IMPACT OF UNTREATED HOSPITAL EFFLUENTS ON DISSEMINATION OF ANTIBIOTIC RESISTANCE GENES

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submitted by **OSMAN KAYALI** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Assoc. Prof. Dr. Can Özen
Head of Department, **Biotechnology** _____

Prof. Dr. Bülent İçgen
Supervisor, **Environmental Eng., METU** _____

Examining Committee Members:

Prof. Dr. Filiz Bengü Dilek
Environmental Eng., METU _____

Prof. Dr. Bülent İçgen
Environmental Eng., METU _____

Prof. Dr. Özlem Osmanağaoğlu
Biology, Ankara University _____

Assoc. Prof. Dr. Çağdaş Devrim Son
Biology, METU _____

Assist. Prof. Dr. Yasemin Dilşad Yılmazel Tokel
Environmental Eng., METU _____

Date: 12.09.2019

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Surname: Osman Kayalı

Signature:

ABSTRACT

IMPACT OF UNTREATED HOSPITAL EFFLUENTS ON DISSEMINATION OF ANTIBIOTIC RESISTANCE GENES

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Hospital wastewaters (HWWs) were reported to be hotspots for antibiotics and antibiotic-resistant bacteria. However, little is known about the impact of these effluents on the dissemination of antibiotic-resistance genes (ARGs). In this study, therefore, HWWs were monitored for 16S rRNA gene for overall bacterial genes and seven ARGs of *aadA, tetA, cmlA, sul1, qnrS, ermB* and $bla_{\text{CTX-M}}$ corresponding to commonly used antibiotics aminoglycosides, tetracyclines, amphenicols, sulfonamides, quinolones, macrolide-lincosamide-streptogramin group and β-lactams, respectively. Seasonally collected effluents of six different hospitals were analysed in terms of overall bacterial genes and seven ARGs by using quantitative polymerase chain reaction. Overall bacterial gene copy numbers were found to be the lowest with 10^3 copies/mL in winter, while the highest copy numbers with 10^5 copies/mL were observed in both summer and spring. All the hospitals tested displayed almost similar seasonal ARG copy number profile of *aadA* > *tetA* > *cmlA* ≈ *sul1* > *ermB* ≈ *qnrS* > $bla_{\text{CTX-M}}$. One-way analysis of variance elucidated that seasonal

changes in ARG copy numbers were significant ($p<0.05$). The results indicated that untreated HWWs were hotspots for ARGs and required on-site treatment before discharging into public sewer.

Keywords: Antibiotic resistance, hospital wastewaters, antibiotic resistance genes

ÖZ

ARITILMAMIŞ HASTANE ATIK SULARININ ANTİBİYOTİK DİRENÇ GENLERİNİN YAYILIMINA ETKİSİ

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Hastane atık suları antibiyotik ve antibiyotik dirençli bakteriler açısından noktasal kaynaklar olarak bildirilmiştir. Ancak bu atık suların antibiyotik direnç genlerinin yayılımına etkisi çok fazla bilinmemektedir. Bu nedenle, bu çalışmada hastane atık sularında toplam bakteriyel genler için 16S rRNA geni ve yaygın kullanıma sahip aminoglikozit, tetrasiklin, amfenikol, sülfonamit, kinolon, makrolid-linkozamit-streptogramin ve β-laktam antibiyotik gruplarına yönelik sırasıyla *aadA, tetA, cmlA, sul1, qnrS, ermB* and $bla_{\text{CTX-M}}$ yedi antibiyotik direnç geni incelendi. Altı farklı hastaneden mevsimsel olarak toplanılan atık sularda toplam bakteriyel genler ve yedi antibiyotik direnç geni kantitatif polimeraz zincir reaksiyonu yöntemi ile analiz edildi. En düşük toplam bakteriyel gen sayısı 10^3 kopya/mL ile kış mevsiminde, en yüksek bakteriyel gen sayısı ise 10^5 kopya/mL ile hem yaz hem de ilkbahar mevsimlerinde gözlendi. Bütün hastane örneklerinde benzer olan direnç geni miktarlarının *aadA* > *tetA* > *cmlA* ≈ *sul1* > *ermB* ≈ *qnrS* > $bla_{\text{CTX-M}}$ olarak sıralandığı belirlendi. Tek yönlü varyans analizi, antibiyotik direnç geni miktarlarındaki değişikliklerin mevsimsel

olarak anlamlı olduğunu ortaya koydu ($p<0.05$). Bu sonuçlar, arıtılmamış hastane atık sularının antibiyotik direnç genleri için noktasal kaynak olduğunu ve genel kanalizasyon sistemine dahil edilmeden önce yerinde arıtılması gerektiğini göstermektedir.

Anahtar Kelimeler: Antibiyotik direnci, hastane atık suları, antibiyotik direnç genleri

To my parents

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LIST OF ABBREVIATIONS

ARB: Antibiotic Resistant Bacteria

ARG: Antibiotic Resistance Gene

BOD: Biochemical Oxygen Demand

COD: Chemical Oxygen Demand

DHFR: Dihydrofolate Reductase

DHPS: Dihydropteroate Synthase

ESBL: Extended Spectrum Beta Lactamase

HGT: Horizontal Gene Transfer

HWW: Hospital Wastewater

ICE: Integrative Conjugative Element

IR: Inverted Repeat

IS: Insertion Sequence

MGE: Mobile Genetic Element

MLS: Macrolide-Lincosamide-Streptogramin

PABA: Para-aminobenzoic Acid

PBP: Penicillin Binding Protein

PCR: Polymerase Chain Reaction

PRP: Pentapeptide Repeat Protein

qPCR: Quantitative Polymerase Chain Reaction

TSS: Total Suspended Solids

CHAPTER 1

INTRODUCTION

1.1. Antibiotics: origins, resistance and importance

Almost a century ago a medical revolution that has miraculous effects on the health and life span of humans was introduced. The new drugs called antibiotics enabled the treatment of infectious diseases which were the leading causes of death for the thousands of years (Aslam *et al.,* 2018). Chemical agents used as therapeutics act by killing or inhibiting the growth of microorganisms. Since Sir Alexander Fleming accidentally discovered penicillin, antibiotics have saved the lives of millions of people (Madigan *et al.,* 2015). The first modern antibiotic was discovered in 1909 by Paul Ehrlich. It was named Salvarsan and became very successful for treating syphilis infections. Then the second modern antibiotic, Prontosil, marketed by Bayer. Prontosil was a precursor to the active compound sulfanilamide which was used commonly in the dye industry. Penicillin was discovered by Alexander Fleming in 1928. However, it took place in the market with 12 years delay due to the difficulties in purification and mass production. The beginning of the new 'antibiotic era' started after the successful introduction of these first antibiotics and let to the discovery of many novel antibiotic classes (Aminov, 2010).

The early antibiotics were produced from their natural origin, for example, streptomycin from the genus *Streptomyces*. Today, production method of most of the antibiotics are by chemical synthesis or by chemical modification of naturally occurring compounds (Kümmerer, 2009). More than 20 classes of antibiotics created until early 60s since their first discovery. These different kinds of antibiotics can be classified based on the differences on their molecular structure, mode of action and

spectrum of activity (Etebu & Arikekpar, 2016). Spectrum of activity classification is useful when it comes to treating infections. This classification depends on the target bacterial group of antibiotics. While narrow spectrum antibiotics are specific for certain group of bacteria, broad spectrum antibiotics may target more than one group of bacteria (Figure 1.1).

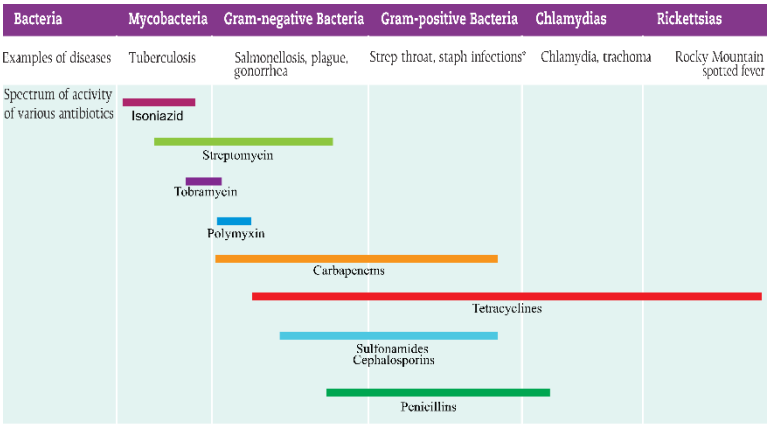

Figure 1.1. Spectrum of activity for various antibiotics (Cowan, 2013)

However, mechanism of action is one of the most popular classifications and the common groups include: β-lactams, sulfonamides, aminoglycosides, macrolides, tetracyclines, chloramphenicol and quinolones (Gothwal & Shashidhar, 2015). Today, antibiotics are mostly used as medicine for humans but also has a widespread usage in animal husbandry, agriculture and aquaculture. For the last decades antibiotic usage in the livestock industry have become a common practice, as they promote animal growth besides prevention and curation of diseases (Sarmah *et al.,* 2006; Sui *et al.*, 2018). The intensive use of antibiotics for medical, veterinary and agricultural purposes increases the dissemination of antibiotic resistance and limits the ability to treat various infections (Berglund, 2015). The threat that bacteria becoming resistant to antibiotics had been predicted by Alexander Fleming, in his Nobel Prize speech in 1945 (WHO, 2014).

Antibiotic resistance is the ability of inactivation of antibiotics or the ability to bypass their effects. In simple terms, antibiotic resistance occurs when regular amount of antibiotics become ineffective against bacteria. There are two main kinds of resistance mechanisms as intrinsic and acquired. Some bacterial species are intrinsically resistant to specific antibiotics due to cellular structure of certain species (Gualerzi & Brandi, 2014). For instance, Gram-negative bacteria are intrinsically more resistant than Gram-positive bacteria to some hydrophilic antibiotics. Because of the difference in outer membrane structure, Gram-negative bacteria become less permeable for hydrophilic antibiotics (Blair *et al.,* 2015). Absence of target structure, increased efflux or differences in expression levels could be the reasons for the intrinsic resistance. Acquired resistance named after the process of acquisition of the new antibiotic resistant phenotypes by the susceptible bacteria. There are two mechanisms behind the ability to acquire advantageous new traits against antibiotics as mutations and horizontal gene transfer (HGT). HGT among different bacterial species is the main reason for the widespread dissemination of antibiotic resistance genes in the various environmental settings (Amábile-Cuevas, 2015). In the 20th century, resistance to the new antibiotic classes have been revealed in a short period of time after the introduction of most antibiotics (Zaman *et al.,* 2017). While the antibiotic resistance increasing dramatically, new classes of antibiotics had not been discovered for the last 40 years. Antibiotic resistance poses a significant threat for the treatment of communicable diseases like, tuberculosis and malaria (WHO, 2014). A report on antibiotic resistance by British Government estimates 10 million deaths annually by 2050 if no action taken against this threat (O'Neill, 2014).

1.2. Mechanisms of action & resistance mechanisms

Bacteria are affected by antibiotics in two different way; bactericidal and bacteriostatic. Bactericidal antibiotics simply kill the bacteria while bacteriostatic antibiotics only inhibit the growth of the bacteria (Manyi-Loh *et al.,* 2018). Both effects can be achieved by targeting metabolic processes in the bacteria and antibiotics

can be categorized based on these action sites like inhibition of cell wall synthesis, inhibition of nucleic acid function, inhibition of protein synthesis, disruption of membrane integrity and inhibition of folic acid synthesis (Cowan, 2013) (Figure 1.2).

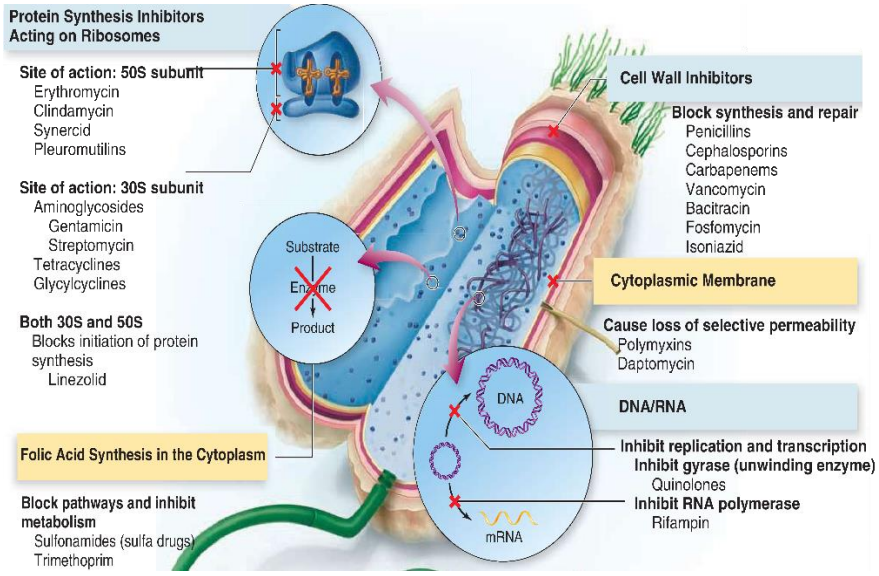

Figure 1.2. Antibiotics shown with their modes of action (Cowan, 2013)

Antibiotic resistance has four main mechanisms; neutralization of the antibiotic, altered target site, bypassing the antibiotic effect and keeping antibiotic concentrations lowest in the cell. Neutralization of the antibiotic can be achieved through modification or inactivation by using certain enzymes. The target site of the antibiotic may be modified by bacteria to reduce the affinity or binding capacity of antibiotics. Some enzymes enable bacteria to utilize alternative pathways for the affected metabolic processes. Antibiotics can be kept below the inhibitory concentrations through increased efflux of antibiotic or by decreasing membrane permeability (Schmieder & Edwards, 2012). These antibiotic resistance mechanisms and affected antibiotics are summarized in Figure 1.3.

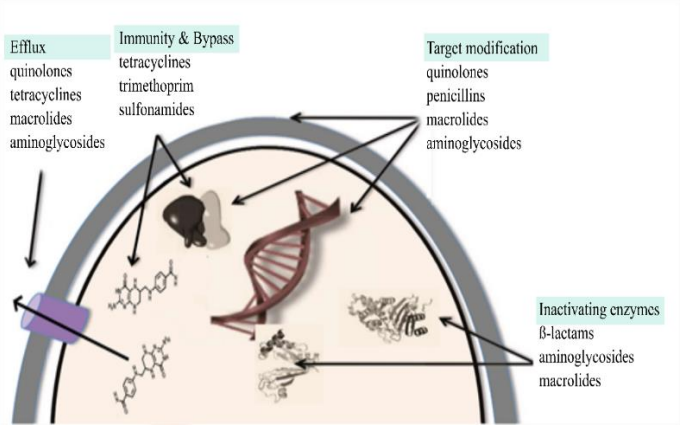

Figure 1.3. Resistance mechanisms (Wright, 2010)

1.3. Antibiotic classes

Antibiotics can be classified in several ways like the chemical structure, mode of action and activity spectrum are common properties used for classification. Some common classes of antibiotics based on chemical or molecular structures include aminoglycosides, β-lactams, macrolides, tetracyclines, quinolones, sulphonamides and amphenicols (Etebu & Arikekpar, 2016). These antibiotic classes also align with the consumption data obtained from national antibiotic consumption surveillance report published by Türkiye İlaç ve Tıbbi Cihaz Kurumu (2017)(Figure 1.4).

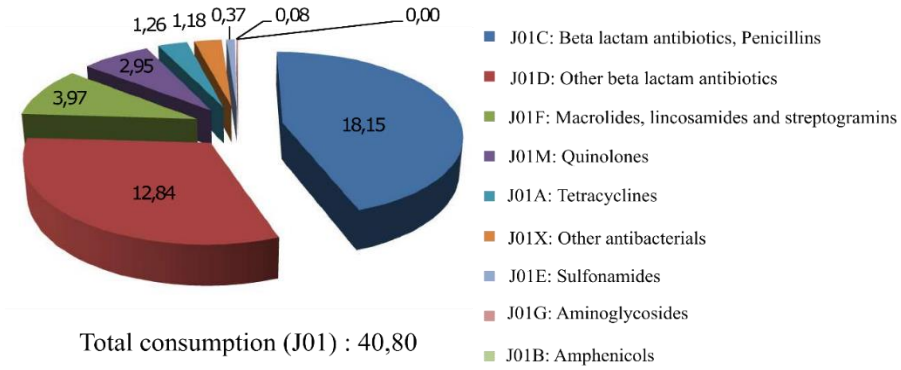

Figure 1.4. Distribution of systematically consumed antibiotics in Turkey in 2013 (Türkiye İlaç ve Tıbbi Cihaz Kurumu, 2017)

1.3.1. Aminoglycosides

Aminoglycosides are broad-spectrum antibiotics that act through inhibition of protein synthesis. They were first established in the 1940s and are still widely used worldwide. They inhibit protein synthesis with an irreversible interaction on the ribosome; and are especially effective against Gram-negative bacteria (Gualerzi & Brandi, 2014). Streptomycin was first isolated and clinically introduced aminoglycoside. Several other members of the class were introduced over the years including neomycin, kanamycin, gentamicin, netilmicin and amikacin. Structure of representative aminoglycosides shown in the Figure 1.5. The systematic use of the class began to drop in the 1980s with the availability of antibiotics that are less toxic and provide broader coverage than the aminoglycosides (Krause *et al.*, 2016).

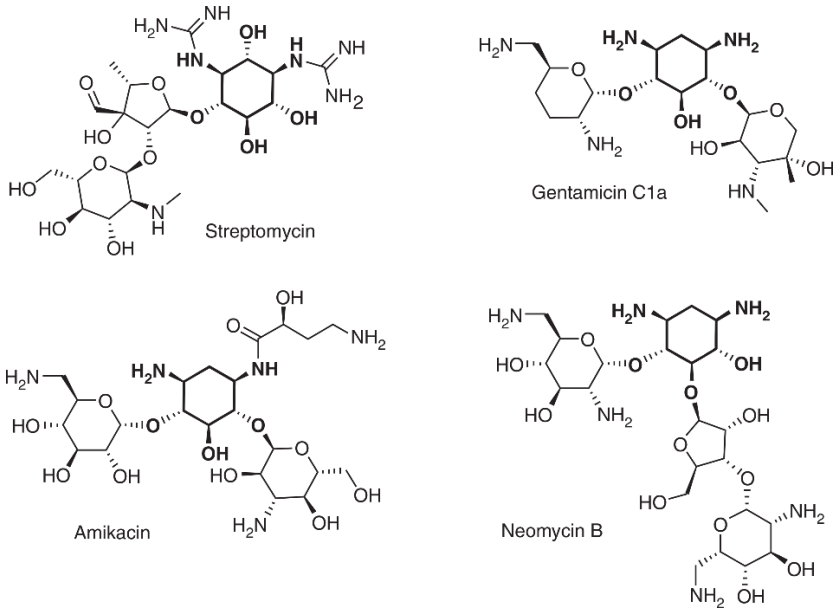

Figure 1.5. Structures of aminoglycoside antibiotics (Krause *et al*., 2016)

Protein synthesis is inhibited by binding aminoglycosides on the A-site on the 16S rRNA of the 30S ribosome. As a result of this interaction, mistranslations occur in the protein synthesis and resulting polypeptides cause damage to the cell. Some

aminoglycosides can impact on the protein synthesis by blocking elongation or by inhibiting initiation (Amábile-Cuevas, 2015). Aminoglycosides resistance occur based on four main mechanisms: Modifications of the 30S ribosomal subunit; enzymatic modification and inactivation of the aminoglycosides, by aminoglycoside acetyltransferases, nucleotidyltransferases, or phosphotransferases; increased efflux and decreased permeability (Doi *et al.,* 2016). Resistance to aminoglycosides depending on membrane function like increased efflux or decreased permeability generally have intrinsic nature of resistance (Krause *et al.,* 2016). The general mechanism of resistance to an antibiotic is the target modification. Aminoglycosides target the A-site of the bacterial ribosome to prevent inhibition by aminoglycosides two possible resistance mechanisms exist as mutations and the modifications of the ribosome. 16S rRNA methyltransferases can modify the binding site of aminoglycosides. These enzymes provide resistance by methylation of a nucleotide in the A-site of the 16S rRNA (Garneau-Tsodikova & Labby, 2016).

The other common mechanism of aminoglycoside resistance is chemical modification by aminoglycoside-modifying enzymes (AMEs). These enzymes contain three subclasses, based on the type of chemical modification they apply to aminoglycosides; aminoglycoside acetyltransferases, nucleotidyltransferases and phosphotransferases. AMEs are highly mobile since their genes are transferred on plasmids, integrons, transposons, and other transposable gene elements usually accompanied by other antibiotic resistance genes (Krause *et al.,* 2016).

1.3.2. Tetracyclines

The discovery of the tetracycline family of antibiotics emerged with the discovery of chlortetracycline in the late 1940s. Since it has a broad spectrum effect on many bacterial disease at the time of discovery rapidly gain the miracle drug status (Dougherty & Pucci, 2014). Tetracyclines are one of the important classes of

antibiotics that have widespread use against both Gram-positive and Gram-negative bacteria and additional activity against rickettsial organisms and mycoplasmas. Tetracyclines were named after the four rings in their chemical structure (Figure 1.6). Tetracyclines interact with 16S rRNA by binding the 30S subunit. This interaction blocks the binding of aminoacyl-tRNAs and inhibits the peptide elongation (Walsh & Wencewicz, 2015).

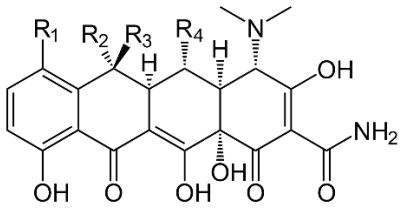

Figure 1.6. Structural body of tetracycline antibiotics (Sultan *et al*., 2018)

The genes that provide tetracycline resistance are associated with mobile elements. Tetracycline resistance genes determine three resistance mechanisms; antibiotic efflux pumps, target modification and antibiotic inactivation (Makowska *et al.,* 2016). Tetracycline resistance gene *tetA* is one of the most common drug specific efflux pump type which belong to the major facilitator superfamily (Møller *et al.,* 2016). There are several tetracycline ribosomal protection proteins and two proteins encoded by *tetO* and *tetM* are well studied genes found in both Gram-positive and Gram-negative bacteria. These proteins act on the direct interaction of tetracycline and ribosome and release the tetracycline from its binding site (Grossman, 2016). Tetracycline inactivation is another mechanism of resistance provided by *tetX*. The *tetX* gene is mostly disseminated by mobile genetic elements and encodes a tetracycline destructase that inactivates tetracyclines by covalent interactions (Markley & Wencewicz, 2018).

1.3.3. Amphenicols

The first and most common member of amphenicol antibiotics is chloramphenicol. Chloramphenicol was first naturally produced from *Streptomyces* species in 1947. It has broad spectrum bacteriostatic activity against some Gram-positive and Gram-negative bacteria including anaerobic species (Fair & Tor, 2014). The other member of the class, florfenicol is a synthetic chloramphenicol derivative that has good tissue penetration due to lipophilic characteristics. The structure of chloramphenicol is shown in the Figure 1.7.

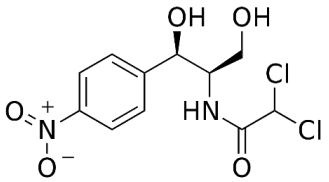

Figure 1.7. Structure of chloramphenicol (Schwarz *et al*., 2004)

Chloramphenicol inhibits protein synthesis with high specificity. It binds peptidyl transferase site at the 50S ribosomal subunit and prevent peptide elongation. Resistance to amphenicols may be due to enzymatic inactivation, target site modification, decreased membrane permeability and active efflux (Fernández *et al.,* 2012; Schwarz *et al.,* 2004). The *cfr* gene encoded methylase causes resistance through the methylation of target on the 23S rRNA (Fair & Tor, 2014). Chloramphenicols are inactivated by acetylation with various chloramphenicol acetyltransferases (*cat* genes). Another most common mechanisms of resistance to chloramphenicol in bacteria is the presence of efflux pumps. The chloramphenicol resistance gene *cmlA* that encodes chloramphenicol exporter is disseminated on transferable plasmids (Bischoff *et al.,* 2005).

1.3.4. Sulfonamides and trimethoprim

The folate coenzyme is essential for eukaryotes, but bacteria can synthesize using enzyme in folate pathway. Dihydropteroate synthase (DHPS) is well known enzyme in the folic acid synthesis pathway and targeted by sulfonamides. Sulfonamides have similar structure of para-amino-benzoic acid (PABA) (Figure 1.8) and interact as substrate on dihydropteroate synthase. This interaction results in the inhibition of dihydrofolic acid formation (Walsh & Wencewicz, 2015). The sulfonamide sulfamethoxazole is usually combined with trimethoprim, another pathway inhibitor, which makes the complex more efficient and sustainable. Trimethoprim inhibits the enzyme dihydrofolate reductase (DHFR) which acts on the conversion of folic acid to dihydrofolate (AlRabiah *et al*., 2018).

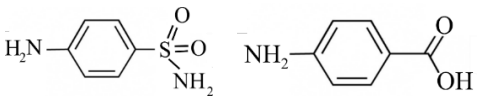

Figure 1.8. Structures of sulfanilamide and PABA (Dougherty & Pucci, 2014)

Introduction of sulfonamides with precursor drug Prontosil in the beginning of 20th century made sulfonamides most popular drug at that time. This early widespread usage of the sulfonamides made the resistance genes spread almost all around the world in association with class 1 integrons (Aminov, 2010). Resistance to sulfonamides occurred by mutations in the DHPS gene or acquired by an alternative DHPS encoding *sul* gene (Byrne-Bailey *et al.,* 2009). Three types of mobile genes are known coding alternative DHPS as *sul*1, *sul*2, and *sul*3. Resistance to trimethoprim transferred by mobile genetic elements are encode for alternative DHFR enzymes like *dfr*1, *dfr*5, *dfr*6, *dfr*7, and *dfr*14 (Skold, 2017).

1.3.5. Quinolones

Nalidixic acid was the first member of quinolones, discovered in the early 60s. The most popular members are known as floxacin, ciprofloxacin and levofloxacin (Etebu & Arikekpar, 2016). The essential bacterial enzymes DNA gyrase and DNA topoisomerase IV are targeted by quinolones. Both enzymes bind to make single strand breaks then religates DNA to relieve from the superhelical twists. These enzymes control the topology of the bacterial chromosome and act on DNA replication, recombination, and transcription. Quinolones act as bacteriostatic antibiotic blocking the replication (Dougherty & Pucci, 2014). Structures of main quinolone core, nalidixic acid and ciprofloxacin are shown in the Figure 1.9.

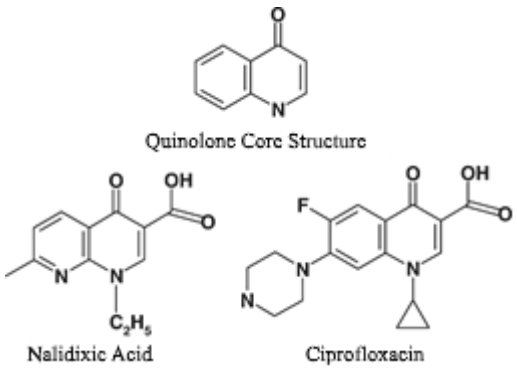

Figure 1.9. Representative quinolone structures (Aldred *et al*., 2014)

The quinolone resistance genes have been located on plasmids in various bacteria. The *qnr* genes translated into pentapeptide repeat proteins (PRPs). These proteins protect from the quinolones by binding topoisomerase IV and DNA gyrase. PRPs act on topoisomerase–quinolone complexes after quinolone binding and facilitate the release of quinolone from the complex. This enables freed enzyme to complete its normal activity and prevent the release of double-stranded DNA breaks resulting from quinolone–topoisomerase interaction (Blair *et al.,* 2015). Plasmid-mediated quinolone resistance confers topoisomerase protection and include different families of Qnr proteins; QnrA, QnrB and QnrS (Cantón, 2009). A variant of acetyltransferase;

AAC(6')-Ib-cr which is active against many aminoglycosides, may also responsible for quinolone acetylation. QepA and OqxAB are efflux pump proteins from two different protein families provide resistance to quinolone antibiotics (Hooper & Jacoby, 2015).

1.3.6. Macrolide/lincosamide/streptogramin group

Macrolide/lincosamide/streptogramin (MLS) group antibiotics are chemically different inhibitors of protein synthesis. While macrolides have multiple lactone rings, lincosamides are derived from proline and lack of a lactone ring. Mixture of cyclic peptide compounds form streptogramin antibiotics (Amábile-Cuevas, 2015). Macrolides, lincosamides and streptogramins are different antibiotic groups but due to similarities in spectrum of activity and mode of action, they are generally classified together. They all have bacteriostatic effect by targeting 50S subunit of the bacterial ribosome to inhibit protein synthesis (Dinos, 2017).

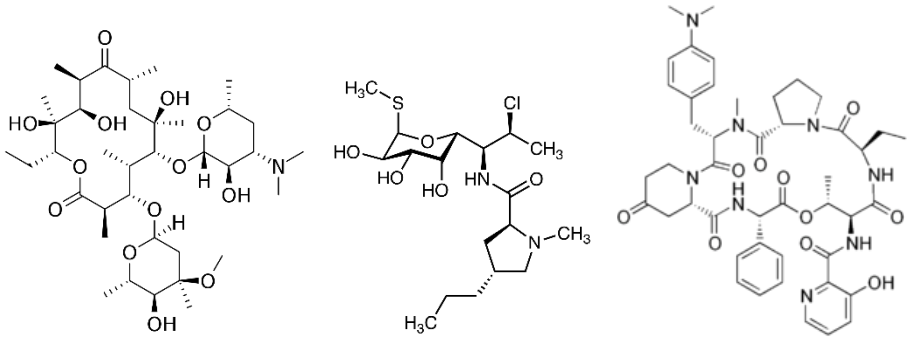

Figure 1.10. Structures of erythromycin (left), clindamycin (middle) and mikamycin B (right) (Amábile-Cuevas, 2015)

The common mechanisms of resistance to macrolides are caused by, efflux pump proteins, ribosomal protection proteins, 23S rRNA modifiers, macrolide esterases and macrolide phosphotransferases (Golkar *et al*., 2018). One of the most common resistance mechanisms developed by bacteria against macrolides is ribosomal modification encoded by the *erm* genes. These genes encode 23S rRNA methylases that are usually encoded on transposons or plasmids. (Reyes *et al.*, 2007). Mef family

efflux pumps have two subgroups encoded by *mefA* and *mefE*. Ribosomal protection conferred by Msr protein family and these proteins act same as tetM/tetO proteins unbind the quinolones from the ribosome complex (Dinos, 2017). Macrolide phosphotransferases encoded by *mph* genes and macrolide esterases encoded by *ere* genes are both present on mobile genetic elements which enable widespread dissemination of these resistance genes into various environments (Van Hoek *et al.,* 2011).

1.3.7. β-lactams

Members of β-lactams contain a β-lactam ring which is very reactive (Figure 1.11). Based on their ring structure, penicillins, cephalosporins, monobactams and carbapenems are the most common members of the β-lactam class antibiotics (Etebu & Arikekpar, 2016). Clinically used first β-lactam was the penicillin G. Today ampicillin and amoxicillin are the most popular penicillin drugs and usually applied with an β-lactamase inhibitor (Fernandes *et al.,* 2013). With the discovery of a new pathway and the first stable cephalosporin dozens of this class were introduced in the 1950s. Cephalosporins are divided into five subclasses depending on their microbial activity. Cefotaxime, cefoperazone, ceftriaxone and ceftazidime have been used clinically as extended spectrum β-lactams (Bush & Bradford, 2016).

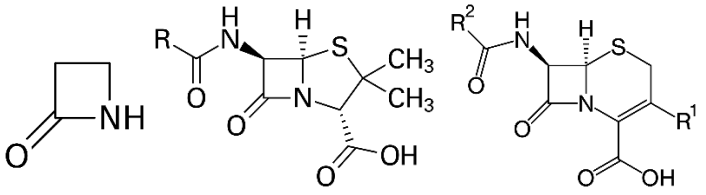

Figure 1.11. Structures of β-lactam ring, penicillins and cephalosporins (Etebu & Arikekpar, 2016)

Monobactams are resistant to β-lactamases and effective against many Gram-negative bacteria. However, Gram-positive bacteria and anaerobes are intrinsically resistant to monobactams. Imipenem and meropenem are two members of carbapenems which

have similar effects on bacteria with different side effects (Fernandes *et al.*, 2013). Structures of a monobactam aztreonam, carbapenems imipenem and meropenem are given in the Figure 1.12. They interact with essential proteins that act on the bacterial cell wall synthesis. Some of these bacterial proteins are named penicillin binding protein (PBP). They are responsible for cross linking peptides of peptidoglycan layer. β-lactam antibiotics bind to these PBP and inhibit the cell wall production by blocking the synthesis of peptidoglycan.

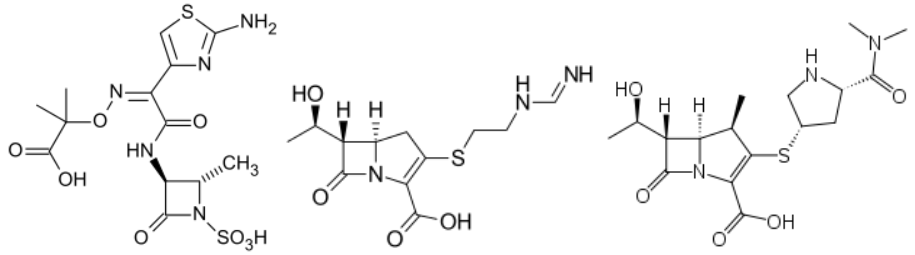

Figure 1.12. Chemical structures of aztreonam, imipenem and meropenem (Etebu & Arikekpar, 2016)

Resistance to β-lactam antibiotics has become a worldwide public health problem. β-lactamases act by hydrolysing the β-lactam ring. A number of β-lactamases has been identified since the early 80s and they were classified into four classes A to D, based on their amino acid sequences (Bonnet, 2004). Among these classed A β-lactamases are called extended-spectrum β-lactamases (ESBLs) which can hydrolyse expanded-spectrum β-lactams represent a public health concern. ESBLs of class A mainly include TEM, SHV, and CTX-M enzymes (Cantón *et al.*, 2012). Many of the β-lactamase genes conferring resistance to the penicillin, cephalosporin and monobactam antibiotics are founded on mobile genetic elements (Wright, 2010). Resistance conferred by Class B, C and D β-lactamases were mediated by bla_{IMP}, *ampC*, and bla_{OXA} genes, respectively (Kong *et al.*, 2010).

1.4. Horizontal gene transfer and mobile genetic elements

Maintaining acquired genes in microbial world, is possible via two different ways. First, vertical transfer of a gene from a bacterium to its lineage (Wright, 2010). Second is the HGT that enables sharing genetic material among different species. The HGT is regarded to be the main reason for the global antibiotic resistance phenomenon. HGT takes place by three well-known mechanisms as shown in Figure 1.13; transformation, conjugation and transduction (Gillings, 2017).

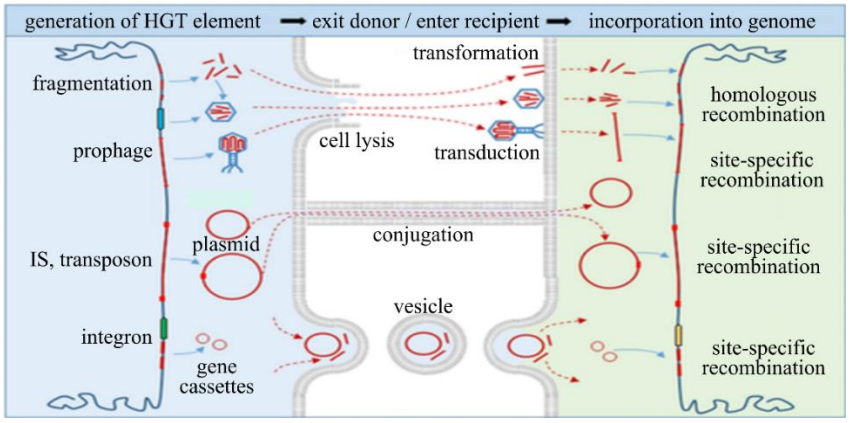

Figure 1.13. Horizontal gene transfer mechanisms (Gillings, 2017)

Transformation is the uptake and use of free DNA fragments in the environment. However, some conditions are required for the occurrence of transformation. Presence of the free DNA and the competent recipient bacteria are necessary. Because many bacterial species are capable of transformation under specific states (von Wintersdorff *et al.*, 2016). These requirements may give the impression that the transformation is a rarely occurring event. However, transformation may take place more frequently in biofilms (Berglund, 2015).

Conjugation considered as the main mechanism of resistance transfer since the discovery of antibiotic resistance gene (ARG) transfer in the 1950s. Bacterial conjugation requires direct contact of cells or the contact of pili between two bacteria, namely a donor and a recipient. The donor cell must contain a conjugative plasmid. The transfer regions of these plasmids encode proteins for the formation of pilus and the DNA transfer proteins that facilitate the export of the DNA strand. As a result of conjugation both cells become donor cell for the transferred conjugative plasmid (Partridge *et al.,* 2018).

Transduction is the transfer of genetic information between bacteria through bacteriophages. Bacteriophages have broad host ranges and they are more persistent than the free DNA. These properties make bacteriophages an important source of gene transfer in the environment (Berglund, 2015). Recent studies indicated that bacteriophages have significant effect on the dissemination of ARGs. Many studies show the presence of various ARGs in bacteriophages from different aquatic environments (Lluch *et al.*, 2011; Brown-Jaque *et al.*, 2015; Lood *et al.*, 2017).

ARGs have increased chance of dissemination when they are integrated with mobile genetic elements (MGEs) such as plasmids, transposons and integrons. Plasmids are extra-chromosomal circular DNA, with the ability of self-replication and most of them have conjugative properties that allow them to transfer among bacteria (Madigan *et al.,* 2015). They also contain adaptive genes like resistance genes and related MGEs like transposons, integrons or insertion sequences within their structure. These MGEs are usually contained one or more ARGs and involved in intracellular mobility (Partridge *et al.,* 2018). Therefore, plasmids harboring these structures are responsible for the widespread dissemination of ARGs. The main structure of *qnrS1*-carrying plasmid pINF5 isolated from *Salmonella* spp. is given in Figure 1.14.

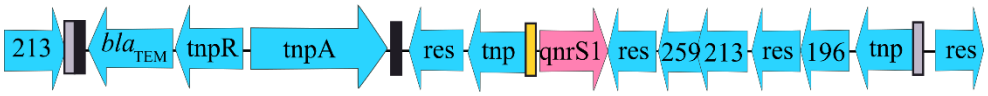

Figure 1.14. The composition of pINF5 plasmid harboring quinolone resistance gene qnrS1 (Léophonte *et al.*, 2004)

Transposition is a genetic exchange by translocation of a sequence between different regions of the same DNA or another source of DNA (Figure 1.15). Genetic materials that have ability for self-transposition are named transposable elements. Most transposable elements encode a basic structure of a transposase that mediates transposition. They also have sequences of inverted repeats (IRs) at the both end acting as recognising sites for the transposase enzyme (Bennett, 2004).

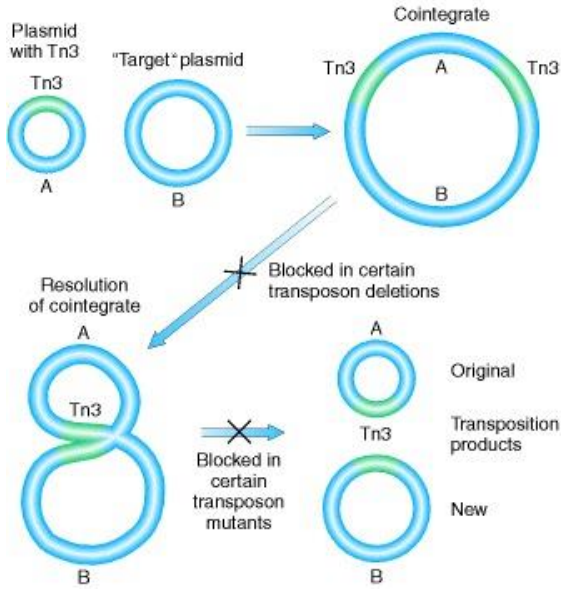

Figure 1.15. Transposition mechanism (Griffiths *et al.*, 2000)

Insertion sequences (ISs) are the simplest form of transposable elements. ISs are composed of a transposase enzyme coding region surrounded by IRs. They can contain various passenger genes including ARGs in their upstream or downstream regions (Siguier *et al.*, 2014). β-lactam resistance gene bla_{CTX} group is highly associated with

ISs. Schematic structure of the IS, IS*Ecp1* containing sequence of a CTX-M type β-lactamase given in Figure 1.16 (Zong *et al.*, 2010).

Figure 1.16. Schematic structure of IS*Ecp1* (Zong *et al.*, 2010)

Three groups of transposons are used for classification; composite transposons, complex transposons and conjugative transposons (Hegstad *et al.*, 2010). Structures of these groups shown in Figure 1.17.

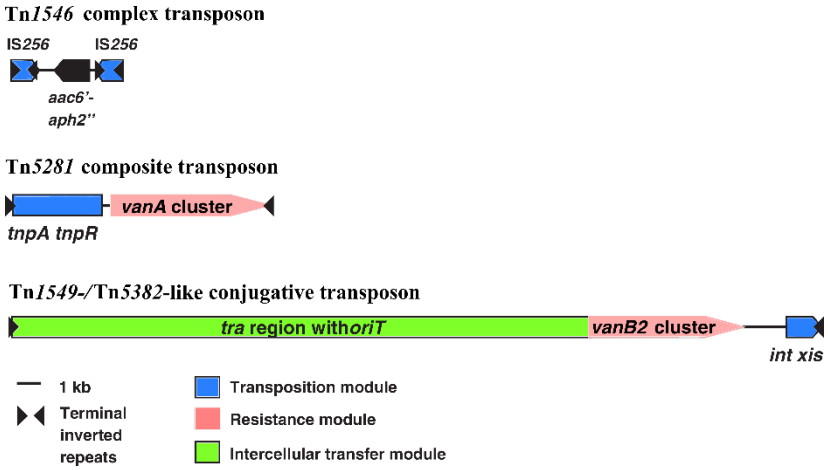

Figure 1.17. Main structures of transposon classes (Hegstad *et al.*, 2010)

Composite transposons are mobilised with two copies of ISs at the flanking sites of the included genes. They are also associated with ARGs and most of them usually encode resistance to at least one antibiotic. A composite transposon *Tn*10 and its structure with tetracycline resistance gene are illustrated in Figure 1.18 (Haniford, 2006).

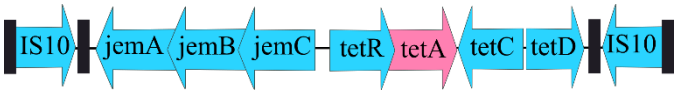

Figure 1.18. Structure of Tn10 and *tetA* resistance gene (Haniford, 2006)

Complex transposons have different genetic organization that enables their mobility with a transposase (TnpA) and a site-specific recombination enzyme (TnpR) that is specific to res site located near TnpR. They are responsible for the dissemination of highly resistant phenotypes to the glycopeptide and MLS group antibiotics (Sultan *et al.,* 2018). The structure of complex transposon *Tn*917 conferring resistance to macrolides is given in the Figure 1.19.

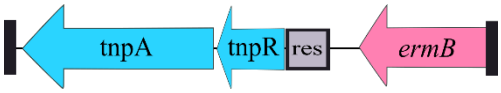

Figure 1.19. The structure of Tn917and ermB resistance gene (Grosso *et al*., 2009)

Conjugative transposons are capable of their own excision, conjugation and integration which provide both intracellular and intercellular mobility (Hegstad *et al.,* 2010). They are considered as integrative conjugative elements (ICEs) and most of them carry ARGs as their accessory genes and cause ARGs dissemination among different species. *Tn*7, discovered as a part of R plasmid, has two resistance genes *dfrI* and *aadA* conferring resistance to trimethoprim and aminoglycosides respectively (Bennett, 2004). The structure of *Tn*7 conjugative transposon is presented in Figure 1.20.

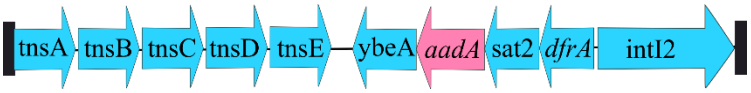

Figure 1.20. The structure of conjugative transposon Tn7 and *aadA* resistance gene (Partridge *et al*., 2018)

A gene cassette is a small mobile element that is usually found in the structure of transposable elements. They are large enough to harbour a gene or two including ARGs and captured by integrons via site specific recombination (Figure 1.21). Integrons are transposable elements that carry an integrase enzyme and a specific recognition site (attI) for recombination and integration of gene cassettes (Sultan *et al.*, 2018).

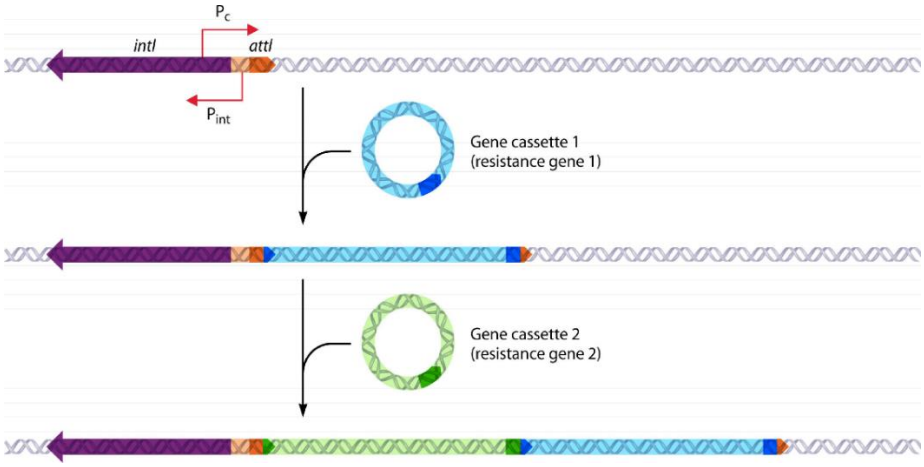

Figure 1.21. Bacterial integrons: capture and release of gene cassettes (Davies & Davies, 2010)

There are several classes of integrons based on the integrase gene type. Class 1 integrases are highly associated with ARGs and they have conserved region containing quaternary ammonium compound resistance gene (*qacEΔ1*), sulfonamide resistance gene (*sul1*) and an open reading frame (*orf5*) (Canal *et al.*, 2016). A class 1 type integron In4 characterized by having an IS IS6100 in its structure together with various ARGs including chloramphenicol resistance gene *cmlA* (Figure 1.22).

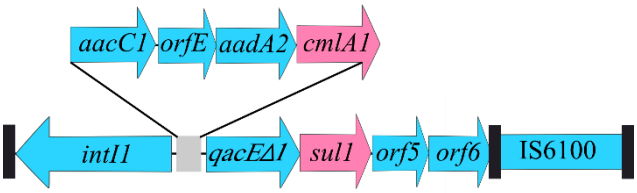

Figure 1.22. The structure of In4 integron with sul1 and cmlA ARGs (Partridge *et al*., 2002)

Occurrence of antibiotics and antibiotic resistance is old as the bacteria itself. Antibiotics are used by bacteria to increase their survival and to be advantageous against their rivals for the use of resources. Also, antibiotic resistance emerged for the same purpose (Berglund, 2015). After the discovery of the antibiotics, these miracle drugs were started to be used extensively. The antibiotic usage was not limited to clinical application. The extensive use of antibiotics in medicine, agriculture, and aquaculture eventually led to the widespread emergence of resistant bacteria in various settings. Increased antibiotic levels created selective pressure on microbial consortia resulting increased antibiotic resistance rates (Von Wintersdorff *et al*., 2016). HGT is the main mechanism that facilitates and ARGs dissemination among bacteria under the selective pressure of contaminated water habitats. This leads an enhanced dissemination of antibiotic resistant bacteria (ARB) and ARGs from anthropogenic sources to the natural environment (Figure 1.23) (Vikesland *et al*., 2017).

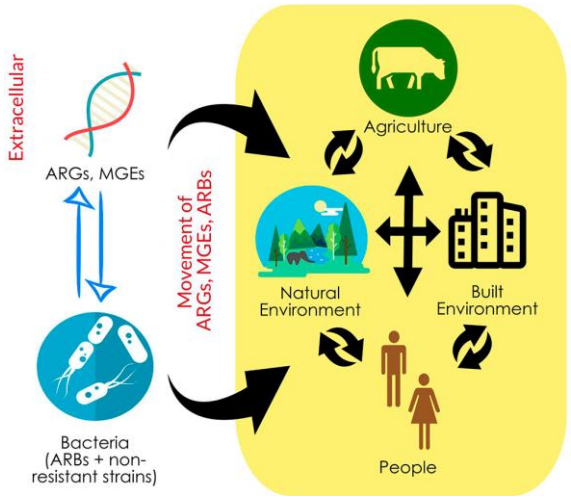

Figure 1.23. Potential dissemination routes for the ARGs (Vikesland *et al*., 2017)

Among all anthropogenic pollution sources hospitals display an important role for the ARG dissemination. Constant use of antibiotics and other therapeutic chemicals create a unique blend of wastewaters with toxic characteristics. These toxic wastewaters enhance HGT among clinical resistant pathogens and environmental susceptible bacteria (Rodriguez-Mozaz *et al*., 2015).

1.5. Hospital wastewaters

Hospitals are health care facilities providing short- and long-term medical care for patients. They typically have wide range of departments such as; emergency, laboratory, radiology, pathology, intensive care units and also non-medical units like kitchen and laundry (Orias & Perrodin 2013). Mainly hospitals generate a significant amount of these wastewaters from these units, ranging from 400 to 1200 L daily per bed (Gautam *et al.,* 2007). The wide range of services produce wastewaters with various toxic pollutants. Therefore, Hospital wastewaters (HWWs) have specialized characteristics due to pollutants including; ARB, antibiotics, pharmaceutical residues, laboratory chemicals, radioactive elements, heavy metals and organic materials (Kumarathilaka *et al.*, 2015). HWWs may be characterized by using chemical and bacteriological parameters. These parameters include pH, temperature and pollutants like antibiotics, ARB, pharmaceutical residues, heavy metals and toxic chemicals (Kusuma *et al.,* 2013). Biochemical oxygen demand (BOD), chemical oxygen demand (COD) and total suspended solids (TSS) are routine tests to determine pollution levels of a water source. BOD, COD and TSS values were found to have 2-3 times higher in HWWs than in domestic wastewaters (Verlicchi, 2017). In many countries, there are no regulations specific about HWWs (Carraro *et al*., 2018). In Turkey, there are no regulations specific to HWWs and they are considered as domestic wastewaters. Due to regulations these domestic wastewaters including HWWs can be discharged directly to the receiving waters when they met criteria given in Table 1.1 (Su kirliliği kontrolü yönetmeliği, 2004). However, these criteria have no limitations on the ARGs. Also there is no country that has regulation on the ARGs discharge standards.

Table 1.1. Discharge limits for domestic wastewaters for 24h composite samples (Su kirliliği kontrolü yönetmeliği, 2004)

Organic water pollutant emissions (Kg/day)	Equivalent population	BOD_5 (mg/L)	COD (mg/L)	TSS (mg/L)
5-120	84-2000	45	120	45
120-600	2000-10000	45	110	30
600-6000	10000-100000	45	100	30
>6000	>100000	35	90	25

BOD_5, biochemical oxygen demand; COD, chemical oxygen demand; TSS, total suspended solids.

Hospital effluents should be considered as different type of wastewaters since their pollutant content increase their cytotoxicity and genotoxicity potentials (Sharma *et al.*, 2014). Also the mixture of pollutants create a perfect environment for the dissemination of antibiotic resistance genes among clinical pathogens and environmental bacteria (Rodriguez-Mozaz *et al.,* 2015). These antimicrobials in wastewater habitats create a selective pressure on microbial populations and increase spread of antibiotic resistance by facilitating HGT (von Wintersdorff *et al.,* 2016). Most of the resistant bacteria released from HWWs can survive and persist in aquatic environments even after treatment (Katouli *et al.,* 2012). It is proved that non-antibiotic disinfectants found in HWWs can induce the emergence of antibiotic resistance and increase resistance rates via cross resistance (Lu *et al.*, 2018). Therefore, HWWs become a great threat for environment since HWWs act as a main vector for transportation of ARB and ARGs. Hospital effluents can contaminate water bodies used in agriculture and households. When untreated hospital effluents are discharged into receiving water bodies, ARGs and ARB can easily disseminate and increase antibiotic resistance exposure which cause great danger to the public health (Lucas *et al.,* 2016).

1.6. Aim of the study

Antibiotics are the most important drugs for treating infectious diseases and are also used for growth promoters and prophylactics in livestock. Intensive usage of antibiotics increases the exposure of ARB and ARGs to the natural habitats. HGT is responsible for the dissemination of ARGs in environment. Emergence and spread of ARGs have become a worldwide health problem in recent decades. Providing a suitable environment for proliferation of ARGs, untreated HWWs may pose great danger to environment and public health. Therefore, the aim of this study to evaluate the potential impact of HWWs on the dissemination of ARGs. For that reason, seasonally collected effluents of six different hospitals were analysed for overall bacterial genes and seven ARGs of *aadA, tetA, sul1, cmlA, qnrS, ermB* and $bla_{\text{CTX-M}}$ corresponding to commonly used antibiotics aminoglycosides, tetracyclines, sulfonamides, amphenicols, quinolones, MLS group and β-lactams, respectively by using quantitative polymerase chain reaction (qPCR).

CHAPTER 2

MATERIALS AND METHODS

2.1. Collection and fixation of HWW effluents

HWW effluents were collected from six major hospitals in Ankara, Turkey and designated as H1, H2, H3, H4, H5 and H6 with bed capacities of 160, 270, 468, 484, 730 and 1140, respectively. The samples were collected on seasonal intervals in between 2016-2017. In an amount of 50 mL samples were taken in triplicate using sterile equipment and immediately transported to the laboratory in a portable ice box for sample fixation process. The samples were centrifuged for 20 min at 4000 rpm then the pellets were collected. Fixation of the collected pellets was done with 50% ethanol-water mixture. (Yang *et al*., 2014). All the samples were stored in -20ºC prior to DNA extraction.

2.2. Total DNA extraction from fixed HWWs

Fixed HWWs were used to extract total DNA using alkaline lysis protocol adopted from Sambrook & Russell (2006) with some modifications. The chemicals used for buffers and solutions in the DNA extraction shown in Table 2.1. Total DNA extraction steps shown in Figure 2.1. Fixed samples were centrifuged using 1.5 mL polypropylene tubes at 4.000 rpm at 4ºC for 10 min. Then the supernatants were removed. Resulting pellets were resuspended in 200 µL ice cold lysis solution I with vigorous vortexing. The amount of 400 µL of lysis solution II was added into the tubes. Tubes were mixed by inverting several times and incubated on ice. An amount of 300 µL of ice-cold lysis solution III was added into the tubes.

Table 2.1. Chemicals and buffers used in total DNA extraction

Chemicals	**Suppliers**
Acetic acid glacial	≥99.85%Sigma Aldrich, Germany
Ethyl alcohol	>99.8 %, Sigma Aldrich, Germany
Ethylenediamine tetra acetic acid	Sigma Aldrich, Germany
Isopropanol	>99.8 %, Merck, Germany
Potassium acetate	Merck, Germany
Sodium dodecyl sulphate (SDS)	Merck, Germany
Sodium hydroxide	Sigma Aldrich, Germany
Tris base	>99.9 %, Sigma Aldrich, Germany
Lysis solution I	50 mM glucose, 25 mM Tris-Cl, EDTA, ph:8.0
Lysis solution II	0.2 N NaOH, 1%(w/v) SDS
Lysis solution III	3 M potassium acetate, 5 M acetic acid
TE buffer	10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0)

Then the tubes were mixed by inverting several times and incubated on ice for 2-3 min. The tubes were centrifuged at 14.000 rpm at 4ºC for 5 min. The supernatants were transferred to new tubes without taking any white precipitates. An amount of 600 µL isopropanol was added into the tubes and mixed by vortexing. Tubes were incubated at room temperature for 2 min and centrifuged at 14.000 rpm for at room temperature 5 min. Resulting pellets were washed with 1 mL of 70% ethanol solution by centrifuging at 14.000 rpm at room temperature for 2 min. After the removal of supernatants, the remaining ethanol air dried completely at room temperature. Finally, the pellets resuspended in 50 µL of TE buffer. Quality and the concentration of samples were measured with Colibri Microvolume Spectrophotometer (Titertek Berthold, Germany). The template concentrations were adjusted to 1-5 ng/µL for polymerase chain reaction (PCR) analyses. The extracted DNA solutions were kept at -20ºC until quantitative analyses.

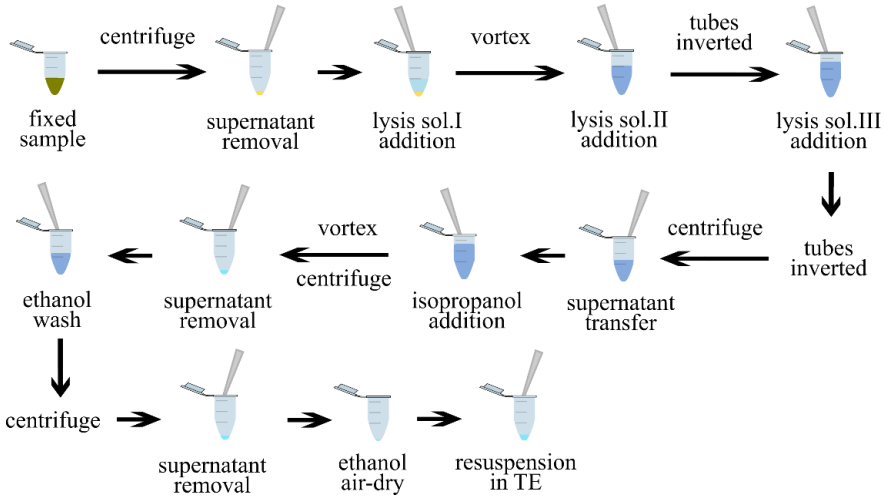

Figure 2.1. Steps followed in total DNA extraction

2.3. Qualitative analyses of ARGs

Specific primer pairs were selected for seven ARGs including *aadA, tetA*, *sul1*, *cmlA, qnrS, ermB* and *bla*$_{CTX-M}$, corresponding to antibiotic classes of aminoglycosides, tetracyclines, sulfonamides, amphenicols, quinolones, MLS group, and β-lactams, respectively. These antibiotics were selected due to their common use in Turkey (PCRTürkiye İlaç ve Tıbbi Cihaz Kurumu, 2017). Copy numbers of 16S rRNA gene were also quantified to monitor total bacterial load. PCR assays were performed for the qualitative analyses of ARGs (Figure 2.2).

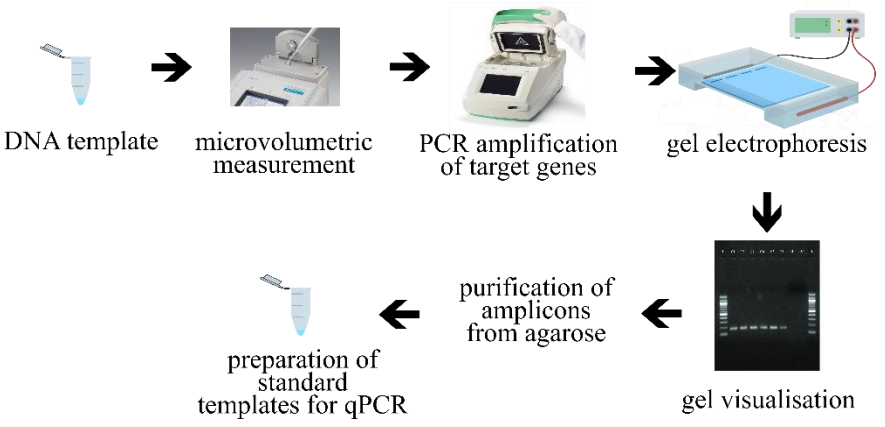

Figure 2.2. Steps followed in qualitative analyses of ARGs

The primer pairs used in this study, their properties and optimum conditions are shown in Table 2.2. PCR reactions were first carried out to optimize annealing temperature and primer concentration. The reactions were performed with the T100 Thermal Cycler (Bio-Rad, USA). The PCR mixture of 25 µL contained 1.4-5 ng template DNA, 2.5 µL of 10X PCR buffer, 1.5 mM of MgCl2, 2 mM dNTPs, forward and reverse primers and 0.125 µL Taq DNA Polymerase (NEB, USA). The thermal cycling steps for PCR amplification were as follows; initial denaturation at 95ºC for 30 sec, 30 cycles of denaturation at 95ºC for 15 sec, annealing at given temperature for 30 sec, elongation at 68ºC for 30 sec and at the end of cycles final extension at 68ºC for 5 min. PCR reactions were performed in duplicates to ensure the reproducibility of the experiments and negative controls were also included in each PCR reaction. Standard curves that were used in qPCR analyses were constructed using PCR amplicons of target genes (Table 2.3) acquired from the DNA templates of resistant bacteria supplied by Icgen & Yilmaz (2014). The amplicons of *Escherichia coli* (DSM 6897) were used for the construction of 16S rRNA standard curve. After the PCR amplification, samples were run in 1.5% agarose gel at 80V for 1 h. For the UV visualization agarose gels were stained with RedSafe (Intron, Korea). The amplicon sizes were determined by using 100 bp DNA ladder (NEB, USA) that was loaded in each gel electrophoresis. The bands corresponding to each target ARGs were visualized under UV light. PCR Clean-up Kit (Macherey-Nagel, Germany) was used for the purification of amplicons from the agarose gel. Purified PCR amplicon concentrations were measured with Colibri Microvolume Spectrophotometer (Titertek Berthold, Germany). The copy number of the ARGs per µL were calculated by using following formula;

$$\text{copy number of ARG / µL} = (L \times C) / (N \times M \times 10^9)$$

where, L is Avogadro's constant (6.02×10^{23}/ mol), C is the mass concentration of amplicon in nanogram per microliter, N is the length of amplicon of ARG and M is the molecular weight of an average base pair of DNA (660 g/mol) (Zhang *et al*., 2009).

Table 2.2. Primers and PCR conditions used in the study

Targeted genes	Primer sequence (F-R) (5'→ 3')	Amplicon size (bp)	Annealing temperature (°C)	Primer concentrations (µM)	References
bla_{CTX-M}	AGTGAAAGCGAACCGAATC CTGTCACCAATGCTTTACC	365	55	0.16	Wen *et al*., 2016
sul1	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	163	56	0.24	Pei *et al.*, 2006
tetA	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	210	60	0.20	Ng *et al*., 2001
aadA	AAATTCTTCCAACTGATCTGCG CCTGAACAGGATCTATTTGAGGC	276	54	0.24	Tian *et al*., 2016
ermB	GATACCGTTTACGAAATTGG GAATCGAGACTTGAGTGTGC	364	60	0.20	Chen *et al*., 2007
qnrS	GTATAGAGTTCCGTGCGTGTGA GGTTCGTTCCTATCCAGCGATT	189	56	0.20	Mao *et al*., 2015
cmlA	GCCAGCAGTGCCGTTTAT GGCCACCTCCCAGTAGAA	158	56	0.28	Li *et al.*, 2013
16S rRNA	CGGTGAATACGTTCYCGG GGWTACCTTGTTACGACTT	143	54	0.50	Suzuki & Taylor, 2000

F, forward; R, reverse; Y, C or T; W, A or T; MLS, macrolide/lincosamide/streptogramin

Table 2.3. Sources for the ARGs used in the study

ARGs	Source	EMBL accession number	References
*bla*CTX-M	*Raoultella planticola* Ag11	KJ395359	Koc *et al.*, 2013
sul1	*Staphylococcus warneri* Co11	KJ395373	Yilmaz *et al.*, 2013
tetA	*Enterococcus faecalis* Cr07	KJ395365	Icgen & Yilmaz, 2014
aadA	*Staphylococcus aureus* Ba01	KJ395371	Yilmaz *et al.*, 2013
ermB	*Delftia acidovorans* Cd11	KJ209817	Icgen & Yilmaz, 2014
qnrS	*Staphylococcus aureus* Al11	KJ395360	Yilmaz *et al.*, 2013
cmlA	*Stenotrophomonas rhizophila* Ba11	KJ395362	Icgen & Yilmaz, 2014

EMBL, The European Molecular Biology Laboratory

2.4. Quantitative analyses of ARGs

Real-time qPCR was used to quantify the abundance of seven ARGs (*aadA, tetA*, *sul1*, *cmlA, qnrS, ermB* and *bla*$_{\text{CTX-M}}$) and total bacterial load (16S rRNA) (Figure 2.3). The qPCR reactions were performed using 2x GoTaq® qPCR Master Mix reagent system (Promega, USA) with TOptical Thermocycler (Biometra GmbH, Germany). Reactions were conducted in 20 µL mixtures containing 1 µL template DNA, 10 µL 2x Master Mix, 300 nM CXR passive reference dye, and forward, reverse primers (Table 2.2). Ten-fold serial dilutions of amplicons (obtained in qualitative analyses)

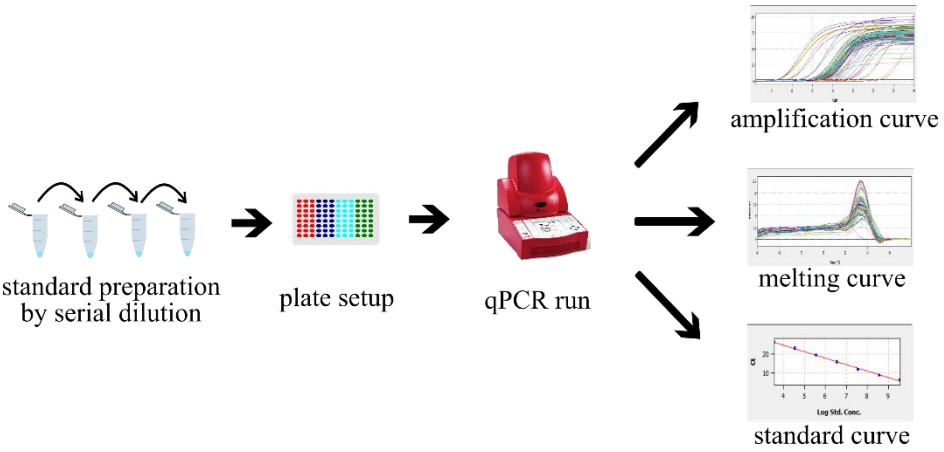

Figure 2.3. Steps followed in quantitative analyses of ARGs

Reactions were conducted in 20 µL mixtures containing 1 µL template DNA, 10 µL 2x Master Mix, 300 nM CXR passive reference dye, and forward, reverse primers (Table 2.2). Ten-fold serial dilutions of amplicons (obtained in qualitative analyses) containing the target gene were used to construct standard curves for each gene (Makowska *et al*., 2016). The DNA samples, standard curves and no template controls were included in every assay and analysed in triplicates. The qPCR data were analysed by using qPCRsoft Software (v. 3,1; Biometra GmbH, Germany). The cycling conditions included; initial denaturation at 95ºC for 2 min, following 40 cycles of denaturation at 95ºC for 15 sec, annealing for 30 sec, elongation at 60ºC for 30 sec. At the end of the cycles, melting curve was plotted by measuring fluorescence intensity of amplicons with each temperature change from 60ºC to 95ºC. Each PCR run was controlled in terms of qPCR efficiencies, R^2 values and melting curves. The qPCR efficiencies were in between 80-100% and R^2 values for all the standard curves constructed were higher than 0.99. Melting curve analysis was also used to determine the specificity of the amplification. The Limit of Quantification (LOQ) values for each qPCR run were taken as the lowest standard concentration that can be quantitatively detected with high precision (Armbruster & Pry, 2008).

2.5. Data analyses

Measurements of all the genes were normalized against the volume of HWW samples and relative abundances of ARGs were normalized against 16S rRNA gene copy numbers (ARG copy number/16S rRNA gene copy number). Seasonal variations of overall bacterial genes and all the ARGs were tested with a significance level of $p<0.05$ through One-way Analysis of Variance (ANOVA). Tukey's Post-hoc tests were also used to compare the variance among seasons separately for each season (SPSS Statics for Windows v.24,0; IBM Corp., Armonk, NY).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Qualitative analyses

Standard PCR assays were conducted with the specific primer pairs of the selected ARGs and 16 rRNA gene. PCR conditions were optimized by changing the annealing temperatures and primer concentrations. The resistant bacteria provided by Icgen and Yilmaz (2014) and *E.coli* (DSM 6897) were used for the extraction of the template DNA. For the construction of standard curves, amplicons purified from agarose gel measured with microvolumetric spectrometer and copy numbers were calculated with the equation given by Zhang *et al*. (2009). Standard curves were prepared with 10-fold dilutions to use for the qPCR assays. Reaction efficiency and specificity parameters were checked with R^2 values and melting curve analyses.

3.1.1. For overall bacterial gene (16S rRNA)

Overall bacterial gene (16S rRNA) specific primer pairs were selected from a study by Suzuki & Taylor (2000). In that study, optimum annealing temperature was 56 ºC and optimum primer concentrations were ranged from 0.1 to 1.5 µM. In the current study, DNA extracted from *Escherichia coli* (DSM 6897) was used as template for the optimization and construction of standard curves. Gradient PCR assays with temperature ranging in between 50- 54 ºC and primer concentrations in between 4.4-6.0 µM were conducted separately to determine the optimum values (Figure 3.1).

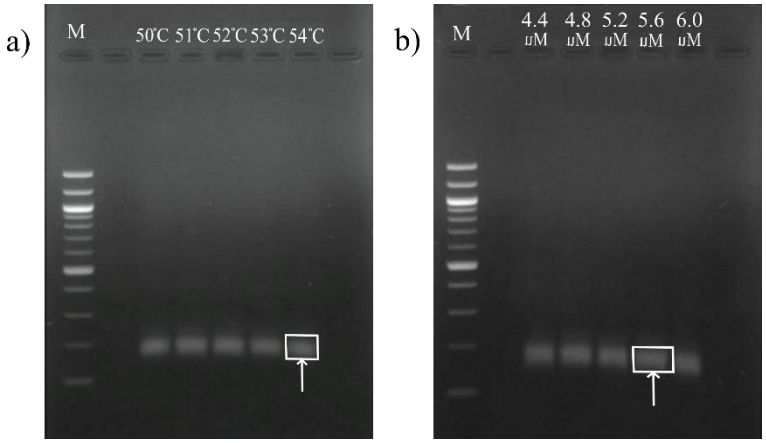

Figure 3.1. 16SrRNA gene optimization results. Annealing temperature gradient (a) and primer concentration gradient (b) reactions were conducted.100 bp DNA ladder were used as a marker (M) with band lengths from top to bottom 1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively.

Optimum annealing temperature was determined as 54 ºC and optimum primer concentration was determined as 5.6 µM (Figure 3.2). Optimized conditions were used in following qPCR assays.

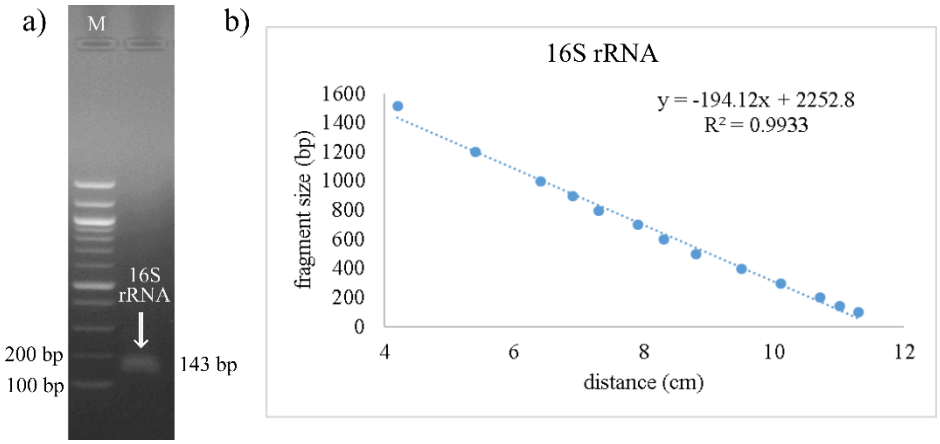

Figure 3.2. Gel electrophoresis results of the 16S rRNA gene with optimum conditions of 54 ºC annealing temperature and 5.6 µM primer concentration. M, 100 bp DNA ladder were used as a marker (a). Molecular weight estimation using agarose gel standard curve (b).

Purification of 16S rRNA gene amplicon from agarose gel resulted in 5.77 ng/µL template DNA which was calculated to have 3.74×10^{10} copy number/µL. Prepared 10-fold serial dilutions of template DNA were used to construct standard curves to use

in quantification for qPCR experiments (Figure 3.3). QPCR assays carried with efficiencies in between 80-100% and R^2 values more than 0.99. LOQs calculated for each qPCR run were at least 3500 copy numbers for the 16S rRNA gene.

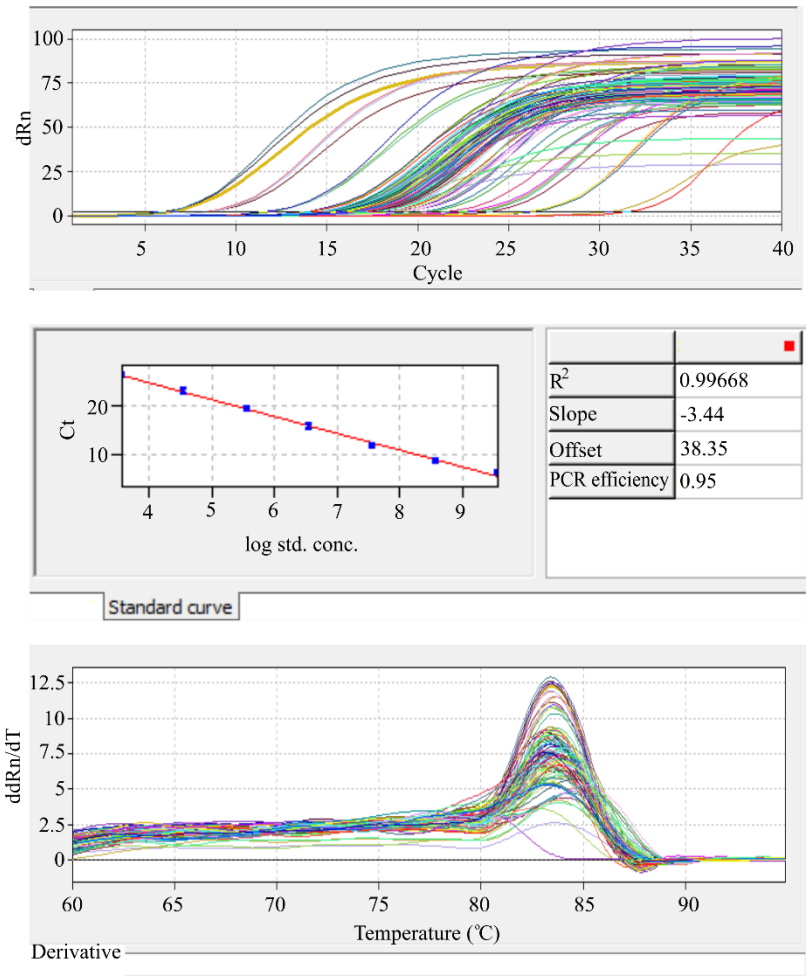

Figure 3.3. 16S rRNA gene qPCR analyses given as amplification (top), standard (middle) and melting (bottom) curve results. Ct, cycle threshold; dRn, fluorescence signal; ddRn/dT, derivative of fluorescence over temperature.

3.1.2. For the *aadA* gene

Aminoglycoside resistance gene *aadA* specific primer pairs were selected from a study by Tian *et al*. (2016). In that study, optimum annealing temperature was 58 ºC and optimum primer concentration was 0.4 µM. In the current study, DNA extracted from

Staphylococcus aureus Ba01 was used as template for the optimization and construction of standard curves. Gradient PCR assays with temperature ranging in between 54-58 ºC and primer concentrations in between 0.12-0.28 µM were conducted separately to determine the optimum values (Figure 3.4).

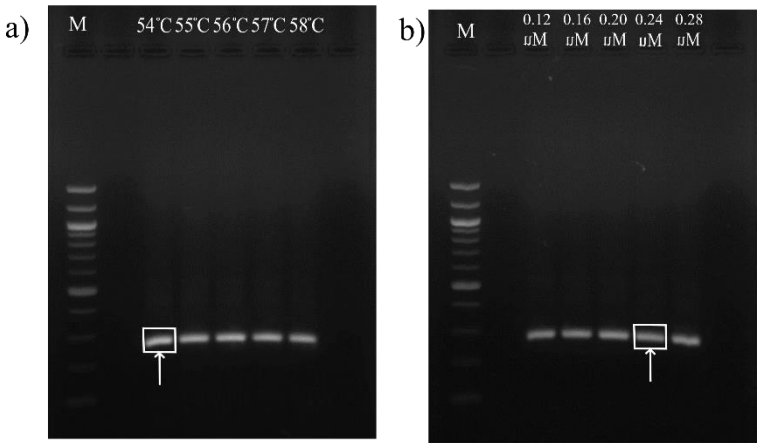

Figure 3.4. The *aadA* gene optimization results. Annealing temperature gradient (a) and primer concentration gradient (b) reactions were conducted.100 bp DNA ladder were used as a marker (M) with band lengths from top to bottom 1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively.

Optimum annealing temperature was determined as 54 ºC and optimum primer concentration was determined as 0.24 µM (Figure 3.5). Optimized conditions were used in following qPCR assays.

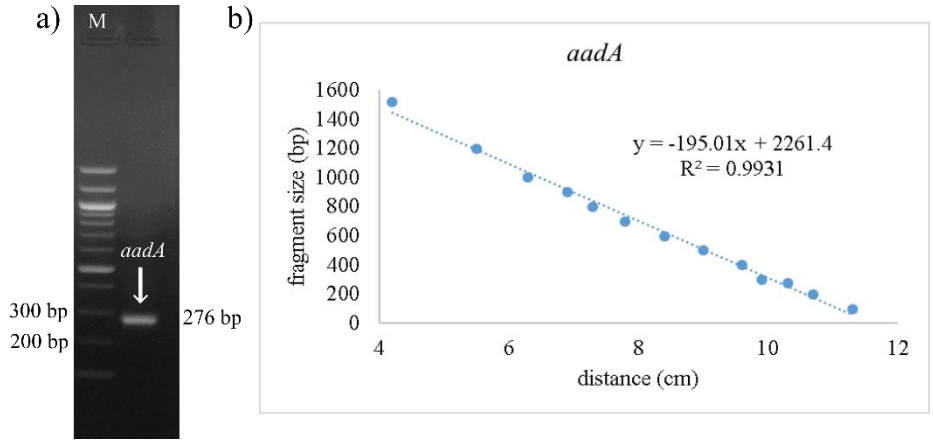

Figure 3.5. Gel electrophoresis results of the *aadA* gene with optimum conditions of 54 ºC annealing temperature and 5.6 µM primer concentration. M, 100 bp DNA ladder were used as a marker (a). Molecular weight estimation using agarose gel standard curve (b).

Purification of *aadA* resistance gene amplicon from agarose gel resulted in 6.8 ng/µL template DNA which was calculated to have 2.28×10^{10} copy number/µL. Prepared 10-fold serial dilutions of template DNA were used to construct standard curves to use in quantification for qPCR experiments (Figure 3.6). QPCR assays run with efficiencies in between 80-100% and R^2 values more than 0.99. LOQs calculated for each qPCR run were at least 1030 copy numbers for the *aadA* resistance gene.

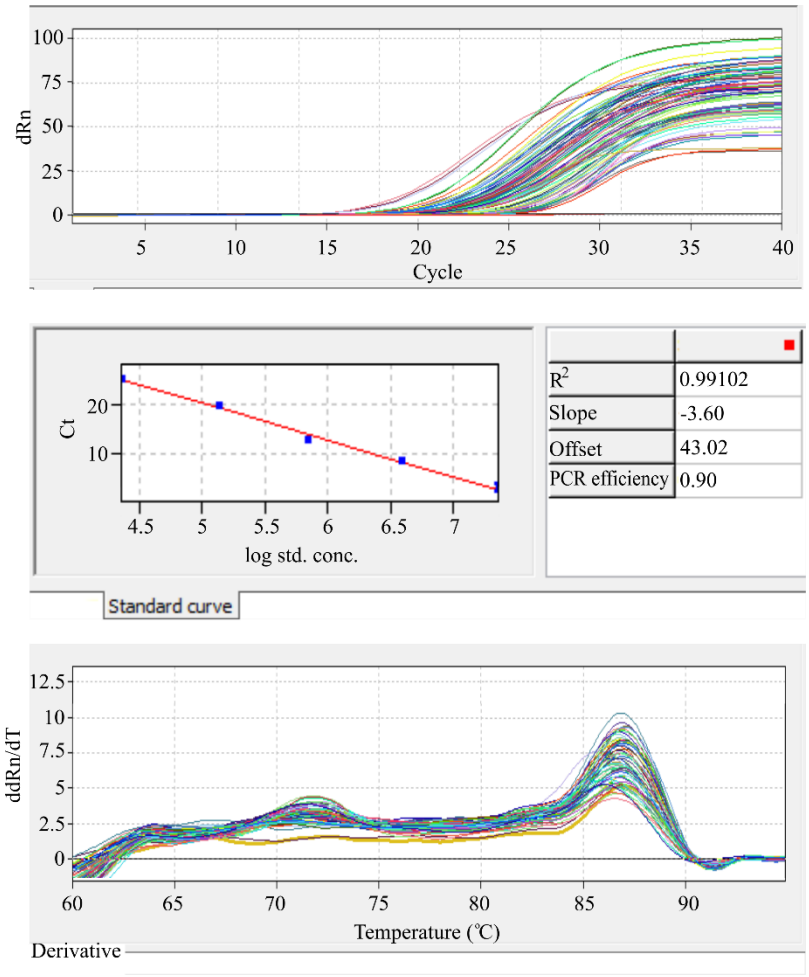

Figure 3.6. The *aadA* gene qPCR analyses given as amplification (top), standard (middle) and melting (bottom) curve results. Ct, cycle threshold; dRn, fluorescence signal; ddRn/dT, derivative of fluorescence over temperature.

3.1.3. For the *tetA* gene

Tetracycline resistance gene *tetA* specific primer pairs were selected from a study by (Ng *et al*., 2001). In that study, optimum annealing temperature was 60 ºC and optimum primer concentration was 0.16 µM. In the current study, DNA extracted from *Enterococcus faecalis* Cr07 was used as template for the optimization and construction of standard curves. Gradient PCR assays with temperature ranging in between 56-60 ºC and primer concentrations in between 0.12-0.28 µM were conducted separately to determine the optimum values (Figure 3.7).

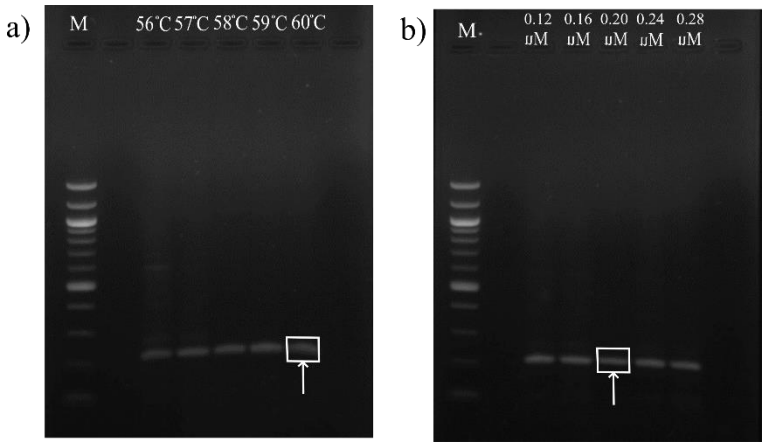

Figure 3.7. The *tetA* gene optimization results. Annealing temperature gradient (a) and primer concentration gradient (b) reactions were conducted.100 bp DNA ladder were used as a marker (M) with band lengths from top to bottom 1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively.

Optimum annealing temperature was determined as 60 ºC and optimum primer concentration was determined as 0.2 µM (Figure 3.8). Optimized conditions were used in following qPCR assays.

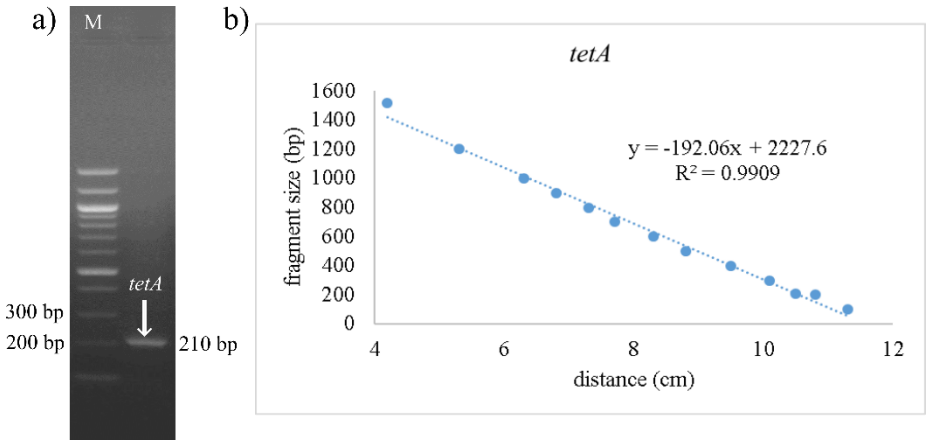

Figure 3.8. Gel electrophoresis results of the *tetA* gene with optimum conditions of 54 ºC annealing temperature and 5.6 µM primer concentration. M, 100 bp DNA ladder were used as a marker (a). Molecular weight estimation using agarose gel standard curve (b).

Purification of *tetA* resistance gene amplicon from agarose gel resulted in 7.5 ng/µL template DNA which was calculated to have 3.31×10^{10} copy number/µL. Prepared 10-fold serial dilutions of template DNA were used to construct standard curves to use in the quantification for qPCR experiments (Figure 3.9). QPCR assays run with efficiencies in between 80-100% and R^2 values more than 0.99. LOQs calculated for each qPCR run were at least 330 copy numbers for the *tetA* resistance gene.

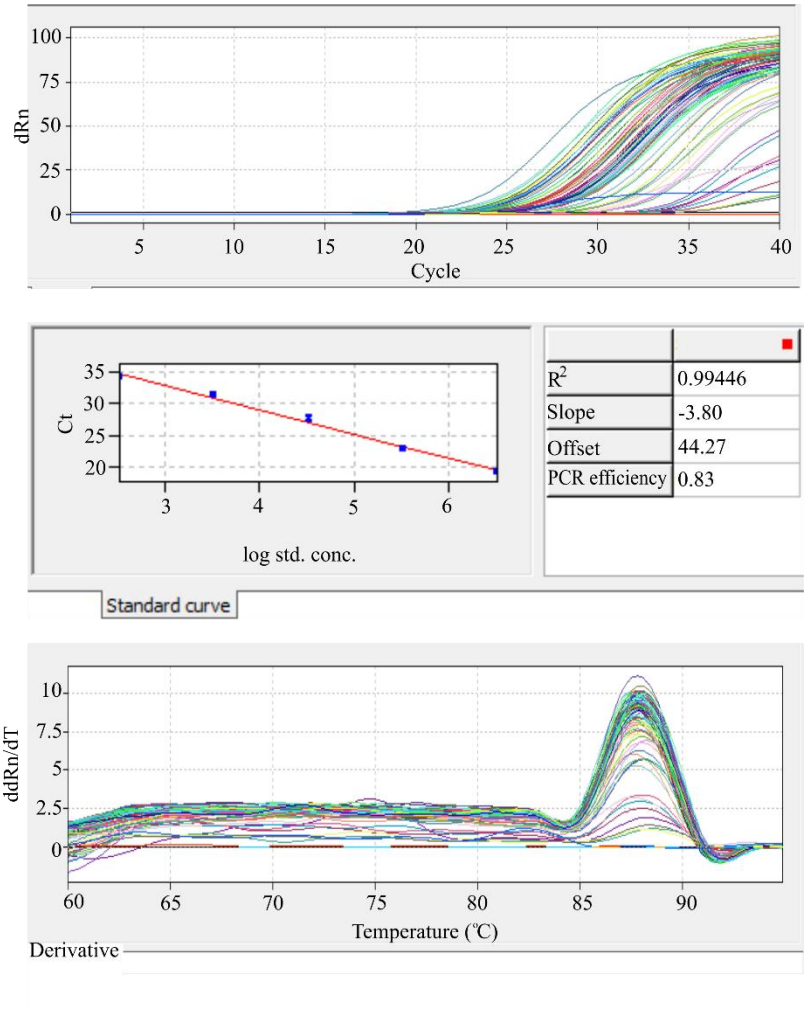

Figure 3.9. The *tetA* gene qPCR analyses given as amplification (top), standard (middle) and melting (bottom) curve results. Ct, cycle threshold; dRn, fluorescence signal; ddRn/dT, derivative of fluorescence over temperature.

3.1.4. For the *cmlA* gene

Chloramphenicol resistance gene *cmlA* specific primer pairs were selected from a study by Li *et al*. (2013). In that study, optimum annealing temperature was 60 ºC and optimum primer concentration was 0.16 µM. In the current study, DNA extracted from *Stenotrophomonas rhizophila* Ba11 was used as template for the optimization and construction of standard curves. Gradient PCR assays with temperature ranging in between 53-57 ºC and primer concentrations in between 0.12-0.28 µM were conducted separately to determine the optimum values (Figure 3.10).

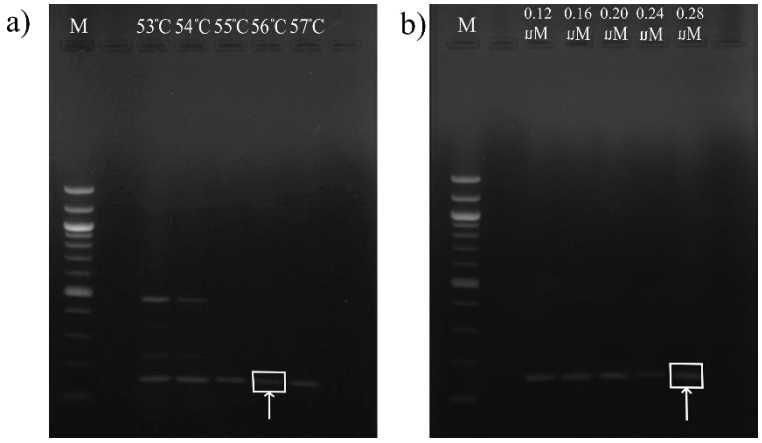

Figure 3.10. The *cmlA* gene optimization results. Annealing temperature gradient (a) and primer concentration gradient (b) reactions were conducted.100 bp DNA ladder were used as a marker (M) with band lengths from top to bottom 1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively.

Optimum annealing temperature was determined as 56 ºC and optimum primer concentration was determined as 0.28 µM (Figure 3.11). Optimized conditions were used in following qPCR assays.

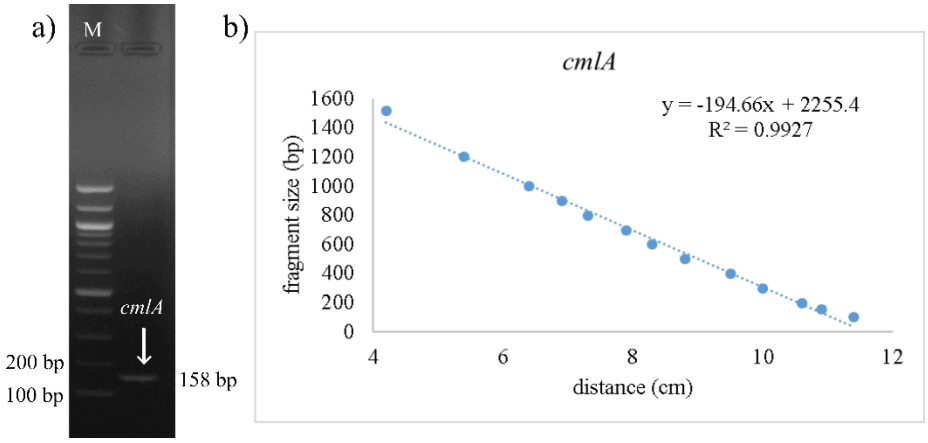

Figure 3.11. Gel electrophoresis results of the *cmlA* gene with optimum conditions of 54 ºC annealing temperature and 5.6 µM primer concentration. M, 100 bp DNA ladder were used as a marker (a). Molecular weight estimation using agarose gel standard curve (b).

Purification of *cmlA* resistance gene amplicon from agarose gel resulted in 7.5 ng/µL template DNA which was calculated to have 4.4×10^{10} copy number/µL. Prepared 10-fold serial dilutions of template DNA were used to construct standard curves to use

in the quantification for qPCR experiments (Figure 3.12). QPCR assays carried with efficiencies in between 80-100% and R^2 values more than 0.99. Quantification limits LOQs calculated for each qPCR run were at least 44 copy numbers for the *cmlA* resistance gene.

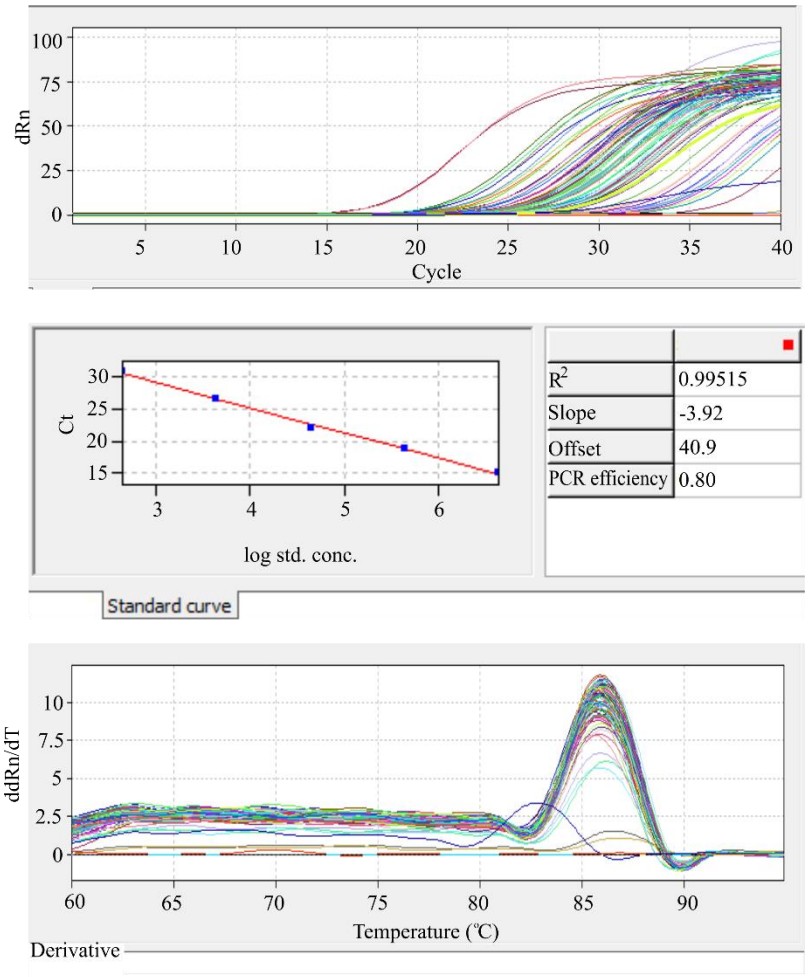

Figure 3.12. The *cmlA* gene qPCR analyses given as amplification (top), standard (middle) and melting (bottom) curve results. Ct, cycle threshold; dRn, fluorescence signal; ddRn/dT, derivative of fluorescence over temperature.

3.1.5. For the *sul1* gene

Sulfonamide resistance gene *sul1* specific primer pairs were selected from a study by Pei *et al*. (2006). In that study, optimum annealing temperature was 55.9 ºC and optimum primer concentration was 0.06 µM. In the current study, DNA extracted from *Staphylococcus warneri* Co11 was used as template for the optimization and construction of standard curves. Gradient PCR assays with temperature ranging in between 54-58 ºC and primer concentrations in between 0.12-0.28 µM were conducted separately to determine the optimum values (Figure 3.13).

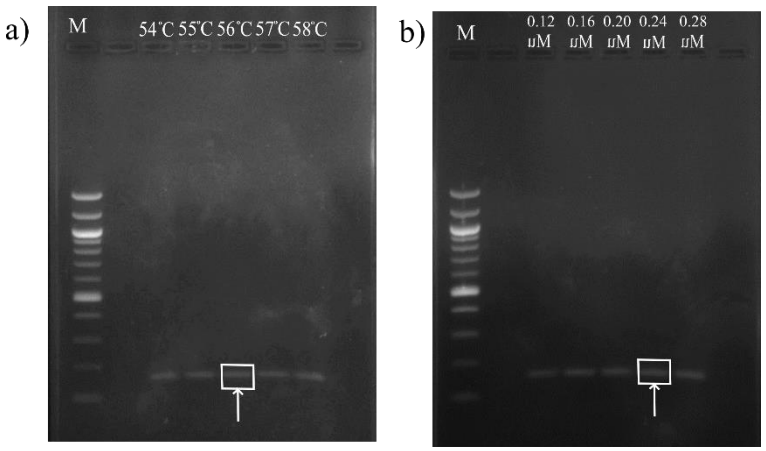

Figure 3.13. The *sul1* gene optimization results. Annealing temperature gradient (a) and primer concentration gradient (b) reactions were conducted.100 bp DNA ladder were used as a marker (M) with band lengths from top to bottom 1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively.

Optimum annealing temperature was determined as 56 ºC and optimum primer concentration was determined as 0.24 µM (Figure 3.14). Optimized conditions were used in following qPCR assays.

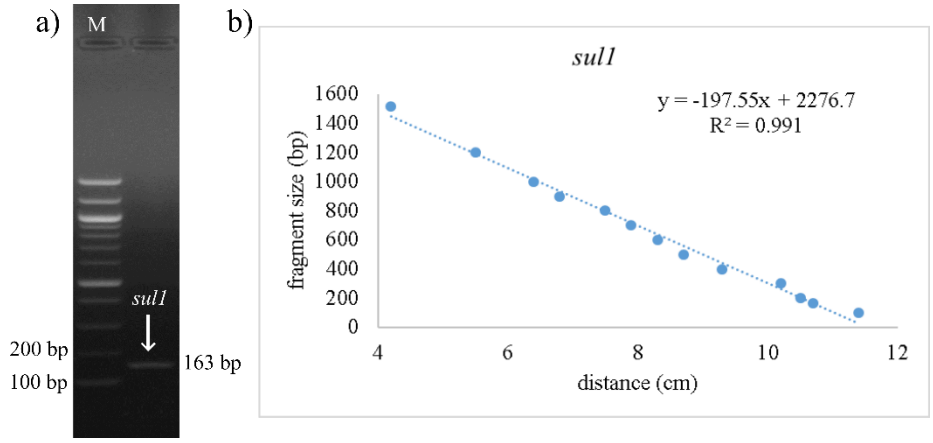

Figure 3.14. Gel electrophoresis results of the *sul1* gene with optimum conditions of 54 ºC annealing temperature and 5.6 µM primer concentration. M, 100 bp DNA ladder were used as a marker (a). Molecular weight estimation using agarose gel standard curve (b).

Purification of *sul1* resistance gene amplicon from agarose gel resulted in 1.95 ng/µL template DNA which was calculated to have 1.11×10^{10} copy number/µL. Prepared 10-fold serial dilutions of template DNA were used to construct standard curves to use in the quantification for qPCR experiments (Figure 3.15). QPCR assays run with efficiencies in between 80-100% and R^2 values more than 0.99. LOQs calculated for each qPCR run were at least 15 copy numbers for the *sul1* resistance gene.

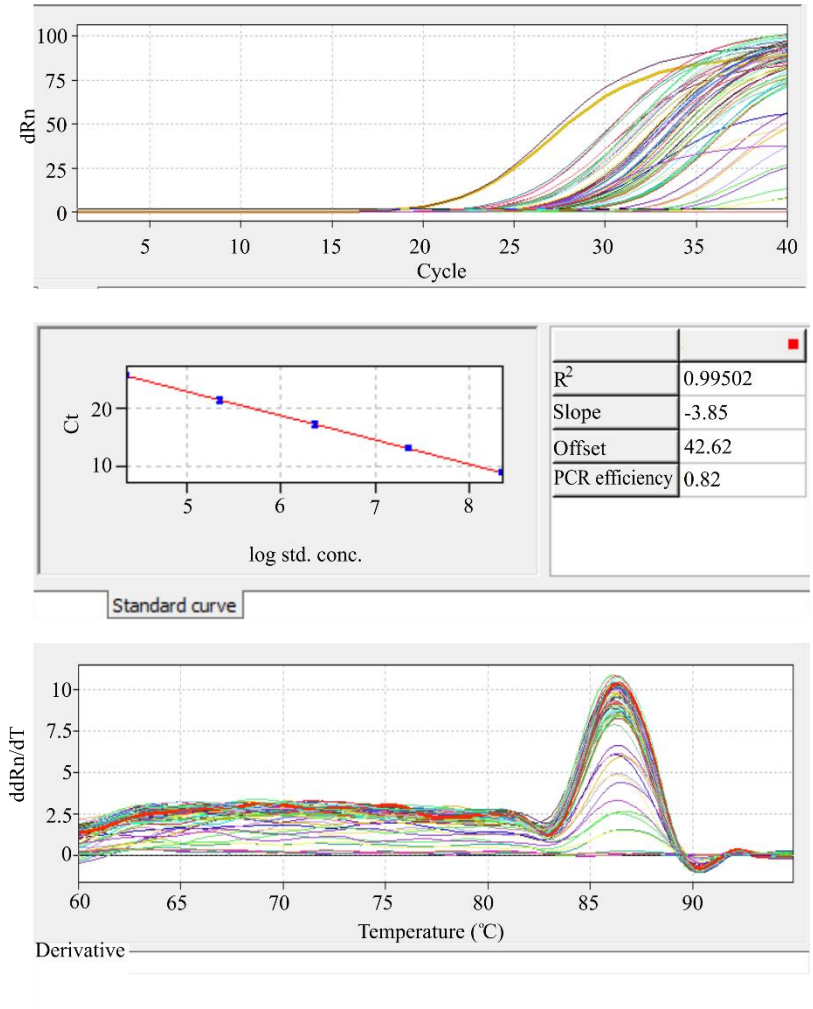

Figure 3.15. The *sul1* gene qPCR analyses given as amplification (top), standard (middle) and melting (bottom) curve results. Ct, cycle threshold; dRn, fluorescence signal; ddRn/dT, derivative of fluorescence over temperature.

3.1.6. For the *qnrS* gene

Quinolone resistance gene *qnrS* specific primer pairs were selected from a study by Mao *et al*. (2015). In that study, optimum annealing temperature was 54.6 ºC and optimum primer concentration was 0.2 µM. In the current study, DNA extracted from *S. aureus* Al11 was used as template for the optimization and construction of standard curves. Gradient PCR assays with temperature ranging in between 53-57 ºC and primer concentrations in between 0.12- 0.28 µM were conducted separately to determine the optimum values (Figure 3.16).

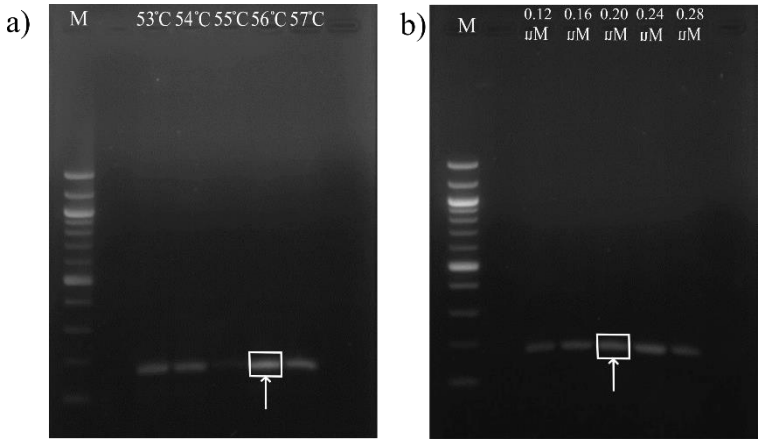

Figure 3.16. The *qnrS* gene optimization results. Annealing temperature gradient (a) and primer concentration gradient (b) reactions were conducted.100 bp DNA ladder were used as a marker (M) with band lengths from top to bottom 1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively.

Optimum annealing temperature was determined as 56 ºC and optimum primer concentration was determined as 0.20 µM (Figure 3.17). Optimized conditions were used in following qPCR assays.

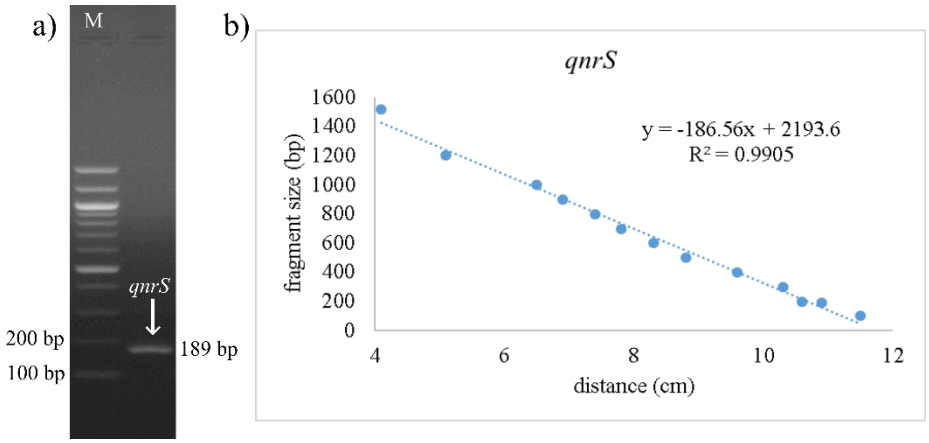

Figure 3.17. Gel electrophoresis results of the *qnrS* gene with optimum conditions of 54 ºC annealing temperature and 5.6 µM primer concentration. M, 100 bp DNA ladder were used as a marker (a). Molecular weight estimation using agarose gel standard curve (b).

Purification of *qnrS* resistance gene amplicon from agarose gel resulted in 10.3 ng/µL template DNA which was calculated to have 5.05×10^{10} copy number/µL. Prepared 10-fold serial dilutions of template DNA were used to construct standard curves to use

in the quantification for qPCR experiments (Figure 3.18). QPCR assays carried with efficiencies in between 80-100% and R^2 values more than 0.99. LOQs calculated for each qPCR run were at least 50 copy numbers for the *qnrS* resistance gene.

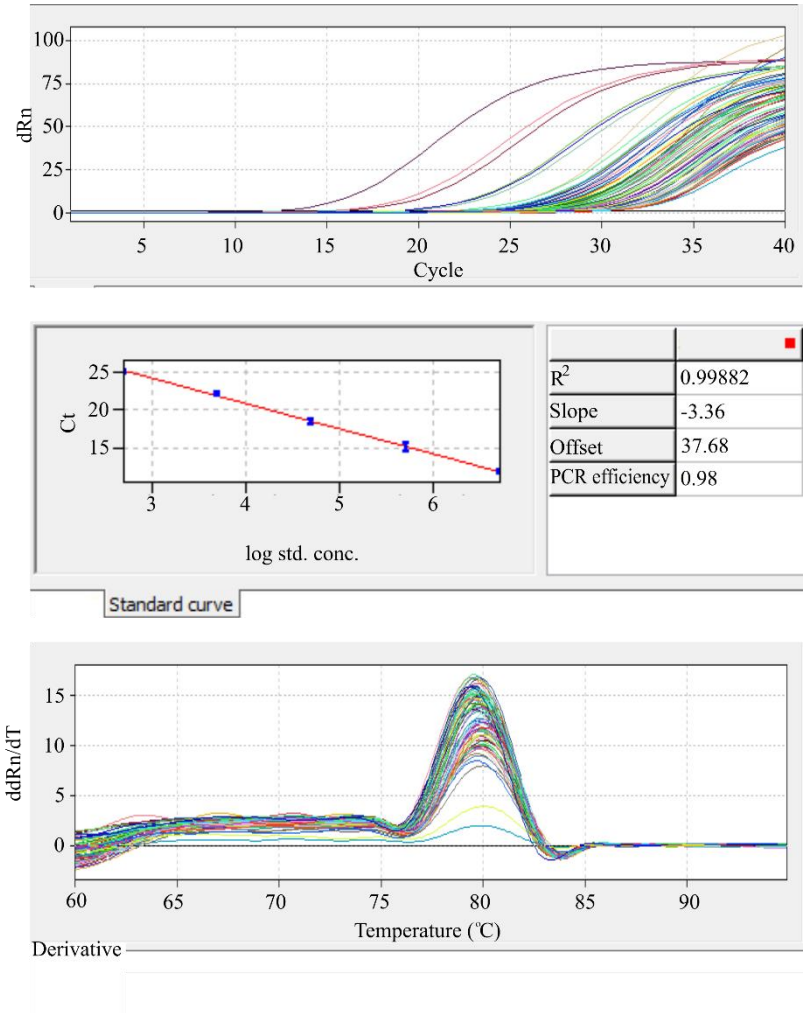

Figure 3.18. The *qnrS* gene qPCR analyses given as amplification (top), standard (middle) and melting (bottom) curve results. Ct, cycle threshold; dRn, fluorescence signal; ddRn/dT, derivative of fluorescence over temperature.

3.1.7. For the *ermB* gene

MLS group antibiotics resistance gene *ermB* specific primer pairs were selected from a study by Chen *et al*. (2007). In that study, optimum annealing temperature was 58

ºC and optimum primer concentration was 0.12 µM. In the current study, DNA extracted from *Delftia acidovorans* Cd11 was used as template for the optimization and construction of standard curves. Gradient PCR assays with temperature ranging in between 56-60 ºC and primer concentrations in between 0.12- 0.28 µM were conducted separately to determine the optimum values (Figure 3.19).

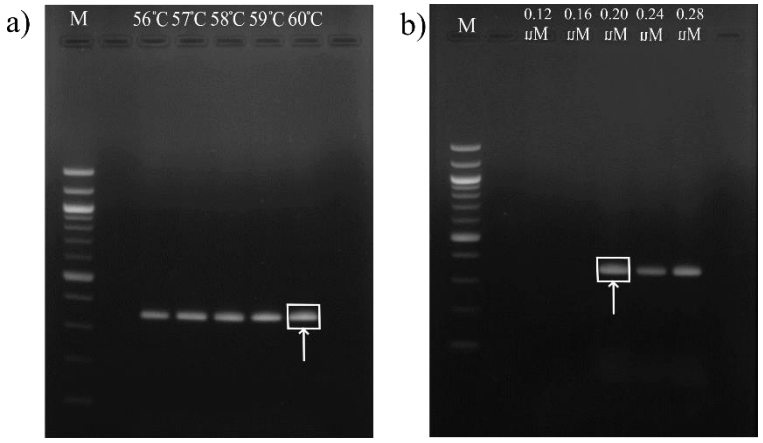

Figure 3.19. The *ermB* gene optimization results. Annealing temperature gradient (a) and primer concentration gradient (b) reactions were conducted.100 bp DNA ladder were used as a marker (M) with band lengths from top to bottom 1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively.

Optimum annealing temperature was determined as 60 ºC and optimum primer concentration was determined as 0.20 µM (Figure 3.20). Optimized conditions were used in following qPCR assays.

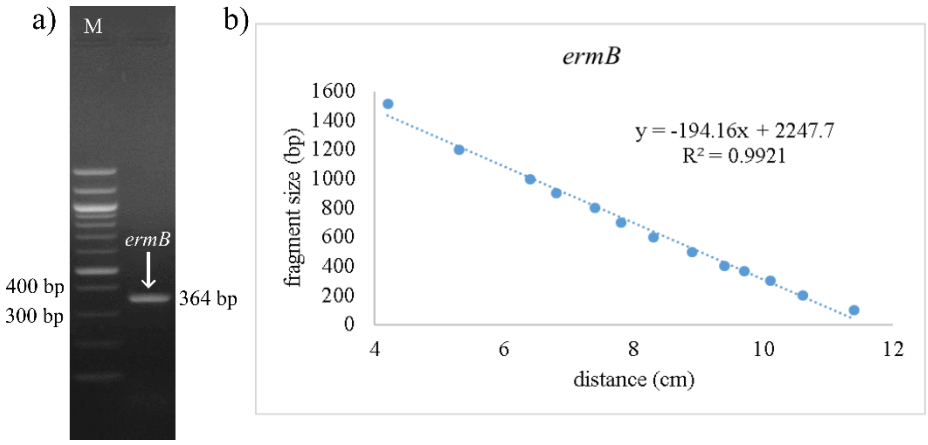

Figure 3.20. Gel electrophoresis results of the *ermB* gene with optimum conditions of 54 ºC annealing temperature and 5.6 µM primer concentration. M, 100 bp DNA ladder were used as a marker (a). Molecular weight estimation using agarose gel standard curve (b).

Purification of *ermB* resistance gene amplicon from agarose gel resulted in 1.43 ng/µL template DNA which was calculated to have 0.364×10^{10} copy number/µL. Prepared 10-fold serial dilutions of template DNA were used to construct standard curves to use in the quantification for qPCR experiments (Figure 3.21). QPCR assays carried with efficiencies in between 80-100% and R^2 values more than 0.99. LOQs calculated for each qPCR run were at least 37 copy numbers for the *ermB* resistance gene.

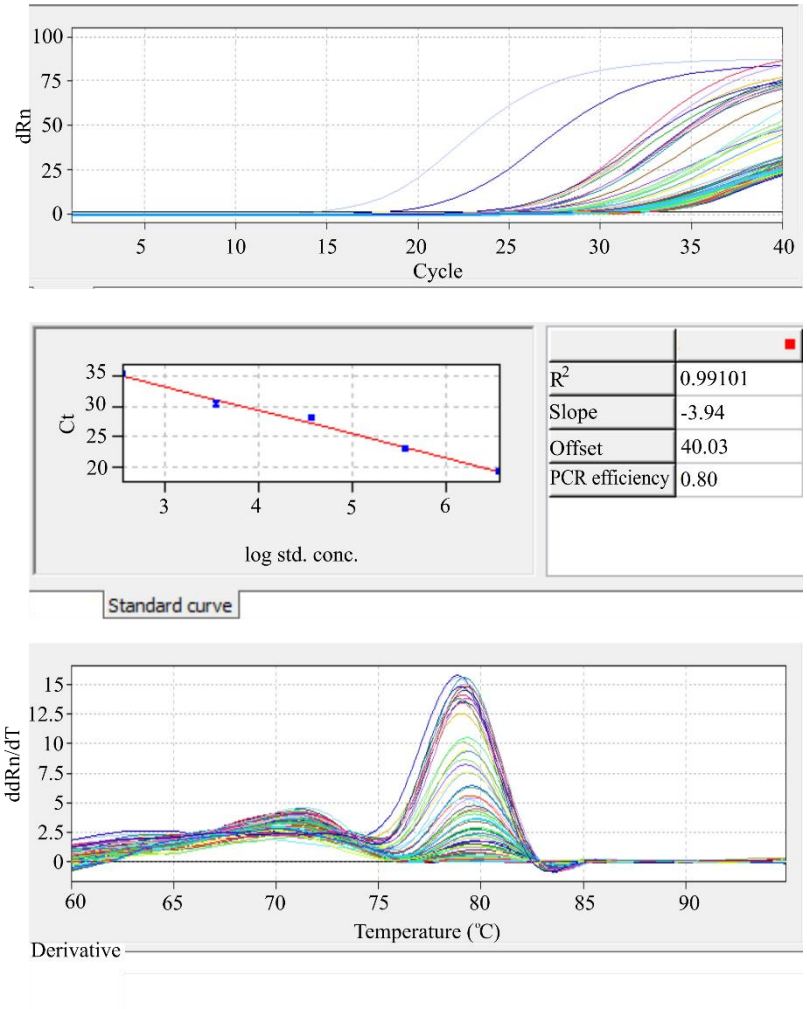

Figure 3.21. The *ermB* gene qPCR analyses given as amplification (top), standard (middle) and melting (bottom) curve results. Ct, cycle threshold; dRn, fluorescence signal; ddRn/dT, derivative of fluorescence over temperature.

3.1.8. For the *bla*$_{\text{CTX-M}}$ gene

β-lactam resistance gene *bla*$_{\text{CTX-M}}$ specific primer pairs were selected from a study by Wen *et al*. (2016). In that study, optimum annealing temperature was 55 ºC and optimum primer concentration was 0.32 µM. In the current study, DNA extracted from *Raoultella planticola* Ag11 was used as template for the optimization and construction of standard curves. Gradient PCR assays with temperature ranging in between 53-57 ºC and primer concentrations in between 0.12-0.28 µM were conducted separately to determine the optimum values (Figure 3.22).

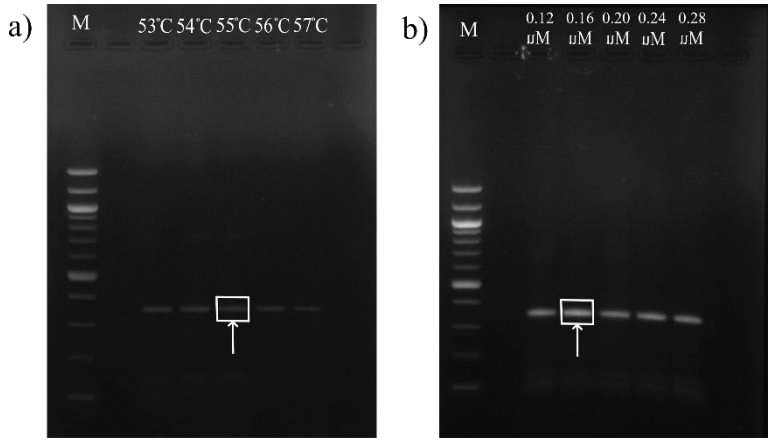

Figure 3.22. The *bla*$_{CTX-M}$ gene optimization results. Annealing temperature gradient (a) and primer concentration gradient (b) reactions were conducted.100 bp DNA ladder were used as a marker (M) with band lengths from top to bottom 1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp, respectively.

Optimum annealing temperature was determined as 55 ºC and optimum primer concentration was determined as 0.16 µM (Figure 3.23). Optimized conditions were used in following qPCR assays.

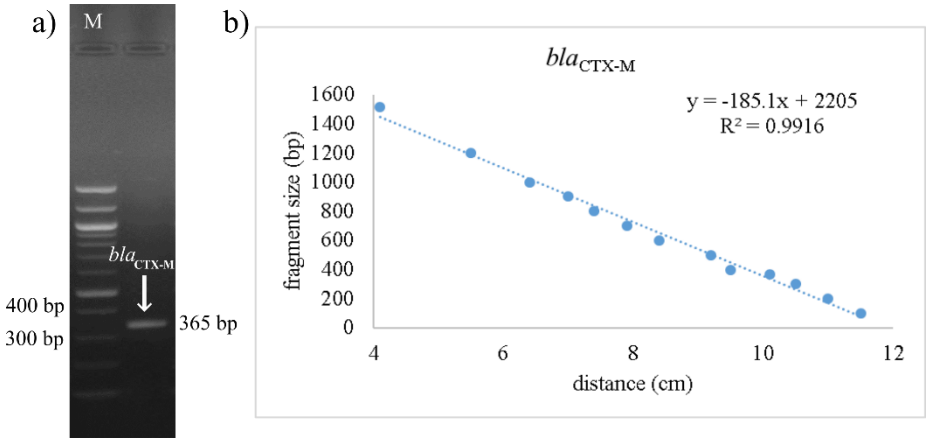

Figure 3.23. Gel electrophoresis results of the *bla*$_{CTX-M}$ gene with optimum conditions of 54 ºC annealing temperature and 5.6 µM primer concentration. M, 100 bp DNA ladder were used as a marker (a). Molecular weight estimation using agarose gel standard curve (b).

Purification of *bla*$_{CTX-M}$ resistance gene amplicon from agarose gel resulted in 5.5 ng/µL template DNA which was calculated to have 1.4×10^{10} copy number/µL. Prepared 10-fold serial dilutions of template DNA were used to construct standard

curves to use in the quantification for qPCR experiments (Figure 3.24). QPCR assays run with efficiencies in between 80-100% and R^2 values more than 0.99. LOQs calculated for each qPCR run were at least 7 copy numbers for the *bla*$_{CTX-M}$ resistance gene.

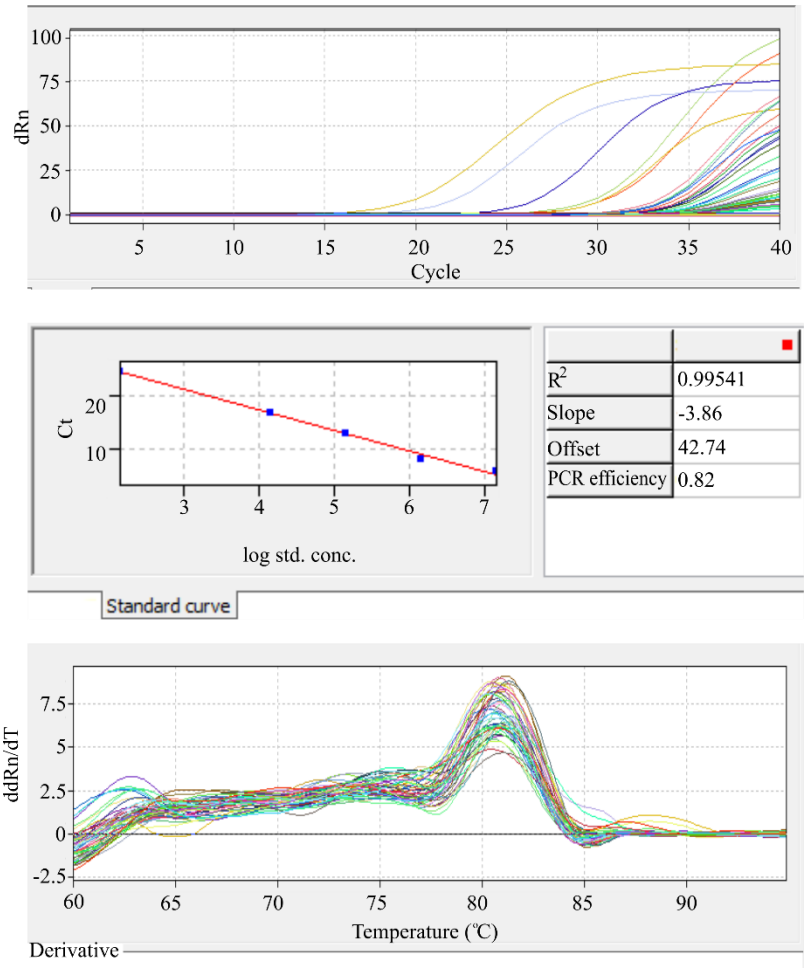

Figure 3.24. The *bla*$_{CTX-M}$ gene qPCR analyses given as amplification (top), standard (middle) and melting (bottom) curve results. Ct, cycle threshold; dRn, fluorescence signal; ddRn/dT, derivative of fluorescence over temperature.

3.2. Quantitative analyses

Seasonally collected effluents of six different hospitals (from the lowest H1 to the highest bed capacity H6) were analysed in terms of overall bacterial genes (16s rRNA) and seven ARGs of *aadA, tetA, cmlA, sul1, qnrS, ermB* and *bla*$_{CTX-M}$ corresponding to

commonly used antibiotics aminoglycosides, tetracyclines, amphenicols, sulfonamides, quinolones, macrolide-lincosamide-streptogramin group and β-lactams, respectively. The ARGs were quantified using qPCR to obtain the absolute values as copy number/mL and these values were normalized to 16S rRNA gene copy numbers to get the relative abundances as target ARG copies/16S rRNA gene copy. In this study *bla*$_{\text{CTX-M}}$ gene were found to have lowest absolute and relative abundances among the studied ARGs. Raw data of the quantification results for each gene and hospital are given in Appendix A. Statistical results for seasonal variations in gene abundances are given in Appendix B. Absolute and relative quantification results are presented separately for each hospital. The results are summarized for each hospital below:

3.2.1. For H1

H1 with 160 bed capacity had the highest abundance with 2.4×10^{5} copies/mL 16S rRNA gene in summer. In H1, the profiles of absolute concentrations and relative abundances followed the same pattern; *aadA* > *tetA* > *cmlA* > *sul1* > *qnrS* > *ermB* > *bla*$_{\text{CTX-M}}$. Among the hospitals analysed, highest copy numbers for the *aadA, cmlA, sul1* and *qnrS* genes detected in H1 (Figure 3.25). Among all wastewater samples, the *aadA* gene had the highest absolute abundance with 9.5×10^{4} copies/mL and relative abundance with 0.92 copies/16S rRNA gene in spring. In summer, the *cmlA* and the *sul1* genes were detected with highest absolute abundance with 1.2×10^{4} and 4.5 copies/mL, respectively. In spring, relative abundances of the *cmlA* and *sul1* genes were 8.3×10^{-2} and 4.6×10^{-2} copies/16S rRNA gene, respectively. Quinolone resistance gene *qnrS* was found to have highest copy numbers in autumn with 1.5×10^{3} copies/mL absolute abundance and 2.3×10^{-2} copies/16S rRNA gene relative abundance. Absolute abundances of the *ermB* and *bla*$_{\text{CTX-M}}$ genes were detected up to 18.99 and 1.35 copies/mL and relative abundances were 2.2×10^{-4} and 3.8×10^{-5}, respectively.

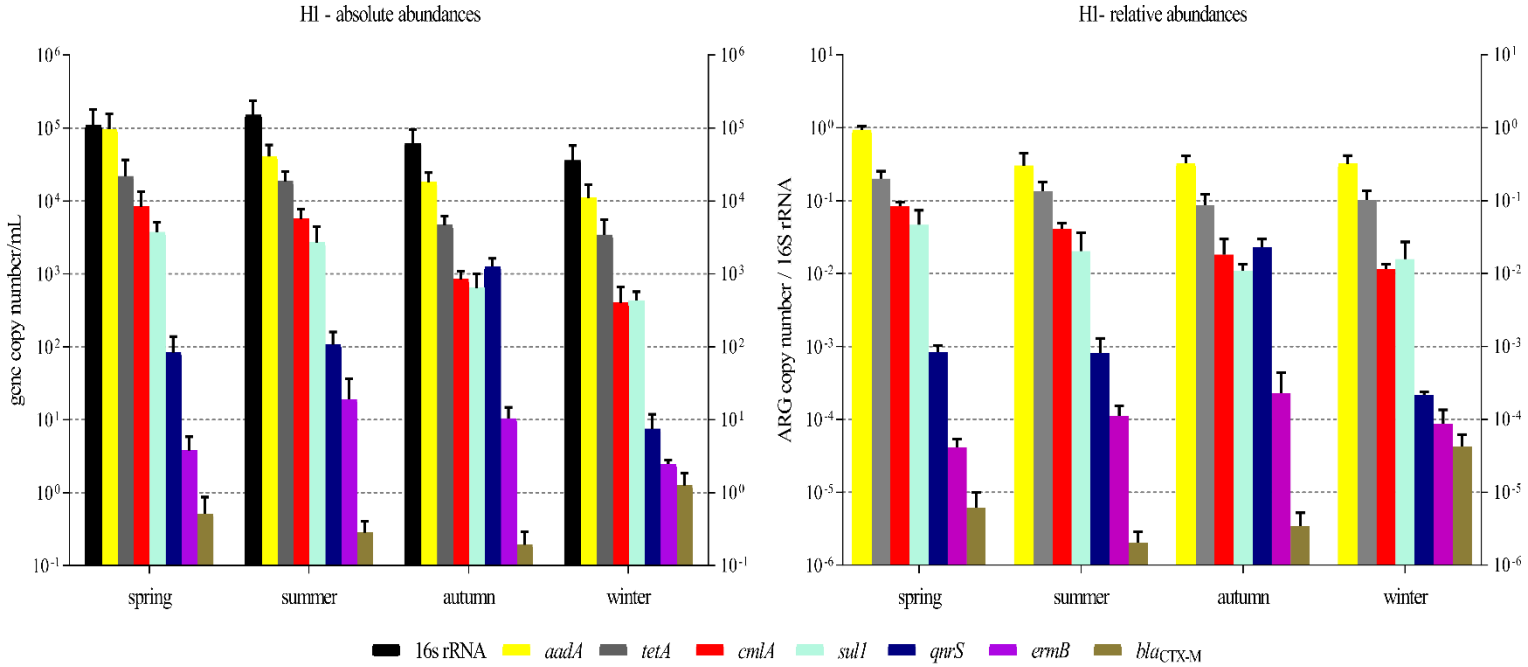

Figure 3.25. H1 absolute and relative abundances. Error bars indicate the standard deviation of three replicates. 16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA*, chloramphenicol resistance gene; *sul1* , sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; $bla_{\text{CTX-M}}$, ß-lactam resistance gene.

3.2.2. For H2

H2 with 270 bed capacity had the highest abundance with 2.8×10^{5} copies/mL 16S rRNA gene in summer season. In H2, the profiles in absolute concentrations and relative abundances followed the same pattern; *aadA* > *tetA* > *sul1* > *cmlA* > *qnrS* > *ermB* > $bla_{\text{CTX-M}}$ (Figure 3.26). The summer was found to be the most ARG abundant season. Highest average absolute copy numbers for the genes *aadA* (5.6×10^{4}), *tetA* (1.7×10^{4}), *cmlA* (2.4×10^{3}), *sul1* (8.3×10^{2}) *and qnrS* (1.3×10^{1}) were detected in summer. Highest relative abundances of *aadA* (3.6×10^{-1}), *tetA* (1.0×10^{-1}) and *sul1* (1.0×10^{-1}) were also found highest in summer. The genes *cmlA* (8.1×10^{-3}) and *qnrS* (1.6×10^{-3}) showed the highest relative abundances in spring. The *ermB* gene was detected between 1.5–5 copies/mL and 3.1×10^{-5}–4.9×10^{-4} copies/16S rRNA. The lowest amount of ARG $bla_{\text{CTX-M}}$ was detected with less than 1.2 copies/mL and 2.8×10^{-5} copies/16S rRNA.

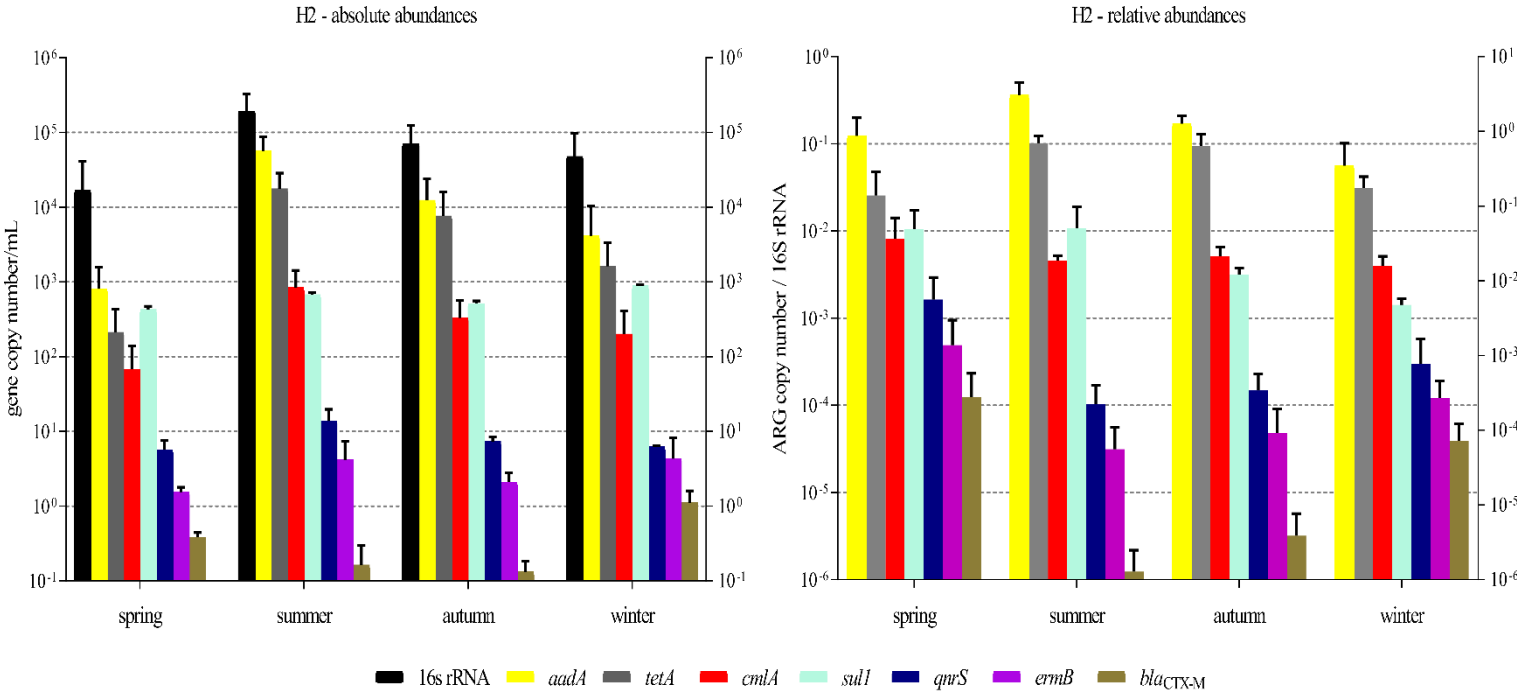

Figure 3.26. H2 absolute and relative abundances. Error bars indicate the standard deviation of three replicates. 16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA*, chloramphenicol resistance gene; *sul1* , sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; $bla_{\text{CTX-M}}$, ß-lactam resistance gene.

3.2.3. For H3

H3 with 468 bed capacity had the highest abundance with 1.0×10^5 copies/mL 16S rRNA gene in spring season. General profile for H3 in absolute concentrations and relative abundances followed the same pattern; *aadA* > *tetA* > *cmlA* > *sul1* > *ermB* > *qnrS* > $bla_{\text{CTX-M}}$. Among the hospitals analysed, the highest copy number for the *ermB* gene was detected for H3 in summer (Figure 3.27). The *ermB* gene had the absolute abundances up to 4.7×10^2 copies/mL and relative abundances up to 5.3×10^{-3} copies/16S rRNA gene. The absolute abundances of *aadA* (2.5×10^4), *cmlA* (2.6×10^3) and *sul1* (9.2×10^2) genes were spotted as highest in winter for H3. The gene *tetA* was detected as highest absolute abundance with 1.7×10^4 for H3 in autumn. Absolute abundances of the *qnrS* and $bla_{\text{CTX-M}}$ genes were detected up to 5.4×10^2 and 2 copies/mL. Relative abundances of the genes *aadA* (3.8×10^{-1}), *tetA* (1.3×10^{-1}) and *sul1* (3.6×10^{-2}) were found to be the highest in summer. Relative abundances were higher in spring for the genes *qnrS* (4.5×10^{-3}), *ermB* (4.5×10^{-3}) and in winter for the genes cmlA (3.1×10^{-2}) and $bla_{\text{CTX-M}}$ (2.3×10^{-5}).

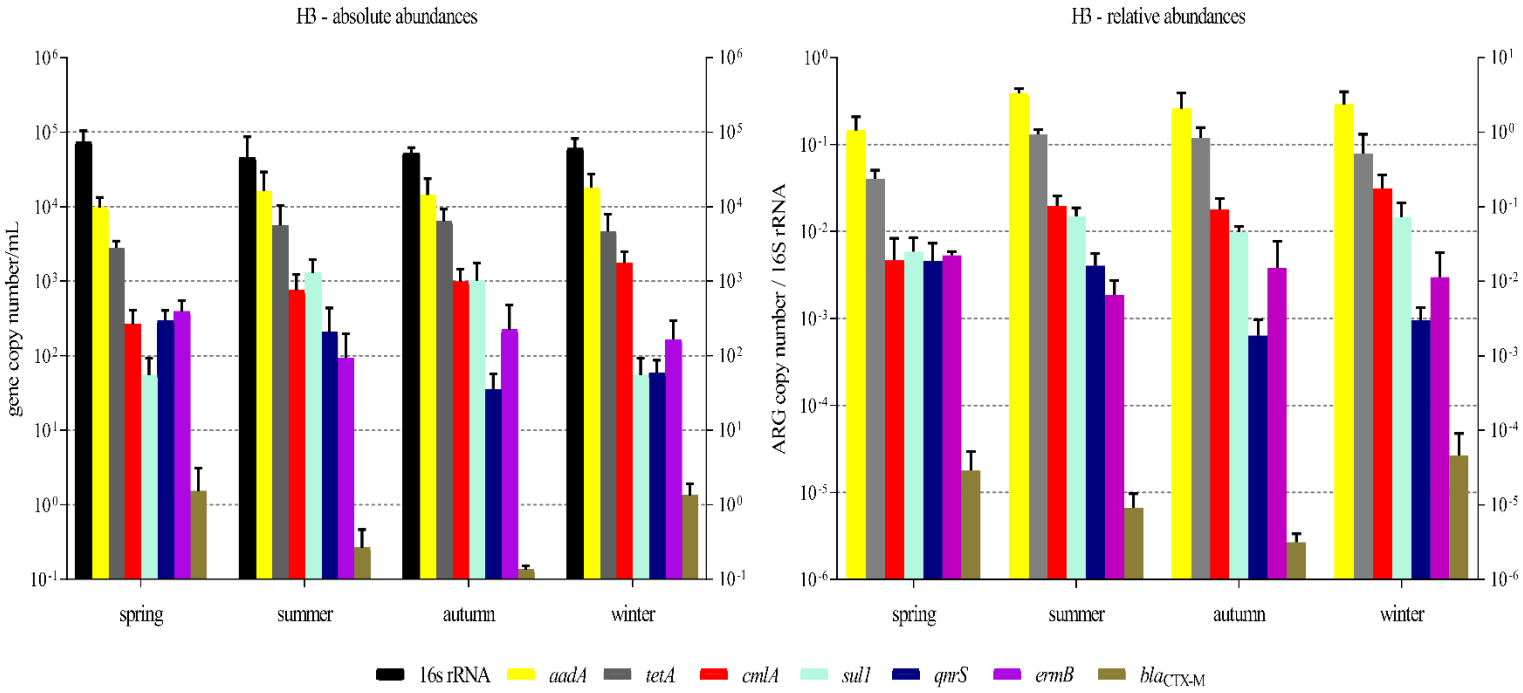

Figure 3.27. H3 absolute and relative abundances. Error bars indicate the standard deviation of three replicates. 16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA*, chloramphenicol resistance gene; *sul1* , sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; $bla_{\text{CTX-M}}$, ß-lactam resistance gene.

3.2.4. For H4

H4 with 484 bed capacity had the highest abundance with 1.4×10^{5} copies/mL 16S rRNA gene in summer season. In H4, the profile in absolute concentrations and relative abundances followed the same pattern; *aadA* > *tetA* > *cmlA* > *sul1* > *qnrS* > *ermB* > $bla_{\text{CTX-M}}$. Among the hospitals analysed, the highest absolute and relative abundance for the *tetA* gene was detected for H4 in spring (Figure 3.28). Among all wastewater samples, the *tetA* gene had the highest absolute abundance with 4.1×10^{4} copies/mL and relative abundance with 2.5×10^{-1} copies/16S rRNA gene in spring. In spring, the *aadA* and the *cmlA* genes were detected with highest absolute abundance with 5.8×10^{4} and 2.2×10^{3} copies/mL, respectively. Absolute abundances of the *sul1, qnrS* and $bla_{\text{CTX-M}}$ genes were detected up to 9.3×10^{2}, 3.2×10^{2} and 2.42 copies/mL in autumn. The *ermB* gene was found to have the highest copy numbers in summer with 1.9×10^{2} copies/mL absolute abundance and 3.0×10^{-2} copies/16S rRNA gene relative abundance. The highest relative abundances were detected for the genes *aadA* (6.6×10^{-1}), *tetA* (2.5×10^{-1}) and *cmlA* (2.7×10^{-2}) in spring, for the genes *sul1* (1.6×10^{-2}) and *qnrS* (5.9×10^{-3}) in autumn and for the $bla_{\text{CTX-M}}$ (5.5×10^{-5}) gene in summer.

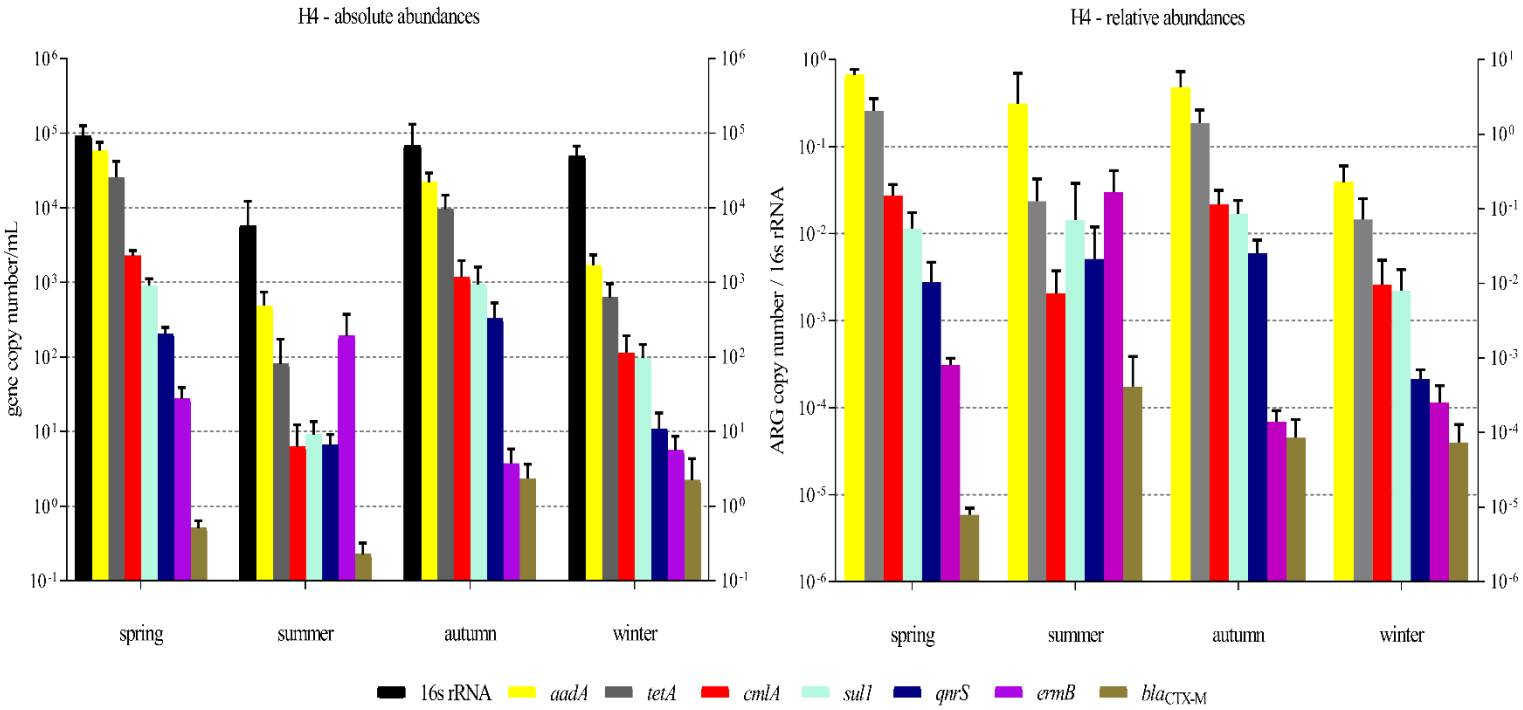

Figure 3.28. H4 absolute and relative abundances. Error bars indicate the standard deviation of three replicates. 16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA*, chloramphenicol resistance gene; *sul1* , sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; $bla_{\text{CTX-M}}$, ß-lactam resistance gene.

3.2.5. For H5

H5 with 730 bed capacity had the highest abundance with 1.7×10^5 copies/mL 16S rRNA gene in summer season. In H5, the profile in absolute concentrations and relative abundances followed the same pattern; *aadA* > *tetA* > *sul1* > *cmlA* > *qnrS* > *ermB* > $bla_{\text{CTX-M}}$ (Figure 3.29). The gene abundance pattern was found same as in H2. The spring was found to be the most ARG abundant season for H5. The highest absolute abundances were for *aadA* (3.6×10^4), *tetA* (2.1×10^4), *cmlA* (2.0×10^3), *sul1* (9.4×10^2) and *qnrS* (8.3×10^2) genes. The *ermB* gene was found to have the highest copy numbers in summer up to 9.4 copies/mL and the $bla_{\text{CTX-M}}$ gene was up to 7.2 copies/mL in winter. Relative abundances for *aadA* (2.8×10^{-1}), *tetA* (1.2×10^{-1}), *sul1* (2.2×10^{-2}) and *ermB* (1.3×10^{-4}) genes were highest in summer. Relative abundances of the genes *cmlA* (1.0×10^{-2}), *qnrS* (4.3×10^{-3}) and $bla_{\text{CTX-M}}$ (1.3×10^{-3}) were detected highest in autumn, spring and winter, respectively.

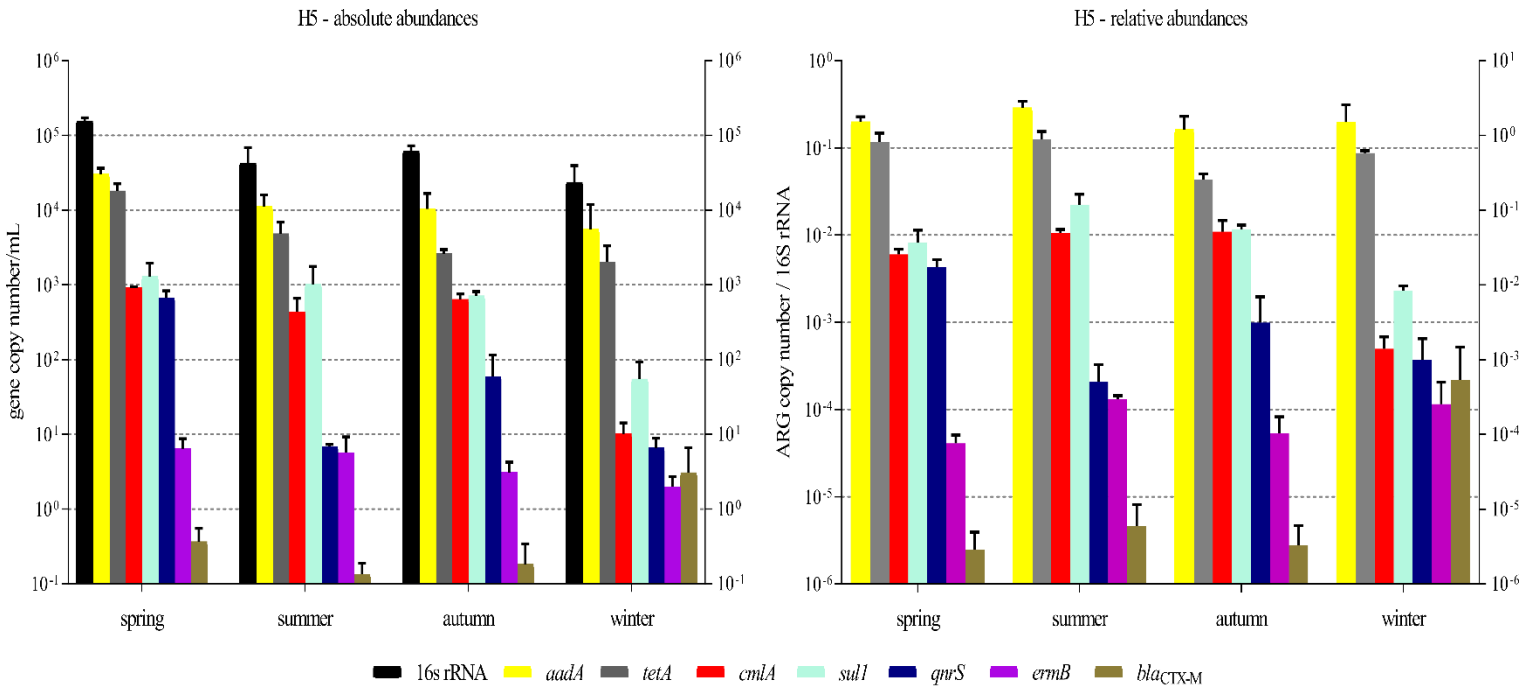

Figure 3.29. H5 absolute and relative abundances. Error bars indicate the standard deviation of three replicates. 16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA*, chloramphenicol resistance gene; *sul1* , sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; bla_{CTX-M}, ß-lactam resistance gene.

3.2.6. For H6

H6 with the highest bed capacity (1140 beds) had the highest abundance with 2.3×10^5 copies/mL 16S rRNA gene in spring season. The profile in absolute concentrations and relative abundances were found to be the same; *aadA* > *tetA* > *cmlA* > *sul1* > *qnrS* > *ermB* > $bla_{\text{CTX-M}}$. Among the hospitals analysed, the highest absolute and relative abundance for the $bla_{\text{CTX-M}}$ gene was detected in spring and winter with 2.2×10^1 copies/mL and 4.2×10^{-4} copies/16S rRNA (Figure 3.30). However, the $bla_{\text{CTX-M}}$ gene had the lowest abundances compared to other ARGs tested for. The spring was also found to be the most ARG abundant season for H6. Spring samples were detected with highest absolute abundances for *aadA* (9.9×10^4), *tetA* (2.0×10^4), *cmlA* (5.2×10^3), *sul1* (2.2×10^2) and *qnrS* (2.5×10^2) genes. The *ermB* gene was found to have the highest copy numbers in autumn up to 7.8×10^1 copies/mL and highest relative abundance with 7.1×10^{-4} copies/16S rRNA. The highest relative abundances were detected for the genes *aadA* (6.6×10^{-1}), *tetA* (1.9×10^{-1}) and *cmlA* (5.0×10^{-2}) in spring and for the genes *sul1* (3.0×10^{-2}) and *qnrS* (1.8×10^{-3}) in winter.

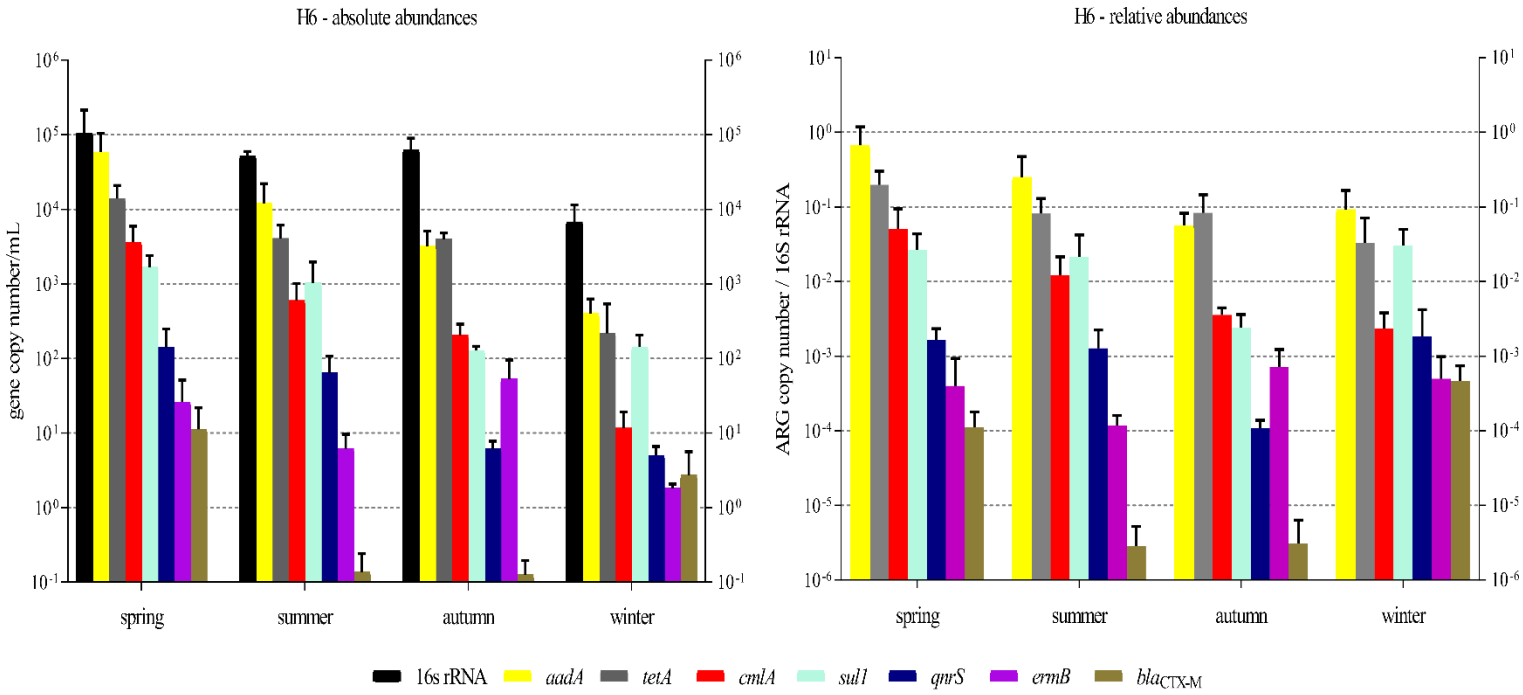

Figure 3.30. H6 absolute and relative abundances. Error bars indicate the standard deviation of three replicates. 16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA*, chloramphenicol resistance gene; *sul1* , sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; $bla_{\text{CTX-M}}$, ß-lactam resistance gene.

Seasonally collected effluents of six different hospitals (from the lowest bed capacity H1 to the highest bed capacity H6) were analysed in terms of overall bacterial genes (16s rRNA) and seven ARGs of *aadA, tetA, cmlA, sul1, qnrS, ermB* and $bla_{\text{CTX-M}}$ corresponding to commonly used antibiotics aminoglycosides, tetracyclines, amphenicols, sulfonamides, quinolones, macrolide-lincosamide-streptogramin group and β-lactams, respectively. The ARGs were quantified using qPCR to obtain the absolute values as copy number/mL and these values were normalized to 16S rRNA gene copy numbers to get the relative abundances as target ARG copies/16S rRNA gene copy. The copy numbers of overall bacterial genes (16S rRNA) ranged in between 5.6 x 10^3 and 1.9 × 10^5 copies/mL. The highest abundances were observed in spring and summer seasons for all of the HWWs tested. Seasonal variations in average gene copy numbers of each hospital were significant ($p<0.05$). H2 and H5 displayed the highest overall gene copy numbers with 1.9 x 10^5 and 1.5 × 10^5 copies/mL, respectively. The lowest overall gene copy numbers were observed in H4 and H6 with 5.6 x 10^3 and 6.6 × 10^3 copies/mL, respectively. The results revealed that there was no correlation between the bed capacity of the hospitals and the copy numbers of the overall genes released through the effluents discharged. Although direct patient exposure to antibiotics is a primary risk factor for getting ARB and ARGs in discharges, studies indicate that the influence of antibiotic use may operate not only at the individual patient level, but also at hospital-level (Aldeyab *et al*., 2012; Lawes *et al*., 2017).

The HWWs tested in this study were found to have relatively high copy numbers of the genes *aadA, tetA, cmlA, sul1,* and *qnrS* in all seasons. Among the ARGs analysed, the *aadA* gene conferring resistance to aminoglycosides had the highest absolute abundance with 9.5×10^4 copies/mL and relative abundance with 0.92 copies/16S rRNA gene copy in H1. The aminoglycoside resistance gene *aadA* is highly associated with MGEs (Wang *et al*., 2018). The integron nature of the *aadA* gene and the plasmid- and/or transposon-associated location of the integrons play role in the excessive horizontal spread of *aadA.* Water environments provide ideal settings for HGT via MGEs. Among them, HWWs provide favourable conditions (Hocquet *et al*., 2016). Therefore, the high concentrations of the *aadA* gene was accounted for the prevalence of MGEs in HWWs tested.

Tetracycline (*tet*), sulfonamide (*sul*), macrolide (*erm*), quinolone (*qnr*), and β-lactams (*bla*) resistance encoding genes are also the most reported genes in effluents due to the extensive use of the corresponding antibiotics (Qiao *et al*., 2018). In the current study, tetracycline resistance gene *tetA* was the second most abundant gene in all of the HWWs tested. The *tetA* gene had the highest absolute abundance with 2.5×10^4 copies/mL and relative abundance with 0.25 copies/16S rRNA gene copy in H4. The *tetA* gene has frequently been described in association with conjugative plasmids. The critical role played by plasmids in the HGT of ARGs has been widely recognized and is particularly prominent (Levy & Marshall, 2004). The primary mechanisms of HGT are conjugation (plasmids are transferred from a donor cell to a recipient cell), transformation (uptake of naked DNA), and transduction (bacteriophages as

transporters of genetic information) (Alekshun & Levy, 2007). Dissemination and propagation of ARGs could occur via HGT from resistant bacteria to susceptible strains either between different species or across genera (Thomas & Nielsen, 2005). This could explain the abundance of the *tetA* gene in the HWWs tested in the current study.

The chloramphenicol resistance gene *cmlA* had the third highest absolute abundance with 8.3×10^3 copies/mL and relative abundance with 0.083 copies/16S rRNA gene in H1. Studies on the *cmlA* gene suggest that conjugation of plasmids encoding *cmlA* is one mechanism for the wide dissemination of chloramphenicol resistance. Since co-resistance to sulfamethoxazole, tetracycline and kanamycin is observed among the majority of chloramphenicol resistant transconjugants, the use of any of these antimicrobials can result in the selection of bacteria resistant not only to that specific agent, but by genetic linkage of resistance genes, to other unrelated antimicrobial agents, in this case chloramphenicol (Bischoff *et al*., 2005).

The sulfonamide resistance gene *sul1* had the fourth highest absolute abundance with 3.7×10^3 copies/mL and relative abundance with 0.046 copies/16S rRNA gene copy in H1. The *sul* genes might be associated with conjugative and/or mobilizable plasmids and integrons, which are also found to be abundant among resistant isolates, promoting dissemination of the sulfonamide resistance. The *sul1* gene is found linked to other resistance genes in class 1 integrons and on large conjugative plasmids (Trobos *et al*., 2008).

The MLS group resistance gene *ermB* had the fifth highest absolute abundance with 2.9×10^2 copies/mL in H3 and relative abundance with 0.030 copies/16S rRNA gene copy in H4. The gene *ermB* encoded methylase methylates 23S rRNA, thereby altering the drug binding site, thus conferring resistance not only to macrolides (erythromycin) but also to lincosamides (clindamycin) and streptogramin B (MLS phenotype) antibiotics. Elements associated with MLS resistance are the Tn*917* transposon that carries the *ermB* gene (Shaw & Clewell, 1985). Transposable elements are specific DNA segments that can repeatedly insert into one or more sites in one or more genomes (Roberts & Mullany, 2011). They can be distributed on both chromosomes and plasmids, and are able to interact by recombination between elements and/or by transposition into other elements, forming all kinds of novel chimeric structures (Li *et al*., 2011). These transposable elements increase the risk for transfer of MLS group resistance as in the case of current study.

The quinolone resistance gene *qnrS* had the highest absolute abundance with 1.2×10^3 copies/mL and relative abundance with 0.022 copies/16S rRNA gene in H1. Transmissible quinolone resistance (*qnrS*) is also attributable to genes encoding plasmid-encoded efflux pumps (Tran & Jacoby, 2002). While plasmid-encoded quinolone-resistance genes generally confer low-level resistance, their overall impact is great because they shield otherwise susceptible bacteria from the lethal effects of the quinolones, allowing them greater time and opportunity to evolve higher-level resistance.

The β-lactam resistance gene *bla*$_{CTX-M}$ had the seventh highest absolute abundance with 1.1×10^1 copies/mL and relative abundance with 0.0004 copies/16S rRNA gene in H6. β lactam resistance gene *bla*$_{CTX-M}$ was found at the lowest concentrations in all the HWWs tested. Resistance to ceftriaxone or other β-lactams is usually due to the CTX-M (cefotaxime-hydrolyzing β-lactamase) group extended-spectrum β-lactamases (ESBL) (Hopkins *et al*., 2006). Genes encoding CTX-M-type ESBLs are usually located in transmissible plasmids (Carattoli, 2009). Therefore, resistance to extended-spectrum β-lactams has been reported in many parts of the world and CTX-M type enzymes have become the most dominant ESBLs (Bonnet, 2004; Cantón & Coque, 2006). The results obtained from the current study also supported this.

After analyses of effluents of six different hospitals (from the lowest bed capacity H1 to the highest bed capacity H6) in terms of overall bacterial genes (16s rRNA) and seven ARGs, following ARG patterns were observed: *aadA* > *tetA* > *cmlA* > *sul1* > *ermB* > *qnrS* > *bla*$_{CTX-M}$ in H3-4 and 6; *aadA* > *tetA* > *sul1* > *cmlA* > *qnrS* > *ermB* > *bla*$_{CTX-M}$ in H2 and H5; *aadA* > *tetA* > *cmlA* > *sul1* > *qnrS* > *ermB* > *bla*$_{CTX-M}$ in H1. The overall pattern of ARGs in absolute and relative abundances was found to show following order *aadA* > *tetA* > *cmlA* ≈ *sul1* > *ermB* ≈ *qnrS* > *bla*$_{CTX-M}$. The abundance of ARGs discharged from the hospitals tested reported in this study can be considered as alarming. The results clearly indicated that untreated HWWs acted as reservoir of ARGs and required on-site treatment.

CHAPTER 4

CONCLUSIONS & RECOMMENDATIONS

4.1. Conclusions

In this study, seasonally collected effluents of six different hospitals with varying bed capacities (160-1140 beds) were analysed for overall bacterial genes and seven ARGs. QPCR method was used to quantify ARGs including *aadA, tetA, cmlA, sul1, qnrS, ermB* and $bla_{\text{CTX-M}}$ corresponding to commonly used antibiotics aminoglycosides, tetracyclines, chloramphenicol, sulfonamides, quinolones, MLS group and β-lactams. The results pointed out that:

- All the HWWs tested were found to have relatively high copy numbers of the ARGs *aadA, tetA, cmlA, sul1,* and *qnrS* in all seasons.
- ARGs abundances displayed almost similar seasonal ARG copy number profile of $aadA > tetA > cmlA \approx sul1 > ermB \approx qnrS > bla_{\text{CTX-M}}$ for all the HWWs tested.
- Seasonal variations in ARGs abundances for each hospital were significant and the highest abundances were observed in both spring and summer.
- No correlation was observed between the bed capacity of the hospitals and the copy numbers of the overall genes discharged.
- Untreated HWWs were found to be hotspots for ARGs and required on-site treatment before discharging into public sewer.

4.2. Recommendations

- Untreated HWWs were found to be hotspots for ARGs. Therefore, hospitals without any treatment system should be equipped with an appropriate one, like membrane and package membrane bioreactor.
- Installations of package treatment systems at the hospitals need to be evaluated in order to prevent ARG dissemination.
- Regulations on discharge criteria must be revised with new parameters that contribute the dissemination of ARB and ARGs.
- For a better understanding of ARG dissemination, MGEs need to be continuously monitored in HWWs.
- Potential hotspots with constant or seasonal high population density such as large hotels, shopping centers or holiday resorts should also be monitored for the ARGs to get a comprehensive view for global antibiotic resistance crisis.

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APPENDICES

A. Raw data of the quantification results for the genes tested

Table A.1. Absolute and relative abundance results for H1

	Absolute abundances				Relative Abundances			
	spring	summer	autumn	winter	spring	summer	autumn	winter
16S rRNA	10^5	10^5	10^4	10^4	n/a	n/a	n/a	n/a
	1.07±0.71	1.49±0.85	6.08±3.49	3.55±2.19	n/a	n/a	n/a	n/a
aadA	10^4	10^4	10^4	10^4	10^0	10^0	10^0	10^0
	9.58±6.02	4.05±1.82	1.79±0.65	1.09±0.58	0.92±0.11	0.29±0.15	0.32±0.08	0.31±0.09
tetA	10^4	10^4	10^3	10^3	10^-1	10^-1	10^-1	10^-1
	2.16±1.50	1.85±0.68	4.65±1.50	3.40±2.11	2.00±0.5	1.30±0.4	0.86±0.35	1.00±0.34
sul1	10^3	10^3	10^2	10^2	10^-2	10^-2	10^-2	10^-2
	8.31±5.00	5.67±2.07	8.40±2.46	3.98±2.61	4.60±2.60	2.00±1.60	1.00±0.25	1.50±1.10
cmlA	10^3	10^3	10^2	10^2	10^-2	10^-2	10^-2	10^-2
	3.71±1.41	2.68±1.78	6.39±3.64	4.28±1.40	8.30±1.20	4.00±0.83	1.80±1.10	1.10±0.20
ermB	10^0	10^1	10^1	10^0	10^-4	10^-4	10^-4	10^-4
	8.30±5.54	1.07±0.52	1.25±0.37	7.44±4.44	0.40±0.12	1.10±0.40	2.28±2.00	0.87±0.47
qnrS	10^1	10^2	10^3	10^0	10^-4	10^-4	10^-2	10^-4
	3.80±2.03	1.89±1.72	1.03±0.45	2.46±0.33	8.30±1.80	8.10±4.80	2.29±0.66	2.10±0.23
bla$_{CTX-M}$	10^0	10^0	10^0	10^0	10^-6	10^-6	10^-6	10^-5
	0.50±0.35	0.28±0.12	0.19±0.10	1.25±0.60	6.21±3.72	2.03±0.86	3.41±1.80	4.10±2.00

16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA* chloramphenicol resistance gene; *sul1*, sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; *bla*$_{CTX-M}$ β-lactam resistance gene; ±, standard deviation; n/a, not applicable.

Table A.2. Absolute and relative abundance results for H2

	Absolute abundances				Relative Abundances			
	spring	summer	autumn	winter	spring	summer	autumn	winter
16S rRNA	10^4	10^5	10^4	10^4	n/a	n/a	n/a	n/a
	1.65±2.48	1.91±1.33	6.91±5.54	4.73±5.01	n/a	n/a	n/a	n/a
aadA	10^2	10^4	10^4	10^3	10^-1	10^-1	10^-1	10^-2
	8.05±7.75	5.64±3.14	1.23±1.15	4.15±6.17	1.23±0.75	3.60±1.41	1.70±0.39	5.60±4.53
tetA	10^2	10^4	10^3	10^3	10^-2	10^-1	10^-2	10^-2
	2.10±2.18	1.76±1.10	7.59±8.41	1.61±1.71	2.56±2.19	1.02±0.21	9.40±3.44	3.13±1.06
sul1	10^1	10^3	10^2	10^1	10^-2	10^-2	10^-3	10^-3
	9.43±10.7	2.40±2.89	2.05±1.43	6.90±7.92	1.04±0.68	1.07±0.82	3.17±0.57	1.41±0.24
cmlA	10^1	10^2	10^2	10^2	10^-3	10^-3	10^-3	10^-3
	6.73±7.13	8.39±5.89	3.30±2.35	1.99±2.06	8.11±6.07	4.57±0.65	5.15±1.40	3.98±1.13
ermB	10^0	10^0	10^0	10^0	10^-4	10^-5	10^-5	10^-4
	1.54±0.24	4.15±3.25	2.09±0.69	4.31±3.86	4.92±4.59	3.14±2.39	4.83±4.23	1.21±0.68
qnrS	10^0	10^1	10^0	10^0	10^-3	10^-4	10^-4	10^-4
	5.76±1.84	1.37±0.58	7.32±1.08	6.20±0.23	1.63±1.30	1.03±0.67	1.48±0.79	2.99±2.78
bla_{CTX-M}	10^0	10^0	10^0	10^0	10^-4	10^-6	10^-6	10^-5
	0.38±0.06	0.16±0.13	0.13±0.05	1.11±0.49	1.24±1.09	1.24±0.93	3.19±2.54	3.90±2.20

16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene*; tetA*, tetracycline resistance gene*; cmlA* chloramphenicol resistance gene*; sul1*, sulfonamide resistance gene*; qnrS*, quinolone resistance gene*; ermB*, MLS group resistance gene*;* bla_{CTX-M} β-lactam resistance gene; ±, standard deviation; n/a, not applicable.

Table A.3. Absolute and relative abundance results for H3

	Absolute abundances				Relative Abundances			
	spring	summer	autumn	winter	spring	summer	autumn	winter
16S rRNA	10^4	10^4	10^4	10^4	n/a	n/a	n/a	n/a
	7.31±3.10	4.48±4.16	5.21±1.00	6.03±2.12	n/a	n/a	n/a	n/a
aadA	10^3	10^4	10^4	10^4	10^-1	10^-1	10^-1	10^-1
	9.70±3.66	1.61±1.31	1.43±0.93	1.79±0.93	1.45±0.62	3.87±0.54	2.57±1.34	2.88±1.17
tetA	10^3	10^3	10^3	10^3	10^-2	10^-1	10^-1	10^-2
	2.79±0.67	5.57±4.71	6.36±2.84	4.61±3.29	4.06±1.02	1.31±0.18	1.18±0.38	7.87±5.26
sul1	10^1	10^3	10^3	10^1	10^-4	10^-2	10^-2	10^-4
	5.44±3.84	1.29±0.66	1.02±0.74	5.44±3.84	7.26±3.04	3.64±1.34	1.92±1.21	8.38±3.18
cmlA	10^2	10^2	10^2	10^3	10^-3	10^-2	10^-2	10^-2
	2.65±1.44	7.51±4.89	9.75±4.67	1.75±0.73	4.65±3.70	1.98±0.58	1.80±0.61	3.11±1.36
ermB	10^2	10^1	10^2	10^2	10^-3	10^-3	10^-3	10^-3
	3.90±1.61	9.34±9.02	2.25±2.50	1.64±1.33	5.33±0.57	1.87±0.84	3.82±3.92	2.99±2.69
qnrS	10^2	10^2	10^1	10^1	10^-3	10^-3	10^-4	10^-4
	2.97±1.05	2.06±2.31	3.52±2.21	5.83±2.87	4.59±2.72	4.05±1.51	6.37±3.37	9.58±3.78
bla_{CTX-M}	10^0	10^0	10^0	10^0	10^-5	10^-6	10^-6	10^-5
	1.52±1.58	0.26±0.19	0.13±0.01	1.34±0.57	1.79±1.19	6.67±3.00	2.67±0.67	2.65±2.12

16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA* chloramphenicol resistance gene; *sul1*, sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; bla_{CTX-M} β-lactam resistance gene; ±, standard deviation; n/a, not applicable.

Table A.4. Absolute and relative abundance results for H4

	Absolute abundances				Relative Abundances			
	spring	summer	autumn	winter	spring	summer	autumn	winter
16S rRNA	10^4	10^3	10^4	10^4	n/a	n/a	n/a	n/a
	9.7±3.52	5.7±6.51	6.74±6.45	4.85±1.85	n/a	n/a	n/a	n/a
aadA	10^4	10^2	10^4	10^3	10^-1	10^-1	10^-1	10^-2
	5.82±1.72	4.83±2.62	2.2±0.73	1.68±6.55	6.67±1.05	3.09±3.88	4.8±2.49	3.86±2.14
tetA	10^4	10^1	10^3	10^2	10^-1	10^-2	10^-1	10^-2
	2.54±1.67	8.5±9.23	9.6±5.22	6.26±3.27	2.58±0.98	2.35±1.92	1.87±0.76	1.45±1.08
sul1	10^3	10^3	10^2	10^2	10^-2	10^-2	10^-2	10^-3
	8.99±2.16	9.14±4.45	9.37±6.69	9.6±5.05	1.14±0.6	1.45±2.33	1.7±0.7	2.21±1.65
cmlA	10^3	10^0	10^3	10^2	10^-2	10^-3	10^-2	10^-3
	2.26±4.14	6.25±6.05	1.17±0.78	1.13±0.79	2.73±0.93	2.05±1.69	2.17±0.97	2.57±2.37
ermB	10^0	10^1	10^1	10^0	10^-4	10^-2	10^-5	10^-4
	2.76±1.15	1.91±1.82	3.7±2.14	5.57±3.1	3.08±0.61	3.02±2.29	6.91±2.38	1.15±0.63
qnrS	10^1	10^2	10^3	10^0	10^-3	10^-3	10^-3	10^-4
	2.4±0.45	6.61±2.5	3.27±2.03	1.9±0.68	2.75±1.95	5.12±6.86	5.94±2.47	2.13±0.6
bla_{CTX-M}	10^0	10^0	10^0	10^0	10^-6	10^-4	10^-5	10^-5
	5.8±0.13	2.26±0.95	2.33±1.32	2.23±2.11	5.88±1.13	1.73±2.15	4.51±2.8	4±2.41

16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA* chloramphenicol resistance gene; *sul1*, sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; bla_{CTX-M} β-lactam resistance gene; ±, standard deviation; n/a, not applicable.

Table A.5. Absolute and relative abundance results for H5

	Absolute abundances				Relative Abundances			
	spring	summer	autumn	winter	spring	summer	autumn	winter
16S rRNA	10^5	10^4	10^4	10^4	n/a	n/a	n/a	n/a
	1.54±0.17	4.21±2.64	6.11±1.17	2.33±1.61	n/a	n/a	n/a	n/a
aadA	10^4	10^4	10^4	10^3	10^-1	10^-1	10^-1	10^-1
	3.05±0.59	1.12±0.48	1.04±0.67	5.54±6.3	1.98±0.29	2.88±0.55	1.62±0.68	1.98±1.13
tetA	10^4	10^3	10^3	10^3	10^-1	10^-1	10^-2	10^-2
	1.79±0.46	4.79±2.08	2.6±0.36	2.01±1.32	1.17±0.3	1.25±0.28	4.32±0.73	8.75±0.53
sul1	10^3	10^3	10^2	10^1	10^-3	10^-2	10^-2	10^-3
	1.29±0.66	1.02±0.74	7.08±1	5.44±3.84	8.13±3.28	2.24±0.69	1.17±0.13	2.32±0.31
cmlA	10^2	10^2	10^2	10^1	10^-3	10^-2	10^-2	10^-4
	9.14±0.26	4.32±2.31	6.37±1.17	1.01±0.4	6.01±0.86	1.07±0.98	1.09±0.38	4.97±1.84
ermB	10^0	10^0	10^0	10^0	10^-5	10^-4	10^-5	10^-4
	6.41±2.28	5.64±3.55	3.06±1.19	1.97±0.72	4.1±0.99	1.33±1.17	5.37±2.89	1.16±8.98
qnrS	10^2	10^0	10^1	10^0	10^-3	10^-4	10^-4	10^-4
	6.67±1.64	6.85±0.5	5.91±5.62	6.58±2.27	4.33±0.88	2.08±1.19	9.9±9.72	3.75±2.77
bla$_{CTX-M}$	10^0	10^0	10^0	10^0	10^-6	10^-6	10^-6	10^-4
	0.36±0.19	0.13±0.05	0.82±0.16	3.05±3.57	2.47±1.46	4.6±3.5	2.77±1.92	2.18±3.02

16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA* chloramphenicol resistance gene; *sul1*, sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; *bla*$_{CTX-M}$ β-lactam resistance gene; ±, standard deviation; n/a, not applicable.

Table A.6. Absolute and relative abundance results for H6

	Absolute abundances				Relative Abundances			
	spring	summer	autumn	winter	spring	summer	autumn	winter
16S rRNA	10^5	10^4	10^4	10^3	n/a	n/a	n/a	n/a
	1.04±1.1	5.09±0.88	6.15±2.79	6.66±4.78	n/a	n/a	n/a	n/a
aadA	10^4	10^4	10^3	10^2	10^-1	10^-1	10^-2	10^-2
	5.82±4.55	1.2±1	3.19±1.92	4±2.27	6.66±5.17	2.47±2.24	5.6±2.58	9.15±7.47
tetA	10^4	10^3	10^3	10^2	10^-1	10^-2	10^-2	10^-2
	1.39±0.69	4.03±2.09	3.99±0.82	2.16±3.22	1.97±1.04	8.11±4.77	8.31±6.21	3.28±3.86
sul1	10^3	10^3	10^2	10^2	10^-2	10^-2	10^-3	10^-2
	1.68±0.7	1.03±0.93	1.28±0.18	1.42±0.63	2.65±1.69	2.12±2.08	2.43±1.18	3.05±1.94
cmlA	10^3	10^2	10^2	10^1	10^-2	10^-2	10^-3	10^-3
	3.6±2.33	5.99±4.11	2.06±0.8	1.16±0.76	5.06±4.4	1.21±0.92	3.55±0.87	2.33±1.47
ermB	10^1	10^0	10^1	10^0	10^-4	10^-4	10^-4	10^-4
	2.6±2.53	6.18±3.43	5.29±4.24	1.81±0.27	3.99±5.3	1.17±0.44	7.13±5.09	4.96±4.89
qnrS	10^2	10^1	10^0	10^0	10^-3	10^-3	10^-4	10^-3
	1.41±1.06	6.42±4.34	6.14±1.66	4.98±1.64	1.65±0.67	1.28±0.95	1.09±0.29	1.83±2.35
bla_{CTX-M}	10^1	10^0	10^0	10^0	10^-4	10^-6	10^-6	10^-4
	1.12±1.05	0.13±1.04	0.12±0.07	2.73±2.87	1.11±0.67	2.84±2.36	3.05±3.31	4.63±2.79

16S rRNA, overall bacterial gene; *aadA*, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA* chloramphenicol resistance gene; *sul1*, sulfonamide resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; bla_{CTX-M} β-lactam resistance gene; ±, standard deviation; n/a, not applicable.

B. Statistical analyses

Table B.1. Analysis of variance for H1

		Sum of Squares	df	Mean Square	F	Sig.
cmlA	Between Groups	.009	3	.003	35.061	.000
	Within Groups	.001	8	.000		
	Total	.010	11			
ermB	Between Groups	.000	3	.000	1.622	.260
	Within Groups	.000	8	.000		
	Total	.000	11			
qnrS	Between Groups	.001	3	.000	33.507	.000
	Within Groups	.000	8	.000		
	Total	.001	11			
sul1	Between Groups	.002	3	.001	2.716	.115
	Within Groups	.002	8	.000		
	Total	.005	11			
tetA	Between Groups	.023	3	.008	4.081	.050
	Within Groups	.015	8	.002		
	Total	.037	11			
bla$_{CTX-M}$	Between Groups	.000	3	.000	10.080	.004
	Within Groups	.000	8	.000		
	Total	.000	11			
aadA	Between Groups	.841	3	.280	20.898	.000
	Within Groups	.107	8	.013		
	Total	.948	11			

aadA, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA* chloramphenicol resistance gene; *sul1*, sulfonamide resistance gene; *bla*$_{CTX-M}$, ß-lactam resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; Sig, significance ($p<0.05$); F, variation among group; df, degrees of freedom.

Table B.2. Analysis of variance for H2

		Sum of Squares	df	Mean Square	F	Sig.
cmlA	Between Groups	.000	3	.000	17.502	.001
	Within Groups	.000	8	.000		
	Total	.000	11			
ermB	Between Groups	.000	3	.000	2.675	.118
	Within Groups	.000	8	.000		
	Total	.000	11			
qnrS	Between Groups	.000	3	.000	24.740	.000
	Within Groups	.000	8	.000		
	Total	.000	11			
sul1	Between Groups	.001	3	.000	13.975	.002
	Within Groups	.000	8	.000		
	Total	.001	11			
tetA	Between Groups	.012	3	.004	9.065	.006
	Within Groups	.004	8	.000		
	Total	.016	11			
bla$_{CTX-M}$	Between Groups	.000	3	.000	1.517	.283
	Within Groups	.000	8	.000		
	Total	.000	11			
aadA	Between Groups	.026	3	.009	1.613	.262
	Within Groups	.043	8	.005		
	Total	.069	11			

aadA, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA* chloramphenicol resistance gene; *sul1*, sulfonamide resistance gene; *bla*$_{CTX-M}$, ß-lactam resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; Sig, significance ($p<0.05$); F, variation among group; df, degrees of freedom.

Table B.3. Analysis of variance for H3

		Sum of Squares	df	Mean Square	F	Sig.
cmlA	Between Groups	.000	3	.000	.995	.443
	Within Groups	.000	8	.000		
	Total	.000	11			
ermB	Between Groups	.000	3	.000	2.566	.127
	Within Groups	.000	8	.000		
	Total	.000	11			
qnrS	Between Groups	.000	3	.000	3.581	.066
	Within Groups	.000	8	.000		
	Total	.000	11			
sul1	Between Groups	.000	3	.000	2.430	.140
	Within Groups	.000	8	.000		
	Total	.000	11			
tetA	Between Groups	.000	3	.000	.551	.662
	Within Groups	.000	8	.000		
	Total	.000	11			
bla$_{CTX-M}$	Between Groups	.006	3	.002	3.726	.061
	Within Groups	.005	8	.001		
	Total	.011	11			
aadA	Between Groups	.154	3	.051	7.068	.012
	Within Groups	.058	8	.007		
	Total	.212	11			

aadA, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA* chloramphenicol resistance gene; *sul1*, sulfonamide resistance gene; *bla*$_{CTX-M}$, ß-lactam resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; Sig, significance ($p<0.05$); F, variation among group; df, degrees of freedom.

Table B.4. Analysis of variance for H4

		Sum of Squares	df	Mean Square	F	Sig.
cmlA	Between Groups	.001	3	.000	5.253	.027
	Within Groups	.001	8	.000		
	Total	.002	11			
ermB	Between Groups	.000	3	.000	1.076	.412
	Within Groups	.000	8	.000		
	Total	.000	11			
qnrS	Between Groups	.000	3	.000	5.056	.030
	Within Groups	.000	8	.000		
	Total	.000	11			
sul1	Between Groups	.003	3	.001	10.771	.003
	Within Groups	.001	8	.000		
	Total	.003	11			
tetA	Between Groups	.015	3	.005	4.302	.044
	Within Groups	.009	8	.001		
	Total	.024	11			
bla$_{CTX-M}$	Between Groups	.000	3	.000	2.351	.148
	Within Groups	.000	8	.000		
	Total	.000	11			
aadA	Between Groups	.089	3	.030	3.105	.089
	Within Groups	.077	8	.010		
	Total	.166	11			

aadA, aminoglycoside resistance gene; ***tetA***, tetracycline resistance gene; ***cmlA*** chloramphenicol resistance gene; ***sul1***, sulfonamide resistance gene; ***bla***$_{CTX-M}$, ß-lactam resistance gene; ***qnrS,*** quinolone resistance gene; ***ermB,*** MLS group resistance gene; Sig, significance ($p<0.05$); F, variation among group; df, degrees of freedom.

Table B.5. Analysis of variance for H5

		Sum of Squares	df	Mean Square	F	Sig.
cmlA	Between Groups	.001	3	.000	5.253	.027
	Within Groups	.001	8	.000		
	Total	.002	11			
ermB	Between Groups	.000	3	.000	1.076	.412
	Within Groups	.000	8	.000		
	Total	.000	11			
qnrS	Between Groups	.000	3	.000	5.056	.030
	Within Groups	.000	8	.000		
	Total	.000	11			
sul1	Between Groups	.003	3	.001	10.771	.003
	Within Groups	.001	8	.000		
	Total	.003	11			
tetA	Between Groups	.015	3	.005	4.302	.044
	Within Groups	.009	8	.001		
	Total	.024	11			
bla$_{CTX-M}$	Between Groups	.000	3	.000	2.351	.148
	Within Groups	.000	8	.000		
	Total	.000	11			
aadA	Between Groups	.089	3	.030	3.105	.089
	Within Groups	.077	8	.010		
	Total	.166	11			

aadA, aminoglycoside resistance gene; *tetA*, tetracycline resistance gene; *cmlA* chloramphenicol resistance gene; *sul1*, sulfonamide resistance gene; *bla*$_{CTX-M}$, ß-lactam resistance gene; *qnrS*, quinolone resistance gene; *ermB*, MLS group resistance gene; Sig, significance ($p<0.05$); F, variation among group; df, degrees of freedom.

Table B.6. Analysis of variance for H6

		Sum of Squares	df	Mean Square	F	Sig.
cmlA	Between Groups	.002	3	.001	10.552	.004
	Within Groups	.000	8	.000		
	Total	.002	11			
ermB	Between Groups	.002	3	.001	5.161	.028
	Within Groups	.001	8	.000		
	Total	.003	11			
qnrS	Between Groups	.000	3	.000	1.399	.312
	Within Groups	.000	8	.000		
	Total	.000	11			
sul1	Between Groups	.000	3	.000	.789	.533
	Within Groups	.001	8	.000		
	Total	.002	11			
tetA	Between Groups	.132	3	.044	10.971	.003
	Within Groups	.032	8	.004		
	Total	.164	11			
bla$_{CTX-M}$	Between Groups	.000	3	.000	1.372	.319
	Within Groups	.000	8	.000		
	Total	.000	11			
aadA	Between Groups	.641	3	.214	3.817	.058
	Within Groups	.448	8	.056		
	Total	1.088	11			

aadA, aminoglycoside resistance gene; ***tetA***, tetracycline resistance gene; ***cmlA*** chloramphenicol resistance gene; ***sul1***, sulfonamide resistance gene; ***bla***$_{CTX-M}$, ß-lactam resistance gene; ***qnrS,*** quinolone resistance gene; ***ermB,*** MLS group resistance gene; Sig, significance ($p<0.05$); F, variation among group; df, degrees of freedom.