# PREVALENCE AND CHARACTERISATION OF INTEGRONS IN CLINICAL ISOLATES OF *ENTEROBACTERIACEAE* AND *PSEUDOMONAS* FROM HOSPITALS IN MALAYSIA

By

**KOR SUE BEE**

A project report submitted to the Department of Biomedical Science
Faculty of Science
Universiti Tunku Abdul Rahman
in partial fulfillment of the requirements for the degree of
Master of Science
December 2011

## ABSTRACT

## PREVALENCE AND CHARACTERISATION OF INTEGRONS IN CLINICAL ISOLATES OF *ENTEROBACTERIACEAE* AND *PSEUDOMONAS* FROM HOSPITALS IN MALAYSIA

**Kor Sue Bee**

Integrons are genetic elements which contain determinants of the site specific recombination system that are capable of integrating and expressing the mobile resistant gene cassettes. The objectives of this study were to investigate the prevalence of integrons and characterise the gene cassettes present in clinical isolates obtained from hospital patients in Malaysia. In this study, one hundred forty-seven of *Enterobacteriaceae* and *Pseudomonas* sp. were collected. Total DNA was extracted using fast-boil method, and PCR amplification of 16S rRNA was carried out to test the integrity of DNA isolated. Subsequently, PCR screenings of integrase (*intI*) and sulfonamide resistant gene (*sul1*) were performed. PCR products amplified using *intI* degenerate primer were then digested with *RsaI* and *HinfI* to determine the classes of integrons present. Integron-positive bacteria were subsequently subjected to PCR amplification of the gene cassette present using specific primers. Lastly, PFGE was used to determine the clonal relationship between high-frequency of integron-positive *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* which carry the same gene cassettes. Through

PCR amplification, *intI* and *sul1* were found in 48.3% (71/147) and 35% (51/147) of the collected isolates, respectively. Class 1 integrons was the most dominant class identified (45.6%, 67/147). By using *chi*-square analysis, integrons were found to be significantly associated with the presence of *sul1* gene ($p<0.001$) and increased the resistance of isolates toward sulfonamide, quinolone, tetracycline, chloramphenicol, tigecycline, but lesser extend towards β-lactam and aminoglycoside antibiotics. Class 1 and class 2 gene cassette regions were successfully amplified in 66.2% of integron-positive isolates (47/71) and the most commonly detected gene cassettes were genes encoding resistance towards aminoglycosides (*aad*) and trimethoprim (*dfr*). In this study, eight *K. pneumoniae*, four *E. coli* and eight *P. aeruginosa* was shown to carry similar gene cassette arrays. Clonal mapping using CHEF-Mapper XA system (PFGE), showed that gene cassette in some of the *K. pneumoniae* and *P. aeruginosa* isolates could be spread through specific clone within the hospitals. In conclusion, this study demonstrated the wide distribution of class 1 integrons with different gene cassette arrays identified among our Malaysian hospital patients. This provides important information on the mechanisms of multiple antibiotic resistance genes acquisition in clinical isolates of Malaysian population.

## ACKNOWLEDGEMENT

My first words of gratitude go to my family, especially to mum and dad, for their support, kindness, and love. I would also like to thank my supervisor, Dr Chew Choy Hoong, for her invaluable guidance and consideration throughout my research project. Special thanks to my co-supervisor, Dr Say Yee How, as well as Dr.Choo Quok Cheong for their advices and kindness throughout this project. Also, thanks to Department of Biomedical Science, Universiti Tunku Abdul Rahman, by offering a great chance to learn and work on my project.

My sincere thanks also go to my fellow labmates, Lim Wyi Sian, Ng Wei Ling, Gwee Pei Shing, Rachel Tham, and others, for their constant encouragement and happiness along the research time. I also enjoyed good times with many of the lab officers, Ms Ng Shel Ling, Mr Saravanan a/l Sivasangaran, Ms Woo Suk Fong, Ms Luke Choy May, and others. Thank you for all the help and assistance during my laboratory work**.** Lastly, I want to thank Ms Saw, Ms Leong, Ms Jennifer Khoo, Ms Yap, and Mr Kam. I spent wonderful times and value dearly my friendship with all of them.

Last but not least, thanks to Malaysia Toray Science Fund (MTSF) and Universiti Tunku Abdul Rahman Research Fund (UTARRF) for supporting this research.

## APPROVAL SHEET

This thesis entitled "**PREVALENCE AND CHARACTERISATION OF INTEGRONS IN CLINICAL ISOLATES OF *ENTEROBACTERIACEAE* AND *PSEUDOMONAS* FROM HOSPITALS IN MALAYSIA**" was prepared by KOR SUE BEE and submitted as partial fulfillment of the requirement for the degree of Master of Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Dr. CHEW CHOY HOONG)
Date: ……....
Assistant Professor
Department of Biomedical Science
Faculty of Science
Universiti Tunku Abdul Rahman

**FACULTY OF SCIENCE**

**UNIVERSITI TUNKU ABDUL RAHMAN**

Date:

**SUBMISSION OF THESIS**

It is hereby certified that **KOR SUE BEE** (ID No: **09UEM08989**) has completed this thesis entitled "**PREVALENCE AND CHARACTERISATION OF INTEGRONS IN CLINICAL ISOLATES OF *ENTEROBACTERIACEAE* AND *PSEUDOMONAS* FROM HOSPITALS IN MALAYSIA**" under the supervision of Dr. Chew Choy Hoong (supervisor) from the Department of Biomedical Science, Faculty of Science, and Dr Say Yee How (Co-supervisor) from the Department of Biomedical Science, Faculty of Science.

I understand that the University will upload softcopy of my thesis in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(KOR SUE BEE)

## DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

KOR SUE BEE

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 59-be | 59-base element |
| bp | Base pair |
| CS | Conserved segment |
| DMSO | Diethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxyribonucleoside triphosphate |
| EDTA | Ethylene diamine-tetra-acetatic acid |
| *et al.* | "et alia" (Italian word referring to 'and others') |
| h | Hour |
| kb | Kilobase |
| LMP | Low melting point |
| M | Molar |
| MDR | Multidrug resistant |
| $MgCl_2$ | Magnesium chloride |
| min | Minute |
| OD | Optical density |
| PCR | Polymerase chain reaction |
| PFGE | Pulsed field gel electrophoresis |
| *Taq* | *Thermus aquatics* |
| TBE | Tris-borate-EDTA |
| U | Unit |
| UPGMA | Unweighted paired group method using arithmetic average |
| V | Volt |
| v/v | Volume per volume |
| w/v | Weight per volume |

# CHAPTER 1

# INTRODUCTION

Dissemination of antibiotic resistance genes by horizontal gene transfer has led to the rapid emergence of multidrug resistance (MDR) among bacteria. Infections caused by MDR gram-negative bacteria are now recognised as important causes of morbidity and mortality among hospitalised patients (Paterson & Bonomo, 2005; Walsh *et al.*, 2005). The family *Enterobacteriaceae*, for example, contains genera that inhabit the intestinal tracts of humans which are frequently exposed to various antibiotics. Thus, it contributes higher potential for the dissemination of antibiotic resistance genes (Goldstein *et al.*, 2001). On the other hand, *Pseudomonas aeruginosa* is also an important pathogen for immunocompromised patients (Driscoll *et al.*, 2007).

In recent years, it has been shown that a substantial portion of resistance genes are integrated into DNA elements called "integrons" (Hall, 2002). Integrons are elements that participate in a powerful site-specific recombination system and thus, play a major role in acquiring and disseminating antibiotic resistance genes in a clinical setting. Many antibiotic resistance genes found in gram-negative bacteria are part of a gene cassette integrated into integrons (Recchia & Hall, 1995a). Integrons can capture individual resistant genes but do not, on their own, mobilise between cells. They were frequently associated with mobile genetic elements, such as transposons and conjugative plasmids, which

contribute to their dissemination through the horizontal gene transfer events (Hall *et al.*, 1999, Holmes *et al.*, 2003). Class 1 integrons are the most prevalent and well characterised integrons (Martinez-Freijo *et al.*, 1999). It possess three essential components in the 5' conserved segment (CS): an *intI* gene, encoding an integrase, a specific recombination site (*attI* site), and a promoter that directs transcription of the gene cassettes. Most class 1 integrons contain an additional resistance gene, *sul1*, in the 3' CS, which confers sulfonamides resistance (Collis & Hall, 1995).

Nowadays, several reports have focused on the genetic structure of integrons and have emphasised their role in the acquisition and dissemination of antimicrobial resistance among gram-negative bacilli (Martinez-Freijo *et al.*, 1998; Leverstein-van Hall *et al.*, 2002a; Nijssen *et al.*, 2005; Norrby, 2005). Integrons are likely to be one of the single most important mechanisms for the acquisition, maintenance, and dissemination of resistance to multiple antibiotics. Therefore, additional studies of the epidemiology of integron-mediated antibiotic resistance are urgently needed. In Malaysia, Lim *et al.* (2009a, 2009b) had investigated class 1 integrons in ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*. However, no similar studies so far have been carried out to elucidate the three classes of integrons (class 1, 2 and 3) in a larger scale for multidrug resistant *Enterobacteriaceae* and *Pseudomonas*, as well as to characterise the gene cassettes present within the integrons in our Malaysian population.

Therefore, this present study was designed:

a) To determine the antimicrobial susceptibility profiles of one hundred forty-seven *Enterobacteriaceae* and *Pseudomonas* sp.;
b) To investigate the prevalence of class 1, 2 and 3 integrons and *sul1* genes among the isolates;
c) To identify and characterise the gene cassettes present in integron-positive isolates;
d) To determine the risk factors and bacterial MDR relationship with presence of integrons; and
e) To investigate the clonal relationship between integron-positive isolates that carried similar gene cassettes by pulsed field gel electrophoresis (PFGE)

Considering the growing evidence of clinical resistance in humans, this research could therefore address the concerns of Malaysian public health, aid medical development in our country and contribute to improve the living standards of our society.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 *Enterobacteriaceae* and *Pseudomonas aeruginosa*

*Enterobacteriaceae* and *Pseudomonas aeruginosa* are both gram-negative bacteria which cause various infections and are frequently isolated from clinical specimens (Holt *et al.*, 1994; Orrett, 2004). Members of the family *Enterobacteriaceae*, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Proteus* sp., *Enterobacter* sp., *Salmonella* sp.., *Shigella* sp., *Yersinia* sp. and *Providencia* sp., are frequently identified as aetiological agents of nosocomial and community-acquired infections (Holt *et al.*, 1994). Data obtained from several university medical centers in Malaysia for the year 2007 showed that members of family *Enterobacteriaceae* were the predominant gram negative bacteria that cause bacteremia, representing approximately 60% of the isolated pathogens (Nirmala *et al.*, 2007).

Within this family, *E. coli* is known as the main cause of urinary tract infections (UTIs) (Gupta *et al.*, 2001; Nicolle, 2001; Gaynes & Edwards, 2005) while *Klebsiella* sp. and *Enterobacter* sp. are important causes of pneumonia. All of the *Enterobacteriaceae* have been implicated in bloodstream infections and in peritonitis, cholangitis, and other intra-abdominal infections (Paterson, 2006; Todar, 2007). In addition, bacteria such as *Salmonella* sp. cause gastroenteritis, and invasive infection in some patients (Martinez *et al.*, 2007).

*P. aeruginosa* is also an important opportunistic nosocomial pathogen, which causes diseases from superficial skin infections to serious systemic infections such as fulminant sepsis (Kipnis *et al.*, 2006). It is also the second most common causative agent of hospital-acquired pneumonia, especially in immunocompromised patients such as those who suffer from AIDS, cancer, burn wounds, cystic fibrosis (CF) (Driscoll *et al.*, 2007) and those with a tracheal cannula, tracheotomy, or under mechanical ventilation (Sarlangue *et al.*, 2006). Both *Enterobacteriaceae* and *Pseudomonas* have been shown to be resistant to many antibiotics, disinfections and also have the ability to acquire resistance (Leverstein-van Hall *et al.*, 2002a; Siegel, 2008). With the emergence of multiple drug resistance, a great problem in clinical settings has emerged due to limited therapeutic options.

## 2.2 Antibiotics resistance development in microbes

There are five different modes of action for antimicrobial agents. These include interference with cell wall synthesis (β-lactams and glycopeptide agents), inhibition of protein synthesis (macrolides and tetracyclines), interference with nucleic acid synthesis (fluoroquinolones and rifampin), inhibition of a metabolic pathway (trimethoprim-sulfamethoxazole), and disruption of bacterial membrane structure (polymyxins and daptomycin) (Neu, 1992; Drlica & Zhao, 1997; McManus, 1997; Tenover, 2006).

Nowadays, the heavy selective pressure of antibiotics has accelerated the development and spread of antibiotics and multidrug resistant (MDR) bacteria

in humans and environment (Amyes & Gemmell, 1992; Ploy *et al.*, 2000b; Ferrara, 2006; Chen *et al.*, 2009). Bacteria may manifest resistance to antibacterial drugs through a variety of mechanisms which include (i) enzymatic modification, (ii) down-regulation or alteration of an outer membrane protein channel that the drug requires for cell entry, (iii) antibiotics target site modification, and (iv) presence of active efflux pumps (McManus, 1997) (Figure 2.1).

Enzymatic modification is an important mechanism of resistance to β-lactam family and aminoglycoside family of antibiotics. Aminoglycosides act by binding at several locations within the 30S ribosome, which results in codon misreading and inhibition of protein synthesis (Fourmy *et al.*, 1996, 1998; Schroeder *et al.*, 2000). Three classes of aminoglycoside modifying enzymes have been identified, which act through the addition of an adenyl-, phospho-, or acetyl-group to several possible sites along the aminoglycoside backbone (Shaw *et al.*, 1993). These modifications interfere with the binding of the aminoglycoside to the 30S ribosome, resulting in increased tolerance to the antibiotic (Davies & Wright, 1997). Another mechanism microbes use to resist antibiotic is to reduce membrane permeability (Ochs *et al.*, 1999). For example, in *P. aeruginosa*, OprD is a porin involved in the transport of basic amino acids and carbapenem antibiotics like imipenem and meropenem. Consequently, mutations in the *oprD* promoter region cause decreased expression of porin *oprD*, hence, mediates carbapenem resistance in *Pseudomonas* (Ochs *et al.*, 1999; Epp *et al.*, 2001).

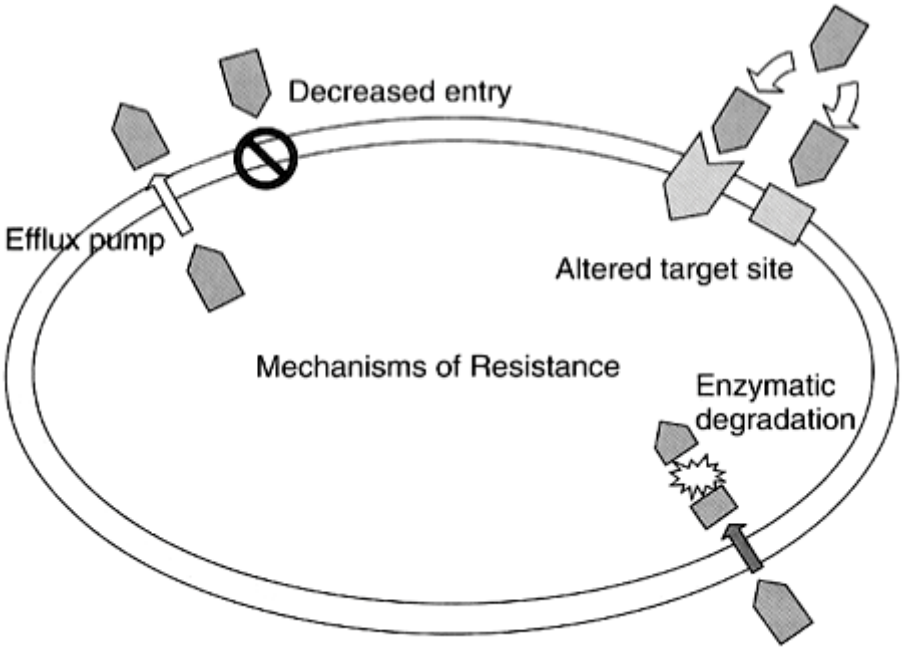



**Figure 2.1: Mechanism of antibiotic resistance in bacteria.** The mechanisms that bacteria exhibit to protect themselves from antibiotics can be classified into four types, including (i) enzymatic modification, (ii) decreased membrane permeability toward antibiotics, (iii) antibiotic target site modification, and (iv) presence of efflux pump. (Image adapted and modified from website, URL: http://www.gwhizmobile.com)

Vancomycin is an important antibiotic used to treat many gram-positive pathogens like the methicillin resistant *Staphylococcus aureus* (MRSA), by inhibiting the transpeptidation process during synthesis of the cell wall by complexing with D-alanine-D-alanine (Bonomo & Gill, 2005). Rapid emergence of vancomycin resistance was discovered in which the mechanism targets to alter the antibiotic target site. Five different genes were determined to be involved. They act in modifying the peptidoglycan terminus from D-alanine-D-alanine to D-alanine-D-lactate, which in turn prevents the binding by vancomycin, as well as decreasing D-alanine-D-alanine pools, but increasing D-alanine-D-lactate pools. These genes were found to be regulated by a two-component regulatory system monitoring gene's transcription (Walsh *et al.*, 1996; Cetinkaya *et al.*, 2000).

Active removal of the antibiotic from the cell by efflux pumps is another method microbes use to resist antibiotic treatments. Many different efflux families have been identified, including four $H^+$ transporter systems which are (i) the major facilitator (MF) superfamily, (ii) the resistance nodulation and cell division (RND) system, (iii) the small multidrug resistance (SMR) family, and (iv) the multidrug and toxic compound extrusion (MATE) family (McKeegan *et al.*, 2002). For instance, *P. aeruginosa* contains an RND system that confers a high degree of intrinsic resistance to many different antibiotics such as chloramphenicol and tetracycline (Poole *et al.*, 1993). Besides, increased expression of the *mexXY* efflux pump proteins confers increased aminoglycoside resistance in *P. aeruginosa* (Westbrock-Wadman *et al.*, 1999;

Mao *et al.*, 2001), while increased expression of the efflux pumps *mexABoprM* and *mexCDoprJ* confer β-lactam resistance (Srikumar *et al.*, 1998).

## 2.3 Integrons

### 2.3.1 Introduction

Development of antibiotic resistance may be mediated by chromosomally located resistance determinants or mutations in a resident gene (Martínez & Baquero, 2000; Leverstein-van Hall *et al.*, 2002a). However, other than the antibiotic resistant mechanism mentioned above, bacteria could also develop resistance through the acquisition of new genetic material from other resistant organisms. Acquisition of resistance genes through horizontal gene transfer, may occur between strains of the same species or between different bacterial species or genera (Leverstein-van Hall *et al.*, 2002a; Tenover, 2006). During the processes of genetic exchange in transformation, conjugation and transduction, plasmids and transposons are involved in facilitating the transfer of resistance genes from one cell to another (Tenover, 2006; Bennett, 2008). In recent years, a third mechanism of resistance gene dissemination has been discovered. It involves a DNA element that mediates the integration of resistance genes by a site-specific recombinational mechanism called "integrons" (Stokes & Hall, 1989; Recchia & Hall, 1997).

### 2.3.2 Definition and classes

Integrons were first described by Stokes & Hall (1989). As reviewed by many researchers, they are genetic elements that contain the specific determinants of a site specific recombination system which is capable of integrating and expressing the mobile gene cassettes (Hall & Collis, 1998; Fluit & Schmitz, 2004; Weldhagen, 2004). These gene cassettes are responsible for encoding resistance determinants to several antimicrobial agents (Fluit & Schmitz, 2004). Since then, more studies have reported that these molecular elements are often found on the plasmids and/or the chromosomes of gram-negative bacteria (Recchia & Hall 1997; Fluit & Schmitz, 1999; Hall *et al.*, 1999; Rowe-Magnus & Mazel, 1999).

There are three essential components of an integron which includes: (i) an integrase gene, *intI,* that encodes for a site-specific recombinase; (ii) an adjacent *attI* site, which is recognised by the integrase and acts as a receptor for gene cassettes; and (iii) a promoter region, known as $P_c$ (Collis & Hall, 1995; Reechia & Hall, 1995a; Leverstein-van Hall *et al.*, 2002a; Fluit & Schmitz, 2004).

Integrons are divided into two major groups: the resistance integrons (RI) and the superintegrons (SI). Resistance integrons carry mostly gene cassettes that encode resistance against antibiotics and disinfectants, and can be located either on the chromosome or on plasmids. The superintegrons contain gene cassettes with a variety of functions, which are always located on bacterial chromosome (Mazel *et al.*, 1998; Norrby, 2005). There were over hundred

classes of integrons have been identified so far, distinguished by their respective integrase genes (*IntI1, IntI2, IntI3,* and *IntI4*) (Arakawa *et al.*, 1995; Reechia & Hall, 1995a; Mazel *et al.*, 1998, Boucher *et al*., 2007). Class 1, 2 and 3 integrons are well known examples of RI with clinical and epidemiological relevance for antibiotic resistance (Fluit & Schmitz, 1999, 2004), whilst class 4 is a distinctive class of integrons located in the *Vibrio cholerae* genome (Mazel *et al.*, 1998) and is now known as *Vibrio cholerae* superintegrons. In the *V. cholerae* strain that has been sequenced, the integron contains an array of one hundred seventy-nine gene cassettes, but only a few of them, for example, the *catB9* gene cassette, which contain genes that are likely to determine resistance to an antibiotic (Heidelberg *et al.*, 2000).

### 2.3.3 Class 1 integrons

Class 1 integrons are the best characterised integrons which are frequently reported in clinical isolates (Martinez-Freijo *et al.*, 1998, 1999; Chang *et al.*, 2000; Schmitz *et al.*, 2001; White *et al.*, 2001; Jones *et al.*, 2003), livestock (Goldstein *et al.*, 2001; Ebner *et al.*, 2004), environmental isolates including rivers (Biyela *et al.*, 2004; Mukherjee & Chakraborty, 2006), fish farm (Schmidt *et al.*, 2001), sewage treatment plant (Da Costa *et al.*, 2006; Lindberg *et al.*, 2007; Zhang *et al.*, 2009) and wastewater sources (Tennstedt *et al.*, 2003). Class 1 integrons, located on plasmids and transposons, are the most prevalent integrons found in clinical isolates and are strongly associated with multi-antibiotic resistance in the hospital environment (Martinez-Freijo *et al.*, 1998). They are now found in 40-70% of gram-negative pathogens

isolated from clinical contexts (Martinez-Freijo *et al.*, 1998; Essen-Zandbergen *et al.*, 2007) as well as in pathogens isolated from livestock (Goldstein *et al.*, 2001; Ebner *et al.*, 2004).

As shown in Figure 2.2, class 1 integrons contain a 5' and 3' conserved segments (CS) and a variable region. Like the other classes of integrons, their 5' conserved segment consists of an *intI1* gene (integrase), *attI1* site, and a promoter region ($P_{ant}$) expressing the inserted gene cassettes (Stokes & Hall, 1989; Levesque *et al.*, 1995; Liebert *et al.*, 1999). In fact, the $P_{ant}$ of class 1 integrons potentially contains two promoters, P1 and P2. On the other hand, the 3' conserved segment contains a truncated antiseptic resistance gene (*qacE1*), a sulfonamide resistance gene (*sul1*) and an open reading frame (*orf5*) of unknown function (Reechia & Hall, 1995a; Levesque *et al.*, 1995; Fluit & Schmitz, 1999). These CS regions flank a variable region, in terms of length and sequence, which contain the gene cassettes of the particular integrons.

Gene cassettes are very simple genetic elements that typically consist of a single promoterless resistant gene and a recombination site called a 59-base element (59-be) or *attC* (Section 2.4). All integrons capture mobile gene cassettes using site-specific recombination mechanism mediated by an integrase gene (*intI*) (Hall *et al.*, 1994). IntI1 is a member of the tyrosine recombinase family (Esposito & Scocca, 1997). It catalyses two types of site-specific recombination reaction which are recombination between two *attC* sites or recombination between *attI1* and a *attC* (Stokes & Hall, 1989; Hall *et al.*, 1991; Holmes *et al.*, 2003; Gillings *et al.*, 2008). However, studies have

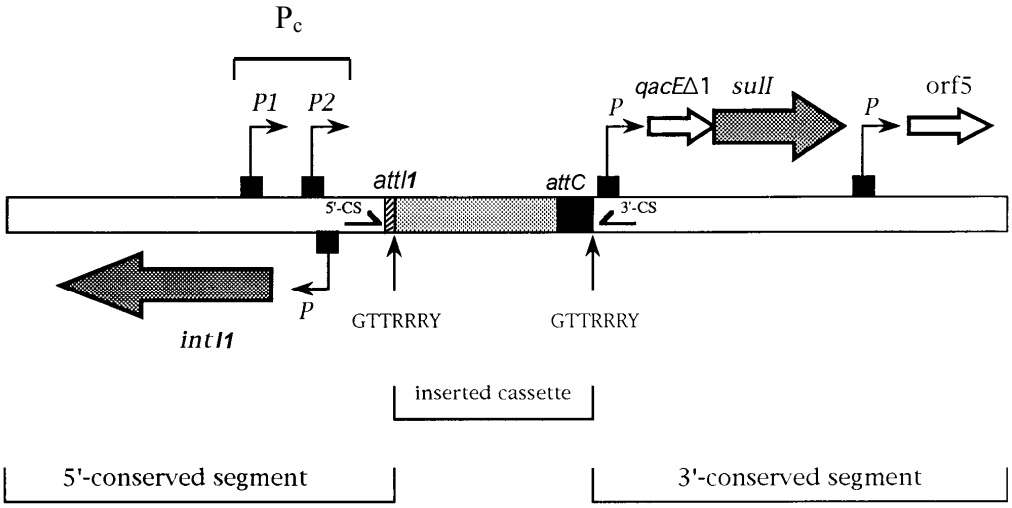



**Figure 2.2: General structure of class 1 integrons.** The location and orientation of different promoters are shown with the arrow showing direction of transcription. The 5'CS consists of integrase gene, *intI1* and recombination site, *attI1*, while 3' CS contains antiseptic resistance gene (*qacEΔ1*), a sulfonamide resistance gene (*sul1*) and an unknown function open reading frame (*orf5*). One inserted cassette is shown, with its associated *attC*. The sequence of GTTRRRY is the point of boundary for integration of gene cassettes. (Image adapted from Levesque *et al.*, 1995)

shown that the interaction between the *attC* and the *attI1* site is the preferred recombination reaction (Collis & Hall, 1992a). The *attI1* site, located at the 3'end of the 5'CS, is less complex than the *attC* (Recchia *et al.*, 1994), whilst it is believed that IntI1 binds stronger to *attI1* than to *attC* site (Fluit & Schmitz, 1999). This recombination results in the assembly of new genes downstream of an integron-associated promoter $P_{ant}$ that directs transcription of the gene cassettes as illustrated in Figure 2.3 (Stokes & Hall, 1989; Hall *et al.*, 1991; Collis & Hall, 1992a, 1995). IntI1 can also act on secondary sites containing a degenerate core site, but this type of recombination is infrequent (Francia *et al.*, 1993).

During recombination, gene cassettes can be inserted one after the other at the recombination site, *attI1*, to produce tandem resistance gene arrays (Bennett, 2008). Therefore, the order of the gene cassettes from 5'CS indicates the order of addition, in which the nearest gene to 5' CS being the latest addition due to cassette insertion at the same point (*attI*). The number of gene cassettes can vary between zero for In0 (Bissonnette & Roy, 1992) to five (Stokes & Hall, 1991) or more.

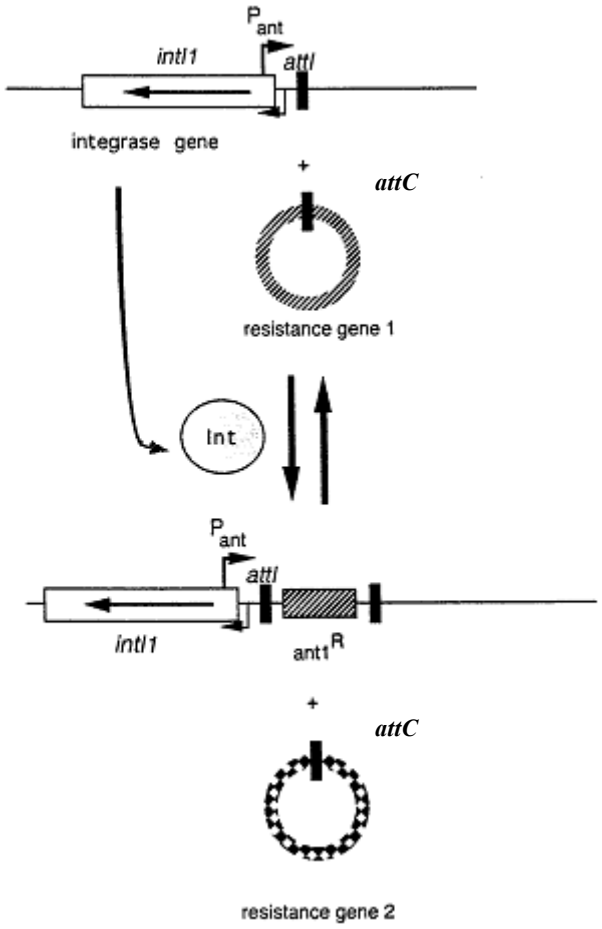



**Figure 2.3: Schematic representation of site specific recombination for class 1 integrons.** Circular antibiotic resistance gene cassettes are inserted into a specific attachment sequence (*attI*) and are placed downstream of a functional promoter element ($P_{ant}$). The excision and integration of gene cassettes are mediated by integrase protein (IntI1). (Image adapted from Mazel & Davies, 1999)

### 2.3.4 Class 2 integrons

Class 2 integrons are found in transposon Tn7 and its related derivatives with the 3' conserved segment of class 2 integrons carry five *tns* genes which are involved in the movement of the transposon (Ploy *et al.*, 2000a). The structure of class 2 integrons has not been fully characterised, but studies have shown that they are made up of a 5' conserved segment containing a pseudo-integrase gene (*intI2*) and gene cassettes encoding resistant to trimethoprim (*dfrA1*), streptothricin (*sat*) and streptomycin (*aadA1*) (Hall & Collis, 1995). In Tn7 itself, the cassettes array appears to end with a truncated cassette known as *orfX*. Consequently, primers covering the *orfX* and the conserved *intI2* region of Tn7 have been used to amplify cassette arrays in class 2 integrons (White *et al.*, 2001).

The *intI2* gene is 40% identical to *intI1* gene (Hall & Collis, 1995). The *intI2* genes are so far described as non-functional because of the presence of an internal stop codon (Hansson *et al.*, 2002). However, studies by Hansson *et al.* (2002) claimed that replacement of the internal stop codon with a codon for glutamic acid yields a functional integrase. He and his colleagues discovered that the functional *IntI2*, however, could not excise gene cassettes from class 1 integrons, even though the gene cassettes found in class 2 integrons are identical to those in class 1 integrons. In contrast, *IntI2* was able to both excise and insert gene cassettes into class 2 integrons. This indicates that *IntI2* is specific to class 2 integrons. The acquisition of new gene cassettes is still possible, but it is dependent on the presence of another type of integrase or natural suppression of stop codon in *IntI2* (Hansson *et al.*, 2002). It has been

proposed that the truncated *IntI2* is in fact a regulatory mechanism in which the DNA-binding domain of the integrase is still present and protein may bind to the same sites as the full protein. However, the advantage of such a function remains unexplained (Hansson *et al.*, 2002; Parks & Peter, 2009). Until now, class 2 integrons have been found in isolates of *Acinetobacter* (Gonzalez *et al.*, 1998), *Shigella* (McIver *et al.*, 2002), and *Salmonella* (Orman *et al.*, 2002).

### 2.3.5 Class 3 integrons

Class 3 integrons were firstly identified associated with a Tn5053-family transposon (Arakawa *et al.*, 1995; Collis *et al.*, 2002). Class 3 integrons have similar structure as class 1 integrons. They contain three essential components of *intI3*, *attI3*, and promoters in 5' conserved segment. The *IntI3* integrase is 59% identical and similar in its properties to *IntI1* integrase (Hall *et al.*, 1999; Collis *et al.*, 2002). It is able to recognise different *attC* and integrate cassettes into the *attI3* site as well catalysing recombination between *attC* and secondary integration sites, but at lower frequencies than *IntI1* integrase (Hall *et al.*, 1999; Collis *et al.*, 2002).

Class 3 integrons have been characterised in *Serratia marcescens*, isolated in Japan, by the identification of the *bla*IMP gene encoding broad-spectrum β-lactam antibiotic resistance. The *bla*IMP gene, first described in a class 3 integrons (Senda *et al.*, 1996; Collis *et al.*, 2002), is also flanked by a *attC*, and an integrase-like gene and the *aac(6')-Ib* (*aacA4*) gene, which encodes aminoglycoside resistance (Arakawa *et al.*, 1995). Class 3 integrons was

subsequently described in *P. aeruginosa*, *Alcaligenes xylosoxidans*, *Pseudomonas putida* and *K. pneumoniae* isolates from Japan (Arakawa *et al.*, 1995; Senda *et al.*, 1996). Generally, *intI3* genes are rarely detected in clinical isolates (White *et al.*, 2001; Reyes *et al.*, 2003; Yu *et al.*, 2003).

## 2.4 Gene cassettes

Gene cassettes include single promoterless open reading frame (ORF) associated with specific recombination site, *attC* or 59-base element at the 3' end (Fluit & Schmitz, 1999). The *attC* sites vary considerably in sequence and length sized from 57 bp to 141 bp (Hall *et al.*, 1991; Recchia & Hall, 1995a; Stokes *et al.*, 1997). The most conserved features of the *attC* sites are the imperfect inverted repeats of 7 nucleotides core site at the 3' end with the sequence of GTTRRRY and the inverse core site displaying the sequence RYYYAAC at 5' end of the element (Hall *et al.*, 1991; Collis & Hall, 1992b; Stokes *et al.*, 1997). Gene cassettes can be excised in a circular form and are then integrated at core sites GTTRRRY with a crossover between the G and the first T (Hall *et al.*, 1991; Collis & Hall, 1992a; Recchia & Hall, 1995b; Hansson *et al.*, 1997; Stokes *et al.*, 1997) (Figure 2.4). It is not yet clear if the cleavage occurs at the same place on both DNA strands or if it is a staggered cleavage. Some proposed that only one strand is cleaved by the integrase and the resulting Holliday junction is then resolved by a cellular enzyme like RuvC (Stokes *et al.*, 1997). After the integration of a circular gene cassette, part of the *attC* would end up at the 5' side of the coding sequence of the gene

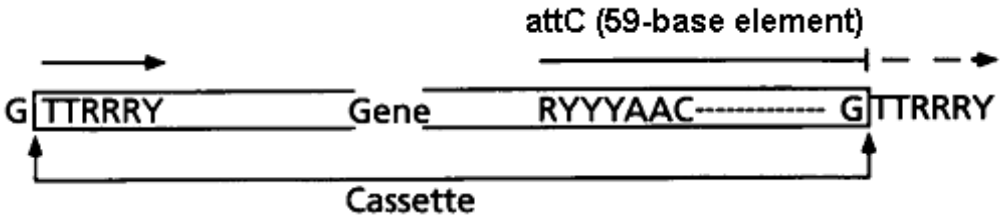



**Figure 2.4: Boundaries of gene cassettes.** The core sites (GTTRRRY) found at each end of the integrated cassette are shown with the crossover located between the G and the first T. The inverse core site (RYYYAAC) is located at the 5' end of the *attC* site (59-base element). Short dashes represent the central region of the *attC* range from 57 to 141 bp (not to scale). (Image adapted from Recchia & Hall, 1995a)

sequence of the gene cassette to which it belongs (Hall *et al.*, 1991; Collis & Hall, 1992a).

Sequences which flank gene cassettes can vary a great deal between the different integron classes (Hall *et al.*, 1994; Stokes *et al.*, 1997; Stokes *et al.*, 2001). However, identical cassettes have been found to be encoded in integrons of different classes (Fluit & Schmitz, 1999, 2004; Rowe-Magnus & Mazel, 1999, 2001). Indeed, there are claimed experimental evidence that the antibiotic resistance genes found in class 1, 2, and 3 integrons were acquired by capturing gene cassettes from a vast pool of diverse cassettes that are prevalent in microbial communities (Stokes *et al.*, 2001; Rowe-Magnus *et al.*, 2002; Michael *et al.*, 2004; Mazel, 2006; Boucher *et al.*, 2007).

Gene cassette mobility between the different integron classes advocates that they may have played an important role in bacterial evolution in order to adapt to unpredictable environmental challenges (Rowe-Magnus & Mazel, 2001; Rowe-Magnus *et al.*, 2002). Cassettes have been observed to "move up" to the first position in an array following exposure to the relevant antibiotic (Rowe-Magnus *et al.*, 2002). This could potentially occur via a number of IntI-mediated processes or by homologous recombination (Hall & Collis, 1995). In fact, deletions, duplications and rearrangements of gene cassettes in integrons have also been observed (Collis & Hall, 1992b). Cassette mobility results in the dissemination of resistance genes with more new cassettes are continually being discovered.

To date, over 100 cassettes that confer resistance to a range of antimicrobial agents have been identified including cassettes resistant to aminoglycosides, penicillins, cephalosporins, carbapenems, trimethoprim, chloramphenicol, rifampin, erythromycin, and quaternary ammonium compounds (Partridge *et al.*, 2009). Table 2.1 summarises some of the recently described gene cassettes, their corresponding protein translated and cassettes length. The origins of the gene cassettes are not known, yet it is suggested that cassettes from multiple resistance integrons (MRIs) may be relatively ancient structures (Recchia & Hall, 1997). Moreover, it is suspected that MRIs evolved from super integrons through the recruitment of super-integrons gene cassettes (Rowe-Magnus *et al.*, 2002; Hall & Stokes, 2004).

## 2.5 Expression of gene cassettes

Integrons is defined as natural bacterial expression vectors (Collis & Hall, 1995; Hall & Collis, 1995). There is a considerable degree of variability in the level of expression of the genes cassettes. This variability of expression can be accounted for by the intrinsic characteristics of both the gene cassettes and the integrons (Levesque *et al.*, 1994; Collis & Hall, 1995; Hall & Collis, 1995; Recchia & Hall, 1995b). Most of gene cassettes do not contain a promoter of their own except for cassettes *qac* (Guerineau *et al.*, 1990), *cmlA* (Bissonnette *et al.*, 1991) and *ereA* (Biskri & Mazel, 2003). A suitably spaced ribosome-binding site (RBS) is usually identified in gene cassette region for regulating its gene expression (Partridge *et al.*, 2009).

**Table 2.1: A representative of gene cassettes, their corresponding encoded protein and cassette length (bp)**

| Gene cassette | Encoded protein | Length of cassette (bp) |
|---|---|---|
| **Resistance to aminoglycosides** | | |
| Aminoglycoside adenylyltransferases | | |
| *aadA1* | AAD(3") | 856 |
| *aadB* | AAD(2") | 591 |
| Aminoglycoside acetyltransferases | | |
| *aacA4* | AAC-(6')-Ib | 637 |
| *aacC1* | AAC-(3)-Ia | 577 |
| **Resistance to β-lactams** | | |
| Class A β-lactamases | | |
| *blaP1* | PSE-1/CARB-2 | 1044 |
| *blaP3* | CARB-4 | >1023 |
| Class B metallo β-lactamases | | |
| *bla*IMP | IMP-1 | 880 |
| *bla*VEB-1 | VEB-1 | 1059 |
| Class D β-lactamases | | |
| *oxa1* | OXA-1 | 1004 |
| *oxa2* | OXA-2 | 876 |
| **Resistance to chloramphenicol** | | |
| Chloramphenicol acetyltransferases | | |
| *catB2* | CATB2 | 739 |
| *catB3* | CATB3 | 715 |
| Chloramphenicol exporter | | |
| *cmlA* | CmlA | 1549 |
| **Resistance to trimethoprim** | | |
| Class A dihydrofolate reductase | | |
| *dfrA5* | DHFRV | 568 |
| *dfrA7* | DHFRVII | 617 |
| Class B dihydrofolate reductase | | |
| *dfrB1* | DHFRIIa | 485 |
| *dfrB2* | DHFRIIb | 384 |
| **Resistance to streptothricin** | | |
| Streptothricin acetyltransferase | | |
| *sat* | SAT-2 | 584 |
| **Resistance to antiseptics and disinfectants** | | |
| Quaternary ammonium compound exporter | | |
| *qacE* | QacE | 587 |
| **Unidentified ORFs** | | |
| *orfA* | - | 501 |
| *orfC* | - | 507 |

(Table adapted and modified from Fluit & Schmitz, 1999; Partridge *et al.*, 2009)

In class 1 integrons, the gene cassettes are expressed from a common promoter region located in the 5' CS. The promoter region contains two potential promoters called P1 and P2. At least four different P1 and two different P2 promoters have been described to date, which may come in varying combination as shown in Table 2.2 (Stokes & Hall, 1989; Bunny *et al.*, 1995; Stokes *et al.*, 2001). These promoters also vary in strength. Levesque *et al.* (1994) and Collis & Hall (1995) have assessed the strength of these promoters relative to the derepressed *E. coli tac* promoter. A *tac* promoter is a functional hybrid from *trp* and *lac* promoter, which are repressed by *lac* repressor but can be derepressed by isopropyl beta-D-thiogalactoside (IPTG) (Boer *et al.*, 1983).

The strong version of the P1 promoter in integrons was found six times more effective than the *tac* promoter, but the *tac* promoter is more efficient than the weak and hybrid promoters. It is located 200 bp upstream from the cassette-encoded genes inserted at the *attI1* site (Levesque *et al.*, 1994; Collis & Hall, 1995). The P2 promoter, with a spacing of only 14 nucleotides between the -35 and -10 boxes, is frequently inactive due to this spacing that unfavourable to expression. Optimum spacing is usually approximate 17 nucleotides (Stokes & Hall, 1989; Levesque *et al.*, 1994; Collis & Hall, 1995; Peters *et al.*, 2001). Research also showed that P2 promoter can be created by insertion of three G residues that increase the spacing between potential -35 and -10 sites to the optimum 17 bp (Collis & Hall, 1995). P2 promoter has been found only in conjunction with the weakest variant of the $P_{ant}$ promoter, and in this configuration P2 is the major promoter utilised (Levesque *et al.*, 1994; Collis & Hall, 1995).

**Table 2.2: The conserved sequences of integron promoters P1 and P2 at position -35 and -10, their spacing and relative strength**

| Promoter | -35 region | -10 region | Spacing (nucleotides) | Strength |
|---|---|---|---|---|
| **P1** | TTGACA | TAAACT | 17 | Strong |
| | TGGACA | TAAGCT | 17 | Weak |
| | TGGACA | TAAACT | 17 | Hybrid 1 |
| | TTGACA | TAAGCT | 17 | Hybrid 2 |
| **P2** | TTGTTA | TACAGT | 14 | Inactive |
| | TTGTTA | TACAGT | 17 | unknown |

(Table adapted from Fluit & Schmitz, 1999)

There are several factors that can affect the expression of the gene cassettes in an integron (Ploy *et al.*, 2000a). Variations in the sequence of $P_{ant}$ can affect the level of expression of a given gene by more than 20-fold as shown by studies by Levesque *et al.* (1994) and Collis & Hall (1995). Thus, mutations and natural variants of $P_{ant}$ have been shown to have an effect on the expression of gene cassettes, as measured by the level of antibiotic resistance conferred by the cassettes encoded downstream of such promoters (Levesque *et al.*, 1994; Ploy *et al.*, 2000a). Studies of Levesque *et al.* (1994) and Collis & Hall (1995) also demonstrated that the order or position of the gene cassettes in the integrons determined the level of resistance observed. Thus, the highest level of resistance for a gene cassette is obtained when the gene cassette was located directly after the 5'CS i.e. latest cassette. As consequence, several RNA transcripts with different length are detected when various cassettes configuration are present (Collis & Hall, 1995). Apparently, premature termination of transcription occurs within the gene cassette. Analysis of transcripts originating from $P_{ant}$ suggests that the stem-loop structures formed by *attC* sites may act as transcription terminators (Levesque *et al.*, 1994; Collis & Hall, 1995; Ploy *et al.*, 2000a). However, little is currently known if *attC* sites of different lengths and sequences have different effects on the expression of downstream genes (Partridge *et al.*, 2009).

## 2.6 Pulsed field gel electrophoresis (PFGE)

PFGE analysis, which was first introduced to study the eukaryotic genome, can separate and resolve significantly larger fragments of DNA as compared to constant field gel electrophoresis (CFGE) (Herschleb *et al.*, 2007). It is now a convenient tool for the epidemiological investigation of bacterial infections (Smith & Canter, 1987). It involves embedding organisms in agarose, lysing the organisms *in situ*, and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently (Finney, 1993; Maslow *et al.*, 1993). Slices of agarose containing the chromosomal DNA fragments are subsequently inserted into the wells of an agarose gel, and the restriction fragments are resolved into a pattern of discrete bands in the gel. The DNA restriction patterns of the isolates are then compared with one another to determine their relationship (Tenover *et al.*, 1995). Criteria for interpreting chromosomal DNA macrorestriction patterns produced by PFGE have been proposed and guidelines have been applied successfully for different bacterial organisms, which are sometimes extended by phylogenetic analysis (Tenover *et al.*, 1995; Privitera *et al.*, 1998; Agodi *et al.*, 1995).

Development of PFGE has increased by two orders of magnitude the size of DNA molecules that can be routinely fractioned and analysed. PFGE pulsing routines use switching frequencies that optimise the periods in which molecules undergo size-dependent reorientation, causing zigzag traversals and enabling the separation of fragments up to 12 Mb (Levene, 1992; Herschleb *et al.*, 2007). Several types of pulsed electrophoresis effect has been introduced to a variety of instruments, including the field-inversion gel electrophoresis

(FIGE), transverse-alternating field gel electrophoresis (TAFE), contour-clamped homogeneous electric fields (CHEF), orthogonal- field alternation gel electrophoresis (OFAGE), rotating gel electrophoresis (RGE) and programmed autonomously controlled electrodes (PACE).

The CHEF Mapper system (Bio-Rad) is based on two leading technologies used, which are CHEF and PACE. Several principle variables in the system are optimised for better resolution or outcome of restriction pattern, including the electrical field strength (voltage), pulse angle, switching time, temperature, electrophoresis run time, agarose concentration, buffer composition, and restriction enzyme used. The migration rate of DNA molecules through an agarose gel depends on switch time, voltage, pulse angle, and run time (Birren *et al.*, 1988; Basim, 2001).

For epidemiological studies, subtyping of bacterial isolates from different sources provides information needed for infection control and it helps to contribute to the risk assessment of bacterial transmission (Tenover *et al.*, 1995). During the last decade, traditional methods of strain typing, such as biochemical profiles (biotyping), bacteriophage typing and serotyping, have been replaced in many laboratories with newer molecular typing methods, such as, plasmid fingerprinting (Hartstein *et al.*, 1990), ribotyping (Brisse *et al.*, 2000), randomly amplified polymorphic DNA analysis (RAPD) (Koeleman *et al.*, 1998), AFLP analysis (Dijkshoorn *et al.*, 1996), multi-locus sequence typing (MLST) (Feil *et al.*, 2000) and pulsed field gel electrophoresis (PFGE) (Bou *et al.*, 2000; Duck *et al.*, 2003). In order to trace

the source and prevent further spread of microorganisms, epidemiological investigation by the use of markers is necessary to discriminate among strains, based on the fact that bacterial isolates of the same transmission route would be clonally related (Speijer *et al.*, 1999; Botes *et al.*, 2003). Among all the methods introduced previously, PFGE is considered to be the reference method for the majority of nosocomial pathogens because of its high discriminatory ability, reproducibility, easy interpretation of banding profiles, and universal application (Versalovic & Lupski, 2002).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

The materials were purchased from the suppliers as listed out:

**Table 3.1 Materials used and their suppliers**

| Materials | Manufacturers |
|---|---|
| DNA loading dye, Oligonucleotides | AIT Biotech |
| Ampicillin | Amresco |
| Agar- agar powder, Calcium chloride | Bendosens |
| Ethidium bromide, IPTG | Bio Basic |
| API 20E bacterial identification kit, Mineral oil, Oxidase reagent | Biomerieux |
| CHEF disposable plug mold, Low melt agarose powder, Pulsed field certified agarose powder, *Saccharomyces cerevisiae* YNN295 yeast DNA size marker | Bio-Rad |
| RNase A, X-gal | Fermentas |
| Oligonucleotides | First Base |
| DMSO, Glacial acetic acid, Glycerol | Fisher Scientific |
| Absolute ethanol | HmbG chemical |
| DNA-spin$^{TM}$ Plasmid DNA Purification Kit | Intron Biotechnology |
| *Taq* Polymerase | Invitrogen |
| Luria-Bertani Agar, Luria-Bertani broth, Proteinase K, Tryptic soy broth | Merck |

Table 3.1, continued:

| | |
|---|---|
| Antibiotic discs, Mueller-Hinton agar | Oxoid |
| dNTPs mix, *E. coli* DH5α, N-lauroylsarcosine, sodium salt, pGEM®-T easy vector system, Restriction enzymes, *Taq* Polymerase, Tris base | Promega |
| QIAquick™ gel extraction kit | QIAGEN |
| EDTA | QRec Chemical |
| LE-agarose powder | SeaKem |
| Isopropanol | Systerm |
| 100 bp DNA molecular weight marker | Vivantis |

## 3.2 Stock solutions

Stock solutions required for DNA gel electrophoresis, bacterial transformation and pulsed field gel electrophoresis were prepared according to Table 3.2, Table 3.3, and Table 3.4, respectively.

**Table 3.2 Solutions used for DNA gel electrophoresis**

| Solution | Composition |
|---|---|
| 25X TAE buffer | 60.5 g of Tris base (MW: 121.1 g/mol), 14.3 ml of Glacial acetic acid and 25 ml of 0.5M EDTA (pH8.0), top up to 500 ml using sterile deionised water |

**Table 3.3 Solution for cloning**

| Solution | Composition |
|---|---|
| X-gal, 50 mg/ml | 50 mg of X-gal in 1 ml of N, N'-dimethyl-formamide (DMF) |
| 100 mM IPTG | 24 mg of IPTG powder dissolved in 1 ml water and filter sterilised |
| Ampicillin, 50 mg/ml | 50 mg of ampicillin dissolved in 1 ml of water and filter sterilised |

**Table 3.4 Solution for pulsed field gel electrophoresis (PFGE)**

| Solution | Composition |
|---|---|
| 1 M Tris, pH8.0 | 48.456 g of Tris base in 400 ml of deionised water, adjust pH to 8.0 |
| 0.5M EDTA, pH8.0 | 74.44 g of EDTA in 400 ml, adjust pH to 8.0 for a complete dissolve |
| TE buffer, pH8.0 | 10 mM Tris, 1 mM EDTA, pH8.0 |
| 10X TBE buffer | 890 mM Tris base, 890 mM Orthoboric acid, 20 mM EDTA in 1 L solution |
| Cell suspension buffer | 100 mM Tris, 100 mM EDTA, pH8.0, sterilised by autoclave |
| Cell lysis buffer | 50 mM Tris, 50 mM EDTA, pH8.0, 1% N-lauroylsarcosine, sodium salt, sterilised by autoclave |
| Proteinase K, 20 mg/ml | 20 mg of proteinase K powder dissolved in 1 ml water |

### 3.3 Sample collection and bacterial identification

A total of one hundred and forty-seven multidrug resistant *Enterobacteriaceae* and *Pseudomonas* isolates were collected from different patients hospitalised at public hospital- Hospital Raja Permaisuri Bainun Ipoh, and several private hospitals including KPJ Ipoh Specialist Hospital, Hospital Pantai Putri Ipoh, Penang Island Hospital and Gribbles Pathology Sdn. Bhd. from May 2009 to October 2010. In detail, samples from Gribbles Pathology Sdn. Bhd. were isolated from patients of the hospitals they served such as Hospital Fatimah (Ipoh, Perak), Damai Service Hospital (Jalan Ipoh, Kuala Lumpur), Lourdes Medical Centre (Jalan Ipoh, Kuala Lumpur), Putra Medical Centre (Sungai Buloh, Selangor), Sentosa Specialist Hospital (Klang, Selangor), Putra Specialist Centre (Melaka), Miri Columbia Asia (Miri, Sarawak), and Bintulu Columbia Asia (Bintulu, Sarawak).

Pertinent information regarding the demographic characteristics of the clinical isolates such as age, gender of the patients, and site of specimens were obtained from the hospitals. The isolates were collected randomly from various sites of the in-patients, and were initially isolated and identified by the microbiology department of the respective hospitals. The bacterial isolates were obtained in stab nutrient agar from the hospitals. Further identification and confirmation of bacteria was carried out using conventional biochemical test and API 20E system assisted by apiweb$^{TM}$ Identification software (Biomeriuex, France) according to manufacturer's instruction.

Prior the use of API strips, orientations test such as oxidase test, mobility test, oxidation/fermentation (OF) test and growth on a selective medium (MacConkey agar) were performed. The results were then recorded as integral part of the final profile for bacterial identification. Briefly, a young culture (18-24 h) on agar plate was removed using sterile Pasteur pipette and suspended in 5 ml of sterile deionised water. The bacterial suspension was then dispensed onto API 20E strips without bubbles formation. For citrate (CIT), Voges-Proskauer (VP), and gelatin (GEL) tests, both tube and cupule were filled with bacterial suspension. Anaerobiosis was created for arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), urease (URE) and $H_2S$ tests by overlaying the cupules with sterile mineral oil. The strip was then placed in incubation box distributed with 5 ml of sterile distilled water and incubated at 37ºC for 18-24 h. Interpretation of the strips was done by obtaining the 7-digits numerical profile. Bacteria were identified using the web-based apiweb$^{TM}$ Identification software. All bacterial strains were preserved at -70ºC as suspension containing 40% (v/v) glycerol for future use.

### 3.4 Antibiotics susceptibility determination

The antibiotic sensitivity profile of each *Enterobacteriaceae* and *Pseudomonas* sp. isolates was tested by the disc diffusion methods on Mueller-Hinton agar (Oxoid, UK) according to Clinical and Laboratory Standard Institute (CLSI) guidelines. The bacterial colony was inoculated in tryptic soy broth and allowed to grow for 4 h at 37ºC. A dilution was made on

the broth culture with sterile broth or deionised water to achieve turbidity equivalent to 0.5 McFarland Standard. The adjusted suspension was then streaked on Mueller-Hinton agar plate with sterile cotton swab and antibiotic discs were then applied. A panel of eighteen antimicrobial agents was tested in this study. The antibiotics included amikacin (AK, 30 µg), amoxicllin/clavulanic acid (AmC, 3 µg), ampicillin (AM, 10 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30 µg), cefuroxime (CXM, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), gentamicin (GN, 10 µg), imipenem (IMP, 10 µg), meropenem (MEM, 10 µg), norfloxacin (NOR, 10 µg), streptomycin (S, 10 µg), sulfamethoxazole (SMZ, 100 µg), tetracycline (TE, 30 µg), tigecycline (TGC, 15 µg), trimethoprim (TMP, 5 µg) and trimethoprim/ sulfamethoxazole (SXT, 25 µg). The zone of inhibition of bacteria was interpreted after 16-18 h of incubation time.

### 3.5 Template DNA preparation

Total DNA of isolates were extracted using fast-boil method. The microorganisms were inoculated into 5 ml of tryptic soy broth (Merck, Germany) and were incubated for 20 h at 37ºC with constant agitation. From the overnight culture, 1.5 ml of bacteria was then harvested by centrifugation at 12,000 rpm for 5 min and the pellet was resuspended in 300 µl of sterile deionised water. Next, the cells were lysed by boiling for 5 min and immediately placed on ice for 2 min. The cell debris was pelleted by centrifugation at 12,000 rpm for 2 min. The supernatant was aspirated and stored at -20ºC until subsequent PCR analysis. The concentration and purity of

extracted total DNA was then tested using Nanodrop 100 spectrophotometer (Thermo Scientific, USA).

## 3.6 Polymerase chain reaction (PCR)

### 3.6.1 16S rRNA amplification

In order to assess the quality of extracted total DNA template, primers were designed to amplify the highly conserved sequences of the bacterial 16S rRNA gene in gram-negative bacteria. The reaction mixture contained 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 250 ng/µl of template DNA and 1 U of *Taq* Polymerase (Invitrogen, USA). The reaction consisted of denaturation at 94ºC for 5 min, followed by an amplification protocol of 30 cycles at 94ºC for 1 min, 56ºC for 1 min, and 72ºC for 1 min, with final elongation at 72ºC for 7 min. Primers used are as shown in Table 3.5.

### 3.6.2 Screening of integron-encoded genes, *intI* and *sul1*

To determine the carriage of integrons in isolates, PCR screening of *intI* and *sul1* genes, commonly associated with integrons, was done in separate reactions using the published primers listed in Table 3.5. A degenerate primer pair hep35 and hep36, which hybridised to conserved region of integron-encoded integrase genes *intI1*, *intI2* and *intI3 (*White *et al.*, 2001) was used. PCR amplification were optimised in 25 µl volume of reaction mix as in 16S rRNA amplification with modification of 1.5 U *Taq* polymerase, 0.5 mM of

**Table 3.5: Primer sequences used for amplification of integrase, *sul1* and 16S rRNA genes**

| Primers | Sequences (5'→ 3') | Expected Size (bp) | References |
|---|---|---|---|
| 16S rRNA_F | GAC GTA CTC GCA GAA TAA GC | 426 | Lin *et al.*, 2008 |
| 16S rRNA_R | TTA GTC TTG CGA CCG TAC TC | | |
| Integrons_F (hep35) | TGC GGG TYA ARG ATB TKG ATT T | 491 | White *et al.*, 2001 |
| Integrons_R (hep36) | CAR CAC ATG CGT RTA RAT | | |
| *sul1*_F | ATG GTG ACG GTG TTC GGC AT | 840 | Stokes & Hall, 1989 |
| *sul1*_R | CTA GGC ATG ATC TAA CCC TC | | |

each primer and 2 µl of DMSO was added to improve the amplification efficiency of degenerate primer. The temperature profile used consist of a pre-denaturation at 94ºC for 5 min, followed by 30 cycles of 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 45 s, with final extension step at 72ºC for 10 min. The PCR products of integrase gene were further digested with *HinfI* and *RsaI* (Promega, USA) to determine the class of integrons present, as described by White *et al.* (2001) (Table 3.6).

Subsequently, the characterisation of 3' conserved region was performed by detecting the sulfonamide resistant genes (*sul1*) in all the bacterial isolates. Using the same reaction mixture as 16S rRNA amplification, *sul1* gene was amplified using the following parameters: 96ºC for 5 min, 30 cycles of 96ºC for 30 s, 60ºC for 30 s, and 70ºC for 1 min, with final elongation at 72ºC for 7 min.

All the amplified products were visualised with ethidium bromide (10 mg/ml) after electrophoresis through 1.5% agarose gel using 1X TAE running buffer, and a 100 bp ladder (Vivantis, Malaysia) was used as the molecular size marker. Specificity of each primer pair was assessed by cloning (Section 3.7) and DNA sequencing of the respective PCR products.

**Table 3.6: RFLP classification of integrase PCR product**

| PCR product | Enzyme | Number of fragments | Fragment Size (bp) |
|---|---|---|---|
| *intI1* | *RsaI* | 1 | 491 |
| | *HinfI* | 1 | 491 |
| *intI2* | *RsaI* | 2 | 334, 157 |
| | *HinfI* | 2 | 300, 191 |
| *intI3* | *RsaI* | 3 | 97, 104, 290 |
| | *HinfI* | 2 | 119, 372 |

Table adapted from White *et al.* (2001)

### 3.6.3 Characterisation of gene cassettes arrays

Following the amplification of integrase gene, characterisations of gene cassettes regions of integrons-positive bacteria isolates were carried out. Class 1 and class 2 integrons cassette regions were amplified with primer pairs of 5'-CS, 3'-CS, and hep74, hep51, respectively (Table 3.7). Besides, the primer Int2F which is specific to the 3'CS region of the integrase gene (approximately 600 bp upstream from the 5'-CS primer site) was used in combination with the 3'-CS primer to show the proximity of inserted gene cassettes to integrons. The graphical representative for the binding sites of primer pairs used to amplify integrase, *sul1*, and gene cassettes arrays in this study are shown in Figure 3.1.

Amplification of gene cassettes was carried out using 1X PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 1 U of *Taq* DNA polymerase and 50 ng/µl of template DNA. The temperature profile was: denaturation at 94ºC for 5 min, followed by 30 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 90 s, final elongation time was 72ºC for 10 min.

Subsequently, PCR-RFLP was performed in order to determine whether the different isolates carried identical gene cassettes. The cassette PCR products were digested with *HinfI* enzyme. Amplicons with the same RFLP pattern were deemed to contain the same gene cassettes. One or two cassette amplicons representative from each distinct restriction digest profiles were purified by using QIAquick™ gel extraction kit (QIAGEN, Germany) or ethanol precipitation, prior to cloning and DNA characterisation (Section 3.7).

**Table 3.7: Oligonucleotide primers used for gene cassettes amplification**

| Primers | Sequences (5'→3') | Expected size (bp) | References |
|---|---|---|---|
| 5'-CS | GGC ATC CAA GCA GCA AG | Variable | Levesque *et al.*, 1995 |
| 3'-CS | AAG CAG ACT TGA CCT GA | | |
| hep74 | CGG GAT CCC GGA CGG CAT GCA CGA TTT GTA | Variable | White *et al.*, 2001 |
| hep51 | GAT GCC ATC GCA AGT ACG AG | | |
| Int2F | CTC GGG TAA CAT CAA GG | - | Mukherjee & Chakraborty, 2006 |

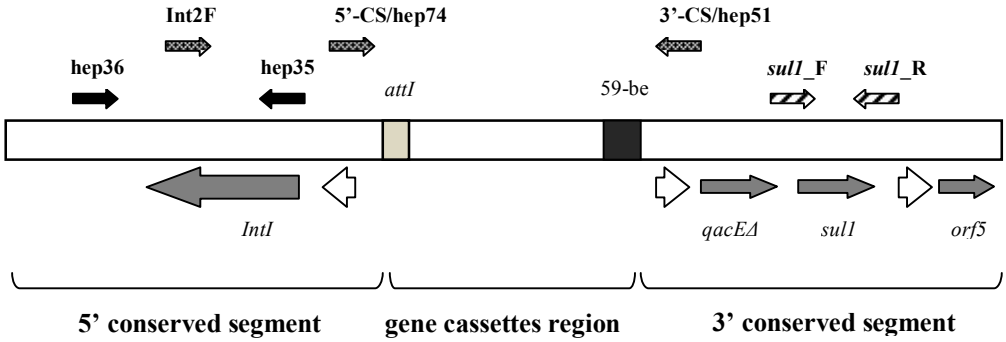



**Figure 3.1: Graphical representation of the binding position for oligonucleotides primers used in this study to amplify integrons' structural genes.** All of the primer pairs used in this study are showed by arrows with patterns. Promoter sites are represented by white arrowheads. Grey arrows represent the encoding genes located within the 5' and 3' conserved segments. Of these, *intI*, encoding an integrase, 3'CS contains antiseptic resistance gene (*qacEΔ1*), a sulfonamide resistance gene (*sul1*) and an open reading frame (*orf5*). The recombination sites and 59 base elements are presented as *attI* and 59-be.

## 3.7 Cloning of PCR products and DNA sequencing

### 3.7.1 Ligation of PCR product to pGEM®-T Easy vector

Purified PCR products were ligated into pGEM®-T Easy Vector System (Promega) according to manufacturer instruction. The ligation mixture of 5 µl of 2X Rapid Ligation buffer, 3 µl of PCR products, 1 µl of pGEM®-T easy vector (50 ng) and 1 µl of T4 DNA ligase (3 U/µl) were incubated overnight at 4ºC. Transformation was then carried out using *Escherichia coli* strain DH5α competent cells (Section 3.7.2).

### 3.7.2 Preparation of competent cells

A single colony of *Escherichia coli* strain DH5α was inoculated into 5 ml of LB broth and incubated overnight at 37ºC, with agitation 200 rpm. Next, 500 µl of the overnight culture was transferred into 25 ml of fresh LB broth and was further grown until it reached optical density (OD) between 0.5-0.6 at wavelength 600 nm. The cells were then pelleted by centrifugation at 6000 *g* for 10 min at 4ºC and resuspended in 2-3 ml of 0.1 M $CaCl_2$ before it was left on ice for 2 h and used for the subsequent transformation process. For long term storage, competent cells were mixed with equal volume of 40% (v/v) sterile glycerol and aliquoted into 1 ml sterile microcentrifuge tubes prior to storage at -70ºC.

### 3.7.3 Transformation of competent cells

In a sterile 1.5 ml microcentrifuge tube, 3-5 µl of ligation mixture was gently mixed with 200 µl of competent cells and incubated on ice for 1 h. A positive and a negative control were included by transforming 200 µl of competent cells with or without pUC19 plasmid, respectively. The mixture was then subjected to heat-shock at 42ºC for exactly 90 s. The tubes were immediately placed on ice for additional 5 min. Eight-hundred µl of LB broth was then added to the tube and the mixture was incubated at 37ºC for 90 min with constant agitation at 150 rpm. Subsequently, cells were pelleted at 6000 *g* for 10 min and resuspended in 100 µl of fresh LB broth. For blue-white screening of transformants, the cells were plated onto LB agar plates pre-spread with 50 µg/ml of ampicillin, 50 µl of IPTG (100 mM), and 20 µl of X-gal (50 mg/ml). Lastly, the plates were incubated overnight at 37ºC.

### 3.7.4 Colony PCR and recombinant plasmid isolation

Following transformation, the positive tranformants were screened by PCR amplification of the inserts using specific primers (SP6 and T7 primers). Briefly, a single white colony was picked up with sterile culture needle and inoculated into a 25 µl of PCR mixture containing: 5 µl of 5X PCR buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of dNTPs (10 mM), 1 µl of each SP6 and T7 primers (10 µM each) and 0.2 µl of *Taq* DNA polymerase (5 U/µl). The PCR mixture was then subjected to amplification using temperature profile consisting of an initial denaturation step at 96ºC for 3 min, followed by 30 cycles of 96ºC for 30 s, annealing at 50ºC for 15 s, and 60ºC for 2.5 min, with

final elongation at 60ºC for 5 min. Finally, the PCR product was assessed in 1.5% agarose gel.

To isolate the recombinant plasmid DNA, DNA-spin$^{TM}$ Plasmid DNA Purification Kit (Intron Biotech) was used according to manufacturer instruction. The efficiency of purification and the concentration of plasmid DNA were assessed by measuring its OD at 260 nm and 280 nm using Nanodrop 100 spectrophotometer (Thermo Scientific, USA).

### 3.7.5 Restriction endonuclease digestion of recombinant plasmid

For the final assessment on size of inserts, the purified recombinant plasmid was digested using *EcoRI*, in which the cloned inserts were released from pGEM®-T Easy vector. Restriction enzyme digestion was carried out as recommended by supplier in a total volume of 10 µl which consists of 1 µl of 10X Restriction enzyme buffer (Buffer H), 0.1 µl of bovine serum albumin (BSA), 0.5 µl of purified plasmid, 0.25 µl *EcoRI* enzyme (12 U/µl) and 8.15 µl of sterile deionised water. The reaction was terminated through incubation at 65ºC for 10 min and the results were assessed by gel electrophoresis.

### 3.7.6 DNA sequencing

The isolated recombinant plasmid was outsourced to First Base Sequencing Laboratory (Malaysia) for the DNA sequencing process. Sequencing reactions

were performed with the universal T7 and Sp6 primers. If the insert size was too large (e.g. gene cassettes) or the sequences obtained from forward and reverse sequencing did not overlap, primer walking was performed by designing new primers for further sequencing reactions.

### 3.8 Computer analysis of sequence data

In this study, alignment of raw sequence data was done using Molecular evolutionary genetics analysis (MEGA) software, version 4 (Tamura *et al.*, 2007) to manually remove the ambiguous regions. The nucleotide sequence analysis was then performed with BLAST-N program available at the homepage of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/).

### 3.9 Statistical analysis

The data were processed and analysed by using the Statistical Package for the Social Sciences (SPSS) software (version 17) for windows. *Chi*-square test was used for analysis on categorical variables and Mann-Whitney U test to analyse continuous variables. All *p*-values were based on 2-tailed tests of significance with $p < 0.05$ is considered statistically significant.

### 3.10 Pulsed field gel electrophoresis (PFGE)

In this study, PFGE was carried out using a contour-clamped homogeneous-field apparatus (CHEF Mapper® XA system, Bio-Rad Laboratories). The protocol used was based on the standardised PulseNet PFGE protocol referencing Centers for Disease Control and Prevention (CDC, USA) with slight modification for self-optimised condition.

#### 3.10.1 Preparation of plug from agar culture

Before the preparation of plug, bacteria culture was streaked on a non-selective medium- tryptic soy agar to obtain single colonies. The plates were incubated at 37ºC for 14-18 h. On the next day, 2% of low-melting point (LMP) agarose (Bio-Rad, USA) was prepared in TE buffer (pH 8.0) and left standing in a water bath set to 55ºC prior to usage. One ml of cell suspension was made from the colonies grown on the agar plates using cotton swab and Cell Suspension buffer to eliminate aerosols and the clumping of cells. The concentration of cells was adjusted to obtain an OD of 1.3-1.4 at wavelength 610 nm. The cell suspension was then placed on ice if more samples were to be processed.

#### 3.10.2 Casting of plug

A disposable plug mold (Bio-Rad, USA) was used for making a agarose plug. An amount of 200 µl of adjusted cell suspension was pre-warmed to 37ºC and transferred into labelled 1.5 ml microcentrifuge tubes. Subsequently, 20 µl of

proteinase K (20 mg/ml stock) and 200 µl of melted 2% LMP agarose (55ºC) was added into tubes and gently resuspended. The mixture was then dispensed immediately into the wells of the plug mold and allowed to solidify at room temperature for 10-15 min or at 4ºC for 5 min.

### 3.10.3 Lysis of cells in agarose plug

Cell lysis/Proteinase K buffer was prepared consisting of 5 ml of Cell lysis buffer and 25 µl of Proteinase K solution (20 mg/ml) for one plug sample. The final concentration of Proteinase K in lysis buffer is 0.1 mg/ml, compared to 0.5 mg/ml added into the cell suspension previously. After mixing, Cell lysis/Proteinase K buffer was transferred into clean 50 ml polypropylene screw-cap tubes. The solidified agarose plug was then pushed out of the mold into appropriate labelled tube and incubated at 54ºC for 2 h with constant agitation at 150-175 rpm.

### 3.10.4 Washing of plug

After cell lysis, the buffer was carefully decanted. Next, 10-15 ml of sterile deionised water pre-heated to 50ºC was added to each tube and incubated at 50ºC for 10-15 min with constant shaking. This step was repeated once. The plugs were then washed with pre-heated (50ºC) sterile TE buffer, pH8.0, for four times at 50ºC. Subsequently, the plugs were ready for restriction digestion (Section 3.10.5) or stored in 1 ml fresh TE buffer at 4ºC until needed.

### 3.10.5 Restriction digestion of DNA in agarose plug

In this study, PFGE patterns were obtained by subjecting the plug to restriction digestion with the enzyme, *XbaI*. The plug was sliced into 10 mm x 2 mm width with sterile razor blade and transferred into 200 µl of restriction digestion mixture which contained 20 µl of 10X restriction enzyme buffer (Buffer D), 2 µl of BSA, 40 U of *XbaI* enzyme in a final volume of 200 µl. The mixture was then incubated at 37ºC for 3 h, as recommended by manufacturer. The rest of the plug was stored in 1 ml of TE buffer and kept at 4ºC.

### 3.10.6 Casting of agarose gel

Before gel loading, 2.2 L of 0.5X TBE was freshly prepared in the pulsed field gel electrophoresis chamber and pre-chilled to 14ºC. One hundred ml of 1% pulsed field certified agarose (Bio-Rad, USA) was prepared in 0.5X TBE buffer and kept in 50-60ºC water bath prior use. The restrict digested plug slices were removed from tubes and carefully loaded onto the comb teeth. *Saccharomyces cerevisiae* YNN295 yeast DNA size marker (Bio-Rad, USA) was loaded on the first and last lane on the comb. The excess buffer on the plug was removed with Kleenex tissue and plug slices were allowed to air-dry on comb for 5 min. Next, pulsed field certified agarose was poured into the gel cast and allowed to harden for at least 45 min.

### 3.10.7 Gel electrophoresis running condition

After gel preparation, the gel was placed in the casting frame in the chamber covered with 0.5X TBE buffer. The flow rate was maintained at ~1 L/min (a setting of ~70 on the pump regulator) to equilibrate buffer at 14ºC. For PFGE, gel electrophoresis variables such as voltage, pulse angle, initial and final switching times, running time and other were optimised. A two-state mode was used with optimised electrophoresis variables to achieve the best DNA separation for different bacterial species as stated in Table 3.8.

### 3.10.8 Staining and gel documentation

After the electrophoresis run, the gel was stained with ethidium bromide (10 mg/ml) for 20-30 min and destained for 30min in approximately 500 ml of distilled water before visualizing the bands using MultiDoc-It™ Imaging System (UVP, LLC$^{TM}$). The DNA fingerprints generated by PFGE were analysed with both manual method according to the criteria proposed by Tenover *et al.* (1995) and digitalised method by BioNumerics Fingerprint types and Cluster Analysis software (Applied Maths, USA).

Dendrograms of similarity were calculated based on the Dice coefficient and clustering by the unweighted paired group method using arithmetic averages (UPGMA), allowing for 1% tolerance in band positions. PFGE patterns of isolates with similarity of 80% or higher, were assigned to the same strain.

**Table 3.8: Parameters used for each bacterial species in PFGE**

Similar running voltage of 6 V/cm and switching angle of 120º was used for all the bacterial species.

| Bacterial species | Enzyme | Initial switch time (s) | Final switch time (s) | Run time (h) |
|---|---|---|---|---|
| *Escherichia coli* | *XbaI* | 2.2 | 44.7 | 20.00 |
| *Klebsiella pneumoniae* | *XbaI* | 2.2 | 63.8 | 20.00 |
| *Pseudomonas aeruginosa* | *XbaI* | 0.47 | 26.29 | 20.18 |

# CHAPTER 4

# RESULTS

## 4.1 Clinical and demographic data of patients

### 4.1.1 Bacteria isolates

As mentioned in Section 3.3, a hundred and forty-seven clinical isolates of multidrug resistant (MDR) *Enterobacteriaceae* and *Pseudomonads* were collected from Hospital Raja Permaisuri Bainun Ipoh, KPJ Ipoh Specialist Hospital, Hospital Pantai Putri Ipoh and Gribbles Pathology Sdn. Bhd. The detailed information of the isolates is listed in Appendix A.

Upon collection of isolates, bacterial identification was carried out by standard biological assays and bacterial identification kit, API 20E system. To provide preliminary identification of microbes, gram-negative *Enterobacteriaceae* and *Pseudomonas* were grown on differential MacConkey media (Figure 4.1A) and confirmed through gram staining in which they were stained pink. They also showed no formation of cytochrome c oxidase in oxidase test. The bacteria were then subjected to twenty reaction tests on the API 20E strip. Supplementary test such as oxidation/fermentation and microbial mobility were performed, as shown in Figure 4.1(B) and (C). Figure 4.2 shows the colour changes of cupules of API 20E strip; which were read as a seven-digit code according to reading table provided by the manufacturer. The genus and species of the bacteria were interpreted by using apiweb$^{TM}$ Identification software (Biomerieux, France) (Appendix B).

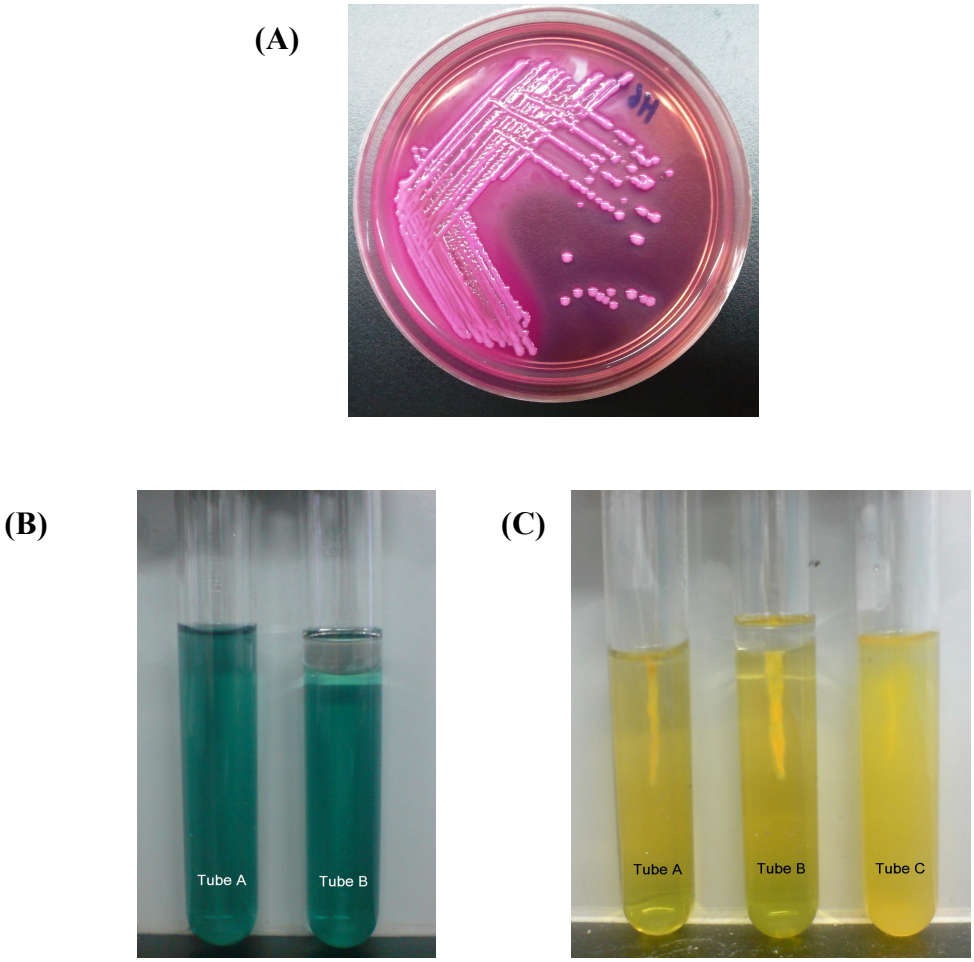



**Figure 4.1: Biochemical assays used in this study**. (A) MacConkey agar streaked with *K. pneumonia* represented one of the members in *Enterobacteriaceae* bacteria family. Only gram-negative bacteria would grow on this selective media with pink colonies formation. (B) Oxidation/Fermentation (OF) media added with glucose before bacteria inoculation. Tube A represents OF media in aerobic condition, while Tube B contained OF media sealed with a layer of sterile mineral oil to create anaerobic environment. (C) Oxidation/Fermentation (OF) media after 72 h incubation. Acidity is shown in both Tube A and B through the yellow colour media formation. Tube C indicates positive result for microbial mobility in aerobic condition in which cloudiness is observed around inoculated bacteria.


**Figure 4.2: API 20E strips with twenty reaction tests**. (A) Negative control of API 20E strips before incubation. Others are API 20E strips result identification for (B) *Klebsiella pneumoniae* (C) *Escherichia coli* (D) *Morganella morganii* and (E) *Proteus mirabilis*

Out of all the clinical isolates, forty isolates were classified under *Klebsiella* genus which comprised mostly of *K. pneumoniae*, *K. oxytoca* and *K. ozanae*. *E. coli* (n= 34) and *Enterobacter* sp. (n= 33) were also highly prevalent among the multidrug resistant isolates in this study. Other clinical isolates obtained include *Pseudomonas* sp. (n= 15), *Serratia* sp. (n= 9), *Providencia* sp. and *Morganella* sp. (n= 5), as presented in Figure 4.3.

### 4.1.2 Site of specimens, Gender, and Age of patients

A number of bacterial samples collected from the microbiological laboratory of hospitals were obtained from various sampling sites such as pus swab (n= 31), normal swab (n= 27), urine (n= 26), wound swabs (n= 12), sputum (n= 11), blood (n= 9), tissue (n= 7), other specimens (n= 20) like bone, endotracheal tubes (ETT), tracheal aspirate, and other sites as represented in Figure 4.4. The nasal, ear, throat swab were grouped as normal swab, for the ease of data analysis.

The gender of patients is summarised in Figure 4.5(A), which suggests that male patients (n= 84, 57%) had higher prevalence in carrying MDR bacteria than female patients (n= 63, 43%). Besides, the distribution of age of the patients amongst the hundred and forty-seven clinical isolates is as reviewed in Figure 4.5(B). The patient's mean age was at 53 ± 20 years old. In addition to this, the patient's age was divided into 5 groups, mainly: 19 and below, 20-39 years old, 40-59 years old, 60-79 years old, and 80 years and above. A dramatic increase in the number of isolates was observed in patients aged

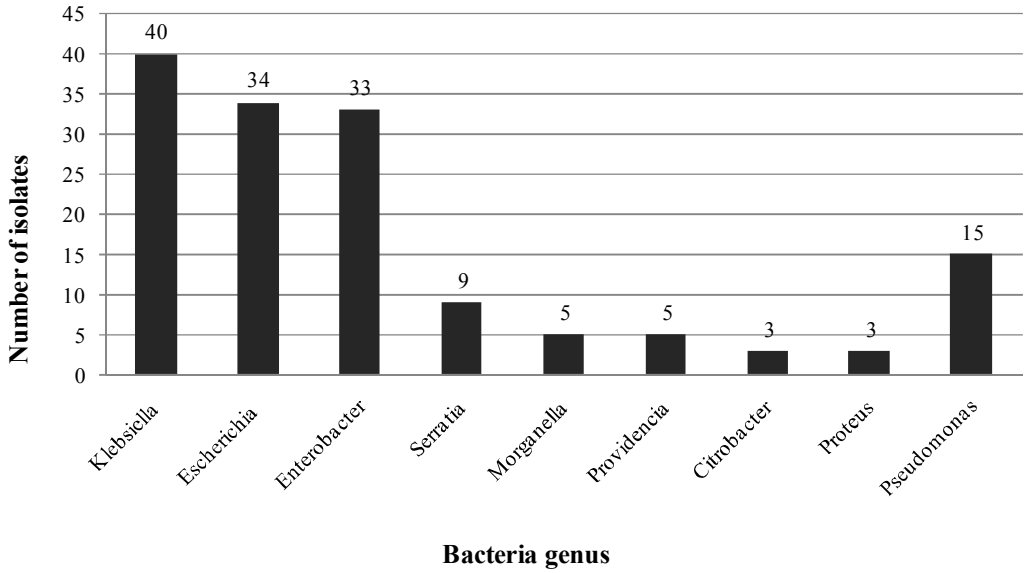



**Figure 4.3: Frequencies of clinical isolates of *Enterobacteriaceae* family and *Pseudomonas* collected from period May 2009 to October 2010.** *Klebsiella* sp. (n= 40) has the highest prevalence among the collected multidrug resistant isolates, followed by *Escherichia* (n= 34), *Enterobacter* sp. (n= 33), *Pseudomonas* sp. (n= 15), *Serratia* sp. (n= 9) and others as indicated above.

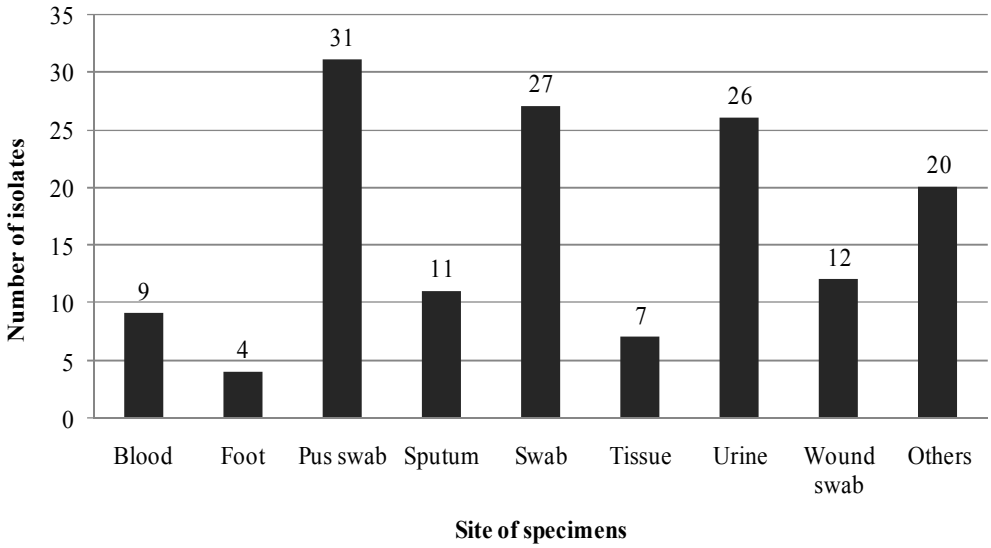



**Figure 4.4: Graph of number of isolates versus site of specimens.** Most of the bacteria isolates were obtained from pus swab (n= 31), normal swab (n= 27) and urine (n= 26). "Others" in the chart indicates sampling sites from bone, endotracheal tubes (ETT), tracheal aspirate and more.

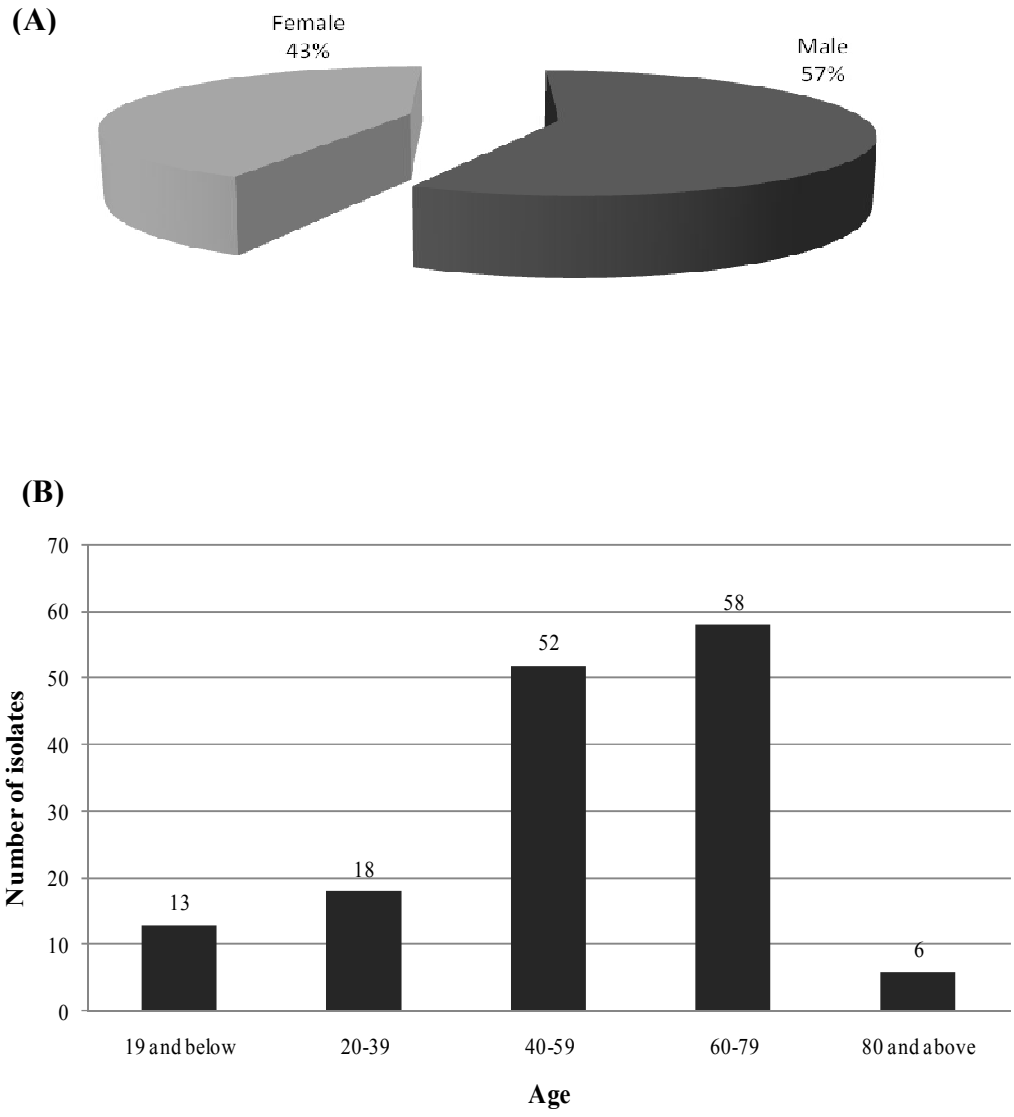


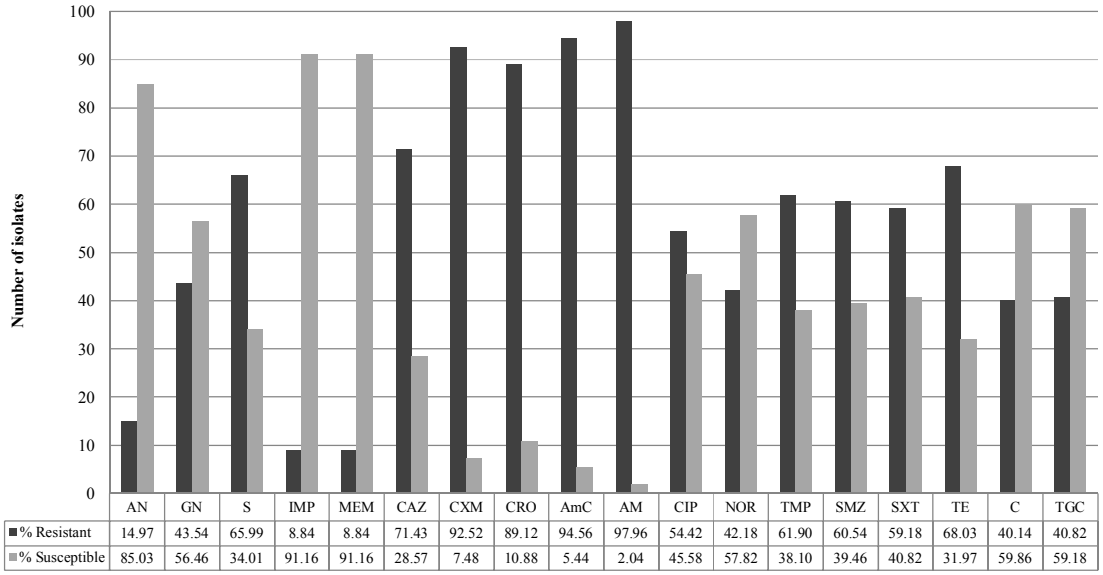

**Figure 4.5: Patient demographic data according to (A) gender and (B) age group.** As shown in (A), 57% were male patients (n= 84) while 43% were female patients (n= 63). In (B), the age group of 60-79 possesed the highest number of isolates (n= 58), followed by 40-59 years old, 20-39 years old, 19 and below, and lastly 80 above. Mean ± SD of patient's age is 53 ± 20 years old.

39 years old and above. The age category of 60-79 years old (n= 58, 39.5%) and 40-59 years old (n= 52, 35.4%) also exhibited high occurrence of carrying MDR isolates. Interestingly, the least number of MDR samples were isolated from patients aged above 80 years old.

## 4.2 Antimicrobial resistance profile

Figure 4.6 displays the graphical analysis on antibiotic susceptibility profiles on one hundred and forty-seven clinical isolates. The eighteen antimicrobial agents used for susceptibility testing were chosen to encompass different antibiotic classes and/or based on resistance genes associated with integrons. Among the isolates, resistance was most often observed towards ampicillin (97.96%), followed by augmentin (94.56%), cefuroxime (92.52%), and ceftriaxone (89.12%). On the other hand, high levels of susceptibility were observed towards antibiotics such as imipenem and meropenem (91.16%), as well as amikacin (85.03%).

Overall, bacterial resistance were observed mostly towards penicillin and cephalosporin groups, but to a lesser extent towards the carbapenems and aminoglycosides antibiotic groups, except streptomycin. Up to 65.99% of the isolates in this study were streptomycin resistant. High levels of resistance of more than 50% were also observed toward tetracycline (68.03%), sulfonamide antibiotics group (60%), and ciprofloxacin (52.42%). Alarmingly, 40.82% of the isolates were observed to be resistant towards the newly synthesised antibiotic group- tigecycline.




| | AN | GN | S | IMP | MEM | CAZ | CXM | CRO | AmC | AM | CIP | NOR | TMP | SMZ | SXT | TE | C | TGC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % Resistant | 14.97 | 43.54 | 65.99 | 8.84 | 8.84 | 71.43 | 92.52 | 89.12 | 94.56 | 97.96 | 54.42 | 42.18 | 61.90 | 60.54 | 59.18 | 68.03 | 40.14 | 40.82 |
| % Susceptible | 85.03 | 56.46 | 34.01 | 91.16 | 91.16 | 28.57 | 7.48 | 10.88 | 5.44 | 2.04 | 45.58 | 57.82 | 38.10 | 39.46 | 40.82 | 31.97 | 59.86 | 59.18 |

**Antibiotics**

**Figure 4.6: Antibiotics susceptibility profiles analysis towards eighteen antibiotics on all one hundred forty-seven isolates.** The percentage of resistance and susceptibility among the 147 clinical isolates are indicated as black and grey bars, respectively. AK, amikacin; GN, gentamicin; S, streptomycin; IMP, imipenem; MEM, meropenem; CAZ, ceftazidime; CXM, cefuroxime; CRO, ceftriaxone; AM, ampicillin; AmC, amoxicillin/ clavulanic acid (Augmentin); CIP, ciprofloxacin; NOR, norfloxacin; TMP, trimethoprim; SMZ, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline; C, chloramphenicol; and TGC, tigecycline.

This study also revealed that nearly 98% of the isolates were resistant to three or more antimicrobial agents, in which 31.3% (46 of 147) of samples were resistant to seven to twelve antibiotics, and 38.1% of them (56 of 147) were resistant to more than twelve types of antimicrobial agents. The details of the antibiotic resistant profile for each clinical isolates are shown in Appendix C.

### 4.3 DNA integrity testing by PCR amplification of 16S rRNA from the bacteria isolates

In this study, 16S rRNA gene were amplified using specific primer (Table 3.3) and it was used as a positive PCR control to ensure the integrity of total DNA extracted from all samples by fast-boil method. If no amplification of the 16S rRNA gene was observed, total DNA would be reextracted and the amplification was repeated in order to reevaluate the total DNA integrity. This conserved primer successfully amplified PCR product with the expected size of 426 bp from various bacterial genus, as shown in Figure 4.7. The specificity of primer was then assessed by cloning one of the PCR products in pGEM-T easy vector and sequencing. DNA sequencing result showed a 99% homology to *Proteus mirabilis* 16S rRNA in the GenBank, and thus confirming the specificity of the primer used (Figure 4.8).

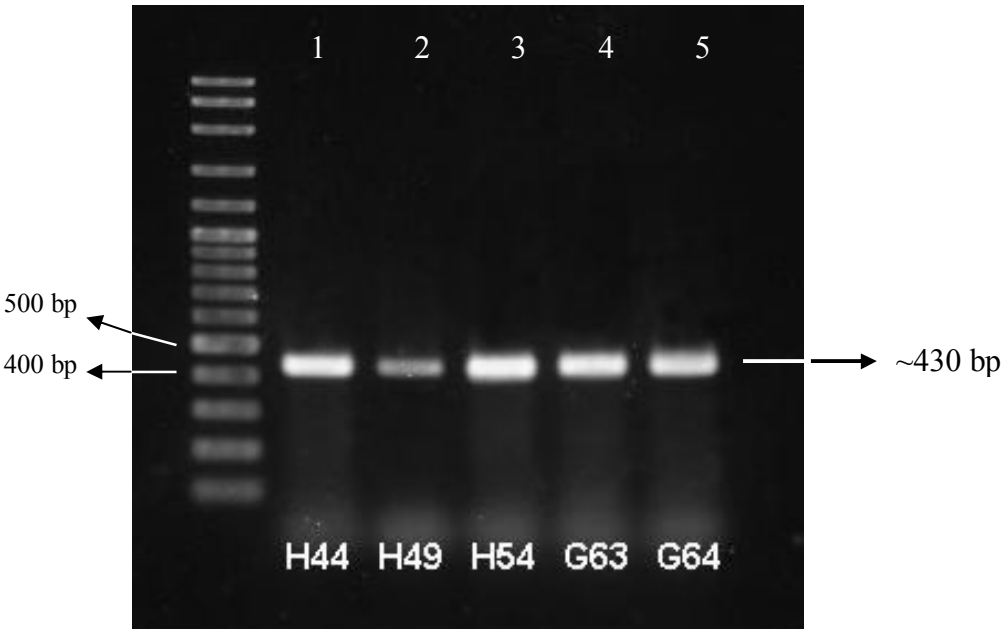



**Figure 4.7: Gel image represents amplification of 16S rRNA gene.** Each lane indicates amplification of PCR products of 426 bp from different bacteria genus as represented by the sample's name, i.e. lane 1: *Enterobacter* sp. (H44), lane 2: *Klebsiella* sp. (H49), lane 3: *E. coli* (H54), lane 4: *Citrobacter* sp. (G63) and lane 5: *Morganella* sp. (G64). Molecular marker used was 100 bp DNA ladder.

```
> gb|JF947362.1| Proteus mirabilis strain 2115 16S ribosomal RNA gene,
partial sequence
Length=1454

 Score = 756 bits (409), Expect = 0.0
 Identities = 418/422 (99%), Gaps = 1/422 (0%)
 Strand=Plus/Plus

Query  1    GACG-TACTCGCAGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG  59
            |||| ||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  433  GACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG  492

Query  60   GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCA  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  493  GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCA  552

Query  120  GATGTGAAAGCCCCGAGCTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTCTT  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  553  GATGTGAAAGCCCCGAGCTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTCTT  612

Query  180  GTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACC  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  613  GTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACC  672

Query  240  GGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCA  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  673  GGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCA  732

Query  300  AACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTAGAGGTTGTGGT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  733  AACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTAGAGGTTGTGGT  792

Query  360  CTTGAACCGTGGCTTCTGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGTCGCA  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  793  CTTGAACCGTGGCTTCTGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCA  852

Query  420  AG  421
            ||
Sbjct  853  AG  854
```

**Figure 4.8: BLASTN DNA sequencing result of purified 16S rRNA PCR products aligned with *Proteus mirabilis* 16S rRNA from GenBank (Accession Number JF947362.1)**

## 4.4 Integrons carriage in isolates

The presence of integrons within all collected isolates was ascertained by PCR detection of integrase gene. Degenerate primer pair hep35 and hep36 was used to amplify conserved region of integrase genes *intI1*, *intI2* and *intI3*. The optimised PCR condition used in this study effectively produced amplicons with the expected size of 491 bp. Alignment of the sequences acquired showed 99% homology with the published sequences in GenBank which corresponded with integrons associated *intI1* and *intI2* gene (as shown in Figure 4.9 and 4.10) and thus, proving the specificity of the PCR amplification using the degenerate primers in amplifying the class 1 and 2 integrase.

PCR amplification of integrase gene showed that seventy-one (48.3%) of the isolates were integron-positive (Appendix D). The class of integron was further determined by analysing integrase PCR products through RFLP following digestion using *HinfI* and *RsaI* (Figure 4.11). The restriction analysis revealed that 94.4% of isolates (67/71) contained a class 1 integrons. Furthermore, 4.2% (3/71) of isolates were found to contain a class 2 integron, while only one isolate harboured both class 1 and 2 integrons. No class 3 integrons was detected.

Among all isolates tested, a high proportion of integrons was detected in 82.5% of *Klebsiella* sp. (33/40), followed by 61.7% of *E. coli* (21/34), 60% of *P. aeruginosa* (9/15), and *Morganella* sp. (3/5), 33.3% of *Citrobacter* sp. and *Proteus* sp. (1/3), and lastly, 9.1% (3/33) of *Enterobacter* sp. isolates (Table 4.1). No integrons were detected in *Providencia* and *Serratia* isolates.

```
>gb|AEH26333.1|  integrase IntI1 [Klebsiella pneumoniae]
Length=337

 Score = 337 bits (863), Expect = 2e-115
 Identities = 161/163 (99%), Positives = 163/163 (100%), Gaps = 0/163
(0%)
 Frame = -3

Query  489  RVKDVDFDHGTIIVREGKGSKDRALMLPESLAPSLREQLSRARAWWLRDQAEGRSGVALP  310
            RVKD+DFDHGTIIVREGKGSKDRALMLPESLAPSLREQLSRARAWWL+DQAEGRSGVALP
Sbjct  154  RVKDLDFDHGTIIVREGKGSKDRALMLPESLAPSLREQLSRARAWWLKDQAEGRSGVALP  213

Query  309  DALERKYPRAGHSWPWFWVFAQHTHSTDPRSGVVRRHHMYDQTFQRAFKRAVEQAGITKP  130
            DALERKYPRAGHSWPWFWVFAQHTHSTDPRSGVVRRHHMYDQTFQRAFKRAVEQAGITKP
Sbjct  214  DALERKYPRAGHSWPWFWVFAQHTHSTDPRSGVVRRHHMYDQTFQRAFKRAVEQAGITKP  273

Query  129  ATPHTLRHSFATALLRSGYDIRTVQDLLGHSDVSTTMIYTHVL  1
            ATPHTLRHSFATALLRSGYDIRTVQDLLGHSDVSTTMIYTHVL
Sbjct  274  ATPHTLRHSFATALLRSGYDIRTVQDLLGHSDVSTTMIYTHVL  316
```

**Figure 4.9: BLAST-X result of purified integrase PCR products aligned with class 1 integrase from GenBank (Accession Number AEH26333.1)**

```
>dbj|BAB12601.1|  intI2 [Escherichia coli]
 gb|AAT72891.1|  IntI2 [Shigella sonnei]
 gb|ADH82143.1|  IntI2 [Klebsiella pneumoniae]
 gb|ADH82148.1|  IntI2 [Klebsiella pneumoniae]
 gb|ADH82153.1|  IntI2 [Escherichia coli]

Length=325

Score = 332 bits (852), Expect = 6e-114
Identities = 161/163 (99%), Positives = 161/163 (99%), Gaps = 0/163
(0%)
Frame = +3

Query  3    RVKDLDFDNGCITVHDGKGGKSRNSLLPTRLIPAIK*LIEQARLIQQDDNLQGVGPSLPF  182
            RVKD DFDNGCITVHDGKGGKSRNSLLPTRLIPAIK LIEQARLIQQDDNLQGVGPSLPF
Sbjct  143  RVKDFDFDNGCITVHDGKGGKSRNSLLPTRLIPAIKXLIEQARLIQQDDNLQGVGPSLPF  202

Query  183  ALDHKYPSAYRQAAWMFVFPSSTLCNHPYNGKLCRHHLHDSVARKALKAAVQKAGIVSKR  362
            ALDHKYPSAYRQAAWMFVFPSSTLCNHPYNGKLCRHHLHDSVARKALKAAVQKAGIVSKR
Sbjct  203  ALDHKYPSAYRQAAWMFVFPSSTLCNHPYNGKLCRHHLHDSVARKALKAAVQKAGIVSKR  262

Query  363  VTCHTFRHSFATHLLQAGRDIRTVQELLGHNDVKTTQIYTHVL  491
            VTCHTFRHSFATHLLQAGRDIRTVQELLGHNDVKTTQIYTHVL
Sbjct  263  VTCHTFRHSFATHLLQAGRDIRTVQELLGHNDVKTTQIYTHVL  305
```

**Figure 4.10: BLAST-X result of purified integrase PCR products aligned with class 2 integrase from GenBank (Accession Number BAB12601.1, AAT72891.1, ADH82143.1, ADH82148.1 and ADH82153.1)**

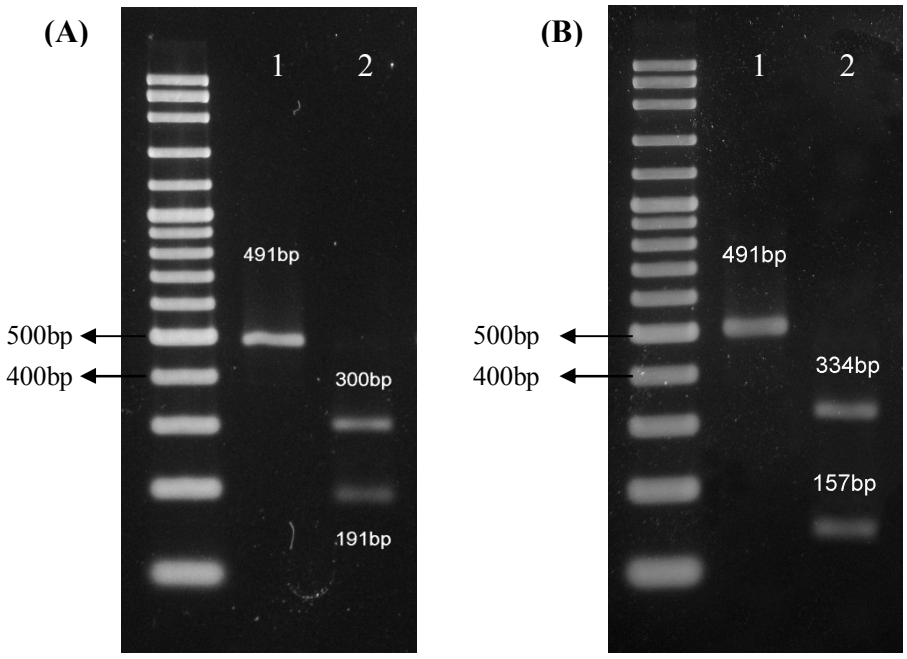



**Figure 4.11: PCR-RFLP gel image digestion on integrase amplified product by the restriction enzymes, (A) *HinfI* and (B) *RsaI.*** (A) Lane 1 and 2 indicate class 1 integrase and class 2 integrase when digested with *HinfI* respectively. Class 1 integrase was determined when no digestion was made, while class 2 integrase produced 300 bp and 191 bp products. (B) Class 1 integrase were not digested by *RsaI* (lane 1); class 2 integrase product was fragmented into 334 bp and 157 bp by *RsaI* (lane 2). Ladder used 100 bp DNA molecular marker (Vivantis, Malaysia).

**Table 4.1: Carriage of integrons by clinical isolates belonging to different bacterial species of *Enterobacteriaceae* and *Pseudomonas* sp.**

| Species | Total samples | Number of isolates carrying integrons | | | |
|---|---|---|---|---|---|
| | | Total | Class 1 | Class 1 and 2 | Class 2 |
| ***Citrobacter* sp.** | 3 | 1 | 1 | | |
| ***Enterobacter* sp.** | 33 | 3 | 2 | | 1 |
| ***Escherichia coli*** | 34 | 21 | 19 | | 2 |
| ***Klebsiella* sp.** | 40 | 33 | 32 | 1 | |
| ***Morganella* sp.** | 5 | 3 | 3 | | |
| ***Proteus* sp.** | 3 | 1 | 1 | | |
| ***Providencia* sp.** | 5 | - | | | |
| ***Pseudomonas* sp.** | 15 | 9 | 9 | | |
| ***Serratia* sp.** | 9 | - | | | |
| **Total** | 147 | 71 | 67 | 1 | 3 |

In this study, *K. pneumoniae* was shown to be the most dominant species to carry a class 1 integrons with 32 out of 67 (47.8%) class 1 integrons identified and followed by *E. coli* (28.4%, 19/67). In other species, the incidence of class 1 integrons carriage ranged from 1.49% (1/67) in *Citrobacter* sp. and *Proteus* sp. to 13.4% (9/67) in *P. aeruginosa*. Only one *K. pneumoniae* isolate carried both class 1 and 2 integrases, while two *E. coli* and one *Enterobacter cloacae* carried a single class 2 integrons.

### 4.5 Association between antimicrobial susceptibility and integrons carriage

In order to assess the effect of integrons carriage on antimicrobial susceptibility profile, the percentage of antibiotic resistance and susceptibility among integron-positive and integron-negative isolates were compared. The statistical significance *p*-value was calculated using Pearson $\chi^2$ test. The antibiotic resistant profile was deemed significantly associated with the presence of integrons as *p*-value was less than 0.05. Fisher exact test was used when at least one cell of the contingency table has an expected cell count smaller than 5.

As shown in Table 4.2, integrons were more commonly and significantly associated with the antibiotics ciprofloxacin, norfloxacin, tetracycline, chloramphenicol, and tigecycline ($p< 0.05$). Similarly, significant association was also found in sulfamethoxazole, trimethoprim, and co-trimoxazole.

**Table 4.2: Association between antimicrobial resistance profiles of isolates to integrons carriage**

| Antibiotics | Integron-positive isolates (N=71) | Integron-negative isolates (N= 76) | $\chi^2$ | *p*-value[a] |
|---|---|---|---|---|
| | Resistant, n (%) | Resistant, n (%) | | |
| Aminoglycosides | | | | |
| Amikacin | 14 (19.7) | 8 (10.5) | 2.437 | 0.118 |
| Gentamicin | 48 (67.6) | 16 (21.1) | 32.361 | **<0.001** |
| Streptomycin | 66 (93) | 31 (40.8) | 44.510 | **<0.001** |
| Carbapenem | | | | |
| Imipenem | 9 (12.7) | 4 (5.3) | 2.502 | 0.114 |
| Meropenem | 10 (14.1) | 3 (3.9) | 4.679 | **0.031** |
| Cephalosporins | | | | |
| Ceftazidime | 60 (84.5) | 45 (59.2) | 11.510 | **0.001** |
| Cefuroxime | 65 (91.5) | 71 (93.4) | 0.186 | 0.666 |
| Ceftriaxone | 63 (88.7) | 68 (89.5) | 0.021 | 0.885 |
| Penicillin | | | | |
| Augmentin[b] | 65 (91.5) | 74 (97.4) | 2.415 | 0.156 |
| Ampicillin[b] | 70 (98.6) | 74 (97.4) | 0.275 | 1.000 |
| Quinolones | | | | |
| Ciprofloxacin | 58 (81.7) | 22 (28.9) | 41.167 | **<0.001** |
| Norfloxacin | 46 (64.8) | 16 (21.1) | 28.791 | **<0.001** |
| Sulfonamide | | | | |
| Sulfamethoxazole | 66 (93.0) | 23 (30.3) | 60.399 | **<0.001** |
| Trimethoprim | 68 (95.8) | 23 (30.3) | 66.803 | **<0.001** |
| Co-trimoxazole | 65 (91.5) | 22 (28.9) | 59.552 | **<0.001** |
| Others | | | | |
| Tetracycline | 60 (84.5) | 40 (52.6) | 17.148 | **<0.001** |
| Chloramphenicol | 38 (53.5) | 21 (27.6) | 10.240 | **0.001** |
| Tigecycline | 40 (56.3) | 20 (26.3) | 13.696 | **<0.001** |

"n" indicates number of isolates.

[a] The statistical significance *p*-value was calculated using Pearson $\chi^2$ test in terms of number of sensitivity and resistance in integron-positive isolates and integron-negative isolates. The significant values are bolded ($p < 0.05$) in this table.

[b] Fisher exact test was applied.

For aminoglycosides drugs, only gentamicin and streptomycin showed significant association with integrons carriage. Amikacin possed very low level of resistance (19.7%) in integron-positive isolates. A similar scenario was observed in carbapenem in which low resistance were seen in imipenem (12.7%) and meropenem (14.1%). Despite the low resistance, meropenem showed marginal significance in association with integrons ($p$= 0.031). On the other hand, the percentage of isolates resistant to amikacin and imipenem was still higher among the integron-positive isolates as compared to integron-negative isolates.

Furthermore, the presence of integrons did not show any significant effect on the percentage of resistance for cefuroxime, ceftriaxone, augmentin and ampicillin. It is observed that the percentages of resistance in these antibiotics were evenly distributed between the integron-positive and integron-negative isolates. Only the third-generation cephalosporin antibiotic, ceftazidime, was found to be significantly associated with the presence of integrons (84.5% resistant, $p$= 0.001).

### 4.6 Relationship between integrons carriage and multiple resistance

As illustrated in Figure 4.12, the number of resistance to the antibiotics for the isolates was categorised into three subgroups; i.e. less than 6 antibiotics, 7 to 12 antibiotics, and lastly, 13 to 18 antibiotics. Interestingly, a greater tendency of resistancy to a higher number of antibiotics was observed in integron-positive isolates. This is evidenced by only two integron-positive isolates

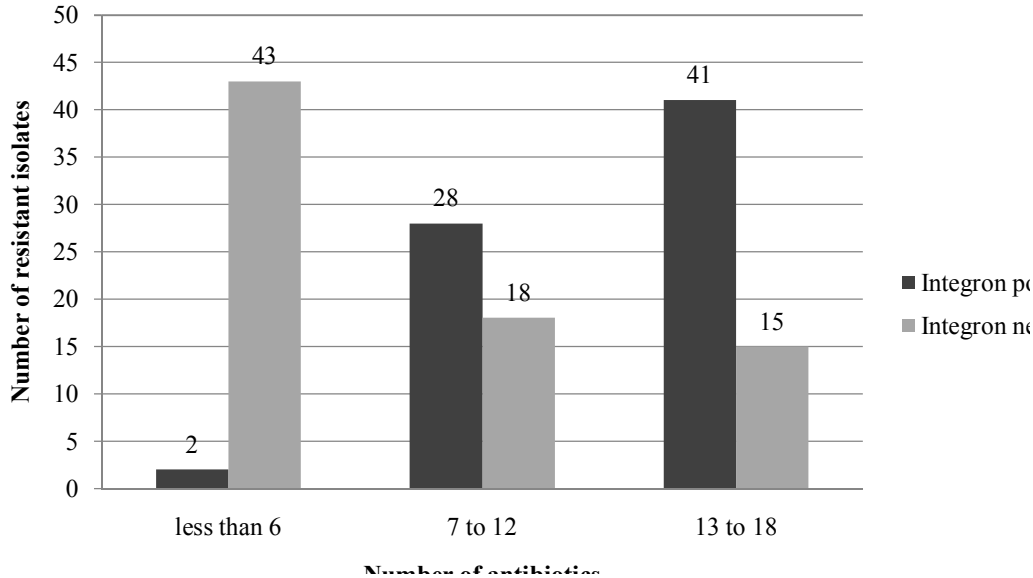



**Figure 4.12: Resistant to increasing number of antibiotics in integron-positive and integron-negative isolates**. The number of antibiotic that the isolates were resistant to were categorised into three sub-groups. Integron-positive and integron-negative isolates are represented by blue and red bars, respectively. *Chi*-square test confirmed that integrons were strongly associated with multidrug resistant in isolates ($p< 0.001$).

showed resistant to less than 6 antibiotics, while twenty-eight isolates were resistant to 7 to 12 types of antibiotics. Lastly, forty-one of the integron-positive isolates were resistant to more than 13 antimicrobial agents.

The highest number of integron-negative isolates was observed to show resistance to less than 6 antibiotics (n= 43). As the number of resistancy towards the antibiotics increased, fewer integron-negative isolates were found and vice versa. This result shows that the integrons were strongly associated with multidrug resistant (MDR) isolates, in which the integron-positive strains demonstrated a greater predilection for antibiotic resistance than integron-negative strains ($p< 0.001$).

### 4.7 Analysis of risk factors associate with integron-carrying isolates

Table 4.3 presents the factors associated with integrons carriage in isolates. To conclude if there was correlation of integrons with the risk factors, analysis of patients' demographics and microbiological data between integron-positive and integron-negative isolates was performed. Risk factors include gender, age, and site of specimen involved. Pearson $\chi^2$ analysis was conducted for categorical variable, i.e. patients' gender and age groups, while Mann-Whitney U test was applied for patients' mean age (continuous variable). No suitable test was found applicable to assess the association of source of specimens toward integrons carriage among isolates. Risk factors were found significantly associated when *p*-value was less than 0.05. Generally, gender and patients' mean age were not associated with presence of integrons, while

**Table 4.3: Factors associated with acquisition of integron-carrying bacteria isolates**

| Factors | Integron-positive (N=71) n/total (%) | Integron-negative (N=76) n/total (%) | $\chi^2$ | *p*-value[a] |
|---|---|---|---|---|
| **Gender** | | | | |
| Male | 36/84 (42.9) | 48/84 (57.1) | 2.325 | 0.127 |
| Female | 35/63 (55.6) | 28/63 (44.4) | | |
| **Age** | | | | |
| Mean ±SD | 54 ± 23 | 52 ± 16 | - | 0.173[b] |
| 19 and below | 9/13 (69.2) | 4/13 (30.8) | 11.277 | **0.028** |
| 20-39 years | 7/18 (38.9) | 11/18 (61.1) | | |
| 40-59 years | 17/52 (32.7) | 35/52 (67.3) | | |
| 60-79 years | 34/58 (58.6) | 24/58 (41.4) | | |
| 80 and above | 4/ 6 (66.7) | 2/6 (33.3) | | |
| **Source of isolates** | | | | |
| Blood | 4/9 (44.4) | 5/9 (55.6) | - | N/A |
| Foot | 1/4 (25) | 3/4 (75) | | |
| Pus swab | 16/31 (51.6) | 15/31 (48.4) | | |
| Sputum | 2/11 (18.2) | 9/11 (81.8) | | |
| Swab | 6/27 (22.2) | 21/27 (77.8) | | |
| Tissue | 4/7 (57.1) | 3/7 (42.9) | | |
| Urine | 24/26 (92.3) | 2/26 (7.7) | | |
| Wound swab | 1/12 (8.3) | 11/12 (91.7) | | |
| Others | 13/20 (65) | 7/20 (35) | | |

[a]The statistical significance *p*-value was calculated using Pearson $\chi^2$ test. The significant values are bolded ($p < 0.05$) in this table. N/A indicates factors are not applicable for Pearson $\chi^2$ test.
[b]Mann-Whitney U test was applied.

an association of marginal significance was found with the five categorised age groups ($p$= 0.028).

## 4.8 *sul1* gene and its association with integrons and bacterial sulfonamide resistant

For additional characterisation of integrons, isolates were screened for the presence of *sul1* gene in 3' conserved region using specific primers (Table 3.3). Figure 4.13 shows the successful amplification of products of approximately 840 bp upon random screening of samples. The nucleotide sequencing and alignment of 840 bp PCR amplified product confirmed and matched 100% in its identity with sulfonamide resistant gene, *sul1* (Figure 4.14).

Out of the one hundred and forty-seven isolates, only 34.7% (n= 51) of the isolates carried sulfonamide resistant gene, *sul1* (Table 4.4). Of the fifty-one *sul1*-positive isolates, 80.4% (n= 41) of them were found to carry integrase genes as well. Furthermore, there were sixty-six isolates which did not harbour either of these integron markers. The association of sulfonamide resistant gene and integrons was statistically significant ($p$< 0.001), which indicates the high incidences of co-existence of *sul1* and *intI* genes in an integron structure.

In Table 4.5, it is evidenced that there is a significant link between *sul1* genes and sulfonamide resistant phenotype present in all isolates ($p$< 0.001). The *sul1*-positive isolates (n= 51) was shown to exhibit more than 90% resistance

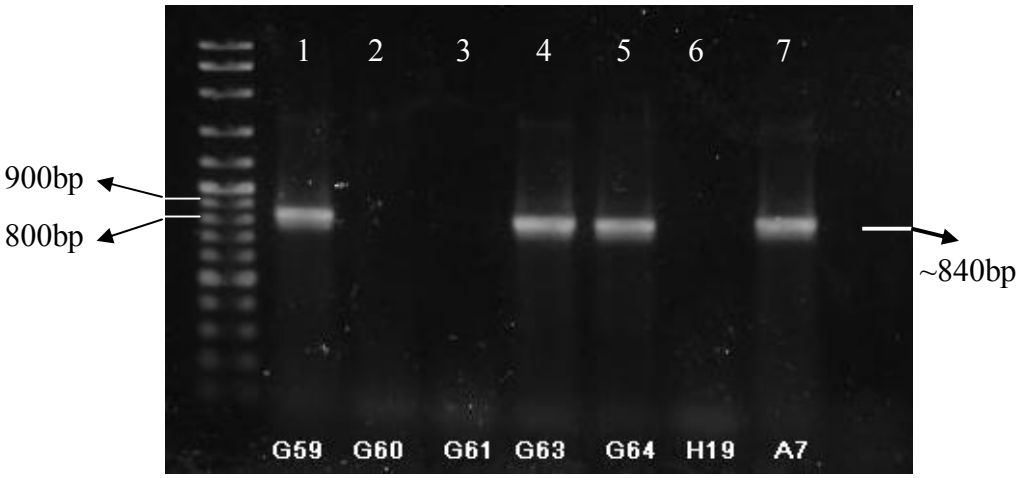



**Figure 4.13: Representative gel image of *sul1* gene amplification.** Random samples were tested and the expected size of approximately 840 bp. PCR products were amplified from random sample G59, G63, G64 and A7. No product was amplified in lanes 2, 3 and 6 which indicates the absence of *sul1* genes.

```
>ref|ZP_06648543.1|  dihydropteroate synthase [Escherichia coli FVEC1412]
 gb|AAF61485.1|AF191564_4  dihydropteroate synthetase type 1
                           [Pseudomonas aeruginosa]
 dbj|BAA78799.1| dihydropteroate synthetase typeI [Plasmid R100]
 gb|ACF06160.1|  dihydropteroate synthase [Klebsiella pneumoniae]
 gb|ACN73425.1|  dihydropteroate synthase [Acinetobacter sp. NFM2]
 gb|EFF01288.1|  dihydropteroate synthase [Escherichia coli FVEC1412]
 gb|AEL16650.1|  dihydropteroate synthase [Acinetobacter baumannii]
Length=288

 Score =  546 bits (1406),  Expect = 0.0
 Identities = 279/279 (100%), Positives = 279/279 (100%), Gaps = 0/279
(0%)
 Frame = +1

Query  1    MVTVFGILNLTEDSFFDESRRLDPAGAVTAAIEMLRVGSDVVDVGPAASHPDARPVSPAD  180
            MVTVFGILNLTEDSFFDESRRLDPAGAVTAAIEMLRVGSDVVDVGPAASHPDARPVSPAD
Sbjct  10   MVTVFGILNLTEDSFFDESRRLDPAGAVTAAIEMLRVGSDVVDVGPAASHPDARPVSPAD  69

Query  181  EIRRIAPLLDALSDQMHRVSIDSFQPETQRYALKRGVGYLNDIQGFPDPALYPDIAEADC  360
            EIRRIAPLLDALSDQMHRVSIDSFQPETQRYALKRGVGYLNDIQGFPDPALYPDIAEADC
Sbjct  70   EIRRIAPLLDALSDQMHRVSIDSFQPETQRYALKRGVGYLNDIQGFPDPALYPDIAEADC  129

Query  361  RLVVMHSAQRDGIATRTGHLRPEDALDEIVRFFEARVSALRRSGVAADRLILDPGMGFFL  540
            RLVVMHSAQRDGIATRTGHLRPEDALDEIVRFFEARVSALRRSGVAADRLILDPGMGFFL
Sbjct  130  RLVVMHSAQRDGIATRTGHLRPEDALDEIVRFFEARVSALRRSGVAADRLILDPGMGFFL  189

Query  541  SPAPETSLHVLSNLQKLKSALGLPLLVSVSRKSFLGATVGLPVKDLGPASLAAELHAIGN  720
            SPAPETSLHVLSNLQKLKSALGLPLLVSVSRKSFLGATVGLPVKDLGPASLAAELHAIGN
Sbjct  190  SPAPETSLHVLSNLQKLKSALGLPLLVSVSRKSFLGATVGLPVKDLGPASLAAELHAIGN  249

Query  721  GADYVRTHAPGDLRSAITFSETLAKFRSRDARDRGLDHA  837
            GADYVRTHAPGDLRSAITFSETLAKFRSRDARDRGLDHA
Sbjct  250  GADYVRTHAPGDLRSAITFSETLAKFRSRDARDRGLDHA  288
```

**Figure 4.14: BLAST-X result of purified *sul1* PCR products aligned with dihydropteroate synthase (type I) from GenBank (Accession Number ZP_06648543.1, AAF61485.1, AF191564_4, BAA78799.1, ACF06160.1, ACN73425.1, EFF01288.1, AEL16650.1)**

**Table 4.4: Cross-tabulation for *chi*-square analysis between integrons and *sul1* genes carriage**

| | | ***sul1* gene** | | **Total** | **$\chi^2$** | ***p*-value** |
|---|---|---|---|---|---|---|
| | | **Positive** | **Negative** | | | |
| **Integrons** | **Positive** | 41 | 30 | 71 | | |
| | **Negative** | 10 | 66 | 76 | 32.21 | 0.001 |
| **Total** | | 51 | 96 | 147 | | |

**Table 4.5: Association between sulfonamides antibiotic resistant profile to *sul1* gene carriage**

| Antibiotics | *sul1*-positive isolates (N=51) | | *sul1*-negative isolates (N= 96) | | $\chi^2$ | *p*-value[a] |
|---|---|---|---|---|---|---|
| | R n(%) | S n(%) | R n(%) | S n(%) | | |
| **Sulfonamides** | | | | | | |
| Trimethoprim | 49 (96.1) | 2 (3.9) | 42 (43.8) | 54 (56.3) | 38.67 | **<0.001** |
| Sulfamethoxazole | 50 (98) | 1 (2) | 39 (40.6) | 57 (59.4) | 45.96 | **<0.001** |
| Co-trimoxazole | 48 (94.1) | 3 (5.9) | 39 (40.6) | 57 (59.4) | 39.45 | **<0.001** |

"R" and "S" indicates resistant and susceptible respectively.
[a]The statistical significance *p*-value was calculated using Pearson $\chi^2$ test. Significant value is shown in bold ($p< 0.05$).

toward all sulfonamides antibiotics tested, i.e. trimethoprim, sulfamethoxazole, and co-trimoxazole. Interestingly, although co-trimoxazole is a combinational antibiotic of trimethoprim and sulfamethoxazole, only a slight difference of between 2-4% was observed in the number of *sul1*-positive isolates when they were exposed to co-trimoxazole than towards stand alone antibiotics. A higher percentage of isolates susceptible toward the three sulfonamide antibiotics was observed in the ninety-six *sul1*-negative isolates.

## 4.9 Identification of integron-borne gene cassettes

The isolates identified carrying either class 1 and 2 integrase genes were further characterised through the PCR amplification of the integron variable region using primer pair 5'-CS, 3'-CS and hep74, hep51. Among seventy-one isolates tested positive for integrase gene, *intI1* and *intI2* gene cassette regions were successfully amplified in forty-two of the isolates (59.2%). As shown in Table 4.6, thirty isolates yielded a single class 1 gene cassettes amplicons and eight isolates yielded two class 1 gene cassettes of different sizes. One isolate, however, harboured both class 1 and 2 gene cassettes, while three isolates carried amplicons for *intI2* gene cassettes region.

The characterisation by PCR-RFLP using *HinfI* separated all the cassette amplicons into a total of nineteen distinct profiles. Figure 4.15 shows a representative gel image for PCR-RFLP patterns of group 1 and 5 amplicons with identical array of 0.7 kb and 1.6 kb. Digestion of PCR products allowed the identification of three subgroups (a, b, c) for each RFLP group 1 and 5.

**Table 4.6: RFLP groups of gene cassettes amplified from seventy-one integron-positive isolates**

| Class of integrons | RFLP groups | Approximate length of amplicons (kb) | Total number of isolates |
|---|---|---|---|
| Class 1 integrons | 1a | 0.7 | 1 |
| | 1b | 0.7 | 2 |
| | 1c | 0.7 | 4 |
| | 2 | 1.0 | 1 |
| | 3a | 1.2 | 1 |
| | 3b | 1.2 | 1 |
| | 4 | 1.3 | 4 |
| | 5a | 1.6 | 1 |
| | 5b | 1.6 | 1 |
| | 5c | 1.6 | 3 |
| | 6 | 1.9 | 4 |
| | 7 | 2.4 | 5 |
| | 8 | 2.6 | 2 |
| Two Class 1 integrons | 9 | 1.0 + 1.6 | 1 |
| | 10 | 1.0 + 2.3 | 1 |
| | 11 | 1.3 + 2.3 | 5 |
| | 12 | 1.6 + 1.9 | 1 |
| Class 1 & 2 integrons | 13 | 1.9 + 2.2 | 1 |
| Class 2 integrons | 14 | 2.2 | 3 |
| "Empty" integrons | 15 | ≈0.15 | 5 |
| No amplification | | | 24 |
| **Total** | **19** | | **71** |

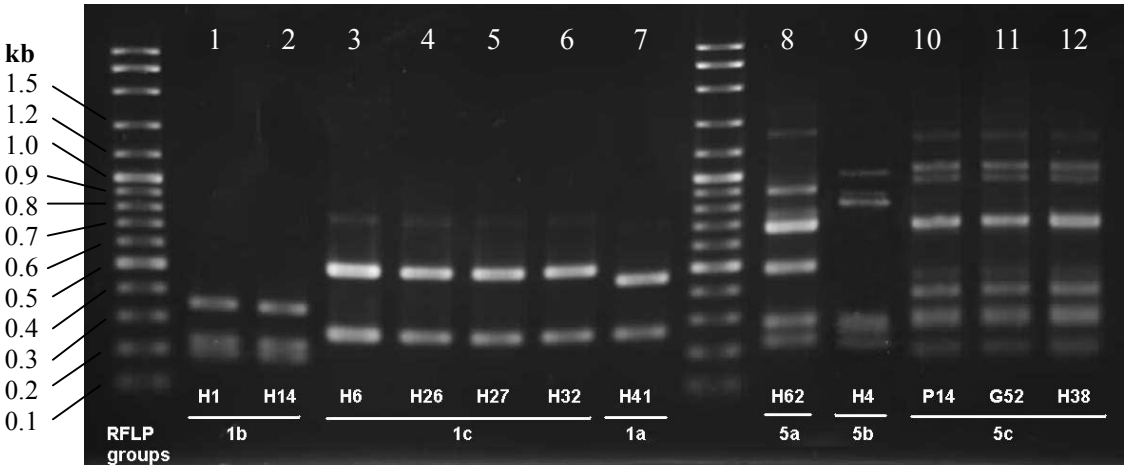



**Figure 4.15: Representative gel image of restriction fragment length polymorphism (PCR-RFLP).** *Hinf1* digestion was performed on identical arrays of 0.7 kb-amplicons (Group 1) and 1.6 kb-amplicons (Group 5). Amplicons with the same restriction pattern imply the presence of the same gene cassettes.

Furthermore, five isolates were shown to harbour only the partial 5' and 3' conserved segments and without the presence of any inserted gene cassettes. This "empty" integron structure was indicated by the amplification of products sized approximately 150 bp (Figure 4.16, lane 1). Surprisingly, the remaining twenty-four isolates (33.8%) were shown to have no amplification of gene cassettes regions (Figure 4.16, lane 3). To double confirm these results were not due to errors during the amplification process, all of the twenty-nine isolates (absence of gene cassette and "empty" integrons) were later subjected to further PCR amplification using the primer combination of Int2F and 3'CS. As expected, the "empty" integrons generated an amplicon larger by an approximately 600 bp (i.e. approximate 750 bp in total) (Figure 4.16, lane 2) than the amplicon derived from amplification of 5'-CS and 3'-CS primers. Besides, primers Int2F and 3'-CS were unable to amplify any gene cassettes from the twenty-four isolates.

The size range of inserted gene cassettes in this study varied in size from 0.7 kb to 2.6 kb. A representative gel image for the gene cassettes amplified was shown in Figure 4.17(A) and (B). The exact size of variable regions and the gene cassettes identified for each group are summarised in Table 4.7. DNA sequencing confirmed the identity of gene cassettes and the data is shown in Appendix E. Figure 4.18 shows a representative of the sequencing result of *aacA4-catB3-dfrA1* gene cassette identified in this study, and the detailed structural regions.

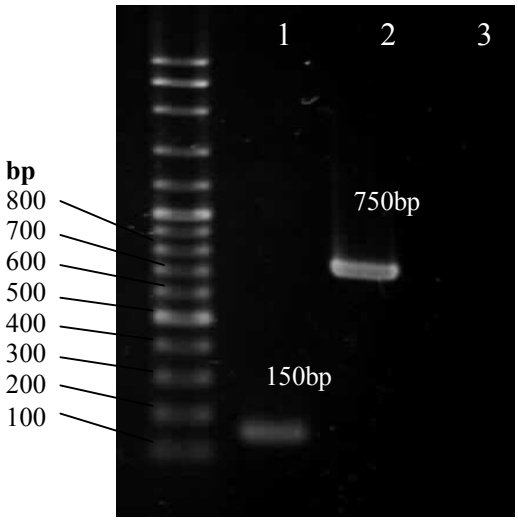



**Figure 4.16: PCR amplification of empty integrons.** Lane 1 shows amplification of "empty integrons" using primer 5'-CS and 3'-CS, in which 150 bp was generated from the amplified partial 5' and 3' conserved segment. The present of "empty integrons" was double confirmed when a 750 bp product was amplified with primer Int2F and 3'-CS. Lane 3 showed no amplification of any gene cassettes and the respective 5' and 3' conserved segment, using either 5'-CS and 3'-CS or Int2F and 3'-CS primer pairs.

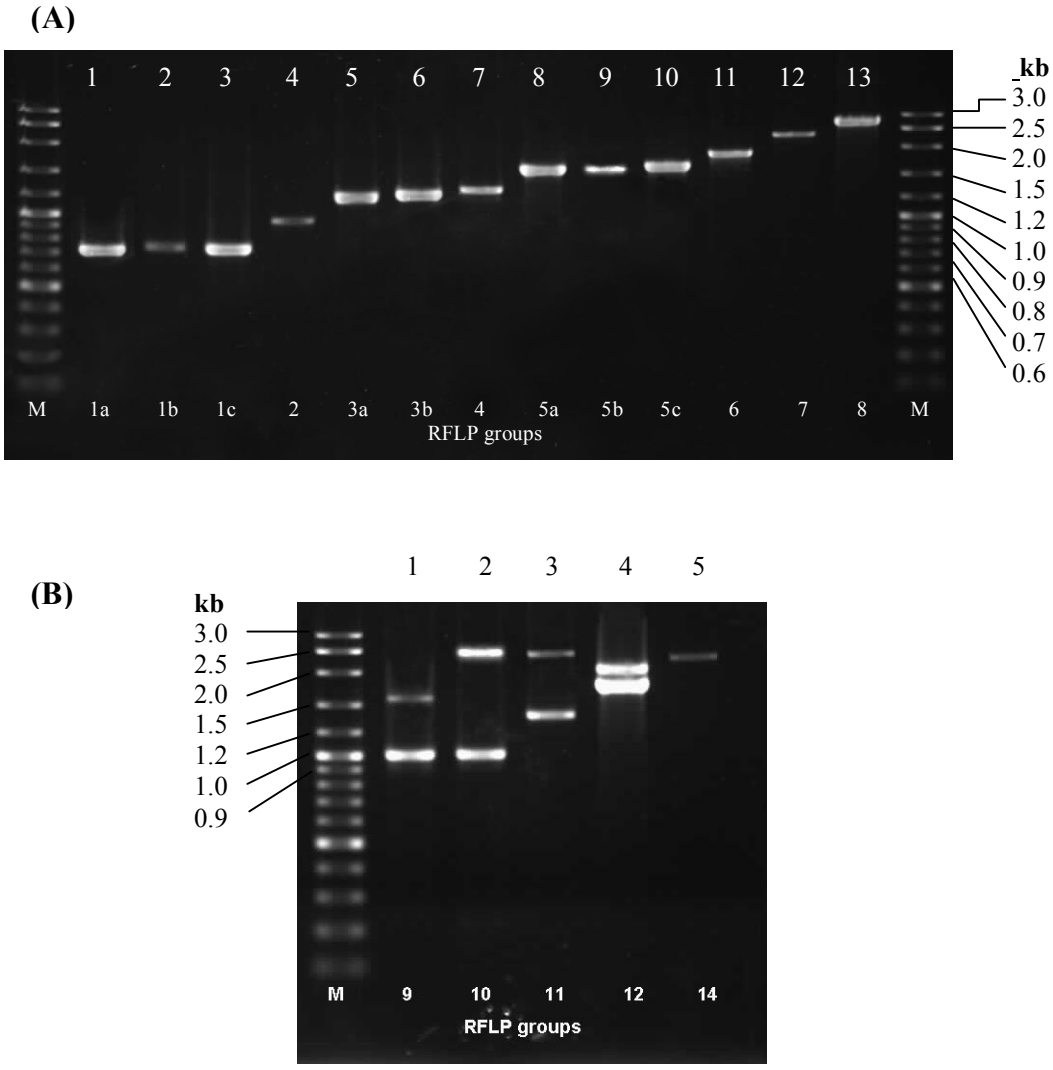



**Figure 4.17: Agarose gels represent PCR amplification of integron-borne gene cassettes.** (A) Amplification of gene cassettes of class 1 integrons using 5'-CS and 3'-CS primer. Lane M is 100 bp size marker. Amplicons were loaded according to respective RFLP groups (see Table 4.6) from left to right lanes. (B) Lane 1 to 4 illustrate gene cassettes of two class 1 integrons and lane 5 represents amplicon of class 2 integrons generated using hep74 and hep51 primer.

**Table 4.7: Characterisation gene cassettes arrays and their respective bacterial species**

| Bacteria species[a] | Sample ID[a] | RFLP groups | Exact array length, bp | Gene cassettes[a,b] |
|---|---|---|---|---|
| *E. aerogenes* | H44 | 6 | 1913 | *dfrA12-orfF-aadA2* |
| *E. cloacae* | K15 | 10 | 1009, 2350 | *aadA2, dfrA1-aadA5* |
| | H3 | 14 | 2224 | *dfrA1-sat1-aadA1* |
| ***E. coli*** | H41 | 1a | 721 | *dfrA5* |
| | H70 | 2 | 1009 | *aadA1* |
| | P14 | 5c | 1664 | *dfrA17-aadA5* |
| | P3 | 6 | 1913 | *dfrA12-orfF-aadA2* |
| | **A7, H54** | 8 | 2655 | ***aacA4-catB3-dfrA1*** |
| | H12 | 9 | 1009, 1586 | *aadA1, dfrA1-aadA1* |
| | H35 | 12 | 1664,1947 | *dfrA17-aadA5, aadA2-linF* |
| | **H5, P4** | 14 | 2224 | ***dfrA1-sat1-aadA1*** |
| *K. oxytoca* | H1 | 1b | 761 | *aadB* |
| | K5 | 4 | 1336 | *aadA6-orfD* |
| ***K. pneumoniae*** | H14 | 1b | 761 | *aadB* |
| | **H6, H26, H27, H32** | 1c | 769 | ***dfrA7*** |
| | H67 | 3a | 1197 | *blaP1* |
| | H62 | 5a | 1596 | *dfrA15-aadA1* |
| | H4 | 5b | 1598 | *dfrA16-aadA2* |
| | H38 | 5c | 1664 | *dfrA17-aadA5* |
| | **H57, H63, H65, H66** | 7 | 2380 | ***aacA4-catB8-aadA1*** |
| | H19 | 13 | 1913, 2224 | *dfrA12-orfF-aadA2, dfrA1-sat1-aadA1* |
| *M. morganii* | G59 | 3b | 1242 | *dfrA1-orfC* |
| | G64 | 6 | 1913 | *dfrA12-orfF-aadA2* |
| | G52 | 5c | 1664 | *dfrA17-aadA5* |
| *P. mirabilis* | H53 | 6 | 1913 | *dfrA12-orfF-aadA2* |

Table 4.7, continued:

| | | | | |
|---|---|---|---|---|
| ***P. aeruginosa*** | **K18, K22,K31** | 4 | 1336 | ***aadA6-orfD*** |
| | K7 | 7 | 2380 | *aacA4-catB8-aadA1* |
| | **K17, K20, K23, K24, K25** | 11 | 1336,2293 | ***aadA6-orfD, aacC3-cmlA5*** |

[a]Sample's name were labelled according to hospitals the isolates were collected respectively. "H", Hospital Raja Permaisuri Bainun Ipoh; "K", KPJ Ipoh Specialist Hospital; "G", Gribbles Pathology "P", Hospital Pantai Putri Ipoh; "A", Penang Island Hospital. Bold indicates the bacterial isolates carrying similar gene cassettes which were further subjected to pulsed field gel electrophoresis (PFGE) for clonal relatedness.
[b]Class 2 cassettes are underlined.

> gb|DQ836057.1| *Escherichia coli* class I integron aminoglycoside 6'-N-acetyltransferase (*aacA4*), chloramphenicol acetyltransferase (*catB3*), and dihydrofolate reductase (*dfrA1*) genes, complete cds
Length=2731

Score = 4887 bits (2646), Expect = 0.0
Identities = 2652/2655 (99%), Gaps = 0/2655 (0%)
Strand=Plus/Plus

```
Query  1     GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  44    GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAA  103
                 -35 promoter region                    |>      attI1

Query  61    CGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCATCACAAAGTACAGCAT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104   CGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCATCACAAAGTACAGCAT  163
                                              <|

Query  121   CGTGACCAACAGCAACGATTCCGTCACACTGCGCCTCATGACTGAGCATGACCTTGCGAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  164   CGTGACCAACAGCAACGATTCCGTCACACTGCGCCTCATGACTGAGCATGACCTTGCGAT  223
             |>      Coding sequences of aacA4 gene cassette

Query  181   GCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGGGGCGGAGAAGAAGCACG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224   GCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTGGTGGGGCGGAGAAGAAGCACG  283

Query  241   CCCGACACTTGCTGACGTACAGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  284   CCCGACACTTGCTGACGTACAGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGT  343

Query  301   CACTCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  344   CACTCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGC  403

Query  361   TCTTGGAAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAATAGA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  404   TCTTGGAAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAATAGA  463

Query  421   CCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTTCGAGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  464   CCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTTCGAGC  523

Query  481   ACTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAGATCCAAACGGACCCGTCGCC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  524   ACTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAGATCCAAACGGACCCGTCGCC  583

Query  541   GAGCAACTTGCGAGCGATCCGATGTTACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGT  600
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  584   GAGCAACTTGCGAGCGATCCGATGCTACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGT  643

Query  601   AACCACCCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAAC  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  644   AACCACCCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAAC  703

Query  661   ACGCAGTGATGCCTAACCCTTCCATCGAGGGGGACGTCCAAGGGCTGGCGCCCTTGGTCG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  704   ACGCAGTGATGCCTAACCCTTCCATCGAGGGGGACGTCCAAGGGCTGGCGCCCTTGGTCG  763
               aacA4          <|                  59 base element

Query  721   CCCCTCATGTCAAACGTTAGACGGCAAAGTCACAGACCGCGGGATCTCTTATGACCAACT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  764   CCCCTCATGTCAAACGTTAGACGGCAAAGTCACAGACCGCGGGATCTCTTATGACCAACT  823
                                                          |>  catB3

Query  781   ACTTTGATAGCCCCTTCAAAGGCAAGCTGCTTTCTGAGCAAGTGAAGAACCCCAATATCA  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  824   ACTTTGATAGCCCCTTCAAAGGCAAGCTGCTTTCTGAGCAAGTGAAGAACCCCAATATCA  883

Query  841   AAGTTGGGCGGTACAGCTATTACTCTGGCTACTATCATGGGCACTCATTCGATGACTGCG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  884   AAGTTGGGCGGTACAGCTATTACTCTGGCTACTATCATGGGCACTCATTCGATGACTGCG  943
```

Figure 4.18, continued:

```
Query  901   CACGGTATCTGTTTCCGGACCGTGATGACGTTGATAAGTTGATCATCGGTAGTTTCTGCT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  944   CACGGTATCTGTTTCCGGACCGTGATGACGTTGATAAGTTGATCATCGGTAGTTTCTGCT  1003

Query  961   CTATCGGGAGTGGGGCTTCCTTTATCATGGCTGGCAATCAGGGGCATCGGTACGACTGGG  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1004  CTATCGGGAGTGGGGCTTCCTTTATCATGGCTGGCAATCAGGGGCATCGGTACGACTGGG  1063

Query  1021  CATCATCTTTCCCGTTCTTTTATATGCAGGAAGAACCTGCATTCTCAAGCGCACTCGATG  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1064  CATCATCTTTCCCGTTCTTTTATATGCAGGAAGAACCTGCATTCTCAAGCGCACTCGATG  1123

Query  1081  CCTTCCAAAAAGCAGGTAATACTGTCATTGGCAATGACGTTTGGATCGGCTCTGAGGCAA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1124  CCTTCCAAAAAGCAGGTAATACTGTCATTGGCAATGACGTTTGGATCGGCTCTGAGGCAA  1183

Query  1141  TGGTCATGCCCGGAATCAAGATCGGGCACGGTGCGGTGATAGGCAGCCGCTCGTTGGTGA  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1184  TGGTCATGCCCGGAATCAAGATCGGGCACGGTGCGGTGATAGGCAGCCGCTCGTTGGTGA  1243

Query  1201  CAAAAGATGTGGAGCCTTACGCTATCGTTGGCGGCAATCCCGCTAAGAAGATTAAGAAAC  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1244  CAAAAGATGTGGAGCCTTACGCTATCGTTGGCGGCAATCCCGCTAAGAAGATTAAGAAAC  1303

Query  1261  GCTTCACCGATGAGGAAATTTCATTGCTTCTGGAGATGGAGTGGTGGAATTGGTCACTGG  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1304  GCTTCACCGATGAGGAAATTTCATTGCTTCTGGAGATGGAGTGGTGGAATTGGTCACTGG  1363

Query  1321  AGAAGATCAAAGCGGCAATGCCCATGCTGTGCTCGTCTAATATTGTTGGCCTGCACAAGT  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1364  AGAAGATCAAAGCGGCAATGCCCATGCTGTGCTCGTCTAATATTGTTGGCCTGCACAAGT  1423

Query  1381  ATTGGCTCGAGTTTGCCGTCTAACAATTCAATCAAGCCGATGCCGCTTCGCGGCACGGCT  1440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1424  ATTGGCTCGAGTTTGCCGTCTAACAATTCAATCAAGCCGATGCCGCTTCGCGGCACGGCT  1483
                          catB3  <|                  59 base element

Query  1441  TATTTCAGGCGTTAACCTCTGAGGAAGAATTGTGAAACTATCACTAATGGTAGCTATATC  1500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1484  TATTTCAGGCGTTAACCTCTGAGGAAGAATTGTGAAACTATCACTAATGGTAGCTATATC  1543
                                                |>  dfrA1

Query  1501  GAAGAATGGAGTTATCGGGAATGGCCCTGATATTCCATGGAGTGCCAAAGGTGAACAGCT  1560
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1544  GAAGAATGGAGTTATCGGGAATGGCCCTGATATTCCATGGAGTGCCAAAGGTGAACAGCT  1603

Query  1561  CCTGTTTAAAGCTATTACCTATAACCAATGGCTGTTGGTTGGACGCAAGACTTTTGAATC  1620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1604  CCTGTTTAAAGCTATTACCTATAACCAATGGCTGTTGGTTGGACGCAAGACTTTTGAATC  1663

Query  1621  AATGGGAGCATTACCCAACCGAAAGTATGCGGTCGTAACACGTTCAAGTTTTACATCTGA  1680
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1664  AATGGGAGCATTACCCAACCGAAAGTATGCGGTCGTAACACGTTCAAGTTTTACATCTGA  1723

Query  1681  CAATGAGAACGTAGTGATCTTTCCATCAATTAAAGATGCTTTAACCAACCTAAAGAAAAT  1740
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1724  CAATGAGAACGTAGTGATCTTTCCATCAATTAAAGATGCTTTAACCAACCTAAAGAAAAT  1783

Query  1741  AACGGATCATGTCATTGTTTCAGGTGGTGGGGAGATATACAAAAGCCTGATCGATCAAGT  1800
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1784  AACGGATCATGTCATTGTTTCAGGTGGTGGGGAGATATACAAAAGCCTGATCGATCAAGT  1843

Query  1801  AGATACACTACATATATCTACAATAGACATCGAGCCGGAAGGTGATGTTTACTTTCCTGA  1860
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1844  AGATACACTACATATATCTACAATAGACATCGAGCCGGAAGGTGATGTTTACTTTCCTGA  1903

Query  1861  AATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGACTTCGCCTCTAACATAAATTATAG  1920
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1904  AATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGACTTCGCCTCTAACATAAATTATAG  1963
```

Figure 4.18, continued:


**Figure 4.18: Representative comparison of the sequences of amplified *aacA4-catB3-dfrA1* gene cassettes with the published *E. coli* cassette array (Genbank Accession Number DQ836057.1).** Identical nucleotides are indicated by vertical lines. The sequence of the cloned product is shown on upper line. Cassette gene *aacA4*, *catB3*, and *dfrA1* were identified, as well as their 59 base element; *attI1* indicates the integron-associated recombination site.

As observed in Table 4.7, the most common gene cassettes detected were the genes encoding resistance towards aminoglycosides and trimethoprim. Genes encoding aminoglycosides adenyltransferase (*aad*), which confers resistance to spectinomycin and streptomycin, were found in 69.2% (27/39) of *intI1*-positive isolates, either alone or in combination with other resistance genes. This is followed by 53.8% (21/39) of *intI1*-positive isolates carrying genes encoding dihydrofolate reductase, which confers resistance to trimethoprim. Amongst these gene cassettes identified, four spectinomycin/ streptomycin determinants (*aadA1*, *aadA2*, *aadA5*, and *aadA6*) and seven trimethoprim resistance determinants (*dfrA1*, *dfrA5*, *dfrA7*, *dfrA12*, *dfrA15*, *dfrA16*, and *dfrA17*) were detected. Other gene cassettes discovered include *aadB* and *aacC*, which encode for gentamicin resistance; *aacA*, encoding amikacin resistance; *catB* and *cmlA*, which encode for chloramphenicol resistance; *linF*, encoding lincosamides resistance and *bla*P1, an encoding PSE-1 for resistance to class A β-lactamase. In addition, gene cassettes encoding unknown products, such as *orfC*, *orfD* and *orfF* were detected as well.

All class 2 integrons were found to contain a similar gene cassettes array, which was the *dfrA1-sat1-aadA1* that confers resistance to trimethoprim, streptothricin and streptomycin/spectinomycin, respectively. The only isolate with the co-existence of class 1 and 2 integrons was shown to harbour a combinatory gene cassettes of group 6 and 14 (*dfrA12-orfF-aadA2* and *dfrA1-sat1-aadA1*).

The characterisation of gene cassettes in this study revealed a significant association between the nature of the gene cassettes and the corresponding antibiotic resistance phenotypes of the isolates. All isolates which harboured dihydrofolate reductase cassette (*dfr*) were resistant to trimethoprim (Table 4.8). Resistance toward co-trimoxazole (trimethoprim + sulfamethoxazole) was also observed in isolates that carried the dihydrofolate reductase cassette amongst the class 1 integrons, but at lesser extent for the isolates of class 2 integrons (1 out of 3 was resistant).

Out of the thirty-one isolates (including class 2 integrons) carrying spectinomycin/ streptomycin resistant cassette, 90% (28/31) of them showed resistance toward streptomycin tested. Besides, *aadB* and *aacC* genes were detected in seven isolates and all of them showed resistance toward gentamicin. In group 12, an isolate with *linF* gene, encoding lincosamides resistance had also showed resistance toward clindamycin (lincosamides class antibiotic). Interestingly, resistance to other antibiotics like carbapenems, cephalosporin, quinolones, tetracycline, and tigecycline did not show any correspondence towards any of the identified gene cassettes.

**Table 4.8: Gene cassettes identified corresponding antibiotics resistant phenotype of gene cassette and its respective isolates**

| RFLP groups | Gene cassettes | Total isolates | Resistant phenotypes | Number of isolates resistant to antibiotics[a] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Ak | Gn | S | Am | AmC | Tmp | C | Smz | Sxt | Imp | Caz | Te | Cip | Tgc |
| 1a | *dfrA5* | 1 | Tmp | 0 | 0 | 1 | 1 | 1 | **1** | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| 1b | *aadB* | 2 | Gn | 0 | **2** | 0 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 2 | 2 | 1 | 2 |
| 1c | *dfrA7* | 4 | Tmp | 0 | 1 | 4 | 4 | 4 | **4** | 2 | 4 | 4 | 1 | 4 | 3 | 3 | 3 |
| 2 | *aadA1* | 1 | S | 0 | 1 | **1** | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| 3a | *blaP1* | 1 | AmAmC | 0 | 0 | 1 | **1** | **1** | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| 3b | *dfrA1-orfC* | 1 | Tmp | 0 | 1 | 1 | 1 | 1 | **1** | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 |
| 4 | *aadA6-orfD* | 4 | S | 4 | 4 | **4** | 4 | 4 | 4 | 4 | 4 | 4 | 2 | 4 | 4 | 4 | 4 |
| 5a | *dfrA15-aadA1* | 1 | TmpS | 0 | 1 | **1** | 1 | 1 | **1** | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| 5b | *dfrA16-aadA2* | 1 | TmpS | 0 | 1 | **1** | 1 | 1 | **1** | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 |
| 5c | *dfrA17-aadA5* | 3 | TmpS | 1 | 3 | **3** | 3 | 3 | **3** | 1 | 3 | 2 | 0 | 1 | 2 | 3 | 2 |
| 6 | *dfrA12-orfF-aadA2* | 4 | TmpS | 0 | 1 | **4** | 4 | 3 | **4** | 2 | 4 | 4 | 0 | 2 | 4 | 2 | 2 |
| 7 | *aacA4-catB8-aadA1* | 5 | AkCS | **2** | 4 | **4** | 5 | 5 | 5 | **4** | 5 | 5 | 0 | 5 | 5 | 5 | 5 |
| 8 | *aacA4-catB3-dfrA1* | 2 | AkCTmp | **0** | 1 | 2 | 2 | 2 | **2** | **2** | 2 | 2 | 0 | 2 | 2 | 2 | 0 |
| 9 | *aadA1+ dfrA1-aadA1* | 1 | S, TmpS | 0 | 1 | **1** | 1 | 1 | **1** | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 |
| 10 | *aadA2 + dfrA1-aadA5* | 1 | S, TmpS | 1 | 1 | **0** | 1 | 1 | **1** | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 |

Table 4.8, continued:

| RFLP groups | Gene cassettes | Total isolates | Resistant phenotypes | Number of isolates resistant to antibiotics[a] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | **Ak** | **Gn** | **S** | **Am** | **AmC** | **Tmp** | **C** | **Smz** | **Sxt** | **Imp** | **Caz** | **Te** | **Cip** | **Tgc** |
| 11 | *aadA6-orfD* + *aacC3-cmlA5* | 5 | S, GnC | 5 | **5** | **5** | 5 | 5 | 5 | **5** | 5 | 5 | 5 | 5 | 5 | 5 | 4 |
| 12 | *dfrA17-aadA5* + *aadA2-LinF* | 1 | TmpS, SDa[b] | 0 | 0 | **1** | 1 | 1 | **1** | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 |
| 13 | *dfrA12-orfF-aadA2* + *dfrA1-sat1-aadA1* | 1 | TmpS, TmpS[†] | 0 | 1 | **1** | 1 | 1 | **1** | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 |
| 14 | *dfrA1-sat1-aadA1* | 3 | TmpS[†] | 1 | 1 | **3** | 2 | 2 | **3** | 0 | 2 | 1 | 0 | 2 | 3 | 2 | 1 |
| | **Total** | 42 | | 14 | 29 | 38 | 41 | 40 | 41 | 25 | 39 | 38 | 8 | 36 | 38 | 35 | 27 |



AK,amikacin; GN, gentamicin; S, streptomycin; AM, ampicilin; AmC, amoxicillin/ clavulanic acid (Augmentin); TMP, trimethoprim; C, chloramphenicol; SMZ, sulfamethoxazole; SXT, trimethoprim/ sulfamethoxazole; IMP, imipenem; CAZ, ceftazidime; TE, tetracycline; CIP, ciprofloxacin; and TGC, tigecycline.

[a]Antibiotic relevance to integron-associated resistant genes were bold. Isolates which did not correlate to antibiotic resistant phenotypes are underlined.
[b]Da represents clindamycin antibiotic.
[†]Antibiotic to test on streptothricin resistant (*sat1*) is not available in market

## 4.10 Epidemiological typing of isolates carrying identical cassette arrays

To determine whether the high prevalence of a particular type of integron in the isolates was caused by the spread of a specific clone, genotypic diversity of the isolates was assessed by PFGE followed by *XbaI* digestion. In particular, *E. coli*, *K. pneumoniae* and *P. aeruginosa*, which were identified to carry identical gene cassettes were selected (Table 4.7, in bold). Their respective PFGE gel images and respective dendrograms generated are illustrated in Figures 4.19, 4.20 and 4.21.

For *E. coli*, isolates carrying the identical gene cassettes arrays showed distinct patterns (Figure 4.19). Dendrogram showed that *E. coli* isolates containing class 1 and 2 gene cassettes array of *aacA4-catB3-dfrA1* and *dfrA1-sat1-aadA1* respectively, has less than 40% in similarity.

On the other hand, *XbaI* restriction digestion of the genomic DNA from eight *K. pneumonia*e yielded approximately twenty DNA fragments measuring from 10 to 700 kb in size (Figure 4.20). Three of the isolates which carried the *dfrA7* gene cassettes showed more than 80% similarity in pattern, in which, sample H26 and H27 posed 90% in similarity. Furthermore, all isolates which contained *aacA4-catB8-aadA1* were deemed to be epidemiological unrelated.

As illustrated in Figure 4.21, two PFGE cluster were developed in which *P. aeruginosa* isolates showed the same unique pattern for the bands generated. The dendrogram analysis showed that there was no clonal relationship

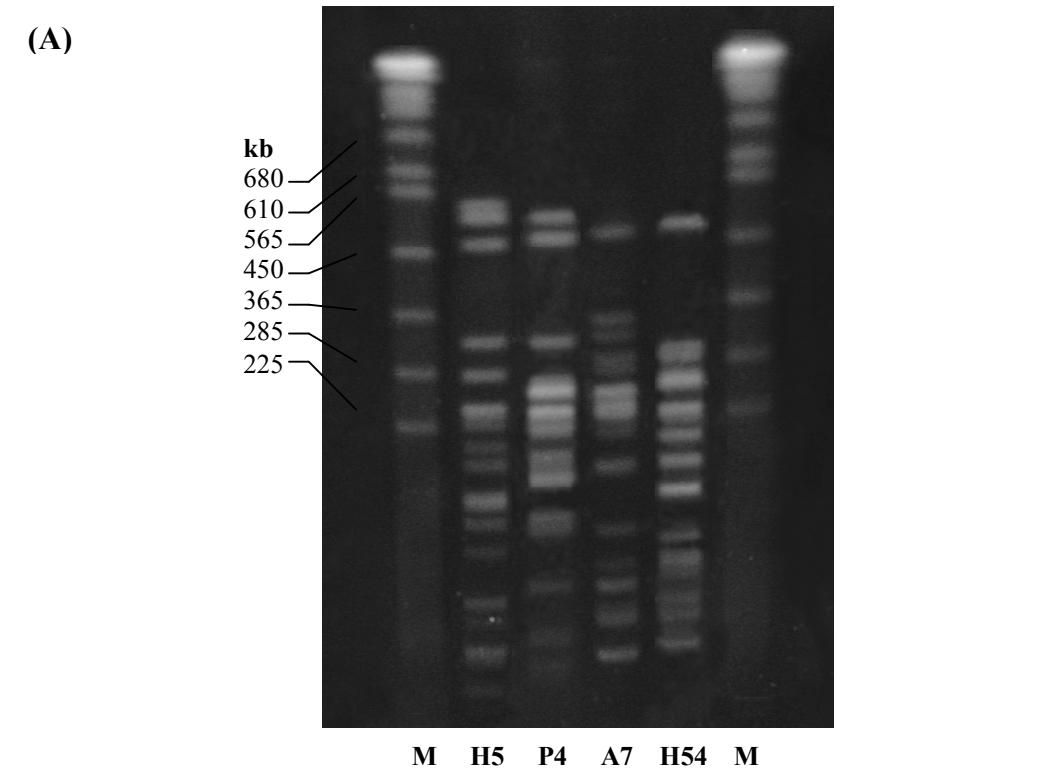
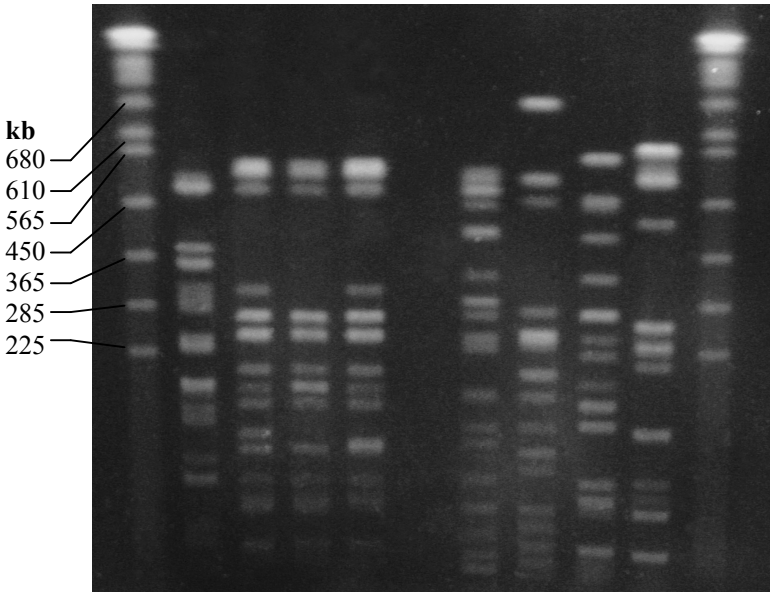



**Figure 4.19: Gel image (A) and dendrogram (B) showing cluster analysis of *XbaI* digested *E. coli* genome with identical cassette arrays, generated by Bionumerics software using UPGMA method.**

**(A)**




**M H6 H26 H27 H32 H57 H63 H65 H66 M**

**(B)**

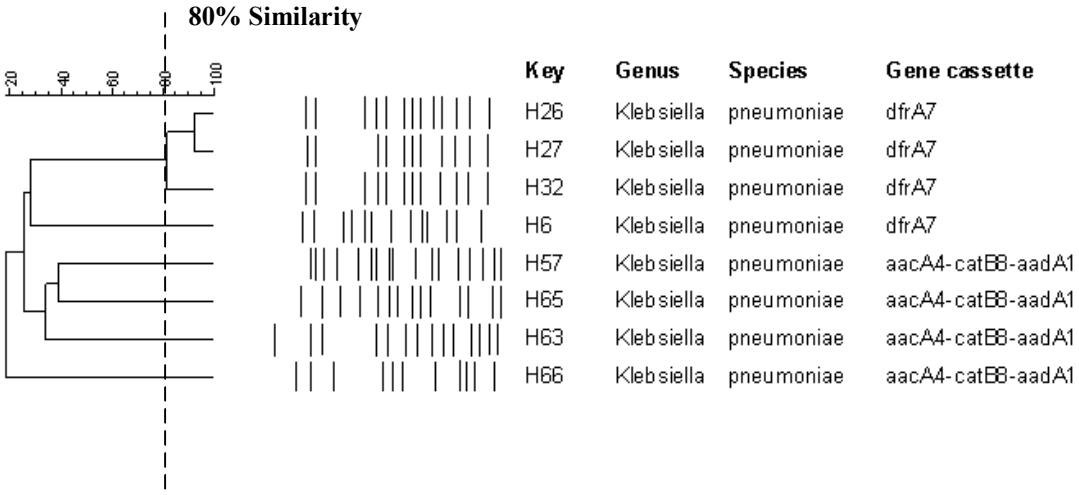
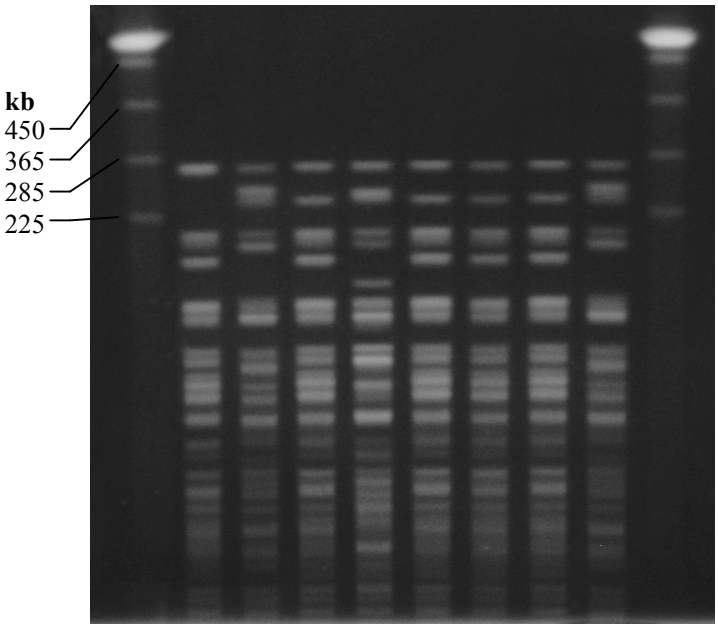



**Figure 4.20: Gel image (A) and dendrogram (B) showing cluster analysis of *XbaI* digested *K. pneumoniae* genome with identical cassette arrays, generated by Bionumerics software using UPGMA method**.

**(A)**




**(B)**

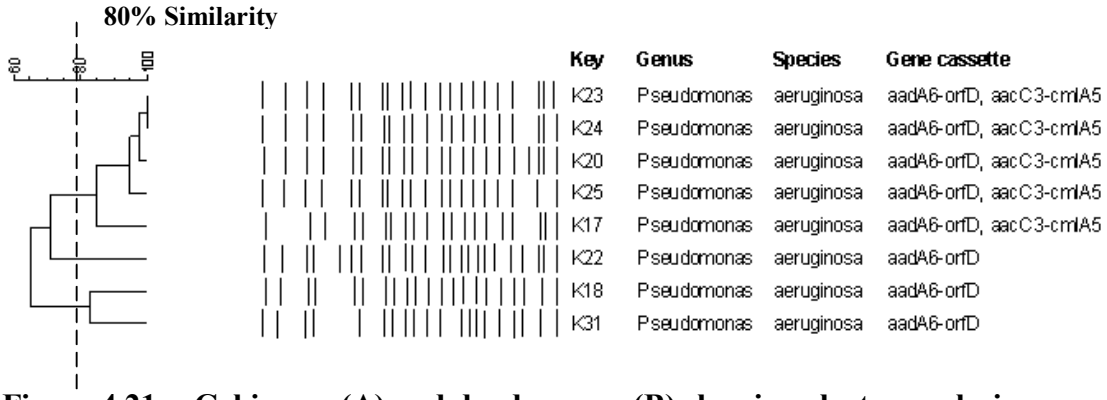



**Figure 4.21: Gel image (A) and dendrogram (B) showing cluster analysis of *XbaI* digested *P. aeruginosa* genome with identical cassette arrays, generated by Bionumerics software using UPGMA method.**

between the isolates which carried the two different combinations of gene cassettes. Of the eight *P. aeruginosa* typed here, five isolates which carried identical *aadA6-orfD* and *aacC3-cmlA5* were grouped into one cluster. PFGE profiles of sample K23 and K24 were likely 100% similar, while sample K20 differed from the two by a difference of one band, suggesting a high clonal relationship among the three samples. Sample K25 also showed more than 90% similarity with the three samples, as compared to K17 with only 85% similarity. Two of the isolates which contained *aadA6-orfD* gene cassettes (K18 and K31) form another cluster with 80% similarity between them.

# CHAPTER 5

# DISCUSSION

## 5.1 Review

There were five objectives in this study. The first objective was to investigate the resistance profile of clinical isolate of *Enterobacteriaceae* and *P. aeruginosa* to several antibiotics. The second objective was to investigate the prevalence of classes of integrons and *sul1* gene in isolates and to identify or characterise the gene cassettes present. Next was to determine the risk factors and association of bacterial MDR with the presence of integrons. The last objective was to investigate the clonal relationship between high frequency integron-positive isolates by PFGE.

## 5.2 Overall antibiotic resistance profile

All one hundred forty-seven *Enterobacteriaceae* and *P. aeruginosa* were tested toward eighteen antimicrobial agents. For the purpose of analysis, both intermediate resistant and resistant were grouped together as resistant. In the present study, majority of the isolates showed resistance to ampicillin (97.96%), augmentin (94.56%), cefuroxime (92.52%), and ceftriaxone (89.12%). However, the isolates were tested sensitive toward imipenem, meropenem (both at 91.16%), and amikacin (85.03%), as illustrated in Figure 4.6. A similar trend of high and low resistance rate in these antibiotics was also observed in earlier study by Gu *et al.* (2008) which investigated isolates

from patients in China. The resistance rates of augmentin and ceftazidime, however, were much higher in present study than those recorded in Gu *et al.* (2008) which is study at 35.2% and 19.7% respectively. Besides, in cephalosporin group antibiotics, lower resistance rates was observed in ceftazidime and ceftriaxone than cefuroxime, supporting the fact that the 3$^{rd}$-generation cephalosporins are more active and effective than 2$^{nd}$-generation drugs against gram-negative organisms (Blaise & Congeni, 2008).

In Malaysia, several studies have shown the high prevalence of multidrug resistance in *Klebsiella* sp. (Lim *et al.*, 2009a), *E. coli* (AlHaj *et al.*, 2007), and *Pseudomonas* sp. (Siva *et al.*, 2009). Table 5.1 compares the antibiotic resistance profile in *E. coli*, *K. pneumoniae* and *P.aeruginosa*. Briefly, the highest resistance rate was toward ampicillin (100%) among these three bacterial species. The three species were also found demonstrating high resistance toward cefuroxime, augmentin, trimethoprim, sulfamethoxazole, co-trimoxazole and tetracycline (>80%). Among the thirty-four *E. coli* isolates, none of them were resistant to amikacin, imipenem and meropenem. This is in concordance with the latest 2009 Malaysian National Surveillance on Antibiotic Resistance Report (available online at http://www.imr.gov.my /report/nsar.htm), which concluded a very low resistance rate of between 0.1% to 1.2% for both carbapenems and amikacin in *E. coli*. Similar to the report, higher rates of resistance towards chloramphenicol, amikacin, imipenem and meropenem were observed in *P. aeruginosa* (86.7%, 66.7%, 53.3% and 40%). The percentage of sensitivities of most antimicrobial agents tested in this study

was very much lower as compared to the other studies in Malaysia (Siva *et al.*, 2009; Fazlul *et al.*, 2011).

**Table 5.1: Antibiotic resistance profile in *E. coli*, *K. pneumoniae* and *P. aeruginosa***

| Antimicrobial agents | Antibiotic resistance rate, n (%) | | |
|---|---|---|---|
| | *E. coli* (N= 34) | *K. pneumoniae* (N= 40) | *P. aeruginosa* (N= 15) |
| Amikacin | 0 (0) | 7 (17.5) | 10 (66.7) |
| Gentamicin | 13 (38.2) | 31 (77.5) | 10 (66.7) |
| Streptomycin | 34 (100) | 34 (85) | 10 (66.7) |
| Imipenem | 0 (0) | 3 (7.5) | 8 (53.3) |
| Meropenem | 0 (0) | 2 (5) | 6 (40) |
| Ceftazidime | 24 (70.6) | 38 (95) | 10 (66.7) |
| Cefuroxime | 29 (85.3) | 39 (97.5) | 14 (93.3) |
| Ceftriaxone | 26 (76.5) | 39 (97.5) | 14 (93.3) |
| Augmentin | 28 (82.4) | 39 (97.5) | 14 (93.3) |
| Ampicillin | 34 (100) | 40 (100) | 15 (100) |
| Ciprofloxacin | 26 (76.5) | 33 (82.5) | 12 (80) |
| Norfloxacin | 22 (64.7) | 24 (60) | 9 (60) |
| Trimethoprim | 30 (88.2) | 36 (90) | 13 (86.7) |
| Sulfamethoxazole | 30 (88.2) | 34 (85) | 12 (80) |
| Co-trimoxazole | 29 (85.3) | 34 (85) | 13 (86.7) |
| Tetracycline | 30 (88.2) | 32 (80) | 14 (93.3) |
| Chloramphenicol | 18 (52.9) | 18 (45) | 13 (86.7) |
| Tigecycline | 2 (5.9) | 30 (75) | 12 (80) |

However, the aminoglycosides (amikacin), the carbapenem (imipenem and meropenem) and cephalosporin (ceftazidime, ceftriaxone) were still the most active antimicrobial agents toward *P. aeruginosa* for Malaysian population. Of note, resistance rate of ceftazidime was lower (66.7%) when compared to ceftriaxone and cefuroxime (93.3%), as ceftazidime is known to be the most

active anti-pseudomonal compound (Greenwood & Eley, 1982). Yet, carbapenem antibiotics remain as the last therapeutic option for treatment of serious infections caused by *P. aeruginosa* (Livermore, 2002).

In this study, the resistance rates against all the antibiotics tested in *E. coli* and *K. pneumoniae* for Malaysia were comparable to those published by AlHaj *et al.* (2007) and Lim *et al.* (2009a). In contrast to *E. coli* and *P. aeruginosa*, *K. pneumoniae* in this study showed higher resistance to gentamicin and cephalosporin. The trend of high cephalosporin resistance corroborated with the data in latest 2009 Malaysian National Surveillance on Antibiotic Resistance Report. The sensitivity rates of *K. pneumoniae* to ceftazidime and ceftriaxone were lower than those reported by Lim *et al.* (2009a), which showed sensitivity rates of 51% and 55%, respectively. This may be due to the varying criteria during sample collection, in which only multidrug resistant isolates were selected for this study, in contrast to other studies which were focused on sporadic cases. Differences in drug resistance among hospitals in Malaysia and also other countries may due to the differential usage of antibiotics in different hospitals. Nevertheless, the resistance pattern of *E. coli* in this study is akin to that observed by AlHaj *et al.* (2007).

### 5.3 Total DNA integrity test by 16S rRNA amplification

The integrity of total DNA isolated was accessed by amplification of 16S rRNA gene in bacteria. As shown in Figure 4.7 and 4.8, a 426 bp product of 16S rRNA was amplified successfully. The 16S rRNA gene sequences are commonly used as housekeeping gene to study bacterial phylogeny and

taxonomy. The reasons include (i) it is present in almost all bacteria, often exists as a multigene family, or operons; (ii) the function of the 16S rRNA gene has not changed over time, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene is large enough for informatics purposes (Patel, 2001). In this study, its amplification is used to check for total DNA integrity before target gene amplification.

## 5.4 Prevalence of integrons among collected isolates

This study gives a representative picture of integron prevalence within the hospital environment in Malaysia. In the present study, we examined one hundred and forty-seven *Enterobacteriaceae* and *Pseudomonas* sp. isolated from Malaysian patients. The existence of integrons was confirmed in 48.3% (71/147) of antibiotic resistant isolates. Class 1 integrons was the dominant class identified, with 45.6% (67/147) of these integron-positive isolates having *intI1*. The proportion of class 1 integrons carriage in this antibiotic resistant isolates is comparable to other studies. Several reports have revealed the prevalence of class 1 integrons in gram-negative clinical isolates to be approximately 43% in Europe (Martinez-Freijo *et al.*, 1998), 36% in Australia (White *et al.*, 2000), 50% in Netherlands (Jones *et al.*, 1997), 59% in France (Sallen *et al.*, 1995), and 52% in Taiwan (Chang *et al.*, 2000). Three (4.2%) of the seventy-one integron-positive isolates were detected to contain class 2 integrase. The result were corresponded to other reports stating that class 2 integrons has much lower prevalence than class 1 integrons (Fluit & Schmitz,

1999; White *et al.*, 2000; Gu *et al.*, 2008). The low incidence of *intI2* in this study was also similar to the distribution of class 2 integrons (Tn7) in the other studies, which ranged from 4 to 5.6% of all screened isolates (White *et al.*, 2000; Gu *et al.*, 2008).

Table 4.1 shows that class 1 and 2 integrons are widespread in different species of *Enterobacteriaceae*. Staggeringly, class 1 integrons was highly detected in 80% of *Klebsiella* sp. collected. A higher percentage of class 1 integrons was observed in this study as compared to another study by Lim *et al.* (2009a) in which class 1 integrase were only found in 41.2% (21/51) of the extended-spectrum β-lactamase-producing (ESBL) *K. pneumoniae* strain. This might be due to the increased in antibiotic resistance in this species or variation in criteria of bacteria sample collection. Besides, none of the single class 2 and 3 integrase was detected in *K. pneumoniae* by Lim *et al.* (2009a), in which similar result was observed in this study. Surprisingly, one of the *K. pneumoniae* isolate in this study was shown to harbour both *intI1* and *intI2*, which to our knowledge, has not been reported by any other studies in Malaysia.

In this present study, 55.9% of (19/34) of *E. coli* was shown to carry class 1 integrons and two isolates were found carrying class 2 integrons (5.9%). This result is comparable to Lim *et al.* (2009b), which reported the prevalence of class 1 integrons in 53.2% (25/47) of the ESBL-*E. coli*, while class 2 integrons was found in 8.5% (4/47) of the isolates.

In this study, class 1 integrons were also found in 60% of *P. aeruginosa*. The high occurrence of integrons in *P. aeruginosa* is in contrast to the study of Yasin *et al.* (2009) in which only 19% of the isolates was shown to carry class 1 integrons. This difference can be explained as merely 69% of the collected isolates were multidrug resistant (MDR) in Yasin *et al.* (2009) as compared to this study in which a significant increase in MDR was observed in this species (100%). However, study carried out by Khosravi *et al.* (2011) in Malaysia showed the high prevalence of class 1 integrons in 60% (54/90) of clinical imipenem-resistant *P. aeruginosa* (IRPA).

### 5.5 Prevalence of *sul1* gene and its association with integron structure

After PCR screening of integrase gene, all of isolates (n= 147) were then screened for *sul1* gene which is believed to be integrated into the 3' conserved segment of an integron structure (Fluit & Schmitz, 1999). Of all isolates, only 34.7% of them (n= 51) contained *sul1* gene. Surprisingly, only 41 of 71 strains tested positive for the integrase gene (57.7%) were shown to harbour *sul1* gene. Out of the fifty-one *sul1*-positive isolates, 80.4% (41/51) of them carried integrase gene. The result is contrary to the relatively high prevalence of *sul1* gene in an integrase positive isolates observed in other clinical settings (Levesque *et al.*, 1995; Antunes *et al.*, 2005). Both of these studies showed that more than 90% of the isolates harbouring integrase gene also carried *sul1* gene. Although the presence of *sul1* gene in integrase-positive isolates is relatively lower than other studies, *chi*-square analysis confirmed a significant link between *sul1* and integrase in an integron structure in this study

($p< 0.001$). Interestingly, the four isolates which harboured class 2 or both class 1 and 2 were *sul1*-negative (Appendix D). This is consistent with a study by Skold (2000) which showed that *sul1* is more often appear in class 1 integrons even though Antunes *et al.* (2005) showed that *sul1* gene could be amplified in isolates carrying both class 1 and 2 integrons.

The *sul1*-positive isolates (n= 51) was shown to exhibit more than 90% resistance toward all sulfonamides antibiotics tested (Table 4.5), indicating the strong contribution of *sul1* gene toward bacterial sulfonamide resistant phenotype. However, antibiotic susceptibility test revealed that approximately 60% of the bacteria in this study were sulfonamide resistant (Figure 4.6). Out of the eighty-nine sulfamethoxazole-resistant isolates, only fifty (56.2%) of them harboured *sul1* gene. This observation can be explained by the fact that the sulfonamide resistance in an isolate may not be a result from *sul1* gene but the presence of *dfr* gene cassettes which are always found in class 1 integrons may also play a part in determining the resistancy (Leverstein-van Hall *et al.*, 2003). A similar scenario was also observed by Antunes *et al.* (2005) in which *sul1* gene was detected in 76% of the sulfonamide-resistant *Salmonella enterica* tested.

## 5.6 Integrons and antibiotic resistance in isolates

As reviewed in Table 4.2, integrons was found to confer significant resistance against the antibiotics compounds tested, with the exception of amikacin, imipenem, cefuroxime, ceftriaxone, augmentin, and ampicillin. Of note, the

presence of integrons significantly affected the susceptibility to the sulfonamide compounds including sulfamethoxazole, trimethoprim, and co-trimoxazole ($p< 0.001$). The strong association can be explained by the occurrence of *sul1* gene as part of the integrons structure in addition to the presence of *dfr*-like gene cassettes carried by integrons (Leverstein-van Hall *et al.*, 2003). These two gene families inactivate trimethoprim and sulfamethoxazole, respectively, and thus, result in resistance to co-trimoxazole.

Resistance to quinolone compounds was also shown to be associated with presence of integrons ($p< 0.001$). This result is surprising as resistance to quinolone compounds is generally believed to be derived through chromosomal point mutation rather than being carried on any mobile genetic elements (Ashraf *et al.*, 1991; Martinez-Freijo *et al.*, 1998). However, several studies have stated that there is a clear association of integrons structures with R-mutator plasmids such as R46, in many *Enterobacteriaceae* (Pinney, 1980; Upton & Pinney, 1983). This particular plasmid increases the chromosomal mutation rate which may then lead to higher fluoroquinolones resistance phenotypes (Ashraf *et al.*, 1991; Martinez-Martinez *et al.*, 1998). Integrons or associated plasmids could carry genes that influence the permeability of cells or efflux of the drug, thereby decreasing susceptibility toward fluoroquinolones (Piddock, 1995).

Furthermore, integrons were also found significantly associated with resistance to aminoglycoside antibiotics tested, except for amikacin ($p= 0.118$). This observation is in concordance with the study by Martinez-Freijo *et al.*

(1998), which revealed that the presence of integrons did not only affect the susceptibility toward amikacin, but it was significantly associated with gentamicin too. The percentages of susceptibility for amikacin in integron-positive isolates in this study was slightly lower (80.3%) as compared to 98.6% reported by Martinez-Freijo *et al.* (1998). Resistance toward aminoglycoside is not surprising, since many *aac*/*aad* genes responsible for aminoglycoside resistance have been reported in integrons. However, the gene cassettes encoding aminoglycoside acetyltransferases (*aacA*), with amikacin as substrate, might encounter low gene expression in integrons (Martinez-Freijo *et al.*, 1998).

The association of the other antibiotics like chloramphenicol, and tetracycline with the presence of an integron is more likely to be due to genetic linkage between integrons and conjugative plasmids and transposons (White *et al.*, 2001).

## 5.7 Integrons and multidrug resistance

Diverse definitions for multidrug resistance have been used by researchers and clinicians worldwide. The term "MDR" was defined as resistance to a number of antibiotics ranging from more than two (Leverstein-van Hall *et al.*, 2002a) or five (Moura *et al.*, 2007) even up to six antibiotics (Gu *et al.*, 2008). In this study, bacterial isolates resistance to more than two antibiotics were termed as MDR isolates.

In this study, antimicrobial resistance patterns revealed that multiple resistance correlated strongly with the presence of integrons ($p< 0.001$). In fact, 99% of isolates appeared resistant toward three or more antibiotics. There are 57.7% (41/71) of integron-positive isolates which were resistant to 13-18 types of antibiotics, whilst 56.6% (43/76) of the integron-negative were resistant to less than six types. Besides, fifteen integron-negative isolates showed low susceptibility, again supporting the possibility of alternative approaches in the mobilization of antibiotic resistance determinants such as chromosomal mutation or other mobile DNA elements acquisition (Chang *et al.*, 2000; Thungapathra *et al.*, 2002). In summary, the prevalence of integron increases along with the number of antibiotic classes for which resistance is expressed, as mentioned in the studies by Martinez-Freijo *et al.* (1998), Schmitz *et al.* (2001) and Leverstein-van Hall *et al.* (2003).

## 5.8 Characterisation of gene cassettes in integron-positive bacteria

Integrase positive bacteria were further characterised in term of their gene cassettes contents. As shown in Table 4.6, gene cassettes in forty-two of the isolates (59.2%) were successfully amplified using 5'-CS, 3'-CS and hep74, hep51 primer pairs respectively. Five of these isolates generated only 150 bp amplicons, were later proven to be an integron structure without any inserted gene cassettes. Furthermore, no amplification product was obtained in 33.8% of the integrase-positive isolates in this study. This is most probably due to the lack of a 3' conserved region (CS) as stated by other authors (White *et al.*, 2001; Moura *et al.*, 2007). This hypothesis can be supported through the

observation that most isolates without any gene cassettes amplification were also lack of *sul1* gene which is generally located at the 3'CS of an integron. Table 5.2 shows the absence of *sul1* gene in 20 out of 24 isolates with no gene cassettes amplification. Others also claimed that this might be due to the modification of primer binding site (Yu *et al.*, 2003) or the variable region is too long to be amplified in these isolates (Barlow *et al.*, 2004; Yao *et al.*, 2007).

**Table 5.2: Cross tabulation between gene cassettes amplification and presence of *sul1* gene**

Gene cassettes * *sul1* cross tabulation

| | | *sul1* | | Total |
|---|---|---|---|---|
| | | negative | positive | |
| Gene Cassettes | No amplification | 20 | 4 | 24 |
| | With amplification | 10 | 37 | 47 |
| Total | | 30 | 41 | 71 |

The size range of inserted gene cassettes varied between 721 bp and 1655 bp. The difference in sizes of cassette inserted between CS regions demonstrates the variable nature of these structures, presumably reflecting the differences in the number and type of inserted gene cassettes as claimed by Martinez-Freijo *et al.* (1998). In this study, integron-positive bacteria having a single amplicon (one PCR fragment) were the most predominant (81%). Nineteen types of gene cassettes arrays were detected, containing one to three cassettes. It is

observed that the gene cassettes of 23.1% (9/39) class 1 integrons isolates were composed of a single cassette gene, while others were mostly composed of more than two cassette genes. The increase in the prevalence of multiple-gene cassettes may be caused by the heavy selective pressure nowadays as more new gene cassettes encoding resistance to antibiotic were introduced (Yu *et al.*, 2003).

Based on the present result, the most common gene cassettes detected in class 1 integrons were the *aadA* and *dfr*-type gene cassettes, which confer resistance to spectinomycin/streptomycin and trimethoprim, respectively. Several studies have reported these two genes were frequently found both in clinical (Chang *et al.*, 2000; Blahna *et al.*, 2006) and environmental (Roe *et al.*, 2003; Henrique *et al.*, 2006) isolates. In the present study, the aminoglycosides adenyltransferase genes (*aad*) were detected in 69.2% (27/39) of *intI1*-positive isolates, alone or in combination with other resistance genes. In Malaysia, spectinomycin and streptomycin are rarely used nowadays. Despite the minimal use as therapeutic agents, resistance to spectinomycin and streptomycin still remains prevalent in *Enterobacteriaceae* isolates as observed here and also those reported by Chiew *et al.* (1998) and Bass *et al.* (1999). Previous studies also showed the presence of streptomycin-resistant bacteria isolated from animals in Denmark (Ridley & Threfall, 1998; Sandvang *et al.*, 1998). The continued usage of spectinomycin/streptomycin in agriculture or animal may result in the selection of resistant genes from streptomycin-resistant bacteria which in turn are transmitted to humans (Wegener *et al.*, 1999; Su *et al.*, 2006).

Beside *aadA*, dihydrofolate reductase (*dfr*) cassettes which confer resistance to trimethoprim were found in 54% (21/39) of *intI1*-positive isolates. Trimethoprim alone, or in combination with sulfamethoxazole, generally act as the empiric drug of choice for many clinical patients worldwide (Navia *et al.*, 2004), including Malaysia. Such specific selection pressure may have supported the acquisition and maintenance of *dfr*-like cassette in class 1 integrons (Sandvang, 1999; Yu *et al.*, 2003).

As observed in Table 4.7, most *aadA* cassettes are usually found present in combination with other gene cassettes. These cassettes are found adjacent to the 5' conserved segment and lie upstream of the *aadA* cassettes. This scenario could further support the suggestion of some researchers claiming that *aadA* may be the first cassette acquired by integrons (Bissonnette & Roy, 1992; Rosser & Young, 1999). This is because the gene cassettes have higher preference to recombine into *attI* site than *attC* site of a former inserted gene cassette. Therefore, the nearest cassette to 5' conserved segment is thought to be the latest addition to the integrons due to the recombination site (Hall & Collis, 1995).

Despite different combinations of gene cassettes were obtained in this study, some gene cassettes were observed as frequently conserved over time. For example, in *E. coli*, gene cassettes such as *aadA1*, *dfrA17-aadA5*, *dfrA1-aadA1* and *dfrA12-orfF-aadA2* found in this study were firstly introduced in the 1990s (Yu *et al.*, 2003). These gene cassettes have also been reported in urinary *E. coli* isolated from Korea, Taiwan, Turkey, Finland and Australia

(Heikkila *et al.*, 1993; Chang *et al.*, 2000; White *et al.*, 2001; Yu *et al.*, 2003). Researchers have proposed that gene cassette is thought to be considerably stable as it is transferred by the mobilisation of the entire integrons, via transposons or plasmids, rather than individual resistant genes (Martinez-Freijo *et al.*, 1999).

In this study, no novel gene cassettes sequences were identified among all the different bacterial species. Ten *E. coli* isolates was found to carry some identical gene cassettes such as *aadA1*, *dfrA17-aadA5*, and *dfrA12-orfF-aadA2*, similar to a previous Malaysia study (Lim *et al.*, 2009b). To our knowledge, cassette arrays like *dfrA5*, *aacA4-catB3-dfrA1* and *dfrA1-aadA1* were first reported in *E. coli* isolated from the Malaysian population, although these genes have been seen in other foreign countries including Asian country i.e. Indonesia and China (Waturangi *et al.*, 2003; Wang *et al.*, 2008). The gene cassette array *aacA4-catB3-dfrA1*, which has been previously reported in Japan (Kumai *et al.*, 2005) and China (Li *et al.*, 2006), was also found in this study, indicating that this resistance cassette is widespread in Asian countries. Surprisingly, a rarely detected combinational cassette of *aadA2-linF* was found in one of the *E. coli* isolate (sample H35) in this study. The stand alone *aadA2* and *linF* cassettes are frequently detected among *Enterobacteriaceae* isolates. However, referring to sequences deposited in GenBank, *aadA2-linF* had only been detected in *Salmonella enterica* (Yan *et al.*, 2009) and *E. coli* (Gonzalez *et al.*, 2005). Our study is the first to report the presence of *aadA2-linF* in *E. coli* from clinical isolates in Malaysia population.

On the other hand, Lim and his colleagues (2009a) also managed to amplify gene cassettes *aadB* and *dfrA12-orfF-aadA2* from 57% (12/21) of the *intI1*-positive ESBL- *K. pneumoniae* isolates in local population. These similar cassettes were also observed in *K. pneumoniae* of this study. Of note, *aadB* is claimed to be frequently associated with $bla_{SHV}$ genes in ESBL-producing *Klebsiella* sp. (Jones *et al.*, 2005). Other cassette arrays, like *dfrA7*, *blaP1*, *dfrA15-aadA1*, *dfrA16-aadA2*, *dfrA17-aadA5*, and *aacA4-catB8-aadA1*, were previously detected by other authors (White & Rawlinson, 2001; Daikos *et al.*, 2007; Gu *et al.*, 2008; Ozgumus *et al.*, 2009). Interestingly, *aacA4-catB8-aadA1* which was firstly reported in clinical *K. pneumoniae* from China (Gu *et al.*, 2008), but never before in Malaysia, was also found widespread in four *K. pneumoniae* isolates in this study. This implicates that fast and wide dissemination of class 1 integrons and their gene cassettes to different parts of the world is going on.

In *P. aeruginosa*, the class 1 integrons variable regions of all isolates (9/9) were successfully amplified. The common gene cassettes detected were *aadA6-orfD* alone or in combination with *aacC3-cmlA5*. Particularly, the cassette *aadA6-orfD* was found in 89% of the isolates. This array seems to be widespread in different regions of the world, such as Mexico, United State of America, France, Poland, and Iran (Naas *et al.*, 1999; Fiett *et al.*, 2006; Shahcheraghi *et al.*, 2009; Garza-Ramos *et al.*, 2010; Borgianni *et al.*, 2011). Unlike *aadA6-orfD* and *aacC3-cmlA5*, the cassette *aacA4-catB8-aadA1* detected in one of the isolates (sample K7) was usually reported in *Acinetobacter baumannii* (Turton *et al.*, 2005), although this gene had been

previously observed in *P. aeruginosa* (Jeong *et al.*, 2009). All the gene cassettes identified in *P. aeruginosa* in this study was deemed to be the first reported incidence in Malaysia, as not much research has been conducted in characterising integrons from *P. aeruginosa* in this country.

Class 2 integrons variable regions were amplified using primers that bind to *attI2* and to *orfX*, which is situated downstream of the cassette region within transposon Tn7 (White *et al.*, 2001). Nucleotide sequencing of the variable region of the four class 2 integrons (including one isolates which harboured both class 1 and 2 integrons) revealed that all four carried the same integrated gene, namely, *dfrA1-sat1-aadA1*, which are analogues to those found in Tn7 (Roe *et al.*, 2003; Gu *et al.*, 2008). The *dfrA1-sat1-aadA1* gene array was said to be stably integrated in the variable region as class 2 integrons is deemed to contain a defective integrase gene which is unable to alter this array of gene cassettes (Hansson *et al.*, 2002). Cassette array *dfrA1-sat1-aadA1* which is associated with *intI2* was reported from several different species, most commonly in *E. coli* (Partridge *et al.*, 2009). However, this class 2 integrons cassette was also successfully amplified in one of the *intI2*-positive *Enterobacter clocae* in this study, which is in agreement with cassette found in Tn7 complete sequence by Ramirez *et al.* (2010).

From Table 4.8, antimicrobial susceptibility tests showed the relationship between the gene cassettes in integrons and bacterial resistancy, as indicated in other studies (Rosser & Young, 1999). For example, 100% of the isolates which harboured dihydrofolate reductase (*dfr*) cassette showed resistance

toward trimethoprim. All the *dfr* cassettes, except for one, were located directly behind the conserved segment, which is closest to the promoter and thereby giving high level of expression and contingent resistance. However, out of the seven isolates carrying *aacA4* which encodes resistant to amikacin, only 28.6% (2/7) of them showed the respective resistant phenotype to the antibiotic. This suggests that *aacA4* associated with integrons carriage mostly provide reduced susceptibility towards amikacin compounds rather than full resistance, probably owing to its low-level of gene expression (Martinez-Freijo *et al*., 1998). A few isolates also exhibited reduced susceptibility to aminoglycoside despite the presence of *aadA* gene cassette in integrons. This may be due to its cassette order and the varying number of promoters used that could cause weakness in the expression of gene cassettes array, as discussed previously (Martinez-Freijo *et al*., 1999). However, the gene cassettes found in this study did not explain the totality of resistance phenotype observed. For instance, no gene cassettes contributing bacterial resistance towards tetracycline, chloramphenicol, and tigecycline were detected. Similar observation were made previously by Chang *et al.* (2000) and Thungapathra *et al.* (2002) which suggest the possible existence of other resistance mechanism responsible for mediating multidrug resistant in many isolates.

### 5.9 Risk factors for integron-carriage

From Table 4.3, it is shown that the presence of integrons is highly associated with patient's age groups ($p$= 0.028). Generally, a marked increased risk to harbour an integron-positive isolates was observed among patients aged 19

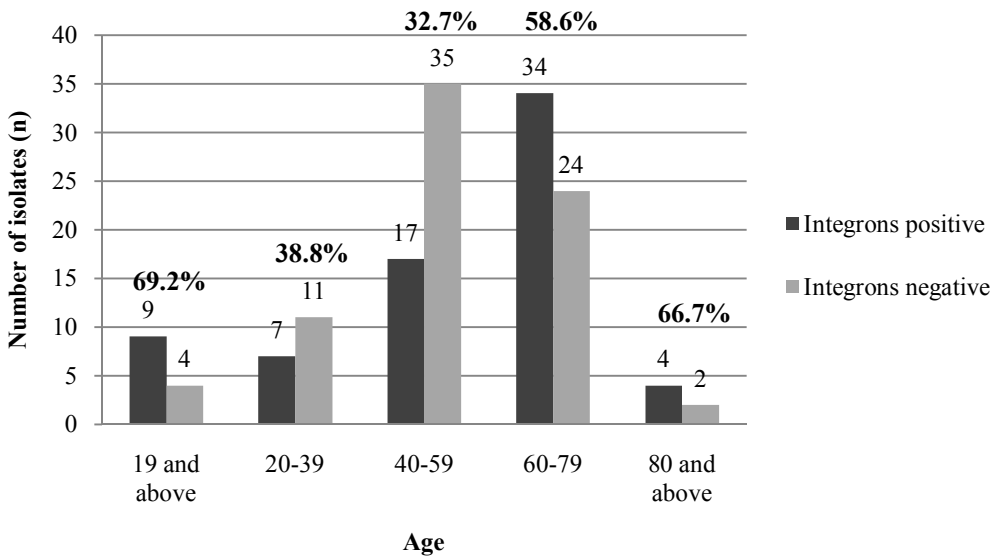



**Figure 5.1: Distribution of integron-positive and integron-negative isolates among different age groups.**

and below (69.2%), 60-79 years old (58.6%), and 80 and above (66.7%) (Figure 5.1). Out of nine integron-positive isolates obtained from patients of younger age (19 and below), 66.7% (6/9) were patients under the age of 10, including two infants. On the contrary, four of the integron-negative isolates in this group were from patients above 13 years old. Similar observation was made in the elderly group, in which 50% (2/4) of the integron-carrying patients was from patients aged 88 years old and above, while the remaining two integron-negative isolates were from patients aged 80 and 81 year old. This result is in agreement with the hypothesis made by Sepp *et al.* (2009). The higher resistance rate and integron-containing bacteria in elderly people is presumably caused by their lifespan selective pressure of antibiotics. However, as suggested by Sepp and colleagues, the possible reason for an infant to carry integron-positive isolates is that their gastrointestinal micro-ecosystem is not

yet fully developed and colonisation resistance against exogenous bacteria is weaker. Hence, high number of *E. coli* from the environment, containing different virulence or resistance genes (e.g. integrons) may be colonising the gastrointestinal system (Sepp *et al.*, 2009).

Besides, the patients' mean age was 54 ± 23 and 52 ± 16 years old for integron-positive and integron-negative isolates, respectively which did not differ significantly. Interestingly, integron carriage is not significantly associated with patient's mean age ($p$= 0.173), and it remains unexplained. Similar observation was made by Nijssen *et al.* (2005) and Daikos *et al.* (2007). However, aging can be considered to be a factor which promotes the acquisition of integron-carrying strain in patients, especially those young and elderly with weakened immune system. Nevertheless, sampling bias has to be taken into account in our study as the size of isolates collected were not equally distributed among each of the defined age groups.

In this study, no significant statistical difference was detected between patients with integron-positive organisms and those without integrons with regards to gender ($p$= 0.127). Despite a close equal number of patients of both genders were observed to carry integrons, females however showed a greater risk of having integron-carrying strains with 12.8% higher than male patients (Figure 5.2). This observation is in agreement with a study by Daikos *et al.* (2007), which investigated the prevalence of integrons in *Enterobacteriaceae* from bloodstream infection. He and his colleagues confirmed that 34.4% of female patients harboured integron-positive isolates, whilst the males only 30.4%.

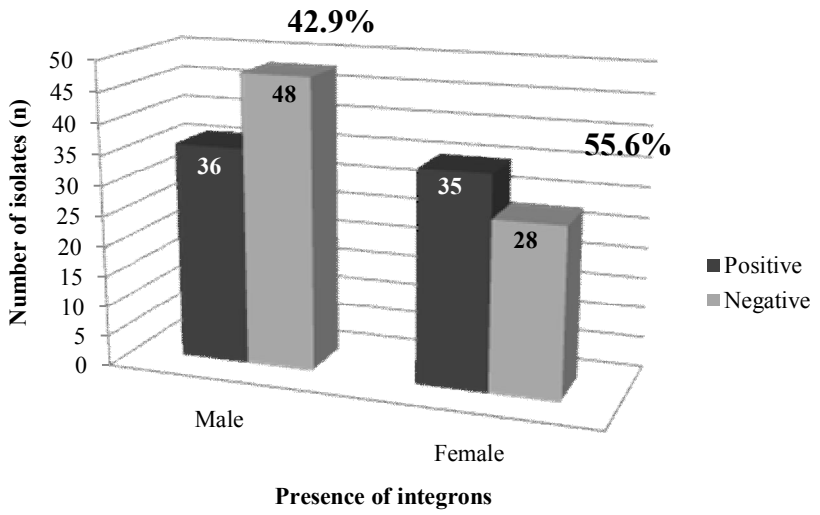



**Figure 5.2: Distribution of integron-positive and integron-negative isolates in male and female patients.**

Our study also investigated the risk of the microbiological data, i.e. sources of collected specimens, contributed to integrons acquisition. *Chi*-square test was shown inappropriate for the analysis due to the small sample size (less than 10) and in excess of independent variables implicated. As shown in Figure 5.3, integrons were more likely present in bacteria isolated from urine, tissue, pus swab and "others" (more than 50%). Of note, 92.3% (24/26) of the urine isolates were integron-positive. The isolates from urine were comprised of twelve samples of *E. coli* and *K. pneumoniae* each, one *E. cloacae* and one *P. aeruginosa*. All of the isolates from urine except two *E. coli* isolates were integron-positive. As far as nosocomial urinary tract infection (UTI) is concerned, *K. pneumoniae* is second only to *E. coli* as the major causative agent and it accounts for 6 to 17% of all infections worldwide (Schembri *et al.*,

2005). Interestingly, our data suggests a comparable high risk of *K. pneumoniae* isolated from urine in acquiring an integron structure than *E. coli*. According to a report by Lina *et al.* (2007), a relatively high occurrence of class 1 integrons was observed in *K. pneumoniae* (88%) than in *E. coli* (54%) despite the later was the most prevalent isolates recovered from UTI patients compared to *K. pneumoniae* (88% versus 12% of the cases). Our result raises the hypothesis that UTI *Klebsiella* sp. is more frequently associated with the lateral antibiotic gene transfer mediator-integrons. However, further studies are needed with a larger scale of sample collection.

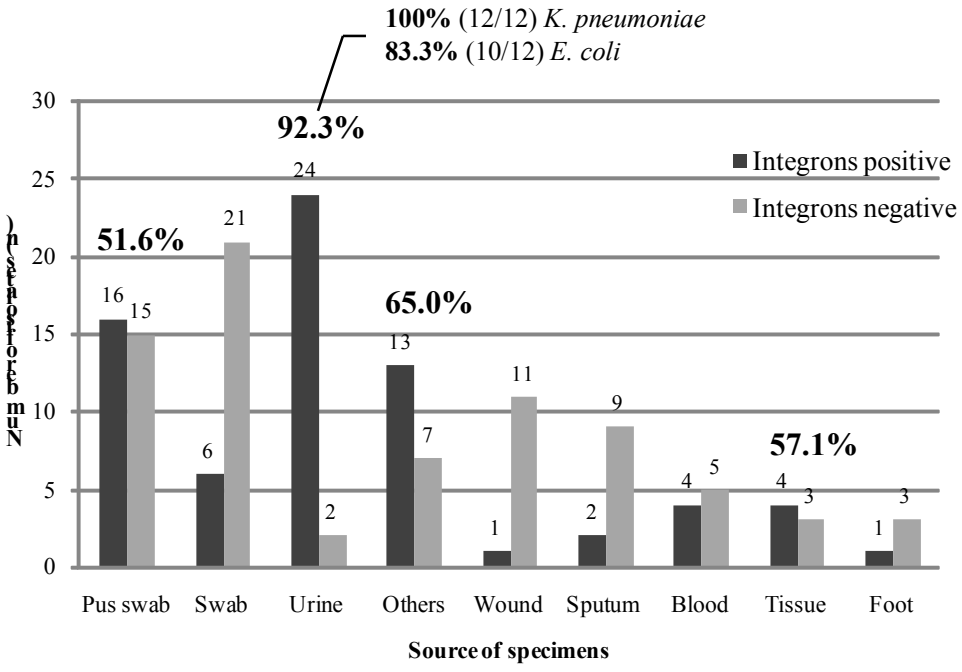



**Figure 5.3: Distribution of integron-positive and integron-negative isolates regards to different site of specimens.** "Others" in the chart indicates sampling sites from bone, endotracheal tubes (ETT), tracheal aspirate and more.

Through this study, twenty-two integron-positive *E. coli* and *K. pneumoniae* isolated from urine were found mostly in females patients (68%) (Table 5.3). In addition, the urinary *E. coli* collected from 50% of female and 20% male patients in this study belonged to the age group 40-79 years old. As mentioned in Dromigny *et al.* (2005) and Ho *et al.* (2010), urinary tract infection is a common phenomenon among women and they are mostly at higher risk of developing UTI during their lifetime. Besides, the incidence of UTI was reported to be progressively increasing in men after middle age owing to the development of prosthetic enlargement and consequent instrumentation (Feliciano *et al.*, 2008). In contrast to *E. coli*, *K. pneumoniae* urine samples were collected from patient of all defined age groups.

**Table 5.3: Cross tabulation of risk factors of gender and age groups with integron-positive *E. coli* and *K. pneumoniae* isolates**

| Gender | Age groups | Integron-positive urine isolates | |
|---|---|---|---|
| | | *E. coli* (n= 10) | *K. pneumoniae* (n= 12) |
| Male | 19 and below | 1 | - |
| | 20-39 years | - | 1 |
| | 40-59 years | 1 | - |
| | 60-79 years | 1 | 2 |
| | 80 and above | - | 1 |
| Female | 19 and below | 2 | 1 |
| | 20-39 years | - | 2 |
| | 40-59 years | 2 | 2 |
| | 60-79 years | 3 | 2 |
| | 80 and above | - | 1 |

## 5.10 Clonal relationship between isolates carried identical gene cassettes

The genetic relatedness for eight *K. pneumoniae*, four *E. coli* and eight *P. aeruginosa* which were identified to contain identical gene cassettes was determined by pulse-field gel electrophoresis (PFGE). PFGE analysis revealed distinct patterns in *E. coli* isolates carrying cassettes *aacA4-catB3-dfrA1* and *dfrA1-sat1-aadA1*. The result suggests that a specific clone did not contribute to dissemination of a particular gene cassette among the *E. coli* isolates. This is expected owing to the isolates were obtained from different sources (blood, pus swab and urine) and hospitals. Thus, it is believed that no inter-hospital or community transmission has occurred in this study for *E. coli*.

Eight *K. pneumonia* isolates in total, however, generated a distinct cluster based on 80% similarity comprising of three isolates carrying *dfrA7* gene cassette. This cluster of three strains with similar *XbaI* profile came from different types of specimens of one particular individual (bone, swab and tissue), suggesting at the probability of the spread of integrons clone within the same host organism (patient). Other isolates which contained *aacA4-catB8-aadA1* were found epidemiological irrelevant to one another.

In *P. aeruginosa*, dendrogram analysis showed less diversity in PFGE pattern and two clusters were generated. It is important to note that all five of the *aadA6-orfD* & *aacC3-cmlA5-* carrying isolates were 80% similar. Interestingly, three of the isolates were isolated from different sites i.e. tracheal aspirate, tissue, and endotracheal tubes (ETT) secretion of the same

patient. On the other hand, two of the isolates carrying *aadA6-orfD* were both collected from tracheal aspirate isolates of two different patients. This result suggests the nosocomial spread of the clone in the respective hospital. Cross-transmission of integron-carrying clones which disseminates bacteria from patient to patient is more often observed in nosocomial environment (Nijssen *et al.*, 2005; Daikos *et al.*, 2007). The use of suboptimal aseptic techniques and infection control practices by healthcare workers, inadequate cleaning and disinfection of the environment and medical equipment, and understaffing may contribute to the dissemination of integron-clones (Chen *et al.*, 2009). Thus, the spread of two isolates carrying *aadA6-orfD* from tracheal aspirate may indicate the dissemination of clones through hospital's ventilation machine as *P. aeruginosa* is commonly found in patients with a tracheal cannula, tracheotomy, or under mechanical ventilation (Sarlangue *et al*., 2006).

Besides intra-species integrons transfer, inter-species transfer of some gene cassettes/integrons is also possible (Daikos *et al.*, 2007). Inter- or intra-species integrons transfer occurs very efficiently among *Enterobacteriaceae* within the hospital settings (Leverstein-van Hall *et al.*, 2002b). For instance, cassette arrays consisting of *dfrA12-orfF-aadA2* have been detected in more than one species (*E. aerogenes*, *E. coli*, *M. morgannii*, and *P. mirabilis*) in this study, despite literature by other researchers claiming that it is mostly found in *E. coli* (Chang *et al.*, 2000; Yu *et al.*, 2003; Kang *et al.*, 2005; Su *et al.*, 2006). In general, a similar PFGE pattern for some isolates may reflect the cross infection or acquisition of integrons from a common source. However, a more

noteworthy conclusion can only be drawn if more data on the specific hospitality setting or patient history were obtained.

### 5.11 Public versus private hospitals

In this study, isolates were collected from one public hospital i.e. Hospital Raja Permaisuri Bainun Ipoh and other private hospitals as mentioned in Section 3.3.

Out of one hundred forty-seven isolates, 44.2% of them (65/147) were obtained from Hospital Raja Permaisuri Bainun Ipoh. Besides, forty-seven isolates (32%) were collected from Gribbles Pathology which was the highest among all private hospitals. The PCR amplification screening of integrase gene showed that integrons was highly prevalence in the public hospital (69%, 45/65). Interestingly, private hospitals showed relatively low integrons carriage (less than 50% of isolates collected), except for isolates from KPJ Ipoh Specialist Hospital (77%, 14/18). Despite the high amount of isolates collected from Gribbles Pathology, only 10.6% of the isolates were integron-positive. In summary, a total of 32% (26/82) of the collected isolates from all combined private hospitals contained integrons.

In the present study, amplification of integrons and its gene cassettes in isolates has act as a tool to investigate the nosocomial spreading in hospitals environment. As mentioned in Section 5.10, PFGE analysis suggests that the two *P. aeruginosa* isolates which carried identical cassette *aadA6-orfD* were

disseminated through specific clone within the hospital, i.e. KPJ Ipoh Specialist Hospital. Both isolates were collected from tracheal aspirate of two different patients, and thus suggests the possible of clone-transmission through the ventilation machine. In Malaysia, inadequate prospective surveillance nosocomial infections were done with all nation hospitals. However, a study by Katherason *et al.* (2008) from Malaysia showed that nosocomial pneumoniae (ventilator-associated pneumonia, VAP) were the major and most prominent nosocomial infections observed in their academic hospitals, related to the usage of mechanical ventilation systems with endotracheal tubes.

In addition, nosocomial dissemination of clone was not detected in isolates from Hospital Raja Permaisuri Bainun Ipoh in this study. Despite higher prevalence of integrons was observed in public hospital, the nosocomial infections control for private hospitals seems to be a concern and needs further addressing. However, a proper conclusion could not be drawn due to the low quantity of samples collected.

## 5.12 Limitations of study

There are several limitations to this study. Firstly, the samples collected were not evenly distributed throughout all the states in Malaysia and the sample size was relatively small.

In addition, insufficient medical history of the patient from whom the isolates were collected might also affect the epidemiological study on prevalence of integrons or gene cassettes in this study. The lack of detailed information such as hospital wards, invasive procedure (catheter or ventilation), and underlying disease of patients restraints the identification of the potential risk for integrons uptake. Besides, data of antibiotics used in preceding months or the response of patients to the antimicrobial therapy which may affect the bacteria antibiotic susceptibility profile are also unknown.

In this study, relatively low proportions (33.8%, 24/71) of gene cassettes contents in integron-positive isolates were unidentifiable. This may be explained by the inability of PCR to amplify integrons with three or more cassettes (>3 kb), which might as well influence the subsequent result.

## 5.13 Future studies

This study has no doubt formed the platform for more promising investigation in future. In order to improve the understanding of how resistance genes flow across bacteria strains, future experimental studies to assess the integrons transfer using conjugation process could be performed. Other genes and transfer mechanisms besides conjugation should also be further investigated. Besides, Southern hybridisation can be done in future to determine the genetic localisation of the detected integrons. This study could help to define whether integrons in this study are plasmid-borne or chromosomally-located.

In addition, PCR amplification of antibiotic resistant genes determinants such as genes encoding resistance towards beta-lactamase (e.g. *bla*TEM, *bla*SHV), tetracycline (e.g. *tet*A, *tet*B, *tet*C), and chloramphenicol (e.g. *cmlA*, *catB2*) could also be carried out. This could help in assessing the relationship between integrons and the specific resistance genes with the bacterial resistance phenotypes. In this study, the gene cassettes for some integron-positive isolates could not be amplified. Therefore, further studies are required to define the most effective methods for screening *Enterobacteriaceae* and *Pseudomonas* sp. carriage in hospitalised patients such as the use of Southern blotting technique.

Furthermore, the sampling size could be increased to reflect the true prevalence of the three classes of integrons and their respective gene cassettes. Details of patients' medical data should also be extensively collected in future. A better understanding of such patient history is crucial in order to assess the

risks of integron-carriage in patients. Due to the clonal dissemination found in this study, a greater attention to environmental decontamination and bacterial colonised patient is needed as a mean for prevention of patient-to-patient transfer. Further studies are required to define the efficacy of the infection control by investigating the way integrons carrying clone spread in hospital to prevent integrons dissemination.

# CHAPTER 6

# CONCLUSION

This study was done to investigate the antibiotic resistant profiles and the prevalence of three classes of integrons in *Enterobacteriaceae* and *Pseudomonas* sp. collected from Malaysia populations. Most of the isolates showed multiple antibiotic resistance, whilst higher resistance was found against ampicillin, augmentin, cefuroxime, and ceftriaxone; whereas, the least resistance was detected against imipenem, meropenem and amikacin.

In this study, integrons were widely distributed in 48.3% out of one hundred and forty-seven isolates collected. Class 1 integrons was the most dominant integrons found, in which it was observed in 45.6% (67/147) of the isolates. On the other hand, 4.2% of the isolates contained class 2 integrons and one isolate harboured both class 1 and 2 integrons. No class 3 integrons was detected. In addition, *sul1* gene was amplified in 35% (51/147) of the isolates and was deemed to be associated with the presence of integrase genes in an integron structure.

PCR amplification and DNA sequencing confirmed the gene cassettes contents in 66.2% (47/71) of integron-positive isolates. The *aadA* and *dfrA*-like cassettes were the most common gene cassettes identified, in addition to many other recently described cassettes that confer antibiotic resistance. No novel gene cassette was identified in this study as compared to other countries;

despite a few of them like *aadA6-orfD*, *aacC3-cmlA5*, and *aacA4-catB8-aadA1* were relatively new to the Malaysian population. The characterisation of *aadA2-linF* represents the first report of an integron-located gene cassette resistant to aminoglycosides and lincosamide in Malaysia. From the present study, a strong association was observed between wide range resistance to antibiotics with the presence of integrons especially towards the sulfonamide antibiotics group. Besides, patients' age group was found as the only significant risk factor for integron-carriage. The epidemiological typing using PFGE also demonstrated the clonal relationship between isolates carrying identical gene cassettes in *K. pneumoniae* and *P. aeruginosa*, but not in *E. coli* isolates obtained in this study.

In conclusion, the present study revealed that integrons are distributed in *Enterobacteriaceae* and *P. aeruginosa* in the hospital patients in Malaysia. The study of integrons and their associated gene cassettes has become a useful epidemiological tool to provide important information on which antibiotics should be administered more carefully in the future to prevent further accumulation of resistance.

## REFERENCES


Agodi, A., Sciacca, A., Giannino, V., Sinatra, F., Campanile, F., Messina, C., Barchitta, M., and Stefani, S. (1995). Ultrastructural and molecular characterization of *Burkholderia cepacia*. *Clinical Microbiology and Infection*, 5, 198.

AlHaj, N., Mariana, N. S., Raha, A. R., and Ishak, Z. (2007). Prevalence of antibiotic resistance among *Escherichia coli* from different sources in Malaysia. *Research Journal of Pharmacology*, 1, 44-49.

Amyes, S., and Gemmell, C. (1992). Antibiotic resistance in bacteria. *Journal of Medical Microbiology*, 36, 4-29.

Antunes, P., Machado, J., Sousa, J. C., and Peixe, L. (2005). Dissemination of sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) in Portuguese *Salmonella enterica* strains and relation with integrons. *Antimicrobial Agents and Chemotherapy*, 49, 836-839.

Arakawa, Y., Murakami, M., Suzuki, K., Ito, H., Wacharotayankun, R., Ohsuka, S., Kato, N., and Ohta, M. (1995). A novel integron-like element carrying the metallo-beta-lactamase gene *bla*IMP. *Journal of Antimicrobial Agents and Chemotherapy*, 39, 1612-1615.

Ashraf, M. M., Ahmed, Z. U., and Sack, D. A. (1991). Unusual association of a plasmid with nalidixic acid resistance in an epidemic strain of *Shigella dysenteriae* type 1 from Asia. *Canadian Journal of Microbiology*, 37, 59-63.

Barlow, R. S., Pemberton, J. M., Desmarchelier, P. M., and Gobius, K. S. (2004). Isolation and characterization of integron-containing bacteria without antibiotic selection. *Antimicrobial Agents and Chemotherapy*, 48, 838-842.

Basim, H. (2001). Pulse-field gel electrophoresis (PFGE) technique and its use in molecular biology. *Turkish Journal of Biology*, 25, 405-418.

Bass, L., Liebert, C. A., Lee, M. D., Summers, A. O., White, D. G., Thayer, S. G. and Maurer, J. J. (1999). Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia col*i. *Antimicrobial Agents and Chemotherapy*, 43, 2925-2929.

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*, 153, 347-357.

Birren, B. W., Lal, E., Clark, S. M., Houd, L., and Simon, M. I. (1988). Optimized conditions for pulse-field gel electrophoretic separations of DNA. *Nucleic Acids Research*, 16, 7563-7581.

Biskri, L., and Mazel, D. (2003). Erythromycin esterase gene *ere(A)* is located in a functional gene cassette in an unusual class 2 integron. *Journal of Antimicrobial Agents and Chemotherapy*, 47, 3326-3331.

Bissonnette, L., and Roy, P. H. (1992). Characterization of In0 of *Pseudomonas aeruginosa* plasmid pVS1, an ancestor of integrons of multiresistance plasmids and transposons of gram-negative bacteria. *Journal of Bacteriology*, 174, 1248-1257.

Bissonnette, L., Champetier, S., Buisson, J. P., and Roy, P. H. (1991). Characterization of the nonenzymatic chloramphenicol resistance (cmlA) gene of the In4 integron of Tn1696: similarity of the product to transmembrane transport proteins. *Journal of Bacteriology*, 173, 4493-4502.

Biyela, P. T., Lin, J., and Bezuidenhout, C. C. (2004). The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Science and Technology*, 50, 45-50.

Blahna, M. T., Zalewski, C. A., Reuer, J., Kahlmeter, G., Foxman, B., and Marrs, C. F. (2006). The role of horizontal gene transfer in the spread of trimethoprim-sulfamethoxazole resistance among uropathogenic *Escherichia coli* in Europe and Canada. *Journal of Antimicrobial and Chemotherapy*, 57, 666-672.

Blaise, L., and Congeni, M. D. (2008). *Antimicrobial therapy*. URL: https://www.pediatriccareonline.org. Accessed on 1st October 2011.

Boer, H. A., Comstock, L. J., and Vasser, M. (1983). The tac promoter: a functional hybrid derived from the trp and lac promoters. *Proceedings of the National Academy of Sciences of U. S. A.*, 80, 21-25.

Bonomo, J., and Gill, R. T. (2005). Antibiotic resistance as a model for strain engineering. *Computers and Chemical Engineering*, 29, 509-517.

Borgianni, L., Prandi, S., Salden, L., Santella, G., Hanson, N. D., Rossolini, G. M., and Docquier, J. D. (2011). Genetic context and biochemical characterization of the IMP-18 metallo-beta-lactamase identified in a Pseudomonas aeruginosa isolate from the United States. *Journal of Antimicrobial Agents and Chemotherapy*, 55, 140-145.

Botes, J., Williamson, G., Sinickas, V., and Gurtler, V. (2003). Genomic typing of *Pseudomonas aeruginosa* isolates by comparison of Riboprinting and PFGE: correlation of experimental results with those predicted from the complete genome sequence of isolate PAO1. *Journal of Microbiological Methods*, 55, 231-240.

Bou, G., Cervero, G., Dominguez, M. A., Quereda, C., and Martinez-Beltran, J. (2000). PCR-based DNA fingerprinting (REP-PCR, AP-PCR) and pulsed-field gel electrophoresis characterization of a nosocomial outbreak caused by imipenem- and meropenem-resistant *Acinetobacter baumannii*. *Clinical Microbiology and Infection*, 6, 635-643.

Boucher, Y., Labbate, M., Koenig, J., and Stokes, H. (2007). Integrons: mobilizable platforms that promote genetic diversity in bacteria. *Trends in Microbiology*, 15, 301-309.

Brisse, S., Milatovic, D., Fluit, A. C., Kusters, K., Toelstra, A., Verhoef, J., and Schmitz, F. J. (2000). Molecular surveillance of European quinolone-resistant clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter* spp. using automated ribotyping. *Journal of Clinical Microbiology*, 38, 3636-3645.

Bunny, K. L., Hall, R. M., and Stokes, H. W. (1995). New mobile gene cassettes containing an aminoglycoside resistance gene, *aacA7*, and a chloramphenicol resistance gene, *catB3*, in an integron in pBWH301. *Journal of Antimicrobial Agents and Chemotherapy*, 39, 686-693.

Centers for Disease Control and Prevention (2008): Standardized laboratory protocol for molecular typing of Escherichia coli O157: H7, non-typical Salmonella serotypes, and Shigella sonnei by pulsed- field gel electrophoresis (PFGE). URL: http://www.cdc.gov/pulsenet/protocols/ecoli_salmonella_shigella_protocols.pdf. Accessed on 1st September 2010.

Cetinkaya, Y., Falk, P., and Mayhall, C. G. (2000). Vancomycin-resistant *enterococci*. *Clinical Microbiology Reviews*, 13, 686-707.

Chang, C. Y., Chang, L. L., Chang, Y. H., Lee, T. M., and Chang, S. F. (2000). Characterisation of drug resistance gene cassettes associated with class 1 integrons in clinical isolates of *Escherichia coli* from Taiwan, ROC. *Journal of Medical Microbiology*, 49, 1097-1102.

Chen, J., Su, Z., Liu, Y., Wang, S., Dai, X., Li, Y., Peng, S., Shao, Q., Zhang, H., Wen, P., Yu, J., Huang, X., and Xu, H. (2009). Identification and characterization of class 1 integrons among *Pseudomonas aeruginosa* isolates from patients in Zhenjiang, China. *International Journal of Infectious Diseases*, 13, 717-721.

Chiew, Y. F., Yeo, S. F., Hall, L. M., and Livermore, D. M. (1998). Can susceptibility to an antimicrobial be restored by halting its use? The case of streptomycin versus *Enterobacteriaceae*. *Journal of Antimicrobial and Chemotherapy*, 41, 247-251.

Collis, C. M., and Hall, R. M. (1992a). Gene cassettes from the insert region of integrons are excised as covalently closed circles. *Molecular Microbiology*, 6, 2875-2885.

Collis, C. M., and Hall, R. M. (1992b). Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. *Journal of Bacteriology*, 174, 1574-1585.

Collis, C. M., and Hall, R. M. (1995). Expression of antibiotic resistance genes in the integrated cassettes of integrons. *Journal of Antimicrobial Agents and Chemotherapy*, 39, 155-162.

Collis, C. M., Kim, M. J., Partridge, S. R., Stokes, H. W., and Hall, R. M. (2002). Characterization of the class 3 integron and the site-specific recombination system it determines. *Journal of Bacteriology*, 184, 3017-3026.

Da Costa, P. M., Vaz-Pires, P., and Bernardo, F. (2006). Antimicrobial resistance in *Enterococcus* spp. isolates in inflow, effluent and sludge from municipal sewage waste treatment plants. *Water Research*, 40, 1735-1740.

Daikos, G. L., Kosmidis, C., Tassios, P. T., Petrikkos, G., Vasilakopoulou, A., Psychogiou, M., Stefanou, I., Avlami, A., and Katsilambros, N. (2007). *Enterobacteriaceae* bloodstream infections: presence of integrons, risk factors and outcome. *Journal of Antimicrobial and Chemotherapy*, 51, 2366-2372.

Davies, J., and Wright, G. D. (1997). Bacterial resistance to aminoglycoside antibiotics. *Trends in Microbiology*, 5, 234-240.

Dijkshoorn, L., Aucken, H., Gerner-Smidt, P., Janssen, P., Kaufmann, M. E., Garaizar, J., Ursing, J., and Pitt, T. L. (1996). Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods. *Journal of Clinical Microbiology*, 34, 1519-1525.

Driscoll, J. A., Brody, S. L., and Kollef, M. H. (2007). The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs*, 67, 351-368.

Drlica, K., and Zhao, X. (1997). DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiology and Molecular Biology Review*, 61, 377-392.

Dromigny, J. A., Nabeth, P., Juergens-Behr, A., and Perrier-Gros-Claude, J. D. (2005). Risk factors for antibiotic resistant *Escherichia coli* isolated from community acquired urinary tract infections in Dakar, Senegal. *Journal of Antimicrobial and Chemotherapy*, 56, 236-239.

Duck, W. M., Steward, C. D., Banerjee, S. N., McGowan Jr., J. E., Tenover, F. C. (2003). Optimization of computer software settings improves accuracy of pulsed-field gel electrophoresis macrorestriction fragment pattern analysis. *Journal of Clinical Microbiology*, 41, 3035-3042.

Ebner, P., Garner, K., and Mathew, K. (2004). Class 1 integrons in various *Salmonella enterica* serovars isolated from animals and identification of genomic island SGI1 in *Salmonella enterica* var. Melagridis. *Journal of Antimicrobial Agents and Chemotherapy*, 53, 1004-1009.

Epp, S. F., Kohler, T., Plesiat, P., Michea-Hamzehpour, M., Frey, J., and Pechere, J. C. (2001). C-terminal region of *Pseudomonas aeruginosa* outer membrane porin OprD modulates susceptibility to meropenem. *Journal of Antimicrobial Agents and Chemotherapy*, 45, 1780-1787.

Esposito, D., and Scocca, J. J. (1997). The integrase family of tyrosine recombinases: evolution of a conserved active site domain. *Nucleic Acids Research*, 25, 3605-3614.

Essen-Zandbergen, A., Smith, H., Veldman, K., and Meviusm, D. (2007). Occurrence and characteristics of class 1, 2 and 3 integrons in *Escherichia coli*, *Salmonella* and *Campylobacter* spp. in the Netherlands. *Journal of Antimicrobial Agents and Chemotherapy*, 59, 746-750.

Fazlul, M. K. K., Zaini, M. Z., Rashid, M. A., and Nazmul, M. H. M. (2011). Antibiotic susceptibility profiles of clinical isolates of *Pseudomonas aeruginosa* from Selayang Hospital, Malaysia. *Biomedical Research*, 22, 263-266.

Feil, E. J., Smith, J. M., Enright, M. C., and Spratt, B. G. (2000). Estimating recombinational parameters in *Streptococcus pneumoniae* from multilocus sequence typing data. *Genetics*, 154, 1439-1450.

Feliciano, J., Teper, E., Ferrandino, M., Macchia, R. J., Blank, W., Grunberger, I., and Colon, I. (2008). The incidence of fluoroquinolone resistant infections after prostate biopsy-are fluoroquinolones still effective prophylaxis? *Journal of Urology*, 179, 952-955.

Ferrara, A. M (2006). Potentially multidrug-resistant non-fermentative Gram negative pathogens causing nosocomial pneumonia. *International Journal of Antimicrobial Agents*, 27, 183-195.

Fiett, J., Baraniak, A., Mrowka, A., Fleischer, M., Drulis-Kawa, Z., Naumiuk, L., Samet, A., Hryniewicz, W., and Gniadkowski, M. (2006). Molecular epidemiology of acquired-metallo-beta-lactamase-producing bacteria in Poland. *Journal of Antimicrobial Agents and Chemotherapy*, 50, 880-886.

Finney, M. (1993). Pulsed-field gel electrophoresis. In: Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (Eds), *Current protocols in molecular biology*. (pp 2.5.9-2.5.17). Current Protocols, Greene-Wiley, New York.

Fluit, A. C., and Schmitz, F. J. (1999). Class 1 integrons, gene cassettes, mobility, and epidemiology. *European Journal of Clinical Microbiology and Infectious Diseases*, 18, 761-770.

Fluit, A. C., and Schmitz, F. J. (2004). Resistance integrons and super-integrons. *Clinical Microbiology and Infection*, 10, 272-288.

Fourmy, D., Recht, M. I., and Puglisi, J. D. (1998). Binding of neomycin-class aminoglycoside antibiotics to the A-site of 16S rRNA. *Journal of Molecular Biology*, 277, 347-362.

Fourmy, D., Recht, M. I., Blanchard, S. C., and Puglisi, J. D. (1996). Structure of the A site of *Escherichia coli* 16S ribosomal RNA complexed with an aminoglycoside antibiotic. *Science*, 274, 1367-1371.

Francia, M. V., Cruz, F., and Garcia Lobo, J. M. (1993). Secondary-sites for integration mediated by the Tn*21* integrase. *Molecular Microbiology*, 10, 823-828.

Garza-Ramos, J. U., Sanchez-Martinez, G., Barajas, J. M., Suarez, S., Sanchez-Perez, A., Rojas-Moreno, T., Carrillo-Quiroz, B., and Silva-Sanchez, J. (2010). Variability of the bla(IMP-15)-Containing Integrons, Highly Related to In95, on an Endemic Clone of *Pseudomonas aeruginosa* in Mexico. *Microbial Drug Resistance*, 16, 191-195.

Gaynes, R., and Edwards, J. R. (2005). Overview of nosocomial infections caused by gram-negative bacilli. *Clinical Infectious Disease*, 41, 848.

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M. and Stokes, H. W. (2008). The evolution of class 1 integrons and the rise of antibiotic resistance. *Journal of Bacteriology*, 190, 5095-5100.

Goldstein, C., Lee, M., Sanchez, S., Hudson, C., Phillips, B., Register, B., Grady, M., Liebert, C., Summers, A., White, D., and Maurer, J. (2001). Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. *enterica* var. Melagridis. *Journal of Antimicrobial Agents and Chemotherapy*, 45, 723-726.

Gonzalez, G., Sossa, K., Bello, H., Dominguez, M., Mella, S., and Zemelman, R. (1998). Presence of integrons in isolates of different biotypes of *Acinetobacter baumannii* from Chilean hospitals. *FEMS Microbiology Letter*, 161, 125-128.

Gonzalez, Z. B., Catalan, A., Escudero, J. A., Dominguez, L., Teshager, T., Porrero, C., and Moreno, M. A. (2005). Genetic basis for dissemination of armA. *Journal of Antimicrobial Agents and Chemotherapy*, 56, 583-585.

Greenwood, D., and Eley, A. (1982). Comparative antipseudomonal activity of some newer beta-lactam agents. *Journal of Antimicrobial and Chemotherapy*, 21, 204-209.

Gu, B., Pan, S., Wang, T., Zhao, W., Mei, Y., Huang, P., and Tong, M. (2008). Novel cassette arrays of integrons in clinical strains of *Enterobacteriaceae* in China. *International Journal of Antimicrobial Agents*, 32, 529-533.

Guerineau, F., Brooks, L., and Mullineaux, P. (1990). Expression of the sulfonamide resistance gene from plasmid R46. *Plasmid*, 23, 35-41.

Gupta, K., Sahm, D. F., and Mayfield, D. (2001). Antimicrobial resistance among uropathogens that cause community-acquired urinary tract infections in women: a nationwide analysis. *Clinical Infectious Disease*, 33, 89-94.

Hall, R. M. (2002). Gene cassettes and integrons: moving single genes. In: Syvanen, M., Kado, C. I. (Eds.), *Horizontal gene transfer*. 2nd Edition. (pp 19-28). San Diego: Academic Press.

Hall, R. M., and Collis, C. M. (1995). Mobile gene cassettes and integrons capture and spread of genes by site-specific recombination. *Molecular Microbiology*, 15, 593-600.

Hall, R. M., and Collis, C. M. (1998). Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. *Drug Resistance*, 1, 109-119.

Hall, R. M., and Stokes, H. W. (2004). Integrons or super integrons? *Microbiology*, 150, 3-4.

Hall, R. M., Brookes, D. E., and Stokes, H. W. (1991). Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. *Molecular Microbiology*, 5, 1941-1959.

Hall, R. M., Collis, C. M., Kim, M. J., Partridge, S. R., Recchia, G. D., and Stokes, H. W. (1999). Mobile gene cassettes and integrons in evolution. *Annals of the New York Academy of Sciences*, 870, 68-80.

Hall, R., Brown, H., Brookes, D., and Stokes, H. (1994). Integrons found in different locations have identical 5'ends but variable 3'ends. *Journal of Bacteriology*, 176, 6286-6294.

Hansson, K., Skold, O., and Sundstrom. L. (1997). Non-palindromic *attl* sites of integrons are capable of site-specific recombination with one another and with secondary targets. *Molecular Microbiology*, 26, 441-453.

Hansson, K., Sundstrom, L., Pelletier, A., and Roy, P. H. (2002). IntI2 integron integrase in Tn7. *Journal of Bacteriology*, 184, 1712-1721.

Hartstein, A. I., Morthland, V. H., Rourke, J. W., Freeman, J. J., Garber, S., Sykes, R., and Rashad, A. L. (1990). Plasmid DNA fingerprinting of *Acinetobacter calcoaceticus* subspecies *anitratus* from intubated and mechanically ventilated patients. *Infection Control and Hospital Epidemiology*, 11, 531-538.

Heidelberg, J. F., Elsen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Umayam, L., Gill, S. R., Nelson, K. E., Read, T. D., Tettelin, H., Richardson, D., Ermolaeva, M. D., Vamathevan, J., Bass, S., Qin, H. Y., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R. D., Nierman, W. C., White, O., Salzberg, S. L., Smith, H. O., Colwell, R. R., Mekalanos, J. J., Venter, J. C., and Fraser, C. M. (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature*, 406, 477-483.

Heikkila, E., Skurnik, M., Sundström, L., and Huovinen, P. (1993). A novel dihydrofolate reductase cassette inserted in an integron borne on a Tn21-like element. *Journal of Antimicrobial and Chemotherapy*, 37, 1297-1304.

Henriques, I., Fonseca, F., Alves, A., Saavedra, M. J., and Correia, A. (2006). Occurrence and diversity of integrons and β-lactamase genes among ampicillin resistant isolates from estuarine waters. *Research Microbiology*, 157, 938-947.

Herschleb, J., Ananiev, G., and Schwartz, D. C. (2007). Pulsed-field gel electrophoresis. *Nature Protocols*, 2, 677-684.

Ho, P., King-Sun, Y., Kin-Hung, C., Janice, Y. C., Tak-Lun, Q., and Kwok-yung, Y. (2010). Antimicrobial resistance among uropathogens that cause acute uncomplicated cystitis in women in Hong Kong: a prospective multicenter study in 2006 to 2008. *Diagnostic Microbiology and Infectious Disease*, 66, 87-93.

Holmes, A. J., Gillings, M. R., Nield, B. S., Mabbutt, B. C., Nevalainen, K. M. H., and Stokes, H. W. (2003). The gene cassette metagenome is a basic resource for bacterial genome evolution. *Environmental Microbiology*, 5, 383-394.

Holt, J., Krieg, N., Sneath, P., Staley, J. and Williams, S. (1994). Bergey's Manual of Determinative Bacteriology. 9th edition. (pp 175-187). Williams and Wilkins, Baltimore, MD, USA.

Jeong, J. H., Shin, K. S., Lee, J. W., Park, E. J., and Son, S. Y. (2009). Analysis of a novel class 1 integron containing metallo-beta-lactamase gene VIM-2 in *Pseudomonas aeruginosa*. *Journal of Microbiology*, 47, 753-759.

Jones, L. A., McIver, C. J., Kim, M. J., Rawlinson, W. D., and White, P. A. (2005). The *aadB* gene cassette is associated with $bla_{SHV}$ genes in *Klebsiella* species producing extended-spectrum β-lactamases. *Journal of Antimicrobial and Chemotherapy*, 49, 794-797.

Jones, L. A., McIver, C. J., Rawlinson, W. D., and White, P. A. (2003). Polymerase chain reaction screening for integrons can be used to complement resistance surveillance programs. *Communicable Diseases Intellegence*, 27, 103-110.

Jones, M. E., Peters, E., Weersink, A. M., Fluit, A., and Verhoef, J. (1997). Widespread occurrence of integrons causing multiple antibiotic resistance in bacteria. *Lancet*, 349, 1742-1743.

Kang, H. Y., Young, S. J., Jae, Y. O., Sung, H. T., Chui, H. C., Dong, C. M., Won, K. L., Yoo, C. L., Sung, Y. S., Dong, T. C., and Je, C. L. (2005). Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* strains from humans and animals in Korea. *Journal of Antimicrobial Agents and Chemotherapy*, 55, 639-644.

Katherason, S. G., Naing, L., Jaalam, K., and Ismail, A. (2008). Baseline assessment of intensive care-acquired nosocomial infection surveillancein three adult intensive care units in Malaysia. *Journal of Infections in Developing Countries*, 2, 364-368.

Khosravi, Y., Tay, S. T., and Vadivelu, J. (2011). Analysis of integrons and associated gene cassettes of metallo-{beta}-lactamase-positive *Pseudomonas aeruginosa* in Malaysia. *Journal of Medical Microbiology*, 60, 988-994.

Kipnis, E., Sawa, T., and Wiener-Kronish, J. (2006). Targeting mechanism of *Pseudomonas aeruginosa* pathogenesis. *Medecine et Maladies Infectieuses*, 36, 78-91.

Koeleman, J. G., Stoof, J., Biesmans, D. J., Savelkoul, P. H., and Vandenbroucke-Grauls, C. M. (1998). Comparison of amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification of *Acinetobacter* genomic species and typing of *Acinetobacter baumannii*. *Journal of Clinical Microbiology*, 36, 2522-2529.

Kumai, Y., Suzuki, Y., Tanaka, Y., Shima, K., Bhadra, R. K., Yamasaki, S., Kuroda, K., and Endo, G. (2005). Characterization of multidrug-resistance phenotypes and genotypes of *Escherichia coli* strains isolated from swine from an abattoir in Osaka, Japan. *Epidemiology and Infection*, 133, 59-70.

Levene, S. D. (1992). Theories of pulse-field gel electrophoresis. In: Methods in molecular biology, Burmeister, M. and Ulanovsky, L. (Eds), *Pulsed-Field Gel Electrophoresis*. (pp 347-365). The Human Press Inc., Totowa, New Jersey.

Leverstein-van Hall, M. A., Blok, H. E. M., Donders, A. R. T., Paauw, A., Fluit, A. C., and Verhoef, J. (2003). Multidrug resistance among *Enterobacteriaceae* is strongly associated with the presence of integrons and is independent of species and isolate origin. *Journal of Infectious Diseases*, 187, 251-259.

Leverstein-van Hall, M. A., Box, A. T., Blok, H. E., Paauw, A., Fluit, A. C., and Verhoef, J. (2002a). Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant *Enterobacteriaceae* in a clinical setting. *Journal of Infectious Disease*, 186, 49-56.

Leverstein-Van Hall, M. A., Paauw, A., Box, A. T., Blok, H. E., Verhoef, J. and Fluit, A. C. (2002b). Presence of integron-associated resistance in the community is widespread and contributes to multidrug resistance in the hospital. *Journal of Clinical Microbiology*, 40, 3038-3040.

Levesque, C., Brassard, S., Lapointe, J., and Roy, P. H. (1994). Diversity and relative strength of tandem promotors for the antibiotic resistance genes of several integrons. *Gene*, 142, 49-54.

Levesque, C., Piche, L., Larose, C., and Roy, P. H. (1995). PCR mapping of integrons reveals several novel combinations of resistance genes. *Journal of Antimicrobial Agents and Chemotherapy*, 39, 185-191.

Li, X., Shi, L. Yang, W. Li, L. and Yamasaki. S. (2006). New array of *aacA4catB3-dfrA1* gene cassettes and a noncoding cassette from a class-1-integron positive clinical strain of *Pseudomonas aeruginosa*. *Journal of Antimicrobial Agents and Chemotherapy*, 50, 2278-2279.

Liebert, C. A., Hall, R. M., and Summers, A. O. (1999). Transposon Tn*21*, flagship of the floating genome. *Microbiology and Molecular Biology Review*, 63, 507-522.

Lim, K. T., Yasin, R., Yeo, C. C., Puthucheary, S., and Thong, K. L. (2009b). Characterization of Multidrug Resistant ESBL-Producing*Escherichia coli* Isolates from Hospitals in Malaysia. *Journal of Biomedicine and Biotechnology*

Lim, K. T., Yeo, C. C., Yasin, R. M., Balan, G., and Thong, K. L. (2009a). Characterization of multidrug-resistant and extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* strains from Malaysian hospitals. *Journal of Medical Microbiology*, 58, 1463-1469.

Lin, L., Ling, B. D., and Li, X. Z. (2009). Distribution of the multidrug efflux pump genes, *adeABC*, *adeDE*, and *adeIJK*, and class 1 integron genes in multiple-antimicrobial-resistant clinical isolates of *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex. *International Journal of Antimicrobial Agents*, 33, 17-32.

Lina, T. T., Rahman, S. R., and Gomes, D. J. (2007). Multiple-Antibiotic Resistance Mediated by Plasmids and Integrons in Uropathogenic *Escherichia coli* and *Klebsiella pneumonia*. *Bangladesh Journal of Microbiology*, 24, 19-23.

Lindberg, R. H., Bjorklund, K., Rendahl, P., Johansson, M. I., Tysklind, M., and Andersson, B. A. V. (2007). Environmental risk assessment of antibiotics in the Swedish environment with emphasis on sewage treatment plants. *Water Research*, 41, 613-619.

Livermore, D. M. (2002). Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? *Clinical Infectious Disease*, 34, 634-640.

Mao, W., Warren, M., Lee, A., Mistry, A., and Lomovskaya, O. (2001). MexXYOprM efflux pump is required for antagonism of aminoglycosides by divalent cations in *Pseudomonas aeruginosa*. *Journal of Antimicrobial Agents and Chemotherapy*, 45, 2001-2007.

Martinez, J. L., and Baquero, F. (2000). Mutation frequencies and antibiotic resistance. *Antimicrobial Agents and Chemotherapy*, 44, 1771.

Martinez, N., Mendoza, M. C., Rodriguez, I., Soto, S., Bances, M., and Rodicio, M. R. (2007). Detailed structure of integrons and transposons carried by large conjugative plasmids responsible for multidrug resistance in diverse genomic types of *Salmonella enterica* serovar Brandenburg. *Journal of Antimicrobial and Chemotherapy*, 60, 1227-1234.

Martinez-Freijo, P., Fluit, A. C., Schmitz, F. J., Verhoef, J., and Jones, M. E. (1999). Many class 1 integrons comprise distinct stable structures occurring in different species of *Enterobacteriaceae* isolated from widespread geographic regions in Europe. *Journal of Antimicrobial and Chemotherapy*, 43, 686-689.

Martinez-Freijo. P., Fluit, A. C., Schmitz, F. J., Grek, V. S. C., Verhoef, J., and Jones, M. E. (1998). Class I integrons in gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. *Journal of Antimicrobial and Chemotherapy*, 42, 689-696.

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

Maslow, J. N., Slutsky, A. M., and Arbeit, R. D. (1993). Application of pulsed-field gel electrophoresis to molecular epidemiology. In: Persing, D. H., Smith, T. F., Tenover, F. C., and White, T. J. (Eds), *Diagnostic molecular microbiology: principles and applications*. (pp 563-572). Washington, D.C: American Society for Microbiology.

Mazel, D. (2006). Integrons: agents of bacterial evolution. *National Review of Microbiology*, 4, 608-620.

Mazel, D., and Davies, J. (1999). Antibiotic resistance in microbes. *Cellular and Molecular Life Science*, 56, 742-754.

Mazel, D., Dychinco, B., Webb, V. A., and Davies, J. (1998). A distinctive class of integron in the *Vibrio cholerae* genome. *Science*, 280, 605-608.

McIver, C. J., White, P. A., Jones, L. A., Karagiannis, T., Harkness, J., Marriott, D., and Rawlinson, W. D. (2002). Epidemic strains of *Shigella sonnei* biotype g carrying integrons. *Journal of Clinical Microbiology*, 40, 1538-1540.

McKeegan, K. S., Borges-Walmsley, M. I., and Walmsley, A. R. (2002). Microbial and viral drug resistance mechanisms. *Trends in Microbiology*, 10, 8-14.

McManus, M. C. (1997). Mechanisms of bacterial resistance to antimicrobial agents. *American Journal of Health-System Pharmacy*, 54, 1420-1433.

Michael, C., Gillings, M., Holmes, A., Hughes, L., Andrew, N., Holley, M., and Stokes, H. (2004). Mobile gene cassettes: A fundamental resource for bacterial evolution. *American Naturalist*, 164, 1-12.

Moura, A., Henriques, I., Ribeiro, R., and Correia, A. (2007). Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *Journal of Antimicrobial Chemotherapy*, 60, 1243-1250.

Mukherjee, S., and Chakraborty, R. (2006). Incidence of class 1 integrons in multiple antibiotic-resistant gram-negative copiotrophic bacteria from the River Torsa in India. *Research in Microbiology*, 157, 220-226.

Naas, T., Poirel, L., and Nordmann, P. (1999). Molecular characterisation of In51, a class 1 integron containing a novel aminoglycoside adenylyltransferase gene cassette, *aadA6*, in *Pseudomonas aeruginosa*. *Biochimica et Biophysica Acta*, 1489, 445-451.

Navia, M. M., Ruiz, J., and Vila, J. (2004). Molecular characterization of the integrons in *Shigella* strains isolated from patients with traveler's diarrhea. *Diagnostic Microbiology and Infectious Disease*, 48, 175-179.

Neu, H. C. (1992). The crisis in antibiotic resistance. *Science*, 257, 1064-1073.

Nicolle, L. E. (2001). Epidemiology of urinary tract infection. *Infections in Medicine*, 18, 153-162.

Nijssen, S., Florijin, A., Top, J., Willem, R., Fluit, A., and Bonten, M. (2005). Unnoticed spread of integron-carrying *Enterobacteriaceae* in intensive care units. *Clinical Infectious Diseases*, 41, 1-9.

Nirmala, D. B., Gin, G. G., Kamarulzaman, A., and Ching, S. (2007). Bacteremia in patients with febrile neutropenia after chemotherapy at a university medical center in Malaysia. *International Journal of Infectious Diseases*, 11, 513-517.

Norrby, S. R. (2005). Integrons: adding another threat to the use of antibiotic therapy. *Clinical Infectious Diseases*, 41, 10-11.

Ochs, M., McCusker, M., Bains, M., and Hancock, R. E. W. (1999). Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. *Journal of Antimicrobial Agents and Chemotherapy*, 43, 1085-1090.

Orman, B. E., Pineiro, S. A., Arduino, S., Galas, M., Melano, R., Caffer, M. I., Sordelli, D. O., and Centron, D. (2002). Evolution of multiresistance in nontyphoid *salmonella serovars* from 1984 to 1998 in Argentina. *Journal of Antimicrobial Agents and Chemotherapy*, 46, 3963-3970.

Orrett, F. A. (2004). Antimicrobial susceptibility survey of *Pseudomonas aeruginosa* strains isolated from clinical sources. *Journal of National Medical Association*, 96, 1065-1069.

Ozgumus, O. B., Sandalli, C., Sevim, A., Celik-Sevim, E. and Sivri, N. (2009). Class 1 and class 2 integrons and plasmid-mediated antibiotic resistance in coliforms isolated from ten rivers in northern Turkey. *Journal of Microbiology*, 47, 19-27.

Parks, A. R., and Peters, J. E. (2009). Tn7 elements: Engendering diversity from chromosomes to episomes. *Plasmid*, 61, 1-14.

Partridge, S. R., Tsafnat, G., Coiera, E., and Iredell, J. R. (2009). Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiology Review*, 33, 757-784.

Patel, J. B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Journal of Molecular Diagnostics*, 6, 313-321.

Paterson, D. L. (2006). Resistance in gram-negative bacteria: *Enterobacteriaceae*. *American Journal of Infections Control*, 34, 20-28.

Paterson, D. L., and Bonomo, R. A. (2005). Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Review*, 18, 657-686.

Peters, E. D. J., Leverstein-van Hall, M. A., Box, A. T. A., Verhoef, J., and Fluit, A. C. (2001). Novel gene cassettes and integrons. *Journal of Antimicrobial Agents and Chemotherapy*, 45, 2961-2964.

Piddock, L. J. (1995). Mechanisms of resistance to fluroquinolones: state-of-the-art 1992-1994. *Drugs*, 49, 29-35.

Pinney, R. J. (1980). Distribution among incompatibility groups of plasmids that confer UV mutability and UV resitance. *Mutation Research*, 72, 155-159.

Ploy, M. C., Denis, F., Courvalin, P., and Lambert, T. (2000a). Molecular characterization of integrons in *Acinetobacter baumannii*: description of a hybrid class 2 integron. *Journal of Antimicrobial Agents and Chemotherapy*, 44, 2684-2688.

Ploy, M. C., Lambert, T., Couty, J. P., and Denis, F. (2000b). Integrons: an antibiotic resistance gene capture and expression system. *Clinical Chemistry and Laboratory Medicine*, 38, 483-487.

Poole, K., Krebes, K., McNally, C., and Neshat, S. (1993). Multiple antibiotic resistance in Pseudomonas aeruginosa: evidence for involvement of an efflux operon. *Journal of Bacteriology*, 175, 7363-7372.

Privitera, A., Rappazzo, G., Sangari, P., Giannin, V., Licciardello, L., and Stefani, S. (1998). Cloning and sequencing of a 16S:23S ribosomal spacer from *Haemophilus parainfluenzae* reveal an invariant, mosaic like organisation of sequence blocks. *FEMS Microbiology Letter*, 164, 289-294.

Ramirez, M. S., Pineiro, S., and Centron, D. (2010). Novel insights about class 2 integrons from experimental and genomic epidemiology. *Journal of Antimicrobial Agents and Chemotherapy*, 54, 699-706.

Recchia, G. D., and Hall, R. M. (1995a). Gene cassettes: a new class of mobile element. *Microbiology*, 141, 3015-3027.

Recchia, G. D., and Hall, R. M. (1995b). Plasmid evolution by acquisition of mobile gene cassettes: plasmid pIE723 contains the *aadB* gene cassette precisely inserted at a secondary site in the IncQ plasmid RSF1010. *Molecular Microbiology*, 15, 179-187.

Recchia, G. D., and Hall, R. M. (1997). Origins of the mobile gene cassettes found in integrons. *Trends in Microbiology*, 5, 389-394.

Recchia, G. D., Stokes, H. W., and Hall, R. M. (1994). Characterization of specific and secondary recombination sites recognised by the integron DNA integrase. *Nucleic Acids Research*, 11, 2071-2078.

Reyes, A., Bello, H., Dominguez, M., Mella, S., Zemelman, R., and Gonzalez, G. (2003). Prevalence and types of class 1 integrons in aminoglycoside-resistant *Enterobacteriaceae* from several Chilean hospitals. *Journal of Antimicrobial Agents and Chemotherapy*, 51, 317-321.

Ridley, A., and Threlfall, E. J. (1998). Molecular epidemiology of antibiotic resistance genes in multiresistant epidemic *Salmonella typhimurium* DT 104. *Microbial Drug Resistance*, 4, 113-118.

Roe, M. T., Vega, E., Pillai, S. D. (2003). Antimicrobial resistance markers of class 1 and class 2 integron-bearing *Escherichia coli* from irrigation waters and sediments. *Emerging Infectious Diseases*, 9, 822-826.

Rosser, S. J., and Young, H. K. (1999). Identification and characterization of class 1 integrons in bacteria from an aquatic environment. *Journal of Antimicrobial and Chemotherapy*, 44, 11-18.

Rowe-Magnus, D. A., and Mazel, D. (1999). Resistance gene capture. *Current Opinion in Microbiology*, 2, 483-488.

Rowe-Magnus, D. A., and Mazel, D. (2001). Integrons: natural tools for bacterial genome evolution. *Current Opinion in Microbiology*, 4, 565-569.

Rowe-Magnus, D., Guerot, A. M., and Mazel, D. (2002). Bacterial resistance evolution by recruitment of super-integron gene cassettes. *Molecular Microbiology*, 43, 1657-1669.

Sallen, B., Rajoharison, A., Desvarenne, S., and Mabilat, C. (1995). Molecular epidemiology of integron-associated antibiotic resistance genes in clinical isolates of *Enterobacteriaceae*. *Microbial Drug Resistance*, 1, 195-202.

Sandvang, D. (1999). Novel streptomycin and spectinomycin resistance gene as a gene cassette within a class 1 Integron isolated from *Escherichia coli*. *Journal of Antimicrobial and Chemotherapy*, 43, 3036-3038.

Sandvang, D., Aarestrup, F. M., and Jensen, L. B. (1998). Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. *FEMS Microbiology Letter*, 160, 37-41.

Sarlangue, J., Brissaud, O., and Labre`ze, C. (2006). Clinical features of *Pseudomonas aeruginosa* infections. *Achieve of Pediatrics*, 13, 13-16.

Schembri, M. A., Blom, J., Krogfelt, K. A., and Klemm, P. (2005). Capsule and fimbria interaction in *Klebsiella pneumonia*. *Infection and Immunity*, 73, 4626-4633.

Schmidt, A. S., Bruun, M. S., Dalsgaard, I., and Larsen, J. L. (2001). Incidence, distribution and spread of tetracycline resistance determinants and integron associated antibiotic resistance genes among motile aeromonads from a fish farming environment. *Applied and Environmental Microbiology*, 67, 5675-5682.

Schmitz, F. J., Hafner, D., Geisel, R., Follmann, P., Kirschke, C., Verhoef, J., Kohrer, K., and Fluit, A. (2001). Increased prevalence of class I integrons in *Escherichia coli*, *Klebsiella* species and *Enterobacter* species isolated over a 7 year period in a German University hospital. *Journal of Clinical Microbiology*, 39, 3724-3726.

Schroeder, R., Waldsich, C., and Wank, H. (2000). Modulation of RNA function by aminoglycoside antibiotics. *EMBO Journal*, 19, 19.

Senda, K., Arakawa, Y., Ichiyama, S., Nakashima, K., Ito, H., Ohsuka, S., Shimokata, K., Kato, N., and Ohta, M. (1996). PCR detection of metallo-beta-lactamase gene (blaIMP) in gram-negative rods resistant to broad-spectrum beta-lactams. *Journal of Clinical Microbiology*, 34, 2909-2913.

Sepp, E., Stsepetova, J., Lõivukene, K., Truusalu, K., Kõljalg, S., Naaber, P. (2009). The occurrence of antimicrobial resistance and class 1 integrons among commensal Escherichia coli isolates from infants and elderly persons. *Annals of Clinical Microbiology and Antimicrobials*, 8, 34.

Shahcheraghi, F., Badmasti, F., and Feizabadi, M. M. (2009). Molecular characterization of class 1 integrons in MDR *Pseudomonas aeruginosa* isolated from clinical settings in Iran, Tehran. *FEMS Immunology and Medical Microbiology*, 58, 421-425.

Shaw, K., Rather, P., Hare, R. S., and Miller, G. H. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside modifying enzymes. *Microbiology Reviews*, 57, 138-163.

Siegel, R. E. (2008). Emerging Gram-negative antibiotic resistance: Daunting challenges, declining sensitivities and dire consequences. *Respiratory Care*, 53, 471-479.

Siva, G. P., Samat, N. A., and Mohamed, R. (2009). Antimicrobial susceptibility of clinical isolates of *Pseudomonas aeruginosa* from a Malaysian Hospital. *Malaysian Journal of Medical Sciences*, 16, 27-32.

Skold, O. (2000). Sulfonamide resistance: mechanisms and trends. *Drug Resistance Updates*, 3, 155-160.

Smith, C. L., and Canter, C. R. (1987). Purification, specific fragmentation, and separation of large DNA molecules. *Methods Enzymology*, 155, 449-467.

Speijer, H., Savelkoul, P. H., Bonten, M. J., Stobberingh, E. E., and Tihie, J. H. (1999). Application of different genotyping methods for *Pseudomonas aeruginosa* in a setting of endemicity in an intensive care unit. *Journal of Clinical Microbiology*, 37, 3654-3661.

Srikumar, R., Kon, T., Gotoh, N., and Poole, K. (1998). Expression of *Pseudomonas aeruginosa* multidrug efflux pumps MexAMexBOprM and MexCMexDOprJ in a multidrug-sensitive *Escherichia coli* strain. *Journal of Antimicrobial Agents and Chemotherapy*, 42, 65-71.

Stokes, H. W., and Hall, R. M. (1989). A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Molecular Microbiology*, 3, 1669-1683.

Stokes, H. W., and Hall, R. M. (1991). Sequence analysis of the inducible chloramphenicol resistance determinant in the Tn*1696* integron suggests regulation by translational attenuation. *Plasmid*, 26, 10-19.

Stokes, H. W., Gorman, D. B. O., Recchia, G. D., Parsekhian, M., and Hall, R. M. (1997). Structure and function of 59-base element recombination sites associated with mobile gene cassettes. *Molecular Microbiology*, 26, 731-745.

Stokes, H. W., Holmes, A. J., Nield, B. S., Holley, M. P., Nevalainen, K. M. H., Mabbutt, B. C., and Gillings, M. R. (2001). Gene cassette PCR: sequence independent recovery of entire genes from environmental DNA. *Applied and Environmental Microbiology*, 67, 5240-5246.

Su, J., Shi, L., Yang, L., Xiao, Z., Li, X., and Yamasaki, S. (2006). Analysis of integrons in clinical isolates of *Escherichia coli* in China during the last six years. *FEMS Microbiology Letter*, 254, 75-80.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24, 1596-1599.

Tennstedt, T., Szczepanowski, R., Braun, S., Pühler, A., and Schlüter, A. (2003). Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. *FEMS Microbiology Ecology*, 45, 239-252.

Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *The American Journal of Medicine*, 119, 3-10.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Nurray, B. E., Persing, D. H., and Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by PFGE: criteria for bacterial strain typing. *Journal of Clinical Microbiology*, 33, 2233-2239.

Thungapathra, M., Amita, Sinha, K. K., Chaudhuri, S. R., Garg, P., Ramamurthy, T., Nair, G. B., and Ghosh, A. (2002). Occurrence of antibiotic resistance gene cassettes *aac(6)-Ib*, *dfrA5*, *dfrA12*,andereA2 in class I integrons in Non-O1, Non-O139 *Vibrio cholerae* strains in India. *Antimicrobial Agents and Chemotherapy*, 46, 2948-2955.

Todar, K. (2007). "Pathogenic *E. coli*". *Online Textbook of Bacteriology*. University of Wisconsin-Madison Department of Bacteriology. URL: http://www.textbookofbacteriology. net/e.coli.html. Accessed on October 2011.

Turton, J., Kaufmann, M. E., Glover, J., Coelho, J. M., Warner, M., Pike, R., and Pitt, T. L. (2005). Detection and typing of integrons in epidemic strains of *Acinetobacter baumannii* found in the United Kingdom. *Journal of Clinical Microbiology*, 43, 3074-3082.

Upton, C., and Pinney, R. J. (1983). Expression of eight unrelated $Muc^{+}$ plasmids in eleven DNA repair-deficient *E. coli* strains. *Mutation Research*, 112, 261-273.

Versalovic, J., and Lupski, J. R. (2002). Molecular detection and genotyping of pathogens: more accurate and rapid answers. *Trends in Microbiology*, 10, 15-21.

Walsh, C. T., Fisher, S. L., Park, I. S., Prahalad, M., and Wu, Z. (1996). Bacterial resistance to vancomycin: Five genes and one missing hydrogen bond tell the story. *Chemistry and Biology*, 3, 21-28.

Walsh, T. R., Toleman, M. A., Poirel, L., and Nordmann, P. (2005). Metallo-beta-lactamases: the quiet before the storm? *Clinical Microbiology Review*, 18, 306-325.

Wang, G. Q., Wu, C. M., Du, X. D., Shen, Z. Q., Song, L. H., Chen, X., and Shen, J. Z. (2008). Characterization of integrons-mediated antimicrobial resistance among *Escherichia coli* strains isolated from bovine mastitis. *Veterinary Microbiology*, 127, 73-78.

Waturangi, D. E., Suwanto, A., Schwarz, S., and Erdelen, W. (2003). Identification of class 1 integrons-associated gene cassettes in *Escherichia coli* isolated from *Varanus* spp. in Indonesia. *Journal of Antimicrobial and Chemotherapy*, 51, 175-177.

Wegener, H., Aarestrup, F., Jensen, L., Hammerum, A., and Bager, F. (1999). Use of antimicrobial growth promoters in food animals and *Enterococcus faecium* resistance to therapeutic antimicrobial drugs in Europe. *Emerging Infectious Diseases*, 5, 329-335.

Weldhagen, G. F. (2004). Integrons and β-lactamases: a novel perspective on resistance. *International Journal of Antimicrobial Agents*, 23, 556-562.

Westbrock-Wadman, S., Sherman, D., Hickey, M. J., Coulter, S. N., Zhu, Y. Q., Warrener, P., Nguyen, L. Y., Shawar, R. M., *et al.* (1999). Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. *Journal of Antimicrobial Agents and Chemotherapy*, 43, 2975-2983.

White, P. A., and Rawlinson, W. D. (2001). Current status of the *aadA* and *dfr* gene cassette families. *Journal of Antimicrobial and Chemotherapy*, 47, 495-496.

White, P. A., McIver, C. J., and Rawlinson, W. D. (2001). Integrons and gene cassettes in the *Enterobacteriaceae*. *Journal of Antimicrobial Agents and Chemotherapy*, 45, 2658-2661.

White, P. A., McIver, C. J., Deng, Y. M., and Rawlinson, W. D. (2000). Characterisation of two new gene cassettes, *aadA5* and *dfrA17*. *FEMS Microbiology Letter*, 182, 265-269.

Yan, J. J., Wu, J. J., Ko, W. C., Chiou, C. S., and Wang, L. R. (2009). The emergence of AAC(6')-Ib-cr among human isolates of *Escherichia coli*, *Salmonella enterica*, and *Klebsiella pneumoniae* and food animal isolates of *E. coli* in Taiwan, unpublished.

Yao, F., Qian, Y. S., Chen, S. Z., Wang, P. F., and Huan, Y. C. (2007). Incidence of extended-spectrum β-lactamases and characterization of integrons in extended-spectrum β-lactamase-producing *Klebsiella pneumonia* isolated in Shantou, China. *Acta Biochimica Biophysica Sinica (Shanghai)*, 39, 527-532.

Yasin, R. M., Lim, K. T., Yeo, C. C., Puthucheary, S. D., Balan, G., Maning, N., Wahab, Z. A., Ismail, N., Tan, E. A., Mustaffa, A., and Thong, K. L. (2009). Genetic fingerprinting and antimicrobial susceptibility profiles of *Pseudomonas aeruginosa* hospital isolates in Malaysia. *Journal of Microbiology, Immunology and Infection*, 42, 197-209.

Yu, H. S., Lee, J. C., Kang, H. Y., Ro, D. W., Chung, J. Y., Jeong, Y. S., Tae, S. H., Choi, C. H., Lee, S. Y., Seol, S. Y., Lee, Y. C., and Cho, D. T. (2003). Changes in gene cassettes of class 1 integrons among *Escherichia coli* isolates from urine specimens collected in Korea during the last two decades. *Journal of Clinical Microbiology*, 41, 5429-5433.

Zhang, X., Zhang, T., Zhang, M., Fang, H. H. P., and Cheng, S. P. (2009). Characterization and quantification of class 1 integrons and associated gene cassettes in sewage treatment plants. *Applied Microbiology and Biotechnology*, 82, 1169-1177.

# APPENDICES

## Appendix A

**Patient's data for one hundred forty-seven *Enterobacteriaceae* and *Pseudomonas***

| Sample ID | Bacteria species | Sites of specimens[a] | Gender | Age |
|---|---|---|---|---|
| G2 | *Providencia rettgeri* | Pus swab | F | 39 |
| G5 | *Pseudomonas species* | Tissue | F | 60 |
| G7 | *Escherichia coli* | Swab | M | 46 |
| G8 | *Enterobacter cloacae* | Blood | F | 54 |
| G10 | *Enterobacter aerogenes* | Pus swab | M | 49 |
| G12 | *Enterobacter cloacae* | Swab | M | 40 |
| G13 | *Klebsiella pneumoniae* | Others | M | 37 |
| G21 | *Enterobacter cloacae* | Swab | M | 58 |
| G22 | *Enterobacter cloacae* | Pus swab | M | 61 |
| G23 | *Enterobacter aerogenes* | Swab | M | 68 |
| G24 | *Enterobacter cloacae* | Sputum | F | 58 |
| G26 | *Proteus vulgaris* | Pus swab | F | 52 |
| G27 | *Serratia marcescens* | Swab | F | 22 |
| G28 | *Enterobacter cloacae* | Sputum | F | 32 |
| G31 | *Enterobacter cloacae* | Swab | M | 47 |
| G33 | *Morganella morganii* | Sputum | F | 76 |
| G36 | *Providencia rettgeri* | Wound swab | M | 32 |
| G39 | *Serratia marcescens* | Swab | M | 19 |
| G42 | *Enterobacter cloacae* | Others | F | 16 |
| G44 | *Serratia marcescens* | Sputum | M | 50 |
| G46 | *Enterobacter cloacae* | Swab | M | 44 |
| G47 | *Enterobacter cloacae* | Foot | F | 65 |
| G49 | *Enterobacter cloacae* | Pus swab | M | 34 |
| G50 | *Enterobacter cloacae* | Tissue | F | 61 |
| G51 | *Enterobacter cloacae* | Pus swab | M | 52 |
| G52 | *Morganella morganii* | Foot | F | 62 |
| G53 | *Enterobacter cloacae* | Swab | M | 67 |
| G54 | *Citrobacter koseri* | Foot | M | 61 |
| G55 | *Providencia rettgeri* | Swab | M | 53 |
| G56 | *Citrobacter koseri* | Foot | M | 50 |
| G57 | *Serratia marcescens* | Swab | M | 35 |
| G58 | *Serratia marcescens* | Sputum | M | 79 |
| G59 | *Morganella morganii* | Swab | F | 78 |

| | | | | |
|---|---|---|---|---|
| G60 | *Enterobacter cloacae* | Wound swab | F | 47 |
| G61 | *Serratia marcescens* | Wound swab | F | 49 |
| G62 | *Enterobacter cloacae* | Swab | M | 53 |
| G63 | *Citrobacter freundii* | Pus swab | M | 38 |
| G64 | *Morganella morganii* | Swab | F | 49 |
| G65 | *Enterobacter cloacae* | Others | F | 23 |
| G66 | *Enterobacter aerogenes* | Swab | M | 53 |
| G67 | *Enterobacter aerogenes* | swab | M | 72 |
| G68 | *Enterobacter cloacae* | Swab | M | 69 |
| G69 | *Enterobacter cloacae* | Sputum | M | 61 |
| G70 | *Enterobacter aerogenes* | Pus swab | F | 54 |
| G71 | *Providencia rettgeri* | Wound swab | M | 67 |
| G72 | *Enterobacter cloacae* | Wound swab | M | 29 |
| G73 | *Providencia rettgeri* | Swab | M | 67 |
| H1 | *Klebsiella oxytoca* | Blood | F | 60 |
| H2 | *Klebsiella ozanae* | Others | M | 58 |
| H3 | *Enterobacter cloacae* | Others | M | 0 |
| H4 | *Klebsiella pneumoniae* | Urine | F | 88 |
| H5 | *Escherichia coli* | Pus swab | M | 49 |
| H6 | *Klebsiella pneumoniae* | Urine | F | 59 |
| H8 | *Escherichia coli* | Pus swab | M | 70 |
| H9 | *Escherichia coli* | Pus swab | F | 78 |
| H10 | *Enterobacter cloacea* | Pus swab | M | 49 |
| H11 | *Enterobacter cloacea* | Others | M | 49 |
| H12 | *Escherichia coli* | Pus swab | F | 21 |
| H14 | *Klebsiella pneumoniae* | Others | F | 7 |
| H15 | *Klebsiella pneumoniae* | Pus swab | F | 56 |
| H16 | *Klebsiella pneumoniae* | Urine | F | 27 |
| H18 | *Enterobacter gergoviae* | Blood | M | 17 |
| H19 | *Klebsiella pneumoniae* | Pus swab | M | 79 |
| H21 | *Escherichia coli* | Pus swab | F | 69 |
| H22 | *Klebsiella pneumoniae* | Others | M | 79 |
| H23 | *Enterobacter amnigenus* | Others | M | 79 |
| H26 | *Klebsiella pneumoniae* | Others | M | 60 |
| H27 | *Klebsiella pneumoniae* | Swab | M | 60 |
| H28 | *Klebsiella pneumoniae* | Urine | M | 53 |
| H31 | *Klebsiella pneumoniae* | Urine | M | 25 |
| H32 | *Klebsiella pneumoniae* | Tissue | M | 60 |
| H33 | *Escherichia coli* | Pus swab | M | 29 |
| H34 | *Klebsiella pneumoniae* | Others | F | 75 |
| H35 | *Escherichia coli* | Urine | M | 49 |
| H36 | *Escherichia coli* | Wound swab | M | 56 |
| H37 | *Escherichia coli* | Pus swab | M | 19 |
| H38 | *Klebsiella pneumoniae* | Tissue | M | 67 |
| H39 | *Escherichia coli* | Blood | M | 40 |
| H40 | *Klebsiella pneumoniae* | Blood | F | 2 |

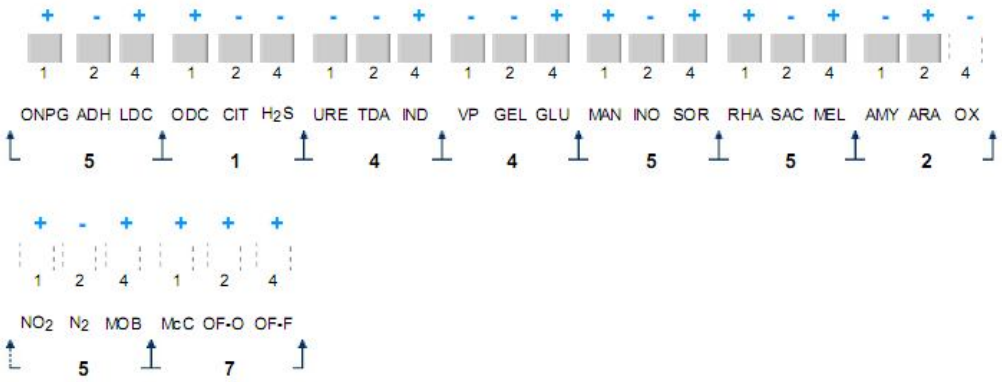
| | | | | |
|---|---|---|---|---|
| H41 | *Escherichia coli* | Pus swab | F | 75 |
| H42 | *Escherichia coli* | Pus swab | M | 80 |
| H43 | *Klebsiella pneumoniae* | Sputum | F | 61 |
| H44 | *Enterobacter aerogenes* | Pus swab | F | 65 |
| H45 | *Klebsiella pneumoniae* | Urine | F | 0 |
| H46 | *Escherichia coli* | Pus swab | F | 67 |
| H47 | *Escherichia coli* | Swab | M | 43 |
| H48 | *Escherichia coli* | Swab | M | 60 |
| H49 | *Klebsiella pneumoniae* | Blood | M | 51 |
| H50 | *Escherichia coli* | Swab | F | 67 |
| H51 | *Escherichia coli* | Urine | F | 71 |
| H52 | *Escherichia coli* | Blood | M | 48 |
| H53 | *Proteus mirabilis* | Wound swab | F | 30 |
| H54 | *Escherichia coli* | Urine | M | 5 |
| H55 | *Klebsiella pneumoniae* | Others | F | 12 |
| H56 | *Klebsiella pneumoniae* | Pus swab | M | 46 |
| H57 | *Klebsiella pneumoniae* | Pus swab | M | 68 |
| H58 | *Klebsiella pneumoniae* | Pus swab | F | 51 |
| H59 | *Escherichia coli* | Pus swab | M | 68 |
| H60 | *Escherichia coli* | Pus swab | F | 48 |
| H62 | *Klebsiella pneumoniae* | Pus swab | M | 62 |
| H63 | *Klebsiella pneumoniae* | Urine | M | 53 |
| H64 | *Klebsiella pneumoniae* | Others | M | 14 |
| H65 | *Klebsiella pneumoniae* | Urine | F | 21 |
| H66 | *Klebsiella pneumoniae* | Pus swab | M | 66 |
| H67 | *Klebsiella pneumoniae* | Tissue | M | 55 |
| H68 | *Klebsiella pneumoniae* | Urine | M | 80 |
| H69 | *Klebsiella pneumoniae* | Urine | F | 47 |
| H70 | *Escherichia coli* | Urine | F | 62 |
| H71 | *Klebsiella pneumoniae* | Blood | F | 50 |
| H72 | *Escherichia coli* | Tissue | F | 52 |
| H73 | *Klebsiella pneumoniae* | Others | M | 63 |
| H74 | *Proteus mirabilis* | Pus swab | F | 72 |
| K1 | *Morganella morganii* | Pus swab | M | 59 |
| K2 | *Pseudomonas aeruginosa* | Wound swab | M | 81 |
| K3 | *Escherichia coli* | Urine | F | 16 |
| K4 | *Serratia liquefaciens* | Swab | M | 52 |
| K5 | *Pseudomonas aeruginosa* | Swab | M | 79 |
| K7 | *Pseudomonas aeruginosa* | others | M | 71 |
| K15 | *Enterobacter cloacae* | Urine | M | 83 |
| K16 | *Escherichia coli* | Urine | F | 60 |
| K17 | *Pseudomonas aeruginosa* | Swab | M | 88 |
| K18 | *Pseudomonas aeruginosa* | others | M | 20 |
| K20 | *Pseudomonas aeruginosa* | Sputum | M | 66 |
| K21 | *Klebsiella pneumoniae* | Sputum | F | 77 |
| K22 | *Pseudomonas aeruginosa* | Urine | M | 56 |

| | | | | |
|---|---|---|---|---|
| K23 | *Pseudomonas aeruginosa* | others | M | 72 |
| K24 | *Pseudomonas aeruginosa* | Tissue | M | 72 |
| K25 | *Pseudomonas aeruginosa* | others | M | 72 |
| K26 | *Klebsiella pneumoniae* | Urine | F | 71 |
| K31 | *Pseudomonas aeruginosa* | others | F | 69 |
| P1 | *klebsiella pneumoniae* | Urine | F | 77 |
| P2 | *Pseudomonas sp* | Wound swab | F | 59 |
| P3 | *Escherichia coli* | Urine | F | 8 |
| P4 | *Escherichia coli* | Others | F | 49 |
| P6 | *klebsiella pneumoniae* | Swab | F | 58 |
| P7 | *Serratia plymuthica* | Wound swab | F | 21 |
| P8 | *Escherichia coli* | Urine | F | 56 |
| P9 | *Serratia marcescens* | Swab | F | 57 |
| P10 | *Escherichia coli* | Urine | M | 72 |
| P11 | *Escherichia coli* | Urine | F | 73 |
| P12 | *Enterobacter cloacae* | Wound swab | M | 55 |
| P13 | *Pseudomonas aeruginosa* | Wound swab | M | 54 |
| P14 | *Escherichia coli* | Urine | F | 59 |
| P16 | *Escherichia coli* | Urine | F | 49 |
| A5 | *Pseudomonas aeruginosa* | Sputum | M | 68 |
| A6 | *Pseudomonas aeruginosa* | Sputum | F | 79 |
| A7 | *Escherichia coli* | Blood | M | 79 |

[a]Nasal, ear, and throat swab were classified in normal "Swab". "Others" indicates sampling sites from bone, endotracheal tubes (ETT), tracheal aspirate and more

## Appendix B

**Seven digits numerical profile of isolates interpreted using apiweb™ Identification software.** Result showed 99.9% ID and T- index of 1.0 with excellent identification of *Escherichia coli*. No tests were shown against to the reference profile of *Escherichia coli* isolates.




**EXCELLENT IDENTIFICATION**

| | |
|---|---|
| **Strip** | API 20 E V4.1 |
| **Profile** | 5 1 4 4 5 5 2 5 7 |
| **Note** | |

| **Significant taxa** | **% ID** | **T** | **Tests against** |
|---|---|---|---|
| **Escherichia coli 1** | **99.9** | **1.0** | |

| **Next taxon** | **% ID** | **T** | **Tests against** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Kluyvera spp** | **0.1** | **0.38** | **LDC** | **25%** | **SOR** | **25%** | **SAC** | **89%** | **AMY** | **99%** |

## Appendix C

**Antibiotic resistant profile in one hundred forty-seven *Enterobacteriaceae* and *Pseudomonas***

| Sample ID | AN | GN | S | IMP | MEM | CAZ | CXM | CRO | AmC | AM | CIP | NOR | TMP | SMZ | SXT | TE | C | TGC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G2 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | S | S | R |
| G5 | R | R | S | R | S | R | S | S | S | R | R | S | S | S | S | S | S | S |
| G7 | S | S | R | S | S | S | R | R | S | R | S | S | R | R | R | R | S | S |
| G8 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | S | S | R |
| G10 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G12 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | S | S | S |
| G13 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | R | R | S |
| G21 | R | R | R | S | S | R | R | R | R | R | R | R | S | R | S | R | R | S |
| G22 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G23 | S | S | R | S | S | R | R | R | R | R | S | S | S | S | S | S | S | R |
| G24 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G26 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | R | S | S |
| G27 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | R | S | S |
| G28 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G31 | S | S | R | S | S | R | R | R | R | R | S | S | S | S | S | R | S | S |
| G33 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G36 | S | R | S | S | S | S | R | R | R | R | S | S | S | S | S | R | S | R |
| G39 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | R | S | R |
| G42 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | S | S | S |
| G44 | S | S | R | S | S | S | R | R | R | R | S | S | S | S | S | R | S | S |

| Sample ID | AN | GN | S | IMP | MEM | CAZ | CXM | CRO | AmC | AM | CIP | NOR | TMP | SMZ | SXT | TE | C | TGC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G46 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | R | S | S | R |
| G47 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | S | S | S |
| G49 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G50 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | S | S | S |
| G51 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G52 | S | R | R | S | S | S | R | R | R | R | R | R | R | R | R | S | S | R |
| G53 | S | S | R | S | S | S | R | R | R | R | S | S | S | S | S | S | S | S |
| G54 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G55 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | R | S | S |
| G56 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G57 | S | S | R | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G58 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | R | S | S |
| G59 | S | R | R | S | S | S | S | R | R | R | R | S | R | R | R | R | R | S |
| G60 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G61 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | S | S | S |
| G62 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | S | S | R |
| G63 | S | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| G64 | S | S | R | S | S | S | R | R | R | R | S | S | R | R | R | R | R | R |
| G65 | S | S | R | S | S | S | S | R | R | R | S | S | S | S | S | S | S | S |
| G66 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G67 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | S | S | S | S |
| G68 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G69 | S | S | S | S | S | R | R | R | R | R | S | S | R | R | R | R | S | R |
| G70 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |

| Sample ID | AN | GN | S | IMP | MEM | CAZ | CXM | CRO | AmC | AM | CIP | NOR | TMP | SMZ | SXT | TE | C | TGC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G71 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | R | R | S |
| G72 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| G73 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | R | R | S |
| H1 | S | R | S | S | S | R | R | R | R | R | R | R | R | R | R | R | S | R |
| H2 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H3 | R | R | R | S | R | R | R | R | R | S | R | R | R | R | S | R | S | R |
| H4 | S | R | R | S | S | R | R | R | R | R | R | S | R | R | R | R | S | S |
| H5 | S | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | S |
| H6 | S | S | R | R | S | R | R | R | R | R | R | R | R | R | R | R | R | R |
| H8 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | S |
| H9 | S | S | R | S | S | R | R | R | R | R | R | R | S | S | R | R | R | S |
| H10 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | R | S | S |
| H11 | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| H12 | S | R | R | S | S | R | R | R | R | R | R | S | R | R | R | R | R | S |
| H14 | S | R | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | R |
| H15 | S | R | R | S | S | R | R | R | R | R | R | S | R | R | R | R | R | R |
| H16 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | R |
| H18 | S | S | R | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S |
| H19 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | S | S | R |
| H21 | S | S | R | S | S | S | R | R | S | R | R | R | R | R | R | R | R | S |
| H22 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | S | S | R |
| H23 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| H26 | S | S | R | S | S | R | R | R | R | R | S | R | R | R | R | R | S | R |
| H27 | S | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | R |

| Sample ID | AN | GN | S | IMP | MEM | CAZ | CXM | CRO | AmC | AM | CIP | NOR | TMP | SMZ | SXT | TE | C | TGC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H28 | S | R | R | S | S | R | R | R | R | R | S | S | R | R | R | S | S | S |
| H31 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | R |
| H32 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | S | S | S |
| H33 | S | S | R | S | S | R | R | R | R | R | S | S | R | R | R | R | S | S |
| H34 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | R |
| H35 | S | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H36 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H37 | S | R | R | S | S | R | R | R | R | R | S | S | S | R | S | S | S | S |
| H38 | R | R | R | S | S | R | R | R | R | R | R | R | R | R | S | R | R | R |
| H39 | S | S | R | S | S | R | R | R | R | R | R | R | S | S | S | R | S | S |
| H40 | S | R | R | S | S | R | R | R | R | R | R | S | R | R | R | R | S | R |
| H41 | S | S | R | S | S | R | R | S | R | R | S | S | R | R | R | R | R | S |
| H42 | S | S | R | S | S | R | R | R | R | R | R | S | S | S | S | S | S | S |
| H43 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | S |
| H44 | S | S | R | S | R | R | R | R | R | R | S | S | R | R | R | R | S | S |
| H45 | S | S | R | S | S | R | R | R | R | R | R | S | R | R | R | R | R | R |
| H46 | S | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H47 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H48 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H49 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H50 | S | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H51 | S | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H52 | S | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | S |
| H53 | S | S | R | S | S | R | R | S | R | R | R | S | R | R | R | R | R | R |

| Sample ID | AN | GN | S | IMP | MEM | CAZ | CXM | CRO | AmC | AM | CIP | NOR | TMP | SMZ | SXT | TE | C | TGC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H54 | S | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H55 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | R |
| H56 | R | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | R |
| H57 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | R |
| H58 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | S | S | S |
| H59 | S | S | R | S | S | R | R | R | R | R | R | S | R | R | R | R | R | S |
| H60 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |
| H62 | S | R | R | S | S | R | R | R | R | R | S | S | R | S | R | R | S | R |
| H63 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | R |
| H64 | S | R | R | S | S | R | R | R | R | R | R | S | R | S | R | R | R | R |
| H65 | S | S | S | S | S | R | R | R | R | R | R | S | R | R | R | R | R | R |
| H66 | R | R | R | S | S | R | R | R | R | R | R | S | R | R | R | R | R | R |
| H67 | S | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | R |
| H68 | S | S | R | S | S | R | R | R | R | R | R | S | R | R | R | R | S | R |
| H69 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | R |
| H70 | S | R | R | S | S | R | R | S | R | R | R | S | R | R | R | R | R | R |
| H71 | R | R | R | S | S | R | R | R | R | R | R | S | R | R | R | R | R | R |
| H72 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | S |
| H73 | S | R | R | S | S | R | R | R | R | R | R | S | R | R | R | S | R | S |
| H74 | S | R | S | S | S | R | R | S | R | R | S | S | S | R | S | R | S | S |
| K1 | S | S | R | S | S | S | S | S | R | S | S | S | R | R | S | R | R | S |
| K2 | S | S | R | S | S | S | R | R | R | R | R | S | R | R | R | R | R | R |
| K3 | S | R | R | S | S | S | S | S | S | R | R | R | R | R | R | R | R | S |
| K4 | R | R | R | S | R | R | R | R | R | R | R | R | S | S | R | R | R | S |

| Sample ID | AN | GN | S | IMP | MEM | CAZ | CXM | CRO | AmC | AM | CIP | NOR | TMP | SMZ | SXT | TE | C | TGC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| K5 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| K7 | R | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | R |
| K15 | R | R | S | S | S | R | R | R | R | R | R | R | R | R | R | S | S | S |
| K16 | S | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | R |
| K17 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| K18 | R | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | R |
| K20 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| K21 | R | R | R | S | S | R | R | R | R | R | R | R | S | R | S | R | R | R |
| K22 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| K23 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | S |
| K24 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| K25 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| K26 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | R |
| K31 | R | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | R |
| P1 | S | R | S | S | S | R | R | R | S | R | S | S | R | S | S | R | S | S |
| P2 | S | S | S | S | S | S | R | R | R | R | S | S | R | S | S | R | R | R |
| P3 | S | R | R | S | S | S | S | S | S | R | R | R | R | R | R | R | S | S |
| P4 | S | S | R | S | S | S | S | S | S | R | S | S | R | S | S | R | S | S |
| P6 | S | S | S | S | S | S | S | S | R | R | S | S | S | S | S | S | S | R |
| P7 | S | S | S | S | S | S | S | S | R | R | S | S | S | S | S | S | S | S |
| P8 | S | S | R | S | S | S | S | S | R | R | S | S | R | R | R | R | S | S |
| P9 | S | S | R | S | S | S | R | S | R | R | S | S | R | S | S | R | S | R |
| P10 | S | S | R | S | S | S | S | S | R | R | S | S | R | R | R | S | S | S |
| P11 | S | S | R | S | S | S | R | S | R | R | S | S | R | R | R | R | S | S |

| Sample ID | AN | GN | S | IMP | MEM | CAZ | CXM | CRO | AmC | AM | CIP | NOR | TMP | SMZ | SXT | TE | C | TGC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P12 | S | S | S | S | S | S | R | S | R | S | S | S | S | S | S | S | S | R |
| P13 | S | S | S | S | S | S | R | R | R | R | S | S | S | S | R | R | S | S |
| P14 | S | R | R | S | S | S | R | R | R | R | R | R | R | R | R | R | S | S |
| P16 | S | S | R | S | S | S | R | R | S | R | R | R | R | R | S | S | S | S |
| A5 | S | S | S | R | S | S | R | R | R | R | S | S | R | R | R | R | R | R |
| A6 | S | S | S | S | S | S | R | R | R | R | R | S | R | R | R | R | R | R |
| A7 | S | R | R | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S |

## Appendix D

### PCR amplification of integrase and *sul1* genes among all isolates

| Sample ID | Integrase gene | *sul1* gene |
|---|---|---|
| G2 | - | - |
| G5 | - | - |
| G7 | + | - |
| G8 | - | - |
| G10 | - | - |
| G12 | - | - |
| G13 | - | - |
| G21 | - | + |
| G22 | - | - |
| G23 | - | - |
| G24 | - | - |
| G26 | - | - |
| G27 | - | - |
| G28 | - | - |
| G31 | - | - |
| G33 | - | - |
| G36 | - | - |
| G39 | - | - |
| G42 | - | - |
| G44 | - | - |
| G46 | - | - |
| G47 | - | - |
| G49 | - | - |
| G50 | - | - |
| G51 | - | - |
| G52 | + | + |
| G53 | - | - |
| G54 | - | - |
| G55 | - | - |
| G56 | - | - |
| G57 | - | - |
| G58 | - | - |
| G59 | + | + |
| G60 | - | - |
| G61 | - | - |
| G62 | - | - |
| G63 | + | + |
| G64 | + | + |
| G65 | - | - |
| G66 | - | - |
| G67 | - | - |
| G68 | - | - |

| | | |
|---|---|---|
| G69 | - | + |
| G70 | - | - |
| G71 | - | - |
| G72 | - | - |
| G73 | - | - |
| H1 | + | - |
| H2 | - | - |
| H3 | + | - |
| H4 | + | + |
| H5 | + | - |
| H6 | + | - |
| H8 | - | - |
| H9 | + | - |
| H10 | - | - |
| H11 | - | - |
| H12 | + | + |
| H14 | + | - |
| H15 | + | + |
| H16 | + | - |
| H18 | - | - |
| H19 | + | - |
| H21 | + | - |
| H22 | + | - |
| H23 | - | - |
| H26 | + | + |
| H27 | + | + |
| H28 | + | - |
| H31 | + | - |
| H32 | + | + |
| H33 | - | + |
| H34 | + | - |
| H35 | + | + |
| H36 | - | - |
| H37 | + | + |
| H38 | + | + |
| H39 | - | - |
| H40 | + | + |
| H41 | + | - |
| H42 | - | - |
| H43 | + | - |
| H44 | + | + |
| H45 | + | + |
| H46 | - | + |
| H47 | - | + |
| H48 | - | + |
| H49 | + | + |
| H50 | - | - |
| H51 | + | + |
| H52 | - | - |
| H53 | + | + |

| | | |
|---|---|---|
| H54 | + | + |
| H55 | + | - |
| H56 | - | - |
| H57 | + | + |
| H58 | + | + |
| H59 | + | - |
| H60 | + | - |
| H62 | + | - |
| H63 | + | + |
| H64 | - | + |
| H65 | + | + |
| H66 | + | + |
| H67 | + | + |
| H68 | + | - |
| H69 | + | - |
| H70 | + | + |
| H71 | - | + |
| H72 | - | + |
| H73 | + | + |
| H74 | - | - |
| K1 | - | - |
| K2 | - | - |
| K3 | + | - |
| K4 | - | - |
| K5 | + | + |
| K7 | + | + |
| K15 | + | + |
| K16 | + | - |
| K17 | + | + |
| K18 | + | + |
| K20 | + | + |
| K21 | - | - |
| K22 | + | + |
| K23 | + | + |
| K24 | + | + |
| K25 | + | + |
| K26 | + | - |
| K31 | + | + |
| P1 | + | - |
| P2 | - | - |
| P3 | + | - |
| P4 | + | - |
| P6 | - | - |
| P7 | - | - |
| P8 | + | - |
| P9 | - | - |
| P10 | + | - |
| P11 | - | + |
| P12 | - | - |
| P13 | - | - |

| | | |
|---|---|---|
| P14 | + | + |
| P16 | - | - |
| A5 | - | - |
| A6 | - | - |
| A7 | + | + |

## Appendix E

### List of DNA sequences of amplified gene cassettes in this study

Nineteen different gene cassettes were amplified, comprised of single (n= 6), two (n= 9) and three (n= 4) tandem gene arrays. Primer pairs were underlined. Coding sequence of respective gene cassettes were presented in different colors (blue, green, and purple) according to the cassette arrangement listed. All sequences were more than 99% identical compared to sequences from GenBank.

### Class 1 integrons gene cassettes:

>H41_ *dfrA5*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAACCCGGAAC
CAAAATTGTGAAAGTATCATTAATGGCTGCAAAAGCGAAAAACGGAGTGATTGGT
TGCGGTCCACACATACCCTGGTCCGCGAAAGGAGAGCAGCTACTCTTTAAAGCCT
TGACGTACAACCAGTGGCTTTTGGTGGGCCGCAAGACGTTCGAATCTATGGGAGC
ACTCCCTAATAGGAAATACGCGGTCGTTACTCGCTCAGCCTGGACGGCCGATAAT
GACAACGTAATAGTATTCCCGTCGATCGAAGAGGCCATGTACGGGCTGGCTGAAC
TCACCGATCACGTTATAGTGTCTGGTGGCGGGGAGATTTACAGAGAAACATTGCC
CATGGCCTCTACGCTCCATATATCGACGATTGATATTGAGCCGGAAGGAGATGTT
TTCTTTCCGAATATTCCCAATACCTTCGAAGTTGTTTTTGAGCAACACTTTAGCTCA
AACATTAACTATTGCTATCAAATTTGGCAAAAGGGTTAACAAAGCTATGCAATTG
ACGGTAAAAAGCTTCGTTCGCTTCGCTTGCTACGCTTCTTACCGCAATTGATAACG
GCGTTAGATGCACTAAGCACATAATTGCTCACAGCCAAGCTATCAGGTCAAGTCT
GCTT

>H1_ *aadB*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAAACATCATG
AGGGGTTAGGCCGCATGGACACAACGCAGGTCACATTGATACACAAAATTCTAGC
TGCGGCAGATGAGCGAAATCTGCCGCTCTGGATCGGTGGGGGCTGGGCGATCGAT
GCACGGCTAGGGCGTGTAACACGCAAGCACGATGATATTGATCTGACGTTTCCCG
GCGAGAGGCGCGGCGAGCTCGAGGCAATAGTTGAAATGCTCGGCGGGCGCGTCA
TGGAGGAGTTGGACTATGGATTCTTAGCGGAGATCGGGGATGAGTTACTTGACTG
CGAACCTGCTTGGTGGGCAGACGAAGCGTATGAAATCGCGGAGGCTCCGCAGGG
CTCGTGCCCAGAGGCGGCTGAGGGCGTCATCGCCGGGCGGCCAGTCCGTTGTAAC
AGCTGGGAGGCGATCATCTGGGATTACTTTTACTATGCCGATGAAGTACCACCAG
TGGACTGGCCTACAAAGCACATAGAGTCCTACAGGCTCGCATGCACCTCACTCGG
GGCGGAAAAGGTTGAGGTCTTGCGTGCCGCTTTCAGGTCGCGATATGCGGCCTAA
CAATTCGTCCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAGGTGTTAGATG
CACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTT

> H32_ *dfrA7*

GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATTACGG
GGGTTGAATTGAAAATTTCATTGATTTCTGCAACGTCAGAAAATGGCGTAATCGG
TAATGGCCCTGATATCCCATGGTCAGCAAAAGGTGAGCAGTTACTCTTTAAAGCG
CTCACATATAATCAGTGGCTCCTTGTTGGAAGGAAAACATTTGACTCTATGGGTGT
TCTTCCAAATCGAAAATATGCAGTAGTGTCGAGGAAAGGAATTTCAAGCTCAAAT
GAAAATGTATTAGTCTTTCCTTCAATAGAAATCGCTTTGCAAGAACTATCGAAAAT
TACAGATCATTTATATGTCTCTGGTGGCGGTCAAATCTACAATAGTCTTATTGAAA
AAGCAGATATAATTCATTTGTCTACTGTTCACGTTGAGGTTGAAGGTGATATCAAT
TTTCCTAAAATTCCAGAGAATTTCAATTTGGTTTTTGAGCAGTTTTTTTTGTCTAAT
ATAAATTACACATATCAGATTTGGAAAAAAGGCTAACAAGTCGTTCCAGCACCAG
TCGCTGCGCTCCTTGGACAGTTTTTAAGTCGCGGTTTTATGGTTTTGCTGCGCAAA
AGTATTCCATAAAACCACAACTTAAAAACTGCCGCTGAACTCGGCGTTAGATGCA
CTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTT

> H70_ *aadA1*

GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAAACATCATG
AGGGAAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGTCA
TCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAGT
GGATGGCGGCCTGAAGCCACACAGTGATATTGATTTGCTGGTTACGGTGACCGTA
AGGCTTGATGAAACAACGCGGCGAGCTTTGATCAACGACCTTTTGGAAACTTCGG
CTTCCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCA
CGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAA
TGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTG
ATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCC
AGCGGCGGAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTA
AATGAAACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAA
ATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGC
GCCGAAGGATGTCGCTGCCGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAG
CCCGTCATACTTGAAGCTAGACAGGCTTATCTTGGACAAGAGGAAGATCGCTTGG
CCTCCTGCGCAGATCAGTTGGAAGAATTTGTTCACTACGTGAAAGGCGAGATCAC
CAAGGTAGTCGGCAAATAATGTCTAACAATTCGTTCAAGCCGACGCCGCTTCGCG
GCGCGGCTTAACTCAAGCGTTAGATGCACTAAGCACATAATTGCTCACAGCCAAA
CTATCAGGTCAAGTCTGCTT

>H67_ *blaP1*

GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATATTAT
GGAGCCTCATGCTTTTATATAAAATGTGTGACAATCAAAATTATGGGGTTACTTAC
ATGAAGTTTTTATTGGCATTTTCGCTTTTAATACCATCCGTGGTTTTTGCAAGTAGT
TCAAAGTTTCAGCAAGTTGAACAAGACGTTAAGGCAATTGAAGTTTCTCTTTCTGC
TCGTATAGGTGTTTCCGTTCTTGATACTCAAAATGGAGAATATTGGGATTACAATG
GCAATCAGCGCTTCCCGTTAACAAGTACTTTTAAAACAATAGCTTGCGCTAAATTA
CTATATGATGCTGAGCAAGGAAAAGTTAATCCCAATAGTACAGTCGAGATTAAGA
AAGCAGATCTTGTGACCTATTCCCCTGTAATAGAAAAGCAAGTAGGGCAGGCAAT
CACACTCGATGATGCGTGCTTCGCAACTATGACTACAAGTGATAATACTGCGGCA
AATATCATCCTAAGTGCTGTAGGTGGCCCCAAAGGCGTTACTGATTTTTTAAGACA
AATTGGGGACAAAGAGACTCGTCTAGACCGTATTGAGCCTGATTTAAATGAAGGT
AAGCTCGGTGATTTGAGGGATACGACAACTCCTAAGGCAATAGCCAGTACTTTGA
ATAAATTTTTATTTGGTTCCGCGCTATCTGAAATGAACCAGAAAAAATTAGAGTCT
TGGATGGTGAACAATCAAGTCACTGGTAATTTACTACGTTCAGTATTGCCGGCGG
GATGGAACATTGCGGATCGCTCAGGTGCTGGCGGATTTGGTGCTCGGAGTATTAC
AGCAGTTGTGTGGAGTGAGCATCAAGCCCCAATTATTGTGAGCATCTATCTAGCT
CAAACACAGGCTTCAATGGCAGAGCGAAATGATGCGATTGTTAAAATTGGTCATT

CAATTTTTGACGTTTATACATCACAGTCGCGCTGATAAGGCTAACAAGGCCATCA
AGTTGACGGCTTTTCCGTCGCTTGTTTTGTGGTTTAACGCTACGCTACCACAAAAC
AATCAACTCCAAAGCCGCAACTTATGGCGGCGTTAGATGCACTAAGCACATAATT
GCTCACAGCCAAACTATCAGGTCAAGTCTGCTT

> K15_ *aadA2*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGACATCATG
AGGGTAGCGGTGACCATCGAAATTTCGAACCAACTATCAGAGGTGCTAAGCGTCA
TTGAGCGCCATCTGGAATCAACGTTGCTGGCCGTGCATTTGTACGGCTCCGCAGTG
GATGGCGGCCTGAAGCCATACAGCGATATTGATTTGTTGGTTACTGTGGCCGTAA
AGCTTGATGAAACGACGCGGCGAGCATTGCTCAATGACCTTATGGAGGCTTCGGC
TTTCCCTGGCGAGAGCGAGACGCTCCGCGCTATAGAAGTCACCCTTGTCGTGCAT
GACGACATCATCCCGTGGCGTTATCCGGCTAAGCGCGAGCTGCAATTTGGAGAAT
GGCAGCGCAATGACATTCTTGCGGGTATCTTCGAGCCAGCCATGATCGACATTGA
TCTAGCTATCCTGCTTACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTTCG
GCAGCGGAGGAATTCTTTGACCCGGTTCCTGAACAGGATCTATTCGAGGCGCTGA
GGGAAACCTTGAAGCTATGGAACTCGCAGCCCGACTGGGCCGGCGATGAGCGAA
ATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAATAACCGGCAAAATCGC
GCCGAAGGATGTCGCTGCCGACTGGGCAATAAAACGCCTACCTGCCCAGTATCAG
CCCGTCTTACTTGAAGCTAAGCAAGCTTATCTGGGACAAAAAGAAGATCACTTGG
CCTCACGCGCAGATCACTTGGAAGAATTTATTCGCTTTGTGAAAGGCGAGATCAT
CAAGTCAGTTGGTAAATGATGTCTAACAATTCGTTCAAGCCGACCGCGCTACGCG
CGGCGGCTTAACTCCGGCGTTAGATGCACTAAGCACATAATTGCTCACAGCCAAA
CTATCAGGTCAAGTCTGCTT

> G59_ *dfrA1-orfC*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAACCTCTGAGG
AAGAATTGTGAAACTATCACTAATGGTAGCTATATCGAAGAATGGAGTTATCGGG
AATGGCCCTGATATTCCATGGAGTGCCAAAGGTGAACAGCTCCTGTTTAAAGCTA
TTACCTATAACCAATGGCTGTTGGTTGGACGCAAGACTTTTGAATCAATGGGAGC
ATTACCCAACCGAAAGTATGCGGTCGTAACACGTTCAAGTTTTACATCTGACAAT
GAGAACGTATTGATCTTTCCATCAATTAAAGATGCTTTAACCAACCTAAAGAAAA
TAACGGATCATGTCATTGTTTCAGGTGGTGGGGAGATATACAAAAGCCTGATCGA
TCAAGTAGATACACTACATATATCTACAATAGACATCGAGCCGGAAGGTGATGTT
TACTTTCCTGAAATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGACTTCGCCTC
TAACATAAATTATAGTTACCAAATCTGGCAAAAGGGTTAACAAGTGGCAGCAACG
GATTCGCAAACCTGTCACGCCTTTTGTACCAAAAGCCGCGCCAGGTTTGCGATCC
GCTGTGCCAGGCGTTAAGGCTACATGAAAATCGTACATTACGAAGCGAATGCACC
ATGGATAGGAAGAATGAAATGCCCAAACCCAAAGTGTGGGAAGGAAACTCCTGC
CTGGCAATCGAGCGGCATGAGCGACAGTTGCCCGCATTTTTTCTGTGATACTTGCT
CGAATGTAATCCATAGAGAGCAGGACCATGCATTACTGTATGAAAATGAAATCAA
TCAAGAGCTCTTGGATCGAATAGCAGCAACTCTTCCAGATTGCCCTTGCGGGGGT
AGGTTTGTTCCTGGTGCAAACCCAAAGTGTCCGAGTTGCAGGACCGAGTACGTGC
ACCAATGGGATGCAGTGAAAAGGTTGAATGTACCTTTTATGCCAATCTTGGATGG
TTCCTGCTTGATTCGAGATAGGCTGTATTCGTATGAAGTATGCATTGGTTCTAAAC
CAAAATACTGGTGGCGTTTGTTCACAAATGCCTTAACAAGTTTAGGCAAGGGACG
CTCCTGACGTCGCGCCCCTGCTAAAAGCGTTAGATGCACTAAGCACATAATTGCT
CACAGCCAAACTATCAGGTCAAGTCTGCTT

>K18_ *aadA6-orfD*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGACATCATG
AGTAACGCAGTACCCGCCGAGATTTCGGTACAGCTATCACTGGCTCTCAACGCCA

TCGAGCGTCATCTGGAATCAACGTTGCTGGCCGTGCATTTGTACGGCTCTGCACTG
GACGGTGGCCTGAAGCCATACAGTGATATTGATTTGCTGGTTACTGTGGCTGCAC
GGCTCGATGAGACTGTCCGACAAGCCCTGGTCGTAGATCTCTTGGAAATTTCTGCC
TCCCCTGGCCAAAGTGAAGCTCTCCGCGCCTTGGAAGTTACCATCGTCGTGCATG
GTGATGTTGTCCCTTGGCGTTATCCGGCCAGACGGGAACTGCAATTCGGGGAGTG
GCAGCGTAAGGACATTCTTGCGGGCATCTTCGAGCCCGCCACAACCGATGTTGAT
CTGGCTATTCTGCTAACTAAAGTAAGGCAGCATAGCCTTGCATTGGCAGGTTCGG
CCGCAGAGGATTTCTTTAACCCAGTTCCGGAAGGCGATCTATTCAAGGCATTGAG
CGACACTCTGAAACTATGGAATTCGCAGCCGGATTGGGAAGGCGATGAGCGGAA
TGTAGTGCTTACCTTGTCTCGCATTTGGTACAGCGCAGCAACCGGCAAGATCGCA
CCGAAGGATATCGTTGCCAACTGGGCAATGGAGCGTCTGCCAGATCAACATAAGC
CCGTACTGCTTGAAGCCCGGCAGGCTTATCTTGGACAAGGAGAAGATTGCTTGGC
CTCACGCGCGGATCAGTTGGCGGCGTTCGTTCACTTCGTGAAACATGAAGCCACT
AAATTGCTTAGTGCCATGCCAGTGATGTCTAACAATTCATTCAAGCCGACGCCGCT
TCGCGGCGCGGCTTAATTCAGGCGTTAGTACCACTGAAACCCTCCTTTATTTCGCC
CATGTTTATTCAAACGGCATTCAGTTTCTCAAACGCTGTGCAGCGCTGGGTTTGCC
GTTTCTCTGGGCTTCGCCTGGTGGCGTTACGCTGGTTTGTGGTCTTTTTGGCCTCTG
GCCCTTGTGTAGCAAGCGCGAGCAGCTATTTTTTTCGTAGTGCTGTGCCGCCTCGG
TGGCACCGTGCCTTTTCGCAGTTAGCGCCCGTCGCCAAGTTACGGTTATCCGTTTT
GGCTTCTGGCTCTAACATTTCGGTCAAGCCGACCCGCATTCTGCGGTCGGCTTAAC
TCGCCCGTTAGATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAA
GTCTGCTT

>H62_ *dfrA15-aadA1*
GGCATCCAAGCAGCAAGCGCGTCACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAACCCCTAAG
GAAGTATCGTGAAACTATCACTAATGGCAGCAATTTCGAAGAATGGAGTTATCGG
AAATGGCCCAGATATTCCATGGAGTGCCAAAGGGGAACAATTACTCTTCAAAGCG
ATTACCTATAATCAGTGGCTTTTGGTAGGCCGAAAGACTTTCGAGTCAATGGGGG
CTTTACCCAACCGAAAATATGCCGTTGTAACTCGTTCAAGCTTCACTTCCAGTGAT
GAGAATGTATTGGTATTTCCATCTATCGATGAAGCGCTAAATCATCTGAAGACGA
TAACGGATCATGTGATTGTGTCTGGTGGTGGTGAAATATACAAAAGCCTGATCGA
TAAAGTTGATACTTTACATATTTCAACAATCGACATTGAGCCAGAAGGTGATGTCT
ATTTTCCAGAAATCCCCAGTAGTTTTAGGCCAGTTTTTAGCCAAGACTTCGTGTCT
AACATAAATTATAGTTACCAAATCTGGCAAAAGGGTTAACAAGTGGCAGCAACTG
ACCGCCAAAAGTGTCACTTGTTTTGCCAAAAAGCCGGCAAAACAAGCGCCAATTT
TGTCGGCAGCTGTGCCAGGCGTTAAACATCATGAGGGAAGCGGTGATCGCCGAAG
TATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCGCCATCTCGAACCGAC
GTTGCTGGCCGTACATTTGTACGGCTCCGCAGTGGATGGCGGCCTGAAGCCACAC
AGTGATATTGATTTGCTGGTTACGGTGACCGTAAGGCTTGATGAAACAACGCGGC
GAGCTTTGATCAACGACCTTTTGGAAACTTCGGCTTCCCCTGGAGAGAGCGAGAT
TCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTT
ATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGC
AGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAA
GCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTTGATC
CGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATGGAA
CTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGC
ATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGCTGCCGACT
GGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACA
GGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCTCCCGCGCAGATCAGTTGGAA
GAATTTGTTCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTC
TAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAAGCGTTAG
ATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTT

>H4_*dfrA16-aadA2*

GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAACTCGAGGG
AGAAATCGTGAAGTTATCACTAATGGCTGCCAAGTCGAAGAACGGTATTATCGGT
AATGGACCAGATATTCCATGGAGCGCCAAAGGCGAGCAACTTCTATTTAAGGCAA
TTACATATAATCAATGGCTTTTAGTTGGACGCAAAACTTTTGAGTCAATGGGCGCT
CTCCCAAATCGAAAGTATGCAGTTGTAACTCGCTCTAATTTTTCTACGAATGATGA
GGGTGTAATGGTTTTCTCCTCAATTCAGGATGCCTTAATAAATTTAGAGGAAACCA
CGGATCATGTTATCGTTTCTGGTGGTGGTGAAATATACAAAAGCTTGATTTCCAAA
GTAGATACTTTGCATATTTCAACAGTCGACATCGAGCGAGATGGAGACATAGTTT
TTCCTGAAATCCCAGATACATTCAAGTTGGTATTTGAGCAAGATTTCGAGTCTAAC
ATTAACTATTGTTATCAAATCTGGCAAAAGAGTTAACAAGCGCCTGCAATCTGAC
CTCCGGTTACTGTCACCTTTTTTTGCGGTGGAGCTGCAAAAAAGGCGCCATTAACC
TCCGGCAGTTGAGGCGGGCGTTAGACATCATGAGGGTAGCGGTGACCATCGAAAT
TTCGAACCAACTATCAGAGGTGCTAAGCGTCATTGAGCGCCATCTGGAATCAACG
TTGCTGGCCGTGCATTTGTACGGCTCCGCAGTGGATGGCGGCCTGAAGCCATACA
GCGATATTGATTTGTTGGTTACTGTGGCCGTAAAGCTTGATGAAACGACGCGGCG
AGCATTGCTCAATGACCTTATGGAGGCTTCGGCTTTCCCTGGCGAGAGCGAGACG
CTCCGCGCTATAGAAGTCACCCTTGTCGTGCATGACGACATCATCCCGTGGCGTTA
TCCGGCTAAGCGCGAGCTGCAATTTGGAGAATGGCAGCCCAATGACATTCTTGCG
GGTATCTTCGAGCCAGCCATGATCGACATTGATCTAGCTATCCTGCTTACAAAAGC
AAGAGAACATAGCGTTGCCTTGGTAGGTCCGGCAGCGGGGGAATTCTTTGACCCG
GTTCCTGAACAGGATCTATTCGAGGCGCTGAGGGAAACCTTGAAGCTATGGAACT
CGCAGCCCGACTGGGCCGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGCAT
TTGGTACAGCGCAATAACCGGCAAAATCGCGCCGAAGGATGTCGCTGCCGACTGG
GCAATAAAACGCCTACCTGCCCAGTATCAGCCCGTCTTACTTGAAGCTAAGCAAG
CTTATCTGGGACAAAAAGAAGATCACTTGGCCTCACGCGCAGATCACTTGGAAGA
ATTTATTCGCTTTGTGAAAGGCGAGATCATCAAGTCAGTTGGTAAATGATGTCTAA
CAATTCGTTCAAGCCGACCGCGCTACGCGCGGCGGCTTAACTCCGGCGTTAGATG
CACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTT

>G52_ *dfrA17-aadA5*

GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATTAAG
GGAGTTAAATTGAAAATATCATTGATTTCTGCAGTGTCAGAAAATGGCGTAATCG
GTAGTGGTCCTGATATCCCGCGGTCAGTAAAAGGTGAGCAACTACTCTTTAAAGC
GCTCACATATAATCAATGGCTCCTTGTCGGAAGAAAAACATTTGACTCTATGGGT
GTTCTTCCAAATCGCAAATATGCAGTAGTGTCAAAGAACGGAATTTCAAGCTCAA
ATGAAAACGTCCTAGCTTTTCCTTCAATAGAAAATGCTTTGAAAGAGCTATCAAA
AGTTACAGATCATGTATATGTCTCTGGCGGGGGTCAAATCTATAATAGCCTTATTG
AAAAAGCAGATATAATTCATTTGTCTACTGTTCACGTTGAAGTCGAAGGTGATAT
CAAATTCCCTATAATGCCTGAGAATTTCAATTTGGTTTTTGAACAGTTTTTTATGTC
TAATATAAATTATACATACCAGATTTGGAAAAAAGGCTAACAATGCGTTGCAGCA
CCAGTCGCTTCGCTCCTTGGACAGCTTTTAAGTCGCGTCTTTGTGGTTTTGCTGCGC
AAAAGTATTCCACAAAGCCGCAACTTAAAAGCTGCCGCTGAACTTAACGTTAGGC
ATCATGGGTGAATTTTTCCCTGCACAAGTTTTCAAGCAGCTGTCCCACGCTCGCGC
GGTGATCGAGCGCCATCTGGCTGCGACACTGGACACAATCCACCTGTTCGGATCT
GCGATCGATGGAGGGCTGAAGCCGGACAGCGACATAGACTTGCTCGTGACCGTCA
GCGCCGCACCTAACGATTCGCTCCGGCAGGCGCTAATGCTCGATTTGCTGAAAGT
CTCATCGCCGCCAGGCGATGGCGGAACATGGCGACCGCTGGAGCTAACTGTTGTC
GCTCGAAGCGAAGTAGTGCCTTGGCGCTATCCGGCGCGGCGTGAGCTTCAGTTCG
GTGAGTGGCTCCGCCACGACATCCTTTCCGGAACGTTCGAGCCTGCCGTTCTGGAT
CACGATCTTGCGATTTTGCTGACCAAGGCGAGGCAACACAGCCTTGCGCTTCTAG
GCCCATCCGCAGCCACGTTTTTCGAGCCGGTGCCGAAGGAGCATTTCTCCAAGGC
GCTTTTCGACACTATTGCCCAGTGGAATGCAGAGTCGGATTGGAAGGGTGACGAG
CGGAACGTCGTTCTTGCTCTTGCTCGCATTTGGTACAGCGCTTCAACTGGTCTCAT
TGCTCCTAAGGACGTTGCTGCCGCATGGGTATCGGAGCGTTTGCCTGCCGAGCAT
CGGCCCCTCATCTGCAAGGCACGCGCGGTGTACCTGGGTAGCGAGGACGGCGACC

TAGCAATGCGCGTCGAAGAGACGGCCGCGTTCGTTCGATATGCCAAAGCAACGAT
TGAGAGAATCTTGCGTTGAGCGGCATGTGCGAAAAGTGCATCGACCCGCGCCGAG
GGCATCTGATGCCTAACTCGGCGTTCAAGCGGACGGGCTGCGCCCGCCGCTCAAC
TATGCGTTAGATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAG
TCTGCTT

>H12_ *drfA1-aadA1*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAACCTCTGAGG
AAGAATTGTGAAACTATCACTAATGGTAGCTATATCGAAGAATGGAGTTATCGGG
AATGGCCCTGATATTCCATGGAGTGCCAAAGGTGAACAGCTCCTGTTTAAAGCTA
TTACCTATAACCAATGGCTGTTGGTTGGACGCAAGACTTTTGAATCAATGGGAGC
ATTACCCAACCGAAAGTATGCGGTCGTAACACGTTCAAGTTTTACATCTGACAAT
GAGAACGTAGTGATCTTTCCATCAATTAAAGATGCTTTAACCAACCTAAAGAAAA
TAACGGATCATGTCATTGTTTCAGGTGGTGGGGAGATATACAAAAGCCTGATCGA
TCAAGTAGATACACTACATATATCTACAATAGACATCGAGCCGGAAGGTGATGTT
TACTTTCCTGAAATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGACTTCGCCTC
TAACATAAATTATAGTTACCAAATCTGGCAAAAGGGTTAACAAGTGGCAGCAACG
GATTCGCAAACCTGTCACGCCTTTTGTACCAAAAGCCGCGCCAGGTTTGCGATCC
GCTGTGCCAGGCGTTAAACATCATGAGGGAAGCGGTGATCGCCGAAGTATCGACT
CAACTATCAGAGGTAGTTGGCGTCATCGAGCGCCATCTCGAACCGACGTTGCTGG
CCGTACATTTGTACGGCTCCGCAGTGGATGGCGGCCTGAAGCCACACAGTGATAT
TGATTTGCTGGTTACGGTGACCGTAAGGCTTGATGAAACAACGCGGCGAGCTTTG
ATCAACGACCTTTTGGAAACTTCGGCTTCCCCTGGAGAGAGCGAGATTCTCCGCG
CTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCT
AAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCT
TCGAGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGA
ACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTTGATCCGGTTCCT
GAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATGGAACTCGCCGC
CCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGCATTTGGTA
CAGCGCAGTAACCGGCAGAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAAT
GGAGCGCCTGCCGGCCCAGTATCAGCCCGTCGTACTTGAAGCTAGACAGGCTTAT
CTTGGACAAGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTTG
TTCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCTAACAAT
TCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAAGCGTTAGATGCACT
AAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTT

>K15_ *dfrA1-aadA5*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCCGCAGC
TTTGCTGCCGTGCAAATATGGTTCAATCGAGCATCAAGCAACACCGCTGATCCTG
GCTTGCCAAAGTCGGTTCGGCGTAAGGAAGAAAATGTCAGGGCAGGGCTGTTCGC
GTCAACCTCTCGCTGCCTGCGGAACAAATTGGCCGCGGGCCAAAAGCTGCCGCCT
TCGTGCCGCGCGCAGTGGCTGCGCGGTTAAAACCGTGGCCGCCTCCAATCGCCGT
TTCTTCGGCGTTGGGCTCGGCCTAACCAGTTGGTCAAGCCGACGCCCACAAGCTT
CGCTTGTGGGTGCCCTCCGTGCTTCGCACTCCGGCGCGGCTTACCGCGGGCGTTAA
CCTCTGAGGAAGAATTGTGAAACTATCACTAATGGTAGCTATATCGAAGAATGGA
GTTATCGGGAATGGCCCTGATATTCCATGGAGTGCCAAAGGTGAACAGCTCCTGT
TTAAAGCTATTACCTATAACCAATGGCTGTTGGTTGGACGCAAGACTTTTGAATCA
ATGGGAGCATTACCCAACCGAAAGTATGCGGTCGTAACACGTTCAAGTTTTACAT
CTGACAATGAGAACGTAGTGATCTTTCCATCAATTAAAGATGCTTTAACCAACCT
AAAGAAAATAACGGATCATGTCATTGTTTCAGGTGGTGGGGAGATATACAAAAGC
CTGATCGATCAAGTAGATACACTACATATATCTACAATAGACATCGAGCCGGAAG
GTGATGTTTACTTTCCTGAAATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGAC
TTCGCCTCTAACATAAATTATAGTTACCAAATCTGGCAAAAGGGTTAACAAGTGG
CAGCAACGGATTCGCAAACCTGTCACGCCTTTTGTACCAAAAGCCGCGCCAGGTT

TGCGATCCGCTGTGCCAGGCGTTGAACGTCATGAGCAGCTACACCGAAAAAACGG
GAAGGGCTCCGGACTTTAGAGTCGCGTATAAGCGGCTGCCGGCGCGTCAGCGCCT
CTTTCAACACATGCGCTGCAACGTGCATTGGGCGGCCGACGACGACCAGACTCGA
CAGTGGTCTATTTGGCCGGAGTTCGAGTCAGTGGGGCGCGAACCCATTCCGGAGA
CTGATGAACCGCTGGAATGTGGAACCGCCACTATGTGGGTGTTGTTCGACCAGCC
GGAGTACCGACAAGATCTGCGGCAGTGGCTACGCGTGGGCCAGGAGGGGTTCTTC
ATGGCCGGTGCCGAACGAATCGCGCACTTCACCCTTGTGGAGCTACTTGGCCGCG
AAGCAGACGCCCAACCCACCGCTCCAGCGGACGGCCTTCGACCGCCGCTGACCTA
GCACGTTAGGCATCATGGGTGAATTTTTCCCTGCACAAGTTTTCAAGCAGCTGTCC
CACGCTCGCGCGGTGATCGAGCGCCATCTGGCTGCGACACTGGACACAATCCACC
TGTTCGGATCTGCGATCGATGGAGGGCTGAAGCCGGACAGCGACATAGACTTGCT
CGTGACCGTCAGCGCCGCACCTAACGATTCGCTCCGGCAGGCGCTAATGCTCGAT
TTGCTGAAAGTCTCATCACCGCCAGGCGATGGCGGAACATGGCGACCGCTGGAGC
TAACTGTTGTCGCTCGAAGCGAAGTAGTGCCTTGGCGCTATCCGGCGCGGCGTGA
GCTTCAGTTCGGTGAGTGGCTCCGCCACGACATCCTTTCCGGAACGTTCGAGCCTG
CCGTTCTGGATCACGATCTTGCGATTTTGCTGACCAAGGCGAGGCAACACAGCCT
TGCGCTTCTAGGCCCATCCGCAGCCACGTTTTTCGAGCCGGTGCCGAAGGAGCAT
TTCTCCAAGGCGCTTTTCGACACTATTGCCCAGTGGAATGCAGAGTCGGATTGGA
AGGGTGACGAGCGGAACGTCGTTCTTGCTCTTGCTCGCATTTGGTACAGCGCTTCA
ACTGGTCTCATTGCTCCTAAGGACGTTGCTGCCGCATGGGTATCGGAGCGTTTGCC
TGCCGAGCATCGGCCCCTCATCTGCAAGGCACGCGCGGCGTACCTGGGTAGCGAG
GACGACGACCTAGCAATGCGCGTCGAAGAGACGGCCGCGTTCGTTCGATATGCCA
AAGCAACGATTGAGAGAATCTTGCGTTGAGCGGCATGTGCGAAAAGTGCATCGAC
CCGCGCCGAGGGCATCTGATGCCTAACTATGCGTTAGATGCACTAAGCACATAAT
TGCTCACAGCCAAACTATCAGGTCAAGTCTGCTT

>K17_ *aacC3-cmlA5*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCAGCAGC
AGCTAAGATGATCTCTACTCAAACCAAGATTACCCGCCTCAACTCTCAAGACGTT
GGTGTAATGCGGGCAATGCTAGGCATGTTCGGCGAGGCTTTTGAGGACGCTGAGA
ACTATTGCCGCGCTCAACCAAGCGACAGTTACCTACAAGACTTACTGTGTGGCTCT
GGCTTCATCGCAATCGCTGCGTTACAGGGGCAAGAGGTCATCGGTGGGCTCGCCG
CGTATGTGCTCCCAAAGTTTGAACAACAGCGCAAAGAAATCTATATCTACGACTT
AGGCGTGCAGGGAGCCTATCGCCGACGAGGCATCGCCACAGCCTTGATCAATGA
ACTCCAGCGTATCGCACATGATATTGGCGCTTATGTAATTTTTGTCCAGGCTGACT
ATGGGGACGATCCTGCGGTAGCGCTCTACACAAAACTCGGTATCCGGGAGGACGT
GATGCACTTTGACATAGAACCTCAACCTGCTGCCTAACAAGTCGTTCAAGCCGAC
GCTGCCTTCGTGGCAGCAACTGCGTGCATACGCTACGCTAGCACGCGTTGCCGCC
ACTACCGCAGCGCGGCTTAACTCAAGTGTTGGGCGCGCAATAAGGCTCCTCGCAG
AGTTGCTTGAAAGTTGTTACGATTCAAATTCAATCATGAGATAGTCAGCAGATGA
GCACTTCCAAGAACGCAGACAAGTAAGCCGCAGCAACCTTCATTTTTCGGTTGTT
GCGGCGTTCTCATGAATCCTTTTGCTCTACGGGAGCGCCGCCAAATCCTTTGTTCA
AGGAGATGGTTTCGTGCGCTCAAAAAACTTTAGTTGGCGGTACTCCCTTGCCGCC
ACGGTGTTGTTGTTATCACCGTTCGATTTATTGGCATCACTCGGCATGGACATGTA
CTTGCCAGCAGTGCCGTTTATGCCAAACGCGCTTGGTACGACAGCGAGCACAATT
CAGCTTACGCTGACAACGTACTTGGTCATGATTGGTGCCGGTCAGCTCTTGTTTGG
ACCGCTATCGGACCGACTGGGGCGCCGCCCCGTTCTACTGGGAGGTGGCCTCGCC
TACGTTGTGGCGTCAATGGGCCTCGCTCTTACGTCATCGGCTGAAGTCTTTCTGGG
GCTTCGGATTCTTCAGGCTTGTGGTGCCTCGGCGTGCCTTGTTTCCACATTTGCAA
CAGTACGTGACATTTACGCAGGTCGCGAGGAAAGTAATGTCATTTACGGCATACT
CGGATCCATGCTGGCCATGGTCCCGGCGGTAGGCCCATTGCTCGGAGCGCTCGTC
GACATGTGGCTTGGGTGGCGGGCTATCTTTGCGTTTCTAGGTTTGGGCATGATCGC
TGCATCTGCAGCAGCGTGGCGATTCTGGCCTGAAACCCGGGTGCAACGAGTTGCG
GGCTTGCAATGGTCGCAGCTGCTACTCCCCGTTAAGTGCCTGAACTTCTGGTTGTA
CACGTTGTGTTACGCCGCTGGAGTGGGTAGCTTCTTCGTCTTTTTCTCCATTGCGCC
CGGACTAATGATGGGCAGGCAAGGTGTGTCTCAGCTTGGCTTCAGCCTGCTGTTC
GCCACAGTGGCAATTGCCATGGTGTTTACGGCTCGTTTTATGGGGCGCGTGATACC

CAAGTGGGGCAGCCCAAGTGTCTTGCGAATGGGAATGGGATGCCTGATAGCTGGA
GCAGTATTGCTTGCCATCACCGAAATATGGGCTTCGCAGTCCGTGTTAGGCTTTAT
TGCTCCAATGTGGCTAGTGGGTATTGGTGTCGCCACAGCGGTATCTGTGTCGCCCA
ATGGCGCTCTTCGAGGATTCGACCATGTTGCTGGAACGGTCACGGCAGTCTACTTC
TGCTTGGGCGGTGTACTGCTAGGAAGCATCGGAACGTTGATCATTTCGCTGTTGCC
GCGCAACACGGCTTGGCCGGTTGTCGTGTACTGTTTGACCCTTGCAACAGTCGTGC
TCGGTCTGTCTTGTGTTTCCCGAGTGAAGGGCTCTCGCGGCCAGGGGGAGCATGA
TGTGGTCGCGCTACAAAGTGCGGAAAGTACATCAAATCCCAATCGTTGAGAGAAT
GTGGCAAGCTATCGCCCAACAAATCGCTGCAGCCGACCCAAAACCGCTACGCGGT
TTCGGTCGGCTGAGCTCAGGCGTTAGATGCACTAAGCACATAATTGCTCACAGCC
AAACTATCAGGTCAAGTCTGCTT

>H35_ *aadA2-linF*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGACATCATG
AGGGTAGCGGTGACCATCGAAATTTCGAACCAACTATCAGAGGTGCTAAGCGTCA
TTGAGCGCCATCTGGAATCAACGTTGCTGGCCGTGCATTTGTACGGCTCCGCAGTG
GATGGCGGCCTGAAGCCATACAGCGATATTGATTTGCTGGTTACGGTGACCGTAA
GGCTTGATGAAACAACGCGGCTAGCTTTGATCAACGACCTTTTGGAAACTTCGGC
TTCCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCAC
GACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAAT
GGCAGCGCAATGACATTCTTGCGGGTATCTTCGAGCCAGCCATGATCGACATTGA
TCTAGCTATCCTGCTTACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCG
GCAGCGGAGGAATTCTTTGACCCGGTTCCTGAACAGGATCTATTCGAGGCGCTGA
GGGAAACCTTGAAGCTATGGAACTCGCAGCCCGACTGGGCCGGCGATGAGCGAA
ATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAATAACCGGCAAAATCGC
GCCGAAGGATGTCGCTGCCGACTGGGCAATAAAACGCCTACCTGCCCAGTATCAG
CCCGTCTTACTTGAAGCTAAGCAAGCTTATCTGGGACAAAAAGAAGATCACTTGG
CCTCACGCGCAGATCACTTGGAAGAATTTATTCGCTTTGTGAAAGGCGAGATCAT
CAAGTCAGTTGGTAAATGATGTCTAACAATTCGTTCAAGCCGACCGCGCTACGCG
CGGCGGCTTAACTCCGGCGTTGTGCTGACGAAAAAAAACAAAAATCTGCGTATAC
CTTTCCTCATAATCTGTGGCTTCAATAAAAGGATATTTCTATGCTTCAACTGAAAA
TGATCGAACTCTTCAAGGAAGGTTGTCATGAGGATGCACGAATAATCGCGGCATT
GATGTTCGGCTCATTTGCTATCGGAGAGGGTGACGAGTTCTCTGATATCGAATTCG
CAGTGTTCATCCAGGATGACCATTTTGAAAATTTCGATCAGCGCTCGTGGCTTAAT
GCCGTAAGTCCGGTTGCTGCTTACTTTCCGGACGACTTCGGCCACCACACCGCACT
TTTTGAAAACGGCATTCGCGGTGAATTCCATTTCATGCGAAAATCGGACATACCG
GTCATTTCCACTTGGCAAGGCTATGGGTGGTTTCCCTCGCTTGAGGCGGCTGTTTT
GTTGGACCGATCAGGAGAGTTGTCGAGGTACGCAAGCGCTCTCGTGGGCGGTCCC
CCGATACGTGAAGGCGCGCCGCTGGTGGAAGGGCTTGTGTTGAACCTCATCAGCC
TGATGCTCTTTGGGGCCAATCTTTTAAATCGGGGAGAGTACGCTCGCGCCTGGGCT
TTGCTCAGCAAAGCACATGAAAACCTACTCAAGCTGGTTCGACTCCACGAAGGGG
CAACAGACCACTGGCCGACACCTTCACGCGCGCTCGAAAAGGATATCTCGGAGG
ACTCGTATAATCGCTATCTGGCATGCACAAGCAGTGCAGAACCAAGAGCACTATG
TGCAGCCTATCATCAAACGTGGACGTGGAGTCTCGAATTGTTCAAGAGCGTGACA
GAACCTCTGAATATCGAGCTTCCGAGAACTGTAATTGCGCAGGCAAAAAGGTTGC
TCAATGAGTCTGCGACGCCGCACAACAAGTAAATCCAGCGGACGCATAAAAACC
CGCCCGCTGATTTTGACGTTAAATGCACTAAGCACATAATTGCTCACAGCCAAAC
TATCAGGTCAAGTCTGCTT

>H44_ *dfrA12-orfF-aadA2*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGCCATATGA
ACTCGGAATCAGTACGCATTTATCTCGTTGCTGCGATGGGAGCCAATCGGGTTATT
GGCAATGGTCCTAATATCCCCTGGAAAATTCCGGGTGAGCAGAAGATTTTTCGCA
GACTCACTGAGGGAAAAGTCGTTGTCATGGGGCGAAAGACCTTTGAGTCTATCGG

CAAGCCTCTACCGAACCGTCACACATTGGTAATCTCACGCCAAGCTAACTACCGC
GCCACTGGCTGCGTAGTTGTTTCAACGCTGTCGCACGCTATCGCTTTGGCATCCGA
ACTCGGCAATGAACTCTACGTCGCGGGCGGAGCTGAGATATACACTCTGGCACTA
CCTCACGCCCACGGCGTGTTTCTATCTGAGGTACATCAAACCTTCGAGGGTGACG
CCTTCTTCCCAATGCTCAACGAAACAGAATTCGAGCTTGTCTCAACCGAAACCATT
CAAGCTGTAATTCCGTACACCCACTCCGTTTATGCGCGTCGAAACGGCTAACCATT
CCGTCAACGGGACGCCAAAATGCTGCGCATTTTGGTTCCCTCCGCTGCGCTCCGG
CTCTCGTTACGTCCAACGTTAGCGCCACTGAAACCCAGCTTTATTTAGCTCATGTT
TATTCAAACGGCATTTAGCTTTTCAGGCGTTATTCAGTGCCTGTTTTGCCTTTTTTC
CGGGCTTCGCCTGCATGGGCTGCGCAGGTTTTCAGTCTTTTTGGCCTCTAGCCCTT
GCGTAGCAAGCGCAGGCAGCTATCGTTTTTGCAGTGCTGTGCCGCCTCGGTGGCG
CAGCGTTTTTTCACGGTTAGCGCCCGTCGCCAAATTCAAGTTATCCGTTTTGGCTT
CTGGTTCTAACATTTTGGTCAAGCCGACCCGCATTCTGCGGTCGGCTTACCTCGCC
CGTTAGACATCATGAGGGAAGCGGTGACCATCGAAATTTCGAACCAACTATCAGA
GGTGCTAAGCGTCATTGAGCGCCATCTGGAATCAACGTTGCTGGCCGTGCATTTGT
ACGGCTCCGCAGTGGATGGCGGCCTGAAGCCATACAGCGATATTGATTTGTTGGT
TACTGTGGCCGTAAAGCTTGATGAAACGACGCGGCGAGCATTGCTCAATGATCTT
ATGGAGGCTTCGGCTTTCCCTGGCGAGAGCGAGACGCTCCGCGCTATAGAAGTCA
CCCTTGTCGTGCATGACGACATCATCCCGTGGCGTTATCCGGCTAAGCGCGAGCT
GCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCGGGTATCTTCGAGCCAGCC
ATGATCGACATTGATCTAGCTATCCTGCTTACAAAAGCAAGAGAACATAGCGTTG
CCTTGGTAGGTCCGGCAGCGGAGGAATTCTTTGACCCGGTTCCTGAACAGGATCT
ATTCGAGGCGCTGAGGGAAACCTTGAAGCTATGGAACTCGCAGCCCGACTGGGCC
GGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAATAA
CCGGCAAAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAATAAAACGCCTAC
CTGCCCAGTATCAGCCCGTCTTACTTGAAGCTAAGCAAGCTTATCTGGGACAAAA
AGAAGATCACTTGGCCTCACGCGCAGATCACTTGGAAGAATTTATTCGCTTTGTG
AAAGGCGAGATCATCAAGTCAGTTGGTAAATGATGTCTAACAATTCGTTCAAGCC
GACCGCGCTACGCGCGGCGGCTTAACTCCGGCGTTAGATGCACTAAGCACATAAT
TGCTCACNGCCAAACTATCAGGTCAAGTCTGCTT

>K7_ *aacA4-catB8-aadA1*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAA
AACAAAGTTAGGCATCACAAAGTACAGCATCGTGACCAACAGCAACGATTCCGTC
ACACTGCGCCTCATGACTGAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATC
GATCTCATATCGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGA
CGTACAGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATAC
ATTGCAATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTG
GAAGCGGGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAATA
GACCAGTCACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGAACCAAGCTG
GTTCGAGCTCTGGTTGAGTTGCTGTTCGATGATCCCGAGGTCACCAAGATCCAAA
CGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCTACGAGAAAGCGGGGT
TTGAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAGCCGTGTACATGGTTCA
AACACGCCAGGCATTCGAGCGAACACGCAGTGTTGCCTAACCCTTCCATCGAGGG
GGACGTCCAAGGGCTGGCGCCCTTGGCCGCCCCTCATGTCAAACGTTAGACGGCA
AGAAAAGGTTCCACGAACTCTGATGAAAAACTACTTTAACAGCCCTTTCAAAGGG
GAACTTCTTTCTGAGCAAGTGAAAAATCCAAATATCAGAGTAGGCCGGTATAGCT
ATTACTCTGGCTACTATCACGGGCACTCATTTGATGAATGCGCGCGATACTTGCTT
CCAGATCGTGATGACGTTGATAAATTGATCATTGGCAGCTTTTGTTCTATAGGAAG
CGGGGCTTCCTTCATCATGGCTGGCAATCAGGGGCATCGGCATGACTGGGCATCA
TCCTTCCCCTTCTTCTATATGCAAGAGGAGCCTGCTTTCTCAAGAGCACTCGACGC
CTTCCAAAGAGCAGGTGATACCGTCATTGGCAATGATGTCTGGATAGGCTCGGAG
GCAATGATTATGCCTGGCATCAAAATTGGAGACGGTGCCGTGATAGGTAGTCGCT
CGTTGGTGACAAAAGATGTAGAGCCTTATGCCATCATCGGGGGAAATCCCGCAAA
GCAAATTAAGAAGCGCTTCTCCGATGAGGAAATCTCATTGCTCATGGAGATGGAG
TGGTGGAACTGGCCACTAGATAAAATTAAGACAGCAATGCCTCTGCTGTGCTCGT
CAAATATTTTTGGTCTGCATAAGTATTGGCGCGAGTTTGCCGTCTAACAATTCATT

CAAGCCGACGCCGCTTCGCGGCACGGCTTAATTCTGGCGTTAAACATCATGAGGG
AAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGA
GCGCCATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAGTGGAT
GGCGGCCTGAAGCCACACAGTGATATTGATTTGCTGGTTACGGTGACCGTAAGGC
TTGATGAAACAACGCGGCGAGCTTTGATCAACGACCTTTTGGAAACTTCGGCTTC
CCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGAC
GACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGC
AGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCT
GGCTATCTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCG
GCGGAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATG
AAACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGT
AGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCG
AAGGATGTCGCTGCCGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCCCG
TCATACTTGAAGCTAGACAGGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCTC
CCGCGCAGATCAGTTGGAAGAATTTGTTCACTACGTGAAAGGCGAGATCACCAAG
GTAGTCGGCAAATAATGTCTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGC
GGCTTAACTCAAGCGTTAGATGCACTAAGCACATAATTGCTCACAGCCAAACTAT
CAGGTCAAGTCTGCTT

>H54_ *aacA4-catB3-dfrA1*
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCATCACA
AAGTACAGCATCGTGACCAACAGCAACGATTCCGTCACACTGCGCCTCATGACTG
AGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAGTG
GTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAACAGTACTT
GCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCAATGCTGAATGGA
GAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGGAAGCGGGGACGGATGGT
GGGAAGAAGAAACCGATCCAGGAGTACGCGGAATAGACCAGTCACTGGCGAATG
CATCACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTTCGAGCACTGGTTGAGTT
GCTGTTCAATGATCCCGAGGTCACCAAGATCCAAACGGACCCGTCGCCGAGCAAC
TTGCGAGCGATCCGATGTTACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTA
ACCACCCCAGATGGTCCAGCCGTGTACATGGTTCAAACACGCCAGGCATTCGAGC
GAACACGCAGTGATGCCTAACCCTTCCATCGAGGGGGACGTCCAAGGGCTGGCGC
CCTTGGTCGCCCCTCATGTCAAACGTTAGACGGCAAAGTCACAGACCGCGGGATC
TCTTATGACCAACTACTTTGATAGCCCCTTCAAAGGCAAGCTGCTTTCTGAGCAAG
TGAAGAACCCCAATATCAAAGTTGGGCGGTACAGCTATTACTCTGGCTACTATCA
TGGGCACTCATTCGATGACTGCGCACGGTATCTGTTTCCGGACCGTGATGACGTTG
ATAAGTTGATCATCGGTAGTTTCTGCTCTATCGGGAGTGGGGCTTCCTTTATCATG
GCTGGCAATCAGGGGCATCGGTACGACTGGGCATCATCTTTCCCGTTCTTTTATAT
GCAGGAAGAACCTGCATTCTCAAGCGCACTCGATGCCTTCCAAAAAGCAGGTAAT
ACTGTCATTGGCAATGACGTTTGGATCGGCTCTGAGGCAATGGTCATGCCCGGAA
TCAAGATCGGGCACGGTGCGGTGATAGGCAGCCGCTCGTTGGTGACAAAAGATGT
GGAGCCTTACGCTATCGTTGGCGGCAATCCCGCTAAGAAGATTAAGAAACGCTTC
ACCGATGAGGAAATTTCATTGCTTCTGGAGATGGAGTGGTGGAATTGGTCACTGG
AGAAGATCAAAGCGGCAATGCCCATGCTGTGCTCGTCTAATATTGTTGGCCTGCA
CAAGTATTGGCTCGAGTTTGCCGTCTAACAATTCAATCAAGCCGATGCCGCTTCGC
GGCACGGCTTATTTCAGGCGTTAACCTCTGAGGAAGAATTGTGAAACTATCACTA
ATGGTAGCTATATCGAAGAATGGAGTTATCGGGAATGGCCCTGATATTCCATGGA
GTGCCAAAGGTGAACAGCTCCTGTTTAAAGCTATTACCTATAACCAATGGCTGTT
GGTTGGACGCAAGACTTTTGAATCAATGGGAGCATTACCCAACCGAAAGTATGCG
GTCGTAACACGTTCAAGTTTTACATCTGACAATGAGAACGTAGTGATCTTTCCATC
AATTAAAGATGCTTTAACCAACCTAAAGAAAATAACGGATCATGTCATTGTTTCA
GGTGGTGGGGAGATATACAAAAGCCTGATCGATCAAGTAGATACACTACATATAT
CTACAATAGACATCGAGCCGGAAGGTGATGTTTACTTTCCTGAAATCCCCAGCAA
TTTTAGGCCAGTTTTTACCCAAGACTTCGCCTCTAACATAAATTATAGTTACCAAA
TCTGGCAAAAGGGTTAACAAGTGGCAGCAACGGATTCGCAAACCTGTCACGCCTT
TTGTACCAAAAGCCGCGCCAGGTTTGCGATCCGCTGTGCCTGGCGTTATGCAAAA

TGGTGAGTACAAAAAATTGACAACCGGCATTCAGTTTTTGAGAGAGGCCAAAAAA
CATGGTCTAGTTGATAACTTAGCCGATCGTGGTTTTAAACACTTGAAGGGGTTGTA
TTTTTATCGCTTTTATGGGTAAGGTATTCATCAATTAATCTTGGGTTCAGCTTCATA
CGGTGAAGACTTAACATTTGATATAACATGTATTTTTGATGAGATAGATTCACGTT
ATGTCCGTAATTTTCCCATTAATGTCCCACTCCTATGTGGTGGCGGGTTTGGTGAT
GAATATTTCACTCCAGAGTTATGGGAAATAGCGACATTGGACAATGTTGGTGAGA
TATCTCAGTCAATAGTTGATGTGTTCGATGGTTTTCCAAAAAAATGGTTTGATTCG
GTTAGCACAAGGCAAAAATTCGTTGATGAACTGTATCCTCATATTAGAGAAAAAT
ATGAAGAAAGCGGTGCTATTAAGAAAGTTTTATCGGGATGGCCAAAAAATGCATA
ACAAGTGGCTGTTGTCGGACGCACCTGCGCTGGCGCTTCAGTGCGCCGCAAAGCC
AAGCGTTAGATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGT
CTGCTT

**Class 2 integrons gene cassettes:**

>H3_ *dfrA1-sat1-aadA1*
CGGGATCCCGGACGGCATGCACGATTTGTAATAACAGAGTGTCTTGTATTTTTAA
AGAAAGTCTATTTAATACAAGTGATTATATTAATTAACGGTAAGCATCAGCGGGT
GACAAAACGAGCATGCTTACTAATAAAATGTTAACCTCTGAGGAAGAATTGTGAA
ACTATCACTAATGGTAGCTATATCGAAGAATGGAGTTATCGGGAATGGCCCTGAT
ATTCCATGGAGTGCCAAAGGTGAACAGCTCCTGTTTAAAGCTATTACCTATAACC
AATGGCTGTTGGTTGGACGCAAGACTTTTGAATCAATGGGAGCATTACCCAACCG
AAAGTATGCGGTCGTAACACGTTCAAGTTTTACATCTGACAATGAGAACGTATTG
ATCTTTCCATCAATTAAAGATGCTTTAACCAACCTAAAGAAAATAACGGATCATG
TCATTGTTTCAGGTGGTGGGGAGATATACAAAAGCCTGATCGATCAAGTAGATAC
ACTACATATATCTACAATAGACATCGAGCCGGAAGGTGATGTTTACTTTCCTGAA
ATCCCCAGCAATTTTAGGCCAGTTTTTACCCAAGACTTCGCCTCTAACATAAATTA
TAGTTACCAAATCTGGCAAAAGGGTTAACAAGTGGCAGCAACGGATTCGCAAACC
TGTCACGCCTTTTGTACCAAAAGCCGCGCCAGGTTTGCGATCCGCTGTGCCAGGC
GTTAGGCGTCATATGAAGATTTCGGTGATCCCTGAGCAGGTGGCGGAAACATTGG
ATGCTGAGAACCATTTCATTGTTCGTGAAGTGTTCGATGTGCACCTATCCGACCAA
GGCTTTGAACTATCTACCAGAAGTGTGAGCCCCTACCGGAAGGATTACATCTCGG
ATGATGACTCTGATGAAGACTCTGCTTGCTATGGCGCATTCATCGACCAAGAGCTT
GTCGGGAAGATTGAACTCAACTCAACATGGAACGATCTAGCCTCTATCGAACACA
TTGTTGTGTCGCACACGCACCGAGGCAAAGGAGTCGCGCACAGTCTCATCGAATT
TGCGAAAAAGTGGGCACTAAGCAGACAGCTCCTTGGCATACGATTAGAGACACA
AACGAACAATGTACCTGCCTGCAATTTGTACGCAAAATGTGGCTTTACTCTCGGC
GGCATTGACCTGTTCACGTATAAAACTAGACCTCAAGTCTCGAACGAAACAGCGA
TGTACTGGTACTGGTTCTCGGGAGCACAGGATGACGCCTAACAATTCATTCAAGC
CGACACCGCTTCGCGGCGCGGCTTAATTCAGGAGTTAAACATCATGAGGGAAGCG
GTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCGCC
ATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAGTGGATGGCGG
CCTGAAGCCACACAGTGATATTGATTTGCTGGTTACGGTGACCGTAAGGCTTGAT
GAAACAACGCGGCGAGCTTTGATCAACGACCTTTTGGAAACTTCGGCTTCCCCTG
GAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACAT
CATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGC
AATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTAT
CTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAG
GAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTT
AACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTT
ACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATG
TCGCTGCCGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACT
TGAAGCTAGGCAGGCTTATCTTGGACAAGAAGATCGCTTGGCCTCGCGCGCAGAT
CAGTTGGAAGAATTTGTTCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCA
AATAATGTCTAACAATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTC

AAGCGTTAGAGAGCTGGGGAAGACTATGCGCGATCTGTTGAAGGTGGTTCTAAGC
CTCGTACTTGCGATGGCATC

>H45_empty integron (150bp)
GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGC
AGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGATGCACTA
AGCACATAATTGCTCACAGCCAAACTATCAGGTCAAGTCTGCTT