DUPLEX PCR ASSAY FOR SIMULTANEOUS DETECTION OF TWO AMINOGLYCOSIDE RESISTANCE GENES IN CLINICAL SAMPLES OF *Enterobacteriaceae*.

By

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ABSTRACT

DUPLEX PCR ASSAY FOR SIMULTANEOUS DETECTION OF TWO AMINOGLYCOSIDE RESISTANCE GENES IN CLINICAL SAMPLES OF *Enterobacteriaceae*.

Theo Chun Hao

Aminoglycosides include semi-synthetic and natural antibiotics isolated from *Actinomycetes.* They are well-known for their efficacy against the *Enterobacteriaceae* family. Nevertheless, the mass prescription of aminoglycosides in clinical settings has resulted in a significant rise in the number of aminoglycoside-resistant *Enterobacteriaceae* due to the presence of different resistance genes that synthesise aminoglycoside-modifying enzymes. In this study, a total of 60 clinical isolates of *Enterobacteriaceae* obtained from different hospitals in West Malaysia were subjected to eight antibiotics. Subsequently, the bacterial isolates were screened for the presence of *ant(2'')-Ia* and *aph(3')-Ic* genes simultaneously using duplex PCR. Twenty-three (38.33%) isolates of the 60 bacterial isolates were tested positive for *ant(2'')-Ia,* 13 (21.67%) were tested positive for *aph(3')-Ic* and only one (1.67%) bacterial isolate was tested positive for both *ant(2'')-Ic* and *aph(3')-Ic* genes. The prevalence of *ant(2'')-Ia* and *aph(3')-Ic* in *Enterobacteriaceae* was 40.00% and 23.33%, respectively. The

ant(2'')-Ia gene was more prevalent in *Enterobacteriaceae* as compared to the *aph(3')-Ic* gene. Besides, *E. coli* and *K. pneumoniae* were found to host at least one aminoglycoside resistance gene. Statistical analysis determined that there were significant associations between the *ant(2'')-Ia* gene with gentamicin, kanamycin, and imipenem resistance, and the *aph(3')-Ic* gene with kanamycin resistance. However, the association between *ant(2'')-Ia* gene with imipenem resistance may be caused by coincidence, in which the bacterial isolates hosting the *ant(2'')-Ia* gene were carrying other imipenem resistance genes concurrently. This is because aminoglycoside resistance genes were proven to confer cross-resistance only within aminoglycosides, instead of other classes of antibiotics. Nonetheless, the age and gender of the patients have no statistically significant association with the resistance genes studied.

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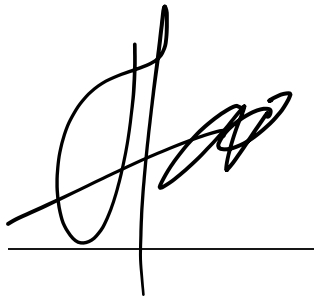
Last but not least, I would like to thank my family members who have been giving me unconditioned love and support in this journey.

DECLARATION

I hereby declare that the project report 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.



THEO CHUN HAO

APPROVAL SHEET

This project report entitled "**DUPLEX PCR ASSAY FOR SIMULTANEOUS DETECTION OF TWO AMINOGLYCOSIDE RESISTANCE GENES IN CLINICAL SAMPLES OF *Enterobacteriaceae*"** was prepared by **THEO CHUN HAO** and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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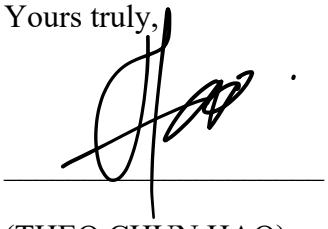
PERMISSION SHEET

It is hereby certified that **THEO CHUN HAO** (ID No: **18ADB05477**) has completed this final year project/ dissertation/ thesis* entitled "**DUPLEX PCR ASSAY FOR SIMULTANEOUS DETECTION OF TWO AMINOGLYCOSIDE RESISTANCE GENES IN CLINICAL SAMPLES OF *Enterobacteriaceae***" under the supervision of Dr Chew Choy Hoong from the Department of Allied Health Sciences, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,



(THEO CHUN HAO)

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LIST OF ABBREVIATIONS

A_{230}	Absorbance at 230 nm
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
bp	Base pair
CIP	Ciprofloxacin
CRO	Ceftriaxone
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ETP	Ertapenem
F	Female
GM	Gentamicin
h	Hour / Hours
I	Intermediately resistant
IMI	Imipenem
K	Kanamycin
M	Male
$MgCl_2$	Magnesium chloride
min	Minutes
mM	Millimolar
MRP	Meropenem
ng	Nanogram

nm	Nanometre
PCR	Polymerase chain reaction
R	Resistant
RNA	Ribonucleic acid
rpm	Revolution per minute
S	Streptomycin
S	Susceptible
Taq	*Thermus aquaticus*
TBE	Tris, boric acid, EDTA
u	Unit
V	Volts
w/v	Weight to volume
µL	Microlitre
µM	Micromolar

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CHAPTER 1

INTRODUCTION

A microorganism is considered resistant when it can survive and continue to reproduce even in the presence of antimicrobial agents. In 1907, the father of modern chemotherapy, Paul Ehrlich, discovered that organisms in trypanosome infections were resistant to the agent prescribed in some cases. Owing to a certain resistance, Ehrlich found out that arsenic compound was effective against fuchsin dye-resistant strain, whereas arsenic-compound-resistant strain preserved its sensitivity to the dye. In 1908, Ehrlich proposed that resistance to antimicrobial agents could possibly be inherited (Naeemmudeen et al., 2021). According to the data from The World Bank (2021), approximately 700,000 deaths worldwide were caused by antimicrobial resistance. Without the implementation of a well defensive approach, it is predicted that the death toll recorded annually may be as high as 10 million by 2050.

Antimicrobial agents, including antibiotics, are considered a cheap therapy to treat various infections caused by bacteria. However, the mass prescription of antibiotics has led to the emergence of antibiotic resistance pathogens due to natural selection. Those pathogens have a high potential to develop into almost indestructible superbugs. As a result, many first-world countries, such as the United States, the United Kingdom, and et cetera, are changing their antimicrobial drugs into ones

that are more expensive with higher potency to overcome the antibiotic resistance pathogens. Nonetheless, second and third world countries are looking for cheaper substitutes due to their financial constraints, thus, leading to higher rates of mortality and morbidity caused by the low potency of alternative drugs (Naeemmudeen et al., 2021).

Aminoglycoside antibiotics are classified as antimicrobials due to their ability to alter the integrity of bacterial cell membranes and/or impede protein synthesis while causing little to no harm to the host (van Hoek et al., 2011). The use of aminoglycosides in treating bacterial infections can be traced back to 50 years ago when they were discovered from *Streptomyces* (Davey et al., 2015). Their primary antibacterial capability lies in their affinity for the 16S rRNA of the 30S ribosomal subunits, causing interference in protein biosynthesis (Chen et al., 2008; Chen et al., 2009). In the 1960s, the first batch of bacterial strains resistant to aminoglycosides were detected. The bacterial strains utilised the mechanisms of R-plasmids, transposons, and integrons dissemination, which led to a surge in resistance to aminoglycosides (Umezawa et al., 1967; Doi et al., 1968). In the 1970s, the first semi-synthetic aminoglycosides, including amikacin, dibekacin, isepamicin, and netilmicin, were formulated with the aim of conquering the first wave of aminoglycoside resistance (Miller et al., 1995). The newly synthesised aminoglycosides were not efficient at eliminating all the resistant bacterial strains. Then, the potency of aminoglycosides in clinical settings was once again established following the discovery of gentamicin, which constitutes another

subfamily of aminoglycosides isolated from *Micromonospora* (Weinstein et al., 1963). Gentamicin is very efficient in targeting *Pseudomonas aeruginosa,* which has very high resistance to the old aminoglycosides. Unfortunately, ANT(2'') enzymes have caused resistance to gentamicin (Benveniste and Davies, 1971; Martin et al., 1971). After that, another aminoglycoside known as butirosine was discovered that is sufficiently competent to prevent the inactivation by ANT(2'') and APH(3') enzymes (Woo et al., 1971).

The efficacy of aminoglycosides towards Gram-negative bacteria is higher and does not require a combination of other antibiotics to achieve synergistic effects. Conversely, a combination of aminoglycosides and other antibiotics that facilitate the entry of aminoglycosides into the cytosol of the target hosts is recommended in the case of Gram-positive bacteria due to the presence of a thick layer of peptidoglycan in their outer plasma membrane. Besides, owing to the mechanism of intake of aminoglycoside antibiotics, which requisites respiration, anaerobic bacteria are said to have intrinsic resistance toward aminoglycosides (Ramirez and Tolmasky, 2010).

Since aminoglycoside antibiotics are the first-line antibiotics used to treat infections caused by *Enterobacteriaceae* in many clinical settings, it is important to discover the current resistance trends and prevalence of the aminoglycoside-resistant genes amongst the clinically isolated *Enterobacteriaceae* in Malaysia.

Therefore, the main objectives of this study were as follows:

1. To screen for the presence of *ant(2'')-Ia* and *aph(3')-Ic* genes simultaneously using duplex PCR amongst the 60 clinical isolates of *Enterobacteriaceae* obtained from different hospitals in West Malaysia.
2. To determine the prevalence of *ant(2'')-Ia* and *aph(3')-Ic* genes.
3. To determine the associations between the resistance genes and the antibiotic resistance phenotype, patients' age, and gender.

CHAPTER 2

LITERATURE REVIEW

2.1 Aminoglycoside Antibiotics

2.1.1 Chemical Structures of Aminoglycosides

The distinctive feature of aminoglycosides is the presence of amino sugars core linked to a dibasic aminocyclitol by glycosidic linkages. The commonly seen dibasic aminocyclitol connected to the amino sugars core is known as 2-deoxystreptamine (Krause et al., 2016). The general four sub-groups of aminoglycosides are based on the identification of the aminocyclitol moiety, as shown in Table 2.1.

Table 2.1: Classification of Aminoglycoside Antibiotics.

Sub-group	Aminocyclitol Moiety	Examples
1	Without deoxystreptamine	Streptomycin
2	Mono-substituted deoxystreptamine ring	Apramycin
3	4,5-di-substituted deoxystreptamine ring	Neomycin Ribostamycin
4	4,6-di-substituted deoxystreptamine ring	Gentamicin Amikacin Tobramycin Plazomicin

Adapted from (Krause et al., 2016).

2.1.2 Antimicrobial Mechanism of Aminoglycosides

The primary antimicrobial mechanisms of aminoglycosides are inhibition and/or alteration of protein synthesis by the bacteria. They have a high affinity for the A-site region of the 16S ribosomal RNA of the 30S ribosome. Different aminoglycoside members have different degrees of explicitness towards the different regions of the A-site; nonetheless, they eventually lead to changes in the conformation of the ribosome. The binding of aminoglycosides to the A-site encourages codon misreading, resulting in a high probability of mistranslation and error-prone protein synthesis. Besides, some members of the aminoglycoside family act by directly impeding the initiation and elongation of the translation process (Kotra et al., 2000). A study by Mehta and Champney (2003) proved that neomycin and paromomycin are capable of inhibiting the assembly of the 30S ribosomal subunit. However, this could be due to the secondary consequence of protein mistranslation. Besides, neomycin B is also capable of impeding the functions of RNase P by disrupting the binding between RNA moiety of RNase P and divalent metal ions (Mikkelsen et al., 1999).

2.1.3 Entry of Aminoglycosides

The entry of aminoglycosides into the cytosol of the bacteria consists of three well-defined steps. The first step is energy-independent, whereas the second and third steps are energy-dependent. In the first stage, the polycationic aminoglycoside molecules are attracted to the negatively charged components of the bacterial cell membrane, such as phospholipids and lipopolysaccharides. This is immediately followed by the displacement of magnesium ions, which are extremely crucial in maintaining the bacterial membrane's integrity (cross bridging and stabilization). As a result, aminoglycoside molecules introduce disruption to the outer membrane and increase the permeability of their uptake into the cytosol. This process is known as "self-promote uptake" (Davis, 1987; Taber et al., 1987).

The second stage is recognised as "energy-dependent phase I" where the aminoglycoside molecules are taken into the bacterial cytosol through the electron transport system with the supplementation of energy from ATP. Anaerobic bacteria do not have an electron transport chain for cellular respiration; thus, they are less or not susceptible to aminoglycosides (Nichols and Young, 1985).

In the third stage, or so-called "energy-dependent phase II," aminoglycoside molecules that have gained access to the cytosol of the bacteria bind to the A-site of the 16S ribosomal RNA and promote errors in protein synthesis, forming non-functional membrane protein molecules. The defected membrane protein molecules inserted into the membrane further decrease the stability of the membrane,

increasing the permeability that promotes the uptake of aminoglycoside molecules. This self-promoting uptake of aminoglycoside molecules is also known as the autocatalytic accelerated rate of uptake that essentially causes bacterial cell death (Hurwitz et al., 1981).

2.2 Toxicities of Aminoglycosides

Aminoglycosides are prescribed to cystic fibrosis patients with critical and recurring pulmonary infections. Nevertheless, despite their high efficacy and potency, aminoglycosides are usually the last resort for Gram-negative bacterial infections in certain situations due to their high prevalence of toxic side effects. The most frequently encountered side effects of aminoglycosides are ototoxicity and nephrotoxicity (Rougier et al., 2004; Selimoglu, 2007).

Under the classification of ototoxicity, aminoglycosides induce damage to the cochlear hair cells and cranial nerve branch controlling hearing, rendering in cochlear toxicity. A study by Jospe-Kaufman et al. (2020) focusing on the destructive effects of geneticin, also known as G-418, on mice has proven the irreversibility of ototoxicity caused by the death of cochlear hair cells. Moreover, the high endocochlear potential of the cochlear hair cells in combination with non-selective transmembrane channels that favour the influx of positively charged ions has facilitated the accumulation of positively charged aminoglycosides within the cochlear hair cells. Other than that, the vestibular cranial nerve branch that manages balance may also be harmed by aminoglycosides, leading to vestibular toxicity.

In the case of nephrotoxicity, aminoglycosides are capable of inducing epithelial cell necrosis that results in tubular necrosis. The high prevalence of nephrotoxicity caused by aminoglycosides could be explained by excessive accumulation in the proximal tubular cells within the kidneys. This is due to the stability of the aminoglycosides, which are resilient towards metabolism, causing the intact antibiotic to collect in a high concentration in the proximal tubular cells, and eventually contribute to acute and/or chronic kidney disease. Nevertheless, nephrotoxicity is reversible most of the time due to the regenerative capability of the proximal tubular cells (Howard et al., 1996).

Numerous research conducted before has concluded that the structural differences amongst the members of aminoglycoside antibiotics have divergent toxicity profiles. As a consequence, chemical modification of the structures of aminoglycosides could be the approach to lower the toxicity without sacrificing their antibacterial potency (Jospe-Kaufman et al., 2020). Another strategy to reduce the toxicity without diminishing the efficacy of aminoglycoside antibiotics is by prescribing higher doses with lower frequency (Krause et al., 2016). Once-daily dosing of aminoglycoside antibiotics has produced superior outcomes and has become the standard dosing schedule to tackle multidrug-resistant bacterial infections in some healthcare facilities in Australia (Avent et al., 2011). Other than that, the systemic toxicity of aminoglycosides could be reduced by administering them through inhalation to treat respiratory infections while providing greater exposure to the lungs (Krause et al., 2016; Jospe-Kaufman et al., 2020).

2.3 Aminoglycoside Modifying Enzymes

2.3.1 Overview

Aminoglycoside modifying enzymes are often coded by genes located in plasmids, which are hypothesised that horizontal gene transfer from *Actinomycetes* to other species of bacteria is the main mechanism that confers resistance to aminoglycoside antibiotics. Aminoglycoside modifying enzymes are classified into three classes according to the mechanism of acetylation, phosphorylation, or adenylation of amino or hydroxyl groups around the aminoglycoside core. The three groups of aminoglycoside-modifying enzymes are aminoglycoside N-acetyltransferases (AACs), aminoglycoside O-nucleotidyltransferases (ANTs) and aminoglycoside O-Phosphotransferases (APHs). Each group contains sub-members based on the position of their target within the aminoglycoside molecules (Shaw et al., 1993; Ramirez and Tolmasky, 2010).

2.3.2 Nomenclatures of Aminoglycoside Modifying Enzymes

The current naming system of the aminoglycoside modifying enzymes is based on two systems. According to the first system, the name contains a three-letter identifier for the general activity, the site of modification enclosed in parenthesis (class), and eventually a roman number representing the subclass of the enzyme. If there are several enzymes conferring the same resistance within the same subclass, a lower-case letter is added to the end of the name as an individual identifier. For instance, ANT(2’’)-I denotes an aminoglycoside O-nucleotidyltransferase targeting the 2’’ position of the aminoglycoside core scaffold under subclass I. Instead of

naming the enzymes, the second nomenclature system denotes the genes that code for the aminoglycoside modifying enzymes. The genes are either designated *aac, aad,* or *aph* with a capital letter at the end that signifies the site of the target. For example, *aadB* indicates aminoglycoside 2''-O-nucleotidyltransferase. In some cases, a number is added to distinguish different genes, such as *aacA1* indicates the *aac(6')-Ia* gene that codes for AAC(6')-I (Ramirez and Tolmasky, 2010).

2.3.3 ANT(2'')-I Enzyme

Aminoglycoside O-nucleotidyltransferases (ANTs) are the smallest group of aminoglycoside-modifying enzymes. They work by bonding an AMP from an ATP donor to hydroxyl groups at 2'', 3'', 4', 6, and 9 positions (Krause et al., 2016).

A study by Kotra et al. (2000) has shown that ANT(2'') and ANT(4') are two of the most clinically relevant ANTs. Based on a study by Ramirez and Tolmasky (2010), ANT(2'')-Ia is the sole member under the subclass of ANT(2'')-I which can be easily found within class 1 and class 2 integrons as a gene cassette. ANT(2'')-Ia confers resistance to gentamicin, tobramycin, dibekacin, sisomicin, and kanamycin. Gene encoding ANT(2'')-Ia can be detected in plasmids and transposons in most cases.

2.3.4 APH(3')-I Enzyme

Aminoglycoside O-Phosphotransferases (APHs) are the largest group of aminoglycoside-modifying enzymes after AACs. They function similar to kinases by catalysing the ATP-dependent phosphorylation of hydroxyl groups (Krause et al., 2016). According to Wright (1999), APHs, serine-threonine, and tyrosine kinases discovered in eukaryotes are alike in terms of functions and structures. All APHs confer resistance to kanamycin and neomycin, with some of the members conferring resistance to other aminoglycosides like gentamicin B and amikacin (Shaw et al. 1993).

Under the subclass of APH(3')-I, there are three enzymes members that are primarily hosted by Gram-negative bacteria, *aph(3')-Ia, aph(3')-Ib,* and *aph(3')-Ic*. The *aph(3')-Ic* gene has a high similarity to the *aph(3')-Ia* gene as they differ only in seven nucleotide substitutions. The genes coding for these enzymes are usually found in plasmids and transposons (Vakulenko and Mobashery, 2003).

CHAPTER 3

MATERIALS AND METHODS

Table 3.1: Chemicals and Reagents Used.

Chemical and reagents	Manufacturer, country
Tryptic soy agar	HiMedia, India
Mueller-Hinton agar	HiMedia, India
Nutrient agar	HiMedia, India
Kanamycin	Thermo Fisher Scientific, England
Gentamicin	Becton, Dickinson and Company, United States of America
Imipenem	Liofilchem, Italy
Meropenem	Liofilchem, Italy
Ertapenem	Liofilchem, Italy
Streptomycin	ThermoFisher, England
Ciprofloxacin	HiMedia, India
Ceftriaxone	Liofilchem, Italy
PCR buffer with loading dye	Promega, United States
GoTaq G2 Flexi DNA Polymerase	Promega, United States
dNTP mix	Promega, United States
Primers	Integrated DNA Technologies, United States
Gel Red (FluoroSafe DNA Stain)	1st Base Laboratories, Singapore
Agarose powder	1st Base Laboratories, Singapore
100 bp DNA ladder	Smobio, Taiwan

Table 3.1 continued: Chemical and Reagents Used.

Chemical and reagents	Manufacturer, country
Tris base	Thermo Fisher Scientific, United States
Boric acid	MERCK, Germany
EDTA disodium salt	Grupo RNM, Portugal

3.1 Bacterial Collection

Multidrug-resistant bacterial isolates were collected from outpatients and inpatients in Hospital Raja Permaisuri Bainun (Ipoh), Fatimah Hospital (Ipoh), Hospital Pantai (Ipoh), Hospital Pantai (Penang), and Island Hospital (Penang). Relevant information from the patients including the gender, age, and the source of bacterial isolate was recorded. For this study, 60 bacterial isolates of four species, including four *Klebsiella aerogenes*, 11 *Enterobacter cloacae*, 19 *Klebsiella pneumoniae,* and 26 *Escherichia coli*, were obtained.

3.2 Bacterial Culture

The bacterial isolates were revived from glycerol by streaking onto tryptic soy agar plates and were incubated at 37 °C overnight. A broth culture of the bacterial isolate was prepared by inoculating one colony of each isolate from their plate culture into a nutrient broth, followed by incubation in a shaking incubator at 200 to 250 rpm for 16 to 24 h at 37 °C.

3.3 Antimicrobial Susceptibility Testing using Kirby-Bauer Method

A pure colony of the bacterial isolates was picked using an inoculation loop and inserted into 0.9% saline to a achieve similar turbidity as the 0.5% McFarland standard. The bacterial suspension was spread evenly onto a Mueller-Hinton agar plate using a cotton swab according to the guidelines of the Clinical Laboratory Standards Institute (CLSI). Each bacterial isolate was subjected to eight antibiotics, including kanamycin, gentamicin, streptomycin, imipenem, meropenem, ertapenem, ciprofloxacin, and ceftriaxone. The bacterial isolates were incubated at 37°C for 16 to 18 h. The resistance phenotype of the bacterial isolates was classified based on the interpretive categories and zone diameter breakpoints provided by CLSI (Cockerill, 2020).

3.4 DNA Extraction (Fast Boil Method)

One and a half millilitres of the bacterial broth culture were aliquoted into a microcentrifuge tube and centrifuged for 5 min at 12000 rpm. The supernatant was discarded, and the pellet was resuspended in 300 µL of sterile distilled water. The suspension was heated to 100 °C for 5 min and immediately placed on ice for 2 min. The suspension was again centrifuged at 12000 rpm for 2 min and the supernatant containing the bacterial DNA was aliquoted into a new microcentrifuge tube to be stored at -20 °C (Kor et al., 2013). The concentration and purity of the DNA extracted were measured using a Nanodrop 100 spectrophotometer (IMPLEN NanoPhotometer ™)

3.5 Optimisation of Duplex PCR

Optimisation of the duplex PCR was conducted to determine the best cycling conditions that ensure the successful annealing of the primers and to minimise the occurrence of unspecific bandings. Gradient PCR was carried out to determine the optimum annealing temperature for both sets of primers used.

3.6 Duplex PCR

The primers used for the detection of the two aminoglycoside modifying enzyme genes are listed in Table 3.2. A duplex PCR assay was utilised to screen for the presence of *ant(2'')-Ia* and *aph(3')-Ic* genes amongst the bacterial isolates. Table 3.3 and 3.4 shows the cycling conditions and components used in the duplex PCR respectively.

Table 3.2: Primer Sequences of the Targeted Genes.

Target Gene	Primer Sequence (5'→3')	Reference
ant(2'')-Ia	F: ATC TGC CGC TCT GGA T R: CGA GCC TGT AGG ACT	(Jouybari et al., 2021)
aph(3')-Ic	F: CGA GCA TCA AAT GAA ACT GC R: GCG TTG CCA ATG ATG TTA CAG	(Navas et al., 2016)

Table 3.3: Cycling Conditions of Duplex PCR.

Steps	Temperature (°C)	Duration (s)	No. of cycle(s)
Initial denaturation	95	180	1
Denaturation	95	30	
Annealing	57.5	30	30
Extension	72	30	
Final extension	72	600	1
Hold	4	∞	-

Table 3.4: Components Used in a Duplex PCR reaction.

Components	**Volume (μL)**	**Final Concentration**
5X Green GoTaq Flexi buffer	5	1X
$MgCl_2$ solution	2	2.0 mM
dNTP mix	1	0.2 mM
ant(2'')-Ia-F	2.5	1.0 μM
ant(2'')-Ia-R	2.5	1.0 μM
aph(3')-Ic-F	2	0.8 μM
aph(3')-Ic-R	2	0.8 μM
GoTaq G2 Flexi DNA Polymerase (5u/μL)	0.3	1.5 u
DNA template	1	100 ng/μL
Nuclease-Free water	0.7	-
Total volume	25	

3.7 Agarose Gel Electrophoresis

A 1.5% (w/v) agarose gel pre-stained with Gel Red was used to electrophorese the PCR products at 90 V for approximately 40 min. 1X TBE buffer was used in the gel electrophoresis. Five microlitres of 100 bp DNA ladder and 5 μL of PCR products were loaded into the well individually. The gel was then viewed under a gel imager by Bio-Rad Laboratories.

3.8 Statistical Analysis

The software IBM SPSS Statistics 22 was used to analyse the association between the aminoglycoside resistance genes with the resistance phenotype, patients' age, and gender using the Chi-Square Test of Independence. A *p*-value of less than 0.05 was considered statistically significant.

CHAPTER 4

RESULTS

4.1 Overview

A total of 60 bacterial isolates of *Enterobacteriaceae* obtained from clinical settings were revived. Amongst the 60 bacterial isolates, there were four *Klebsiella aerogenes,* eleven *Enterobacter cloacae*, nineteen *Klebsiella pneumoniae*, and twenty-six *Escherichia coli*. The relevant information for each bacterial isolate is listed in Appendix A.

4.2 Antimicrobial Susceptibility Testing

All the bacterial isolates were subjected to eight antibiotics, including kanamycin, gentamicin, streptomycin, imipenem, meropenem, ertapenem, ciprofloxacin, and ceftriaxone. The number and percentage of resistance according to antibiotics, followed by their raw resistance phenotypes, are outlined in Tables 4.1 and 4.2 respectively.

Table 4.1: Number and Percentage of Resistance According to Antibiotics.

Antibiotics	Resistance (n %)	Intermediate (n %)	Susceptible (n %)
Kanamycin	43 (71.67%)	0(0.00%)	17 (28.33%)
Gentamicin	39 (65.00%)	9 (15.00%)	12 (20.00%)
Streptomycin	40 (66.67%)	12 (20.00%)	8 (13.33%)
Imipenem	21 (35.00%)	23 (38.33%)	16 (26.67%)
Meropenem	4 (6.67%)	5 (8.33%)	51 (85.00%)
Ertapenem	7 (11.67%)	12 (20.00%)	41 (68.33%)
Ciprofloxacin	33 (55.00%)	18 (30.00%)	9 (15.00%)
Ceftriaxone	48 (80.00%)	2 (3.33%)	10 (16.67%)

*Total number of bacterial isolates = 60.

Table 4.2: Resistance Phenotypes of The Bacterial Isolates.

Isolate	Species	K	GM	S	IMI	MRP	ETP	CIP	CRO
A7	*Escherichia coli*	R	R	R	R	S	S	R	R
G10	*Klebsiella aerogenes*	S	S	I	I	S	S	I	S
G12	*Enterobacter cloacae*	S	S	S	S	S	I	S	R
G13	*Klebsiella pneumoniae*	S	I	R	S	S	R	S	R
G21	*Enterobacter cloacae*	R	R	R	S	S	I	R	R
G23	*Klebsiella aerogenes*	S	S	I	I	S	I	I	R
G24	*Enterobacter cloacae*	R	R	R	R	S	R	S	R
G31	*Enterobacter cloacae*	R	R	R	I	S	S	I	S
G42	*Enterobacter cloacae*	R	R	R	R	S	R	I	R
G50	*Enterobacter cloacae*	R	R	I	R	S	S	S	R
G65	*Enterobacter cloacae*	R	R	I	R	S	S	I	S
G66	*Klebsiella aerogenes*	S	I	I	I	S	S	I	I
G67	*Klebsiella aerogenes*	S	S	I	I	S	S	I	S
G68	*Enterobacter cloacae*	R	R	I	R	I	R	I	R
G69	*Enterobacter cloacae*	R	I	I	R	S	I	R	R
G7	*Escherichia coli*	R	R	R	R	S	S	R	R
H12	*Escherichia coli*	R	R	R	I	S	S	R	R
H14	*Klebsiella pneumoniae*	R	R	S	S	S	S	S	R
H21	*Escherichia coli*	R	R	R	R	S	S	R	R
H28	*Klebsiella pneumoniae*	R	R	R	R	R	R	R	R
H3	*Escherichia coli*	R	R	R	R	R	I	I	R
H31	*Klebsiella pneumoniae*	R	S	I	R	S	S	S	I

Table 4.2 continued: Resistance Phenotype of the Bacterial Isolates.

Isolate	Species	K	GM	S	IMI	MRP	ETP	CIP	CRO
H32	*Klebsiella pneumoniae*	S	S	S	I	S	S	R	R
H33	*Escherichia coli*	R	R	S	R	S	S	S	R
H34	*Klebsiella pneumoniae*	R	R	R	S	S	R	R	R
H35	*Escherichia coli*	R	R	R	I	S	S	R	R
H36	*Escherichia coli*	R	R	R	R	S	I	R	R
H37	*Escherichia coli*	R	R	I	R	R	S	R	R
H38	*Klebsiella pneumoniae*	S	S	R	S	S	I	R	R
H4	*Klebsiella pneumoniae*	R	R	R	R	S	S	I	R
H41	*Escherichia coli*	S	I	R	R	S	S	I	R
H42	*Escherichia coli*	R	R	R	I	I	I	I	R
H43	*Klebsiella pneumoniae*	R	R	R	S	S	S	R	R
H47	*Escherichia coli*	R	R	R	R	I	S	R	R
H48	*Escherichia coli*	R	R	R	I	S	I	R	R
H5	*Enterobacter cloacae*	R	R	R	I	R	S	R	R
H50	*Escherichia coli*	R	S	R	R	S	I	R	R
H52	*Escherichia coli*	R	R	R	S	S	S	R	R
H54	*Escherichia coli*	R	R	R	I	S	S	R	R
H55	*Klebsiella pneumoniae*	R	R	R	S	S	S	R	R
H58	*Klebsiella pneumoniae*	R	R	S	S	S	R	R	R
H59	*Escherichia coli*	R	S	R	S	S	S	S	R
H6	*Klebsiella pneumoniae*	R	S	R	I	S	I	R	R
H62	*Klebsiella pneumoniae*	R	I	I	I	S	S	I	R

Table 4.2 continued: Resistance Phenotype of the Bacterial Isolates.

Isolate	Species	K	GM	S	IMI	MRP	ETP	CIP	CRO
H63	*Klebsiella pneumoniae*	S	R	R	S	S	S	I	R
H65	*Klebsiella pneumoniae*	S	I	R	R	I	S	R	R
H66	*Klebsiella pneumoniae*	R	R	R	I	S	S	I	R
H67	*Klebsiella pneumoniae*	R	S	R	R	S	S	R	S
H72	*Escherichia coli*	R	R	R	I	S	S	R	R
H8	*Escherichia coli*	R	R	R	S	S	S	R	R
H9	*Escherichia coli*	R	R	R	I	S	S	R	R
K3	*Escherichia coli*	R	R	R	I	S	S	R	S
P1	*Klebsiella pneumoniae*	S	R	I	I	S	I	R	R
P11	*Escherichia coli*	S	I	R	I	S	S	I	S
P12	*Enterobacter cloacae*	S	S	S	S	S	S	I	R
P14	*Escherichia coli*	S	R	R	S	S	S	R	R
P3	*Escherichia coli*	R	R	S	I	S	S	R	S
P4	*Escherichia coli*	R	R	R	I	I	S	R	R
P6	*Klebsiella pneumoniae*	S	I	S	S	S	S	S	S
P8	*Escherichia coli*	S	I	R	I	S	S	I	S

*R denotes resistant; I denotes intermediate; S denotes susceptible.

K: Kanamycin; GM: Gentamicin; S: Streptomycin; IMI: Imipenem; MRP: Meropenem; ETP: Ertapenem; CIP: Ciprofloxacin; CRO: Ceftriaxone

Representative images of the antimicrobial susceptibility testing using the Kirby-Bauer method are displayed in Figure 4.1. The diameter of the zone of inhibition was measured for each antibiotic disc and categorised into resistance, intermediate, or susceptible according to the interpretive categories and zone diameter breakpoints provided by CLSI (Cockerill, 2020). For instance, sample H72 was resistant to kanamycin, gentamicin, streptomycin, ciprofloxacin, ceftriaxone, intermediately resistant to imipenem, and susceptible to meropenem and ertapenem.

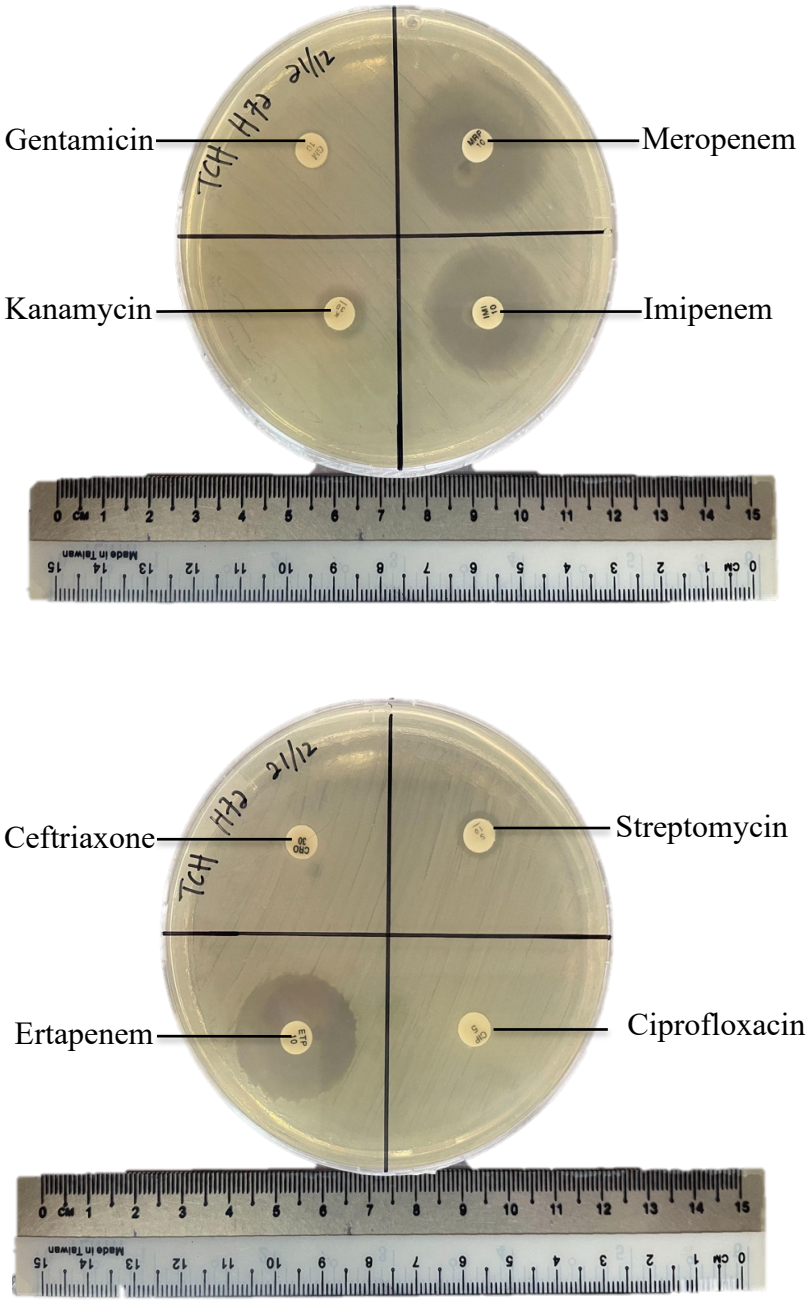

Figure 4.1: Representative Images of Antimicrobial Susceptibility Testing on the Bacterial Isolates.

K: Kanamycin; GM: Gentamicin; S: Streptomycin; IMI: Imipenem; MRP: Meropenem; ETP: Ertapenem; CIP: Ciprofloxacin; CRO: Ceftriaxone

4.3 Optimisation of The Duplex PCR

Figure 4.2 shows the gel image of the gradient PCR conducted with the annealing temperature ranging from 50 °C to 70 °C. Based on the observation of the gel image, an annealing temperature of 57.5 °C was found to be the optimum annealing temperature for the duplex PCR.

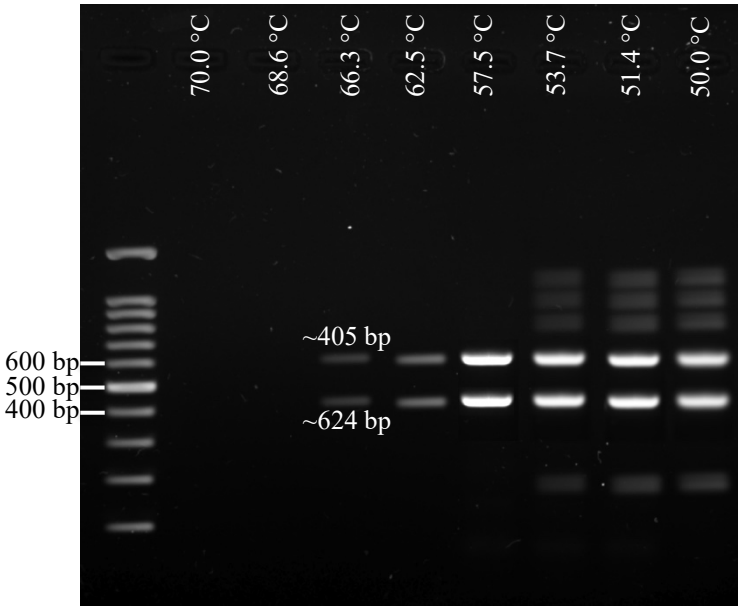

Figure 4.2: Gel Image of Gradient PCR.

The targeted amplicon size for *ant(2'')-Ia* and *aph(3')-Ic* genes was approximately 405 bp and 624 bp, respectively.

4.4 Duplex PCR for The Simultaneous Detection of *ant(2'')-Ia* and *aph(3')-Ic* Genes

All 60 bacterial isolates were subjected to duplex PCR for simultaneous detection of *ant(2'')-Ia* and *aph(3')-Ic* genes. The representative gel image of the duplex PCR is displayed in Figure 4.3. Twenty-three (38.33%) bacterial isolates were positive for *ant(2'')-Ia,* 13 (21.67%) were positive for *aph(3')-Ic,* and only one (1.67%) bacterial isolate was positive for both *ant(2'')-Ic* and *aph(3')-Ic* genes. Table 4.3 concludes the results of duplex PCR on all bacterial isolates.

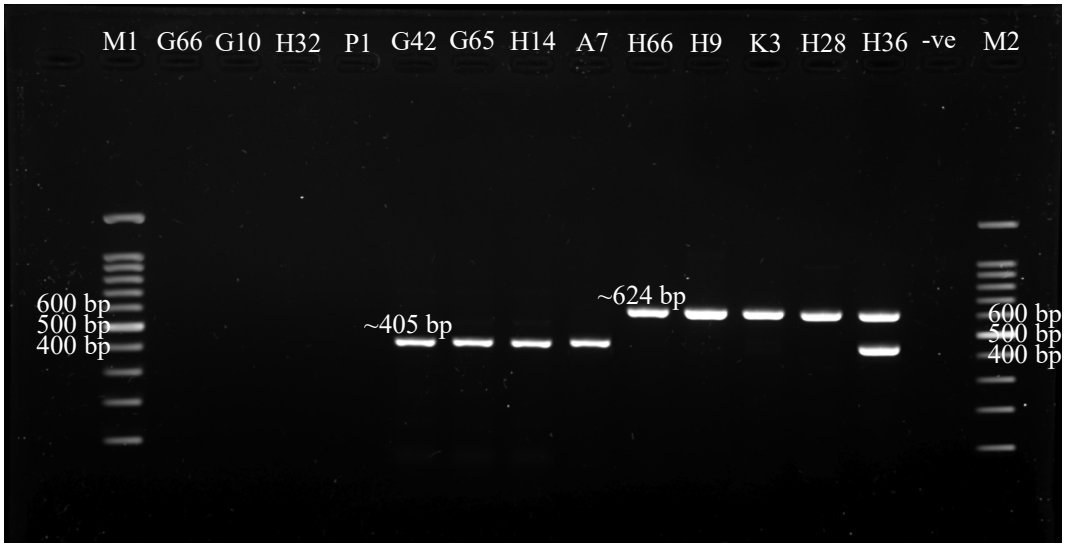

Figure 4.3: Representative Gel Image of Duplex PCR in 1.5% (w/v) Agarose Gel.

The targeted amplicon size for *ant(2'')-Ia* and *aph(3')-Ic* genes was approximately 405 bp and 624 bp, respectively. Lanes M1 and M2 were loaded with 5 µL of 100 bp DNA ladder and the other wells were loaded with the respective samples as labelled.

Table 4.3: Results of Duplex PCR on the Bacterial Isolates.

Isolates	Species	*ant(2'')-Ia*	*aph(3')-Ic*
A7	*Escherichia coli*	+	-
G10	*Klebsiella aerogenes*	-	-
G12	*Enterobacter cloacae*	-	-
G13	*Klebsiella pneumoniae*	-	-
G21	*Enterobacter cloacae*	+	-
G23	*Klebsiella aerogenes*	-	-
G24	*Enterobacter cloacae*	+	-
G31	*Enterobacter cloacae*	+	-
G42	*Enterobacter cloacae*	+	-
G50	*Enterobacter cloacae*	+	-
G65	*Enterobacter cloacae*	+	-
G66	*Klebsiella aerogenes*	-	-
G67	*Klebsiella aerogenes*	-	-
G68	*Enterobacter cloacae*	+	-
G69	*Enterobacter cloacae*	-	-
G7	*Escherichia coli*	+	-
H12	*Escherichia coli*	+	-
H14	*Klebsiella pneumoniae*	+	-
H21	*Escherichia coli*	+	-
H28	*Klebsiella pneumoniae*	-	+
H3	*Escherichia coli*	+	-
H31	*Klebsiella pneumoniae*	-	+
H32	*Klebsiella pneumoniae*	-	-
H33	*Escherichia coli*	+	-
H34	*Klebsiella pneumoniae*	-	+
H35	*Escherichia coli*	-	+
H36	*Escherichia coli*	+	+
H37	*Escherichia coli*	+	-
H38	*Klebsiella pneumoniae*	-	-
H4	*Klebsiella pneumoniae*	+	-
H41	*Escherichia coli*	-	-
H42	*Escherichia coli*	+	-
H43	*Klebsiella pneumoniae*	-	+

Table 4.3 continued: Results of Duplex PCR on the Bacterial Isolates.

Isolates	Species	*ant(2'')-Ia*	*aph(3')-Ic*
H47	*Escherichia coli*	+	-
H48	*Escherichia coli*	+	-
H5	*Enterobacter cloacae*	+	-
H50	*Escherichia coli*	-	+
H52	*Escherichia coli*	+	-
H54	*Escherichia coli*	+	-
H55	*Klebsiella pneumoniae*	-	+
H58	*Klebsiella pneumoniae*	-	-
H59	*Escherichia coli*	-	+
H6	*Klebsiella pneumoniae*	-	+
H62	*Klebsiella pneumoniae*	-	+
H63	*Klebsiella pneumoniae*	-	-
H65	*Klebsiella pneumoniae*	-	-
H66	*Klebsiella pneumoniae*	-	+
H67	*Klebsiella pneumoniae*	-	-
H72	*Escherichia coli*	-	-
H8	*Escherichia coli*	-	-
H9	*Escherichia coli*	-	+
K3	*Escherichia coli*	-	+
P1	*Klebsiella pneumoniae*	-	-
P11	*Escherichia coli*	-	-
P12	*Enterobacter cloacae*	-	-
P14	*Escherichia coli*	-	-
P3	*Escherichia coli*	+	-
P4	*Escherichia coli*	-	-
P6	*Klebsiella pneumoniae*	-	-
P8	*Escherichia coli*	-	-

*+ denotes positive detection of the gene; - denotes negative detection of the gene.

4.5 Statistical Analysis

4.5.1 Association of The Aminoglycoside Resistance Genes with Aminoglycoside Resistance

The resistance phenotypes of the bacterial isolates to aminoglycosides used in this study (kanamycin, gentamicin, and streptomycin) were associated with the aminoglycoside resistance genes detected as shown in Table 4.4.

Table 4.4: Aminoglycoside Resistance Phenotypes and Resistance Genes detected.

Isolate	Species	K	GM	S	*ant(2'')-Ia*	*aph(3')-Ic*
A7	*Escherichia coli*	R	R	R	+	-
G10	*Klebsiella aerogenes*	S	S	I	-	-
G12	*Enterobacter cloacae*	S	S	S	-	-
G13	*Klebsiella pneumoniae*	S	I	R	-	-
G21	*Enterobacter cloacae*	R	R	R	+	-
G23	*Klebsiella aerogenes*	S	S	I	-	-
G24	*Enterobacter cloacae*	R	R	R	+	-
G31	*Enterobacter cloacae*	R	R	R	+	-
G42	*Enterobacter cloacae*	R	R	R	+	-
G50	*Enterobacter cloacae*	R	R	I	+	-
G65	*Enterobacter cloacae*	R	R	I	+	-
G66	*Klebsiella aerogenes*	S	I	I	-	-
G67	*Klebsiella aerogenes*	S	S	I	-	-
G68	*Enterobacter cloacae*	R	R	I	+	-
G69	*Enterobacter cloacae*	R	I	I	-	-
G7	*Escherichia coli*	R	R	R	+	-
H12	*Escherichia coli*	R	R	R	+	-
H14	*Klebsiella pneumoniae*	R	R	S	+	-
H21	*Escherichia coli*	R	R	R	+	-
H28	*Klebsiella pneumoniae*	R	R	R	-	+
H3	*Escherichia coli*	R	R	R	+	-
H31	*Klebsiella pneumoniae*	R	S	I	-	+
H32	*Klebsiella pneumoniae*	S	S	S	-	-
H33	*Escherichia coli*	R	R	S	+	-
H34	*Klebsiella pneumoniae*	R	R	R	-	+
H35	*Escherichia coli*	R	R	R	-	+
H36	*Escherichia coli*	R	R	R	+	+
H37	*Escherichia coli*	R	R	I	+	-
H38	*Klebsiella pneumoniae*	S	S	R	-	-
H4	*Klebsiella pneumoniae*	R	R	R	+	-
H41	*Escherichia coli*	S	I	R	-	-
H42	*Escherichia coli*	R	R	R	+	-

Table 4.4 continued: Aminoglycoside Resistance Phenotype and Resistance Genes detected.

Isolate	Species	K	GM	S	*ant(2'')-Ia*	*aph(3')-Ic*
H43	*Klebsiella pneumoniae*	R	R	R	-	+
H47	*Escherichia coli*	R	R	R	+	-
H48	*Escherichia coli*	R	R	R	+	-
H5	*Enterobacter cloacae*	R	R	R	+	-
H50	*Escherichia coli*	R	S	R	-	+
H52	*Escherichia coli*	R	R	R	+	-
H54	*Escherichia coli*	R	R	R	+	-
H55	*Klebsiella pneumoniae*	R	R	R	-	+
H58	*Klebsiella pneumoniae*	R	R	S	-	-
H59	*Escherichia coli*	R	S	R	-	+
H6	*Klebsiella pneumoniae*	R	S	R	-	+
H62	*Klebsiella pneumoniae*	R	I	I	-	+
H63	*Klebsiella pneumoniae*	S	R	R	-	-
H65	*Klebsiella pneumoniae*	S	I	R	-	-
H66	*Klebsiella pneumoniae*	R	R	R	-	+
H67	*Klebsiella pneumoniae*	R	S	R	-	-
H72	*Escherichia coli*	R	R	R	-	-
H8	*Escherichia coli*	R	R	R	-	-
H9	*Escherichia coli*	R	R	R	-	+
K3	*Escherichia coli*	R	R	R	-	+
P1	*Klebsiella pneumoniae*	S	R	I	-	-
P11	*Escherichia coli*	S	I	R	-	-
P12	*Enterobacter cloacae*	S	S	S	-	-
P14	*Escherichia coli*	S	R	R	-	-
P3	*Escherichia coli*	R	R	S	+	-
P4	*Escherichia coli*	R	R	R	-	-
P6	*Klebsiella pneumoniae*	S	I	S	-	-
P8	*Escherichia coli*	S	I	R	-	-

***** K: Kanamycin; GM: Gentamicin; S: Streptomycin.

To conduct statistical analysis, both resistant and intermediate phenotypes were categorised as resistant. Statistical analysis between the resistance genes and the resistance phenotypes of the bacterial isolates is displayed in Table 4.5. A *p*-value of less than 0.05 was considered statistically significant. Statistical analysis determined that there were significant associations between the *ant(2'')-Ia* gene with kanamycin and gentamicin resistance, and between the *aph(3')-Ic* gene with kanamycin resistance.

Table 4.5: Association between The Aminoglycoside Resistance Genes with Aminoglycoside Resistance.

Antibiotic	**Resistant Isolates (n %)**	***ant(2'')-Ia* (n %)**	***p-value***	***aph(3')-Ic* (n %)**	***p-value***
Kanamycin	43 (71.67%)	24 (40.00%)	**0.000**	14 (23.33%)	**0.005**
Gentamicin	48 (80.00%)	24 (40.00%)	**0.001**	10 (16.67%)	0.287
Streptomycin	52 (86.67%)	21 (35.00%)	0.598	14 (23.33%)	0.102

* *p*-value < 0.05 was considered statistically significant and bolded.

4.5.2 Association of The Aminoglycoside Resistance Genes with Other Classes of Antibiotics (Cross-resistance)

The resistance phenotypes of the bacterial isolates to other classes of antibiotics used in this study (imipenem, meropenem, ertapenem, ciprofloxacin, and ceftriaxone) were associated with the aminoglycoside resistance genes to determine if there was a cross-resistance confer by the aminoglycoside resistance genes to antibiotics of other classes. To conduct statistical analysis, both resistant and intermediate phenotypes were categorised as resistant. Statistical analysis between the aminoglycoside resistance genes and the resistance phenotypes of the bacterial isolates is displayed in Table 4.6. A *p*-value of less than 0.05 was considered statistically significant. Statistical analysis showed that *ant(2'')-Ia* and *aph(3')-Ic* genes did not have any significant association with the antibiotics from other classes, except the association between the *ant(2'')-Ia* gene with imipenem resistance.

Table 4.6: Association of The Aminoglycoside Resistance Genes with Resistance to Other Classes of Antibiotics.

Antibiotics	**Resistant Isolates (n %)**	***ant(2'')-Ia* (n %)**	***p*-value**	***aph(3')-Ic* (n %)**	***p*-value**
Imipenem	44 (73.33%)	21 (35.00%)	**0.039**	10 (16.67%)	0.552
Meropenem	9 (15.00%)	6 (10.00%)	0.082	1 (1.67%)	0.322
Ertapenem	19 (31.67%)	8 (13.33%)	0.520	5 (8.33%)	0.474
Ciprofloxacin	51 (85.00%)	20 (33.33%)	0.522	12 (20.00%)	0.651
Ceftriaxone	50 (83.33%)	21 (35.00%)	0.368	13 (21.67%)	0.259

* *p*-value < 0.05 was considered statistically significant and bolded.

4.5.3 Distribution of The Aminoglycoside Resistance Genes and Their Association with The Patients' Age

According to the Department of Statistics Malaysia Official Portal (2022), Malaysia's population was divided into three main age groups: young age (0 to 14 years old), working age (15 to 64 years old), and old age (above 64 years old). Generally, most of the bacterial isolates hosting resistance genes were isolated from patients of working age. Figure 4.4 shows the overall distribution of *ant(2'')-Ia* and *aph(3')-Ia* genes detected amongst the patients classified into three distinct age groups. Both *ant(2'')-Ia* and *aph(3')-Ic* genes were most prevalent in patients from the working age, followed by old age and young age.

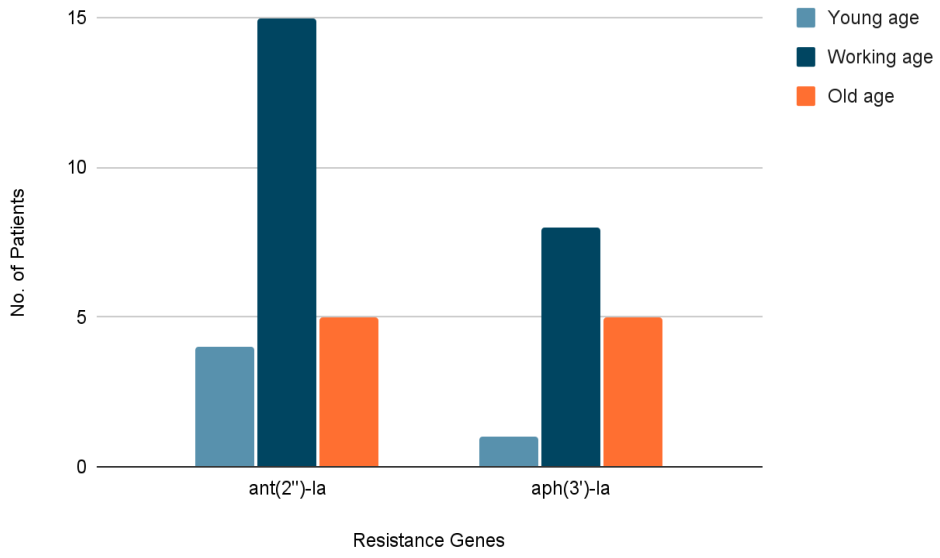

Figure 4.4: Distribution of *ant(2'')-Ia* and *aph(3')-Ic* Genes Based on The Patients' Age Groups.

Table 4.7 tabulates the association between the aminoglycoside resistance genes with the patients' age. A *p*-value of less than 0.05 was considered statistically significant. Statistical analysis showed that there was no significant association between the aminoglycoside resistance genes with patient's age.

Table 4.7: Association between The Age Groups of The Patients with The Resistance Genes.

Resistance genes	**Young age**	**Working age**	**Old age**	**Total**	***p*-value**
ant(2'')-Ia	4 (16.67%)	15(62.50%)	5 (20.83%)	24	0.127
aph(3')-Ic	1 (7.14%)	8 (57.14%)	5 (35.71%)	14	0.782

* *p*-value < 0.05 was considered statistically significant and bolded.

4.5.4 Distribution of The Aminoglycoside Resistance Genes and Their Association with The Patients' Gender

The distribution of *ant(2'')-Ia* and *aph(3')-Ic* genes amongst the bacterial isolates based on the gender of the patients is illustrated in Figure 4.5. The *ant(2'')-Ia* gene was found to be more prevalent amongst the male patients whereas *aph(3')-Ic* gene was distributed equally in both genders.

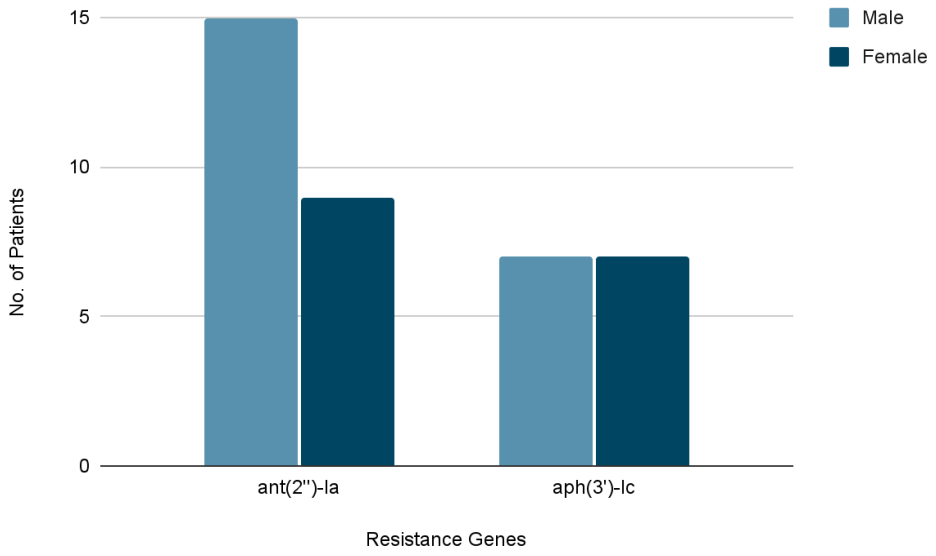

Figure 4.5: Distribution of *ant(2'')-Ia* and *aph(3')-Ic* Genes Based on The Patients' Gender.

The association between the aminoglycoside resistance genes with patients' gender is tabulated in Table 4.8. A *p*-value of less than 0.05 was considered statistically significant. Statistical analysis showed that there was no significant association between the aminoglycoside resistance genes with patients' gender.

Table 4.8: Association between The Gender of The Patients with The Resistance Genes.

Resistance Genes	**Male**	**Female**	**Total**	***p*-value**
ant(2'')-Ia	15 (62.50%)	9 (37.50%)	24	0.317
aph(3')-Ic	7 (50.00%)	7(50.00%)	14	0.392

* *p*-value < 0.05 was considered statistically significant and bolded.

CHAPTER 5

DISCUSSION

5.1 Overview

The objectives of this study were to screen for the presence of *ant(2'')-Ia* and *aph(3')-Ic* genes through duplex polymerase chain reaction (PCR) assay, to determine the prevalence of *ant(2'')-Ia* and *aph(3')-Ic* genes, and to analyse the associations between the resistance genes with the resistance phenotype of the bacterial isolates, patients' age and gender. All the objectives were fulfilled in this study.

5.2 Antimicrobial Susceptibility Testing using Kirby-Bauer Method

Amongst the antibiotics tested, ceftriaxone has the highest resistance rate. Up to 80% (48) of bacterial isolates were resistant to ceftriaxone, followed by kanamycin (71.67%), streptomycin (66.67%), gentamicin (65%), ciprofloxacin (55%), imipenem (35%), ertapenem (11.67%), and meropenem (6.67%). Nevertheless, Hashemi et al. (2013) identified a resistance rate of 45% towards ceftriaxone in Iran. Ceftriaxone, an extended-spectrum third-generation cephalosporin, used to have a higher cure rate, between 72% to 97%. Owing to its high potency in dealing with both Gram-positive and Gram-negative bacteria, the prescription of

ceftriaxone in health facilities was skyrocketing (Tewabe et al., 2021). However, bacteria with resistance to ceftriaxone and other β-lactams isolated from community-acquired infection patients were reported 25 years ago (Al kraiem et al., 2018). Therefore, after years of evolution and the pressure of natural selection, many bacterial isolates from patients are now resistant to ceftriaxone. It was estimated that the resistance towards third generation cephalosporins may increase from 10% to 70% in 2015 (Rosenthal et al., 2012). Not only that, Ruppé et al. (2015) also mentioned that a tremendous increase in resistance towards ceftriaxone may be connected to the transfer of extended-spectrum beta-lactamase producing genes within the plasmids. Moreover, Unemo et al. (2019) also stated that ceftriaxone-resistant bacteria are now spreading globally and are mainly linked to travel to Asian countries.

Jouybari et al. (2021) reported a resistance rate of 94% in the clinical isolates of *Acinetobacter baumannii* to gentamicin, kanamycin, and tobramycin. However, the resistance rate to the aminoglycosides discovered in this study was significantly lower amongst the clinical isolates of *Enterobacteriaceae* used. Moreover, researchers from Turkey and India have reported resistance rates of 94.5% and 32.6%, respectively, against gentamicin amongst the clinical isolates of Gram-negative bacteria (Over et al., 2001; Shahid and Malik, 2005). Not only that, 85% of *Enterobacteriaceae* isolated in Nigeria were resistant to streptomycin (Uzeh et al., 2021). As such, the resistance rate towards gentamicin could vary from country to country due to different geographical distribution.

Fu et al. (2013) reported that the resistance rate of *Enterobacteriaceae* to ciprofloxacin was 78.1%, which is higher than the resistance rate discovered in this study (55%). They found that resistance to ciprofloxacin amongst the *Enterobacteriaceae* was facilitated by single mutations of Ser83Phe, Ser83Leu, Ser83Tyr, and Ser83Ile, and double mutations including Ser83Leu+Asp87Asn, Ser83Leu+Asp87Tyr, and Ser83Phe+Asp87Asn. On the contrary, a study by Daini et al. (2004) revealed a resistance rate of 21.7% towards ciprofloxacin amongst *Enterobacteriaceae,* whereas Reuben et al. (2013) reported that the resistance rate towards ciprofloxacin in *Enterobacteriaceae* was only 16% in Nigeria. Reuben et al., (2013) further justified that the variations in ciprofloxacin resistance rates in different areas may be due to the divergent exposure to various antibiotics and that patients in different areas may have dissimilar attitudes towards the prescription of antibiotics.

Imipenem, ertapenem, and meropenem are categorised as carbapenem antibiotics. Based on Nordmann and Poirel (2019), only 1% of *Enterobacteriaceae* in their study were resistant to carbapenem. Besides, Xu et al. (2015) also indicated that the resistance rate of *Enterobacteriaceae* in Asian countries to imipenem varied between 0.1% to 5.8%. As for meropenem, the resistance rate in *Enterobacteriaceae* in Asian countries varied from 0.9% to 2.6%. The imipenem and meropenem resistance rates obtained in this study were moderately higher than the studies mentioned above, and this may be due to the smaller sample size of this study. For ertapenem resistance, Lob et al. (2018) showed that the resistance to

ertapenem in *Enterobacteriaceae* was 89.5% in Asian countries and 97.3% in the United States and Canada. Nonetheless, only 8.33% of *Enterobacteriaceae* were resistant to ertapenem in this study. As a result, it is clear that the antibiotic profile of the bacteria may differ based on geographical areas.

5.3 Prevalence of *ant(2'')-Ia* and *aph(3')-Ic* Genes

Amongst the 60 bacterial isolates, *ant(2'')-Ia* and *aph(3')-Ic* genes were detected in 24 (40.00%) and 14 (23.33%) bacterial isolates, respectively. Therefore, the prevalence of *ant(2'')-Ia* and *aph(3')-Ic* in *Enterobacteriaceae* is 40% and 23.33%, respectively. The results are contrary to Miró et al. (2013), who identified the prevalence of *ant(2'')-Ia* and *aph(3')-Ia* genes (a close variant to the *aph(3')-Ic* gene) amongst *Enterobacteriaceae* collected from Spain were 3.6% and 13.9% respectively.

In this study, the prevalence of *ant(2'')-Ia* in *K. pneumoniae*, *E. cloacae,* and *E. coli* was 10.53%, 72.73%, and 53.85%, respectively. On the contrary, the prevalence of *aph(3')-Ic* in *E. coli* and *K. pneumoniae* was 23.08% and 42.11%, respectively. The prevalence of *aph(3')-Ic* gene in *E. coli* in this study was slightly higher as compared to a recent research by Bodendoerfer et al. (2020), who showed a 30.3% prevalence of *aph(3')-Ia* gene (a close variant of *aph(3')-Ic* gene that also confers resistance to kanamycin) in *E. coli.* Besides, Mokhtari et al. (2018) reported

that the prevalence of *ant(2'')-Ia* in *K. pneumoniae* was 27.7%, which was almost three times as compared to the current study. The main factor that causes the prevalence of antibiotic resistance genes to vary between different regions or countries is the different protocols in the prescription of antibiotics. Different regions or countries are equipped with different regulations for prescribing antibiotics to patients, not to mention that different antibiotics may be prescribed for similar bacterial infections in different countries. These differences have formed a selective natural selection pressure that tremendously drives the prevalence of resistance to commonly prescribed antibiotics (Goossens et al., 2005). For instance, amikacin and gentamicin are recommended by the National Centre for Disease Control (NCDC) India, whereas amoxicillin and clavulanate are recommended by the Ministry of Health (MOH) Malaysia to treat pyelonephritis (National Centre for Disease Control, 2016; Ministry of Health, 2019).

5.4 Association of The Aminoglycoside Resistance Genes with Aminoglycoside Resistance

Based on the statistical analysis, there was a significant correlation between the *ant(2'')-Ia* gene with kanamycin and gentamicin resistance ($p < 0.05$). This demonstrates that the resistance phenotype of the bacterial isolates to kanamycin and gentamicin was contributed by the presence of the *ant(2'')-Ia* gene in their genome. Nonetheless, the statistical analysis only found a direct association between the *aph(3')-Ic* gene with kanamycin resistance ($p < 0.05$). The results obtained in this study were in concordance with the research by Shaw et al. (1993), that identified that the *ant(2'')-Ia* gene confers resistance to both kanamycin and gentamicin, whereas *aph(3')-Ic* gene confers resistance to kanamycin.

In this study, the *ant(2'')-Ia* gene was detected in *K. pneumoniae, E. cloacae*, and *E. coli.* This discovery conflicts with the result by Cameron et al. (1986), which revealed that *ant(2'')-Ia* was detected in *Pseudomonas aeruginosa, Klebsiella pneumoniae, Morganella morganii, Escherichia coli, Staphylococcus typhimurium, Citrobacter freundii,* and *Acinetobacter baumannii*. Cameron et al. (1986) also outlined that the *ant(2'')-Ia* gene was found in the plasmid and integron, which can be easily transferred to other bacteria. The transmissibility of the resistance genes within the plasmid and integron has resulted in different findings in this study. Conversely, the *aph(3')-Ic* gene was detected in only *E. coli* and *K. pneumoniae*. This finding is also inconsistent with the research by Lee et al. (1990)

and Tauch et al. (2000) who showed the *aph(3')-Ic* gene was only detected in *Klebsiella pneumoniae, Acinetobacter baumannii, Serratia marcescens, Corynebacterium spp., Photobacterium spp.,* and *Citrobacter spp.*. The difference in this finding could be attributed to the fact that *aph(3')-Ic* gene could be found in the plasmid, transposon, and genomic island, which can be easily transferred to other species through different mechanisms of horizontal gene transfer in the clinical environment (Lerminiaux and Cameron, 2019).

5.5 Association of The Aminoglycoside Resistance Genes with Other Classes of Antibiotics (Cross-resistance)

Statistical analysis found that *ant(2'')-Ia* and *aph(3')-Ic* genes did not have any significant association with the antibiotics from other classes, except the association between the *ant(2'')-Ia* gene with imipenem resistance. Houang and Greenwood (1977) and Al-asadi et al. (1981) stated that aminoglycoside resistance genes encoding for aminoglycoside-modifying enzymes exhibit cross-resistance to other aminoglycosides, but could not exhibit resistance to antibiotics of other classes. Nevertheless, the *ant(2'')-Ia* gene was found to have a statistically significant association with imipenem resistance. This may be due to pure coincidence that the bacterial isolates hosting the *ant(2'')-Ia* gene may be hosting other resistance genes concurrently such as bla_{OXA}, bla_{SPM}, and bla_{KPC} genes that confer resistance to imipenem (Mahmoud et al., 2020). The probability of the bacterial isolates hosting other antibiotic resistance genes was high because they

were claimed to be multidrug-resistant strains when collected from the hospital personnel. Nevertheless, screening for imipenem resistance genes amongst the bacterial isolates used in this study ought to be conducted to prove the statement mentioned before. Moreover, there was no study so far that has discovered any cross-resistance to imipenem conferred by *ant(2'')-Ia* gene.

5.6 Correlation between The Aminoglycoside Resistance Gene with The Patients' Age and Gender

Statistical analysis did not find any significant association between the age of the patients with the resistance genes. This result was consistent with the findings by Lee et al. (2016) and Garcia et al. (2017), that identified that there was no statistically significant association between the age of patients with resistance genes. However, it is clear that the prevalence of *ant(2'')-Ia* and *aph(3')-Ic* genes was the highest amongst the working age group in this study. Garcia et al. (2017) also reported a sharp increase in the prevalence of resistance genes amongst patients in their mid-30s. One possible hypothesis for the observation of the highest antibiotic resistance prevalence in the patients of working age is that they have more contact in different environments due to their occupations, causing them to have the greatest exposure to highly resistant bacteria. On the contrary, patients of young age and old age have restricted exposure to different environments due to curtailment. This factor may have tremendously reduced their exposure to highly

resistant bacteria. These statements are presumptive, and further detailed studies on this matter are required to justify them.

Statistical analysis also determined that there was no significant association between the gender of the patients with the resistance genes. This result was in concordance with the study by Hoffmann et al. (2015) who reported that there was no significant association between the gender of the patients and antibiotic resistance.

5.7 Limitations and Future Study

One of the limitations of this study was the relatively small sample size of 60 bacterial isolates as compared to other studies which equipped sample sizes of up to a few thousands. Time constraints and a limited workforce have restricted the sample size of this study. Therefore, a larger sample size should be recruited to test for the prevalence of the resistance amongst the clinical isolates from different hospitals. This is because a larger sample size could give a better statistical result in terms of accuracy and coverage. Furthermore, more resistance genes should be screened to determine the prevalence of other resistance genes amongst the clinical isolates of *Enterobacteriaceae*.

Besides, the bacterial isolates were collected only from hospitals in West Malaysia. A more extensive geographical coverage should be considered for future studies to

determine the prevalence of aminoglycoside resistance throughout Malaysia. Not only that, a collaboration with the National Surveillance of Antimicrobial Resistance (NSAR) Malaysia could be considered to obtain more clinically isolated multidrug-resistant bacteria for research purposes. The results and data of the research can then be published on the NSAR website to raise public awareness of the importance of curbing antibiotic resistance throughout the nation.

Only two aminoglycoside resistance genes were targeted in this study. A higher number of resistance genes from other classes, such as cephalosporin, fluoroquinolones, β-lactams, et cetera, should be screened simultaneously using multiplex PCR to obtain a deeper comprehension on the resistance pattern of clinically isolated *Enterobacteriaceae*.

Sequencing of the duplex PCR products was not conducted in this study due to limited funds from the organisation. Even though the sensitivity of the polymerase chain reaction (PCR) is undeniably high, there may be an unforeseen mistake in the process, such as an incorrect sequence of the primers, causing the wrong amplification of DNA sequences. Therefore, sequencing of the PCR product could be conducted to obtain the sequences of the amplified DNA and compare them to the sequences from the GenBank using the NucleotideBlast (BlastN) programme.

CHAPTER 6

CONCLUSION

The main objective of this study was to screen for the presence of *ant(2'')-Ia* and *aph(3')-Ic* genes simultaneously using duplex PCR amongst the 60 clinical isolates of *Enterobacteriaceae* obtained from different hospitals in West Malaysia. Subsequently, the association between the resistance genes and the antibiotic resistance phenotype, age, and gender of the patients was determined using statistical analysis. Amongst the 60 clinical isolates, 23 (38.33%) were positive for *ant(2'')-Ia,* 13 (21.67%) were positive for *aph(3')-Ic* and only one (1.67%) bacterial isolate was positive for both *ant(2'')-Ic* and *aph(3')-Ic* genes. Overall, the prevalence of the *ant(2'')-Ia* gene was higher in *Enterobacteriaceae* as compared to *aph(3')-Ic* gene.

Statistical analysis has discovered that there were significant associations between the *ant(2'')-Ia* gene with gentamicin, kanamycin, and imipenem resistance, and *aph(3')-Ic* gene with kanamycin resistance. Nonetheless, there was no significant association between the aminoglycoside resistance genes with the patients' age and gender.

This study has provided a deep insight into the distribution of *ant(2'')-Ia* and *aph(3')-Ic* genes amongst the *Enterobacteriaceae* which confer resistance to gentamicin and kanamycin. Therefore, other aminoglycosides or antibiotics of other classes ought to be used when dealing with bacteria hosting these genes. Needless to mention, the prescription of antibiotics must also be closely regulated to decrease the natural selection pressure that drives the emergence of new multidrug-resistant bacteria that can inactivate a broader spectrum of newly synthesised antibiotics.

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APPENDICES

Appendix A

Table 1: Details of The Bacterial Isolates used.

Isolates	Species	Source	Gender	Age
A7	*Escherichia coli*	Blood	M	79
G10	*Klebsiella aerogenes*	Pus swab	M	49
G12	*Enterobacter cloacae*	Bile swab	M	40
G13	*Klebsiella pneumoniae*	Chest tube	M	37
G21	*Enterobacter cloacae*	Swab	M	58
G23	*Klebsiella aerogenes*	Throat swab	M	68
G24	*Enterobacter cloacae*	Mucopurulent sputum	F	58
G31	*Enterobacter cloacae*	Swab	M	47
G42	*Enterobacter cloacae*	Bronchial washing & brushing G	F	16
G50	*Enterobacter cloacae*	Tissue	F	61
G65	*Enterobacter cloacae*	Bronchial washing & brushing G	F	23
G66	*Klebsiella aerogenes*	left ear swab	M	53
G67	*Klebsiella aerogenes*	swab	M	72
G68	*Enterobacter cloacae*	right hip (swab)	M	69
G69	*Enterobacter cloacae*	Mucoid sputum	M	61
G7	*Escherichia coli*	Swab	M	46
H12	*Escherichia coli*	Pus swab	F	21
H14	*Klebsiella pneumonia*	Endotracheal tube aspirate	F	7
H21	*Escherichia coli*	Pus swab	F	69
H28	*Klebsiella pneumonia*	Urine	M	53
H3	*Escherichia coli*	Umbilical venous catheter tip	M	0
H31	*Klebsiella pneumonia*	Urine	M	25
H32	*Klebsiella pneumonia*	Tissue	M	60
H33	*Escherichia coli*	Pus swab	M	29
H34	*Klebsiella pneumonia*	Trachy aspirate	F	75
H35	*Escherichia coli*	Urine	M	49

Table 1 continued: Details of The Bacterial Isolates used.

Isolates	Species	Source	Gender	Age
H36	*Escherichia coli*	Wound swab	M	56
H37	*Escherichia coli*	Pus swab	M	19
H38	*Klebsiella pneumonia*	Tissue	M	67
H4	*Klebsiella pneumonia*	Urine	F	88
H41	*Escherichia coli*	Pus swab	F	75
H42	*Escherichia coli*	Pus swab	M	80
H43	*Klebsiella pneumonia*	Sputum	F	61
H47	*Escherichia coli*	Swab cls	M	43
H48	*Escherichia coli*	Swab cls	M	60
H5	*Enterobacter cloacae*	Pus swab	M	49
H50	*Escherichia coli*	Swab cls	F	67
H52	*Escherichia coli*	Blood	M	48
H54	*Escherichia coli*	Urine	M	5
H55	*Klebsiella pneumonia*	Trachy aspirate	F	12
H58	*Klebsiella pneumonia*	Pus swab	F	51
H59	*Escherichia coli*	Pus swab	M	68
H6	*Klebsiella pneumonia*	Urine	F	59
H62	*Klebsiella pneumonia*	Pus swab	M	62
H63	*Klebsiella pneumonia*	Urine	M	53
H65	*Klebsiella pneumonia*	Urine	F	21
H66	*Klebsiella pneumonia*	Pus swab	M	66
H67	*Klebsiella pneumonia*	Tissue	M	55
H72	*Escherichia coli*	Tissue	F	52
H8	*Escherichia coli*	Pus swab	M	70
H9	*Escherichia coli*	Pus swab	F	78
K3	*Escherichia coli*	Urine	F	16
P1	*Klebsiella pneumoniae*	Urine	F	77
P11	*Escherichia coli*	Urine	F	73
P12	*Enterobacter cloacae*	Wound swab	M	55
P14	*Escherichia coli*	Urine	F	59
P3	*Escherichia coli*	Urine	F	8
P4	*Escherichia coli*	HVS	F	49

Table 1 continued: Details of The Bacterial Isolates used.

Isolates	Species	Source	Gender	Age
P6	*Klebsiella pneumoniae*	Vaginal swab	F	58
P8	*Escherichia coli*	Urine	F	56

Appendix B

Table 2: Absorbance Ratio and Concentration of The DNA extracted.

Isolates	Species	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µL)
A7	*Escherichia coli*	1.94	0.96	305.8
G10	*Klebsiella aerogenes*	1.97	1.04	355.8
G12	*Enterobacter cloacae*	1.69	0.76	219.3
G13	*Klebsiella pneumoniae*	1.82	0.85	208.1
G21	*Enterobacter cloacae*	1.72	0.68	124.6
G23	*Klebsiella aerogenes*	1.93	0.91	138.8
G24	*Enterobacter cloacae*	1.83	0.96	335.0
G31	*Enterobacter cloacae*	1.95	0.88	230.6
G42	*Enterobacter cloacae*	1.90	1.06	477.3
G50	*Enterobacter cloacae*	1.82	0.86	271.0
G65	*Enterobacter cloacae*	1.85	1.03	398.3
G66	*Klebsiella aerogenes*	1.91	0.83	227.9
G67	*Klebsiella aerogenes*	1.72	0.75	133.1
G68	*Enterobacter cloacae*	1.75	0.77	176.1
G69	*Enterobacter cloacae*	1.88	0.86	267.7
G7	*Escherichia coli*	1.82	0.81	192.5
H12	*Escherichia coli*	1.88	0.89	237.0
H14	*Klebsiella pneumoniae*	1.7	0.74	236.7
H21	*Escherichia coli*	2.04	1.13	270.7
H28	*Klebsiella pneumoniae*	1.71	0.72	121.7
H3	*Escherichia coli*	2.00	0.99	258.3
H31	*Klebsiella pneumoniae*	1.81	0.84	150.8
H32	*Klebsiella pneumoniae*	1.92	0.92	285.1
H33	*Escherichia coli*	1.88	0.78	148.3
H34	*Klebsiella pneumoniae*	1.90	0.88	178.3
H35	*Escherichia coli*	1.92	0.94	280.7
H36	*Escherichia coli*	1.80	0.8	194.5
H37	*Escherichia coli*	1.99	0.98	273.8
H38	*Klebsiella pneumoniae*	1.80	0.80	150.4

Table 2 continued: Absorbance Ratio and Concentration of The DNA extracted.

Isolates	Species	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µL)
H4	*Klebsiella pneumoniae*	1.99	0.94	166.6
H41	*Escherichia coli*	2.06	1.14	239.6
H42	*Escherichia coli*	2.05	1.15	331.9
H43	*Klebsiella pneumoniae*	1.80	0.76	140.0
H47	*Escherichia coli*	2.00	1.06	253.3
H48	*Escherichia coli*	2.01	1.05	267.1
H5	*Enterobacter cloacae*	1.97	1.08	375.4
H50	*Escherichia coli*	1.91	0.87	181.5
H52	*Escherichia coli*	2.03	1.28	390.6
H54	*Escherichia coli*	1.92	0.94	311.2
H55	*Klebsiella pneumoniae*	1.76	0.68	122.5
H58	*Klebsiella pneumoniae*	2.00	0.90	161.9
H59	*Escherichia coli*	1.99	0.98	225.4
H6	*Klebsiella pneumoniae*	1.82	1.04	468.1
H62	*Klebsiella pneumoniae*	1.91	0.97	284.7
H63	*Klebsiella pneumoniae*	1.93	0.87	198.4
H65	*Klebsiella pneumoniae*	1.85	0.82	132.2
H66	*Klebsiella pneumoniae*	2.04	1.03	187.2
H67	*Klebsiella pneumoniae*	1.84	0.80	226.5
H72	*Escherichia coli*	2.03	1.30	367.9
H8	*Escherichia coli*	1.99	0.94	208.5
H9	*Escherichia coli*	1.93	0.96	352.4
K3	*Escherichia coli*	1.99	0.87	130.8
P1	*Klebsiella pneumoniae*	1.91	0.91	153.8
P11	*Escherichia coli*	1.93	0.91	122.1
P12	*Enterobacter cloacae*	1.94	0.92	118.9
P14	*Escherichia coli*	1.91	0.96	165.6
P3	*Escherichia coli*	1.92	0.94	288.0
P4	*Escherichia coli*	2.17	1.12	169.1
P6	*Klebsiella pneumoniae*	1.83	0.82	198.2

Table 2 continued: Absorbance Ratio and Concentration of The DNA extracted.

Isolates	Species	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µL)
P8	*Escherichia coli*	2.12	1.28	131.9

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