423	EW-22-R-MAN-3	*E. coli*	2006	Rectal	Community	
424	EW-23-R-MAG-1	*E. coli*	2006	Rectal	Community	
425	EW-23-R-MAC	*E. coli*	2006	Rectal	Community	
426	EW-24-R-MAG-1	*E. coli*	2006	Rectal	Community	
427	EW-24-R-MAG-2	*E. coli*	2006	Rectal	Community	E38
428	EW-24-R-MAN	*E. coli*	2006	Rectal	Community	
429	EW-30-R-MAG	*E. coli*	2006	Rectal	Community	
430	EW-32-R-MAG	*E. coli*	2006	Rectal	Community	
431	EW-32-R-MAN	*E. coli*	2006	Rectal	Community	
432	EW-33-N-MAG	*E. coli*	2006	Nose	Community	
433	EW-33-R-MAG-1	*E. coli*	2006	Rectal	Community	
434	EW-33-R-MAG-2	*E. coli*	2006	Rectal	Community	

435	EW-33-R-MAC-1	*E. coli*	2006	Rectal	Community	
436	EW-33-R-MAN-2	*E. coli*	2006	Rectal	Community	
437	EW-34-R-MAG-1	*E. coli*	2006	Rectal	Community	
438	EW-34-R-MAC-1	*E. coli*	2006	Rectal	Community	
439	EW-34-R-MAN-3	*E. coli*	2006	Rectal	Community	
440	EW-35-R-MAG	*E. coli*	2006	Rectal	Community	
441	EW-41-R-MAG	*E. coli*	2006	Rectal	Community	
442	EW-43-R-MAG-1	*E. coli*	2006	Rectal	Community	
443	EW-43-R-MAG-2	*E. coli*	2006	Rectal	Community	
444	EW-43-R-MAN-1	*E. coli*	2006	Rectal	Community	
445	EW-50-R-MAG	*E. coli*	2006	Rectal	Community	
446	EW-50-R-MAG-1	*E. coli*	2006	Rectal	Community	
447	EW-50-R-MAN-2	*E. coli*	2006	Rectal	Community	
448	EW-56-R-MAG	*E. coli*	2006	Rectal	Community	
449	EW-56-R-MAN	*E. coli*	2006	Rectal	Community	
450	EW-57-R-MAG	*E. coli*	2006	Rectal	Community	
451	EW-57-R-MAN	*E. coli*	2006	Rectal	Community	
452	EW-59-R-MAG	*E. coli*	2006	Rectal	Community	E28
453	EW-60-R-MAG-1	*E. coli*	2006	Rectal	Community	
454	EW-60-R-MAC-1	*E. coli*	2006	Rectal	Community	
455	EW-61-R-MAN	*E. coli*	2006	Rectal	Community	
456	EW-65-R-MAN-1	*E. coli*	2006	Rectal	Community	
457	EW-66-R-MAG	*E. coli*	2006	Rectal	Community	
458	EW-66-R-MAN	*E. coli*	2006	Rectal	Community	
459	EW-68-R-MAG	*E. coli*	2006	Rectal	Community	
460	EW-68-R-MAC-2	*E. coli*	2006	Rectal	Community	
461	EW-68-R-MAN	*E. coli*	2006	Rectal	Community	
462	EW-69-R-MAG	*E. coli*	2006	Rectal	Community	
463	EW-69-R-MAC-2	*E. coli*	2006	Rectal	Community	
464	EW-69-R-MAN-2	*E. coli*	2006	Rectal	Community	
465	EW-71-R-MAG	*E. coli*	2006	Rectal	Community	
466	EW-71-R-MAN	*E. coli*	2006	Rectal	Community	
467	EW-76-R-MAG	*E. coli*	2006	Rectal	Community	
468	EW-76-R-MAN	*E. coli*	2006	Rectal	Community	
469	EW-82-R-MAN	*E. coli*	2006	Rectal	Community	
470	EW-87-R-MAG	*E. coli*	2006	Rectal	Community	
471	EW-88-R-MAN-1	*E. coli*	2006	Rectal	Community	
472	EW-89-R-MAG	*E. coli*	2006	Rectal	Community	
473	EW-89-R-MAN	*E. coli*	2006	Rectal	Community	
474	EW-98-R-MAG	*E. coli*	2006	Rectal	Community	
475	EW-98-R-MAC	*E. coli*	2006	Rectal	Community	
476	EW-98-R-MAN	*E. coli*	2006	Rectal	Community	
477	A-003-I-a-1	*K. pneumoniae*	2005	Stool	Community	K5
478	A-005-I-b-2	*K. pneumoniae*	2005	Stool	Community	
479	A-006-I-a-1	*K. pneumoniae*	2005	Stool	Community	
480	B-013-I-a-2	*K. pneumoniae*	2005	Stool	Community	
481	B-013-I-b-1	*K. pneumoniae*	2005	Stool	Community	
482	C-015-I-b-2	*K. pneumoniae*	2005	Stool	Community	K12
483	C-017-I-a-1	*K. pneumoniae*	2005	Stool	Community	
484	C-021-I-a-2	*K. pneumoniae*	2005	Stool	Community	
485	D-022-I-a-1	*K. pneumoniae*	2005	Stool	Community	
486	D-022-I-b-1	*K. pneumoniae*	2005	Stool	Community	K15
487	D-024-I-a-2	*K. pneumoniae*	2005	Stool	Community	
488	D-026-I-a-2	*K. pneumoniae*	2005	Stool	Community	K9
489	D-026-I-b-1	*K. pneumoniae*	2005	Stool	Community	
490	003-CN-2	*K. pneumoniae*	2007	Stool	Community	
491	008-CN-3	*K. pneumoniae*	2007	Stool	Community	K1
492	015-CN-2	*K. pneumoniae*	2007	Stool	Community	K13
493	018-CAZ	*K. pneumoniae*	2007	Stool	Community	
494	023-CAZ-1	*K. pneumoniae*	2007	Stool	Community	K10
495	025-CN-2	*K. pneumoniae*	2007	Stool	Community	K11
496	029-CN-2	*K. pneumoniae*	2007	Stool	Community	K4
497	033-CAZ-1	*K. pneumoniae*	2007	Stool	Community	

498	033-Na-2	*K. pneumoniae*	2007	Stool	Community	
499	038-CN-2	*K. pneumoniae*	2007	Stool	Community	K2
500	039-Na-2	*K. pneumoniae*	2007	Stool	Community	
501	065-CAZ	*K. pneumoniae*	2007	Stool	Community	
502	065-CN-2	*K. pneumoniae*	2007	Stool	Community	K8
503	073-CN-2	*K. pneumoniae*	2007	Stool	Community	
504	EW-20-N-MAG	*K. pneumoniae*	2006	Nose	Community	K7
505	EW-20-R-MAG-1	*K. pneumoniae*	2006	Rectal	Community	
506	EW-20-R-MAC-1	*K. pneumoniae*	2006	Rectal	Community	
507	EW-29-R-MAG	*K. pneumoniae*	2006	Rectal	Community	K6
508	EW-29-R-MAC-1	*K. pneumoniae*	2006	Rectal	Community	
509	EW-33-R-MAC-2	*K. pneumoniae*	2006	Rectal	Community	
510	EW-34-R-MAG-2	*K. pneumoniae*	2006	Rectal	Community	
511	EW-35-R-MAC	*K. pneumoniae*	2006	Rectal	Community	
512	EW-44-R-MAG-1	*K. pneumoniae*	2006	Rectal	Community	
513	EW-44-R-MAC-1	*K. pneumoniae*	2006	Rectal	Community	
514	EW-51-R-MAG	*K. pneumoniae*	2006	Rectal	Community	K14
515	EW-60-R-MAG-2	*K. pneumoniae*	2006	Rectal	Community	
516	EW-61-R-MAG	*K. pneumoniae*	2006	Rectal	Community	
517	EW-62-R-MAN	*K. pneumoniae*	2006	Rectal	Community	K3
518	EW-67-R-MAC	*K. pneumoniae*	2006	Rectal	Community	
519	EW-68-R-MAC-1	*K. pneumoniae*	2006	Rectal	Community	
520	EW-85-R-MAN	*K. pneumoniae*	2006	Rectal	Community	
521	B-013-I-a-1	*Proteus mirabilis*	2005	Stool	Community	
522	007-Na-1	*Pantoea* spp.	2007	Stool	Community	
523	008-CAZ-1	*Enterobacter cloacae*	2007	Stool	Community	En1
524	008-CN-2	*Citrobacter freundii*	2007	Stool	Community	
525	008-Na-2	*Citrobacter freundii*	2007	Stool	Community	
526	018-CN-3	*Citrobacter koseri*	2007	Stool	Community	
527	030-CN	*Salmonella arizonae*	2007	Stool	Community	
528	034-CN-1	*Kluyvera* spp.	2007	Stool	Community	
529	036-CAZ-1	*Leclercia adecarboxylata*	2007	Stool	Community	En2
530	038-CAZ-1	*Klebsiella terrigena*	2007	Stool	Community	En5
531	038-Na-3	*Enterobacter asburiae*	2007	Stool	Community	
532	056-Na-3	*Leclercia adecarboxylata*	2007	Stool	Community	
533	059-Na-3	*Citrobacter freundii*	2007	Stool	Community	
534	070-CAZ-2	*Enterobacter cloacae*	2007	Stool	Community	
535	EW-11-R-MAG	*Proteus* spp.	2006	Rectal	Community	
536	EW-11-R-MAN-2	*Proteus* spp.	2006	Rectal	Community	
537	EW-22-R-MAG-2	*Proteus* spp.	2006	Rectal	Community	
538	EW-23-R-MAG-2	*Proteus* spp.	2006	Rectal	Community	
539	EW-33-R-MAC-3	*Enterobacter aerogenes*	2006	Rectal	Community	
540	EW-43-R-MAG-3	*Escherichia fergusoni*	2006	Rectal	Community	
541	EW-55-R-MAC	*Enterobacter cloacae*	2006	Rectal	Community	
542	EW-60-R-MAC-2	*Enterobacter aerogenes*	2006	Rectal	Community	
543	EW-65-R-MAG	*Morganella morganii*	2006	Rectal	Community	
544	EW-65-R-MAN-2	*Morganella morganii*	2006	Rectal	Community	
545	EW-69-R-MAN-1	*Citrobacter* spp.	2006	Rectal	Community	En3
546	EW-79-R-MAG	*Citrobacter* spp.	2006	Rectal	Community	En4
547	EW-86-R-MAG	*Enterobacter aerogenes*	2006	Rectal	Community	
548	EW-86-R-MAC	*Enterobacter aerogenes*	2006	Rectal	Community	

9.3. Publications resulting from work conducted during Dphil

1. **Le Thi Minh Vien, Stephen Baker, Le Thi Phuong Thao, Le Thi Phuong Tu, Cao Thu Thuy, Tran Thi Thu Nga, Nguyen Van Minh Hoang, James Iain Campbell, Lam Minh Yen, Nguyen Trong Hieu, Nguyen Van Vinh Chau, Jeremy Farrar and Constance Schultsz (2009).** High prevalence of plasmid-mediated quinolone resistance determinants in commensal members of the *Enterobacteriaceae* in Ho Chi Minh City, Vietnam. *Journal of Medical Microbiology* **58**, 1585-1592.

2. **Nguyen Thi Khanh Nhu, Ha Vinh, Tran Vu Thieu Nga, Richard Stabler, Pham Thanh Duy, Le Thi Minh Vien, H. Rogier van Doorn, Ana Cerdeno-Tarraga, Nicholas Thomson, James Campbell, Nguyen Van Minh Hoang, Tran Thi Thu Nga, Pham Van Minh, Cao Thu Thuy, Brendan Wren, Jeremy Farrar, Stephen Baker (2010).** The sudden dominance $bla_{CTX\text{-}M}$ harbouring plasmids in *Shigella* spp. Circulating in Southern Vietnam. *Plos Neglected Tropical Diseases* **4**(6), e702.

3. **Le Thi Minh Vien, Manal AbuOun, Victoria Morrison, Nicholas Thomson, James I. Campbell, Constance Schultsz and Stephen Baker (2011).** Differential phenotypic and genotypic characterictics of *qnrS1*-harboring plasmids carried by hospital and community commensal *Enterobacteria*. *Antimicrobial Agents and Chemotherapy* **55**(4), 1798-1802.