ANALYSIS OF SELECTED ANTIBIOTICS AND HEAVY METALS IN NORTHERN MALAYSIA SEWAGE TREATMENT FACILITIES USING A WASTEWATER BASED EPIDEMIOLOGY APPROACH

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by

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Master of Science

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CERTIFICATE

This is to certify that the dissertation entitled “ANALYSIS OF SELECTED ANTIBIOTICS AND HEAVY METALS IN NORTHERN MALAYSIA SEWAGE TREATMENT FACILITIES USING A WASTEWATER BASED EPIDEMIOLOGY APPROACH” is the bonafide record of research work done by Mr. Iqbal Iman Bin Ruzi during the period from December 2020 to October 2022 under my supervision.

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DECLARATION

I hereby declare that this dissertation is the result of my study, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other study at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use this dissertation for teaching, research, and promotional purposes.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
<	Less than
=	Equal to
>	More than
±	Plus minus
≥	More or equal to
AAS	Atomic absorption spectrometric
APHA	American Public Health Association
ARB	Antibiotic-resistant bacteria
CDC	Centers for Disease Control
Col A	Colistin A
Col B	Colistin B
DTR	Drug target residue
EPA	United States Environmental Protection Agency
FDA	Food and Drug Administration
HGT	Horizontal gene transfer
ICU	Intensive care unit
LCMS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass-to-charge ratio
MDR	Multidrug-resistant
Mr	Meropenem
MNP	Magnetic Nano Particle
MRSA	Methicillin-resistant Staphylococcus aureus
p	P value
PE	Population equivalents
Poly 1	Polymyxin B1
Poly 2	Polymyxin B2
QC	Quality control
S/N	Signal-to-noise ratio

SPE	Solid phase extraction
STP	Sewage treatment plants
Vm	Vancomycin
WBE	Wastewater-based epidemiology
WHO	World Health Organization

ANALISA ANTIBIOTIK DAN LOGAM BERAT TERPILIH DI PUSAT RAWATAN KUMBAHAN DI UTARA MALAYSIA MENGGUNAKAN PENDEKATAN EPIDEMIOLOGI BERASASKAN AIR KUMBAHAN

ABSTRAK

Antibiotik dan logam berat kebiasaannya ditemui bersama di dalam sistem kumbahan, dan menimbulkan ancaman serius kepada kesihatan manusia dan alam sekitar. Pendedahan dalam jangka masa yang berpanjangan kepada antibiotik dan logam berat sebagai pencemar alam sekitar telah dikaitkan dengan kemunculan rintangan antibiotik. Sebaliknya, loji rawatan kumbahan (STP) telah dikaitkan dengan penyebaran rintangan antibiotik. Walaupun begitu dan fakta bahawa rintangan antibiotik semakin meningkat di seluruh dunia, tiada kajian alam sekitar yang pernah dijalankan di Malaysia mengenai kelaziman antibiotik dan logam berat dalam air sisa. Oleh itu, tujuan kajian ini adalah untuk menganalisis kehadiran dan nasib empat (4) antibiotik pilihan terakhir (Colistin, Polymyxin B, Meropenem, dan Vancomycin) dan lima (5) logam berat terpilih (Kuprum, Kadmium, Nikel, Zink, dan Besi) dalam sampel air kumbahan daripada 18 loji rawatan kumbahan (STP) berbeza di bahagian utara, Malaysia. Untuk kajian ini, pengekstrakan fasa pepejal-kromatografi cecair-spektrometri berjisim (SPE-LCMS) telah dibangunkan untuk menentukan empat antibiotik sasaran dalam air kumbahan dengan sensitiviti dan ketepatan yang memuaskan (had pengesanan di bawah 10 ng/L). Tahap antibiotik adalah di bawah 1 μg/L dengan pelepasan di bawah purata dalam STP, dan kepekatan efluen kadangkala lebih besar daripada influen. Meropenem dan polymyxin B adalah antibiotik yang paling lazim dikesan, diikuti oleh colistin dan vancomycin. Bagi analisis logam berat pula, ia ditentukan dengan menggunakan spektrometri serapan atom (AAS), dengan had kuantifikasi (LOQ) untuk Cd, Cu, Ni, Fe, dan Zn ditetapkan pada 0.01, 0.02, 0.05, 0.1 dan 0.5 (mg/L). Purata kepekatan logam berat dalam sampel air sisa didapati mengikut susunan berikut (unit mg/L): Fe > Ni > Zn > Cu > Cd (influen), dan Fe > Ni > Zn > Cd > Cu (efluen). Memberi perhatian bukan sahaja kepada kejadian dan penyingkirannya tetapi juga kepada pendedahan kepada antibiotik dan logam berat dalam populasi bandar dengan menggunakan pendekatan epidemiologi berasaskan air kumbahan (WBE). Berdasarkan pendekatan WBE, diunjurkan bahawa polymyxin B dan vancomycin adalah antibiotik yang paling lazim terdedahkan manakala besi adalah logam berat yang paling lazim telah terdedah di dalam populasi bandar. Kesimpulannya, kajian ini telah memberi sedikit penerangan terhadap kejadian, nasib, dan anggaran pendedahan antibiotik dan logam berat terpilih dalam populasi bandar di utara Malaysia.

ANALYSIS OF SELECTED ANTIBIOTICS AND HEAVY METALS IN NORTHERN MALAYSIA SEWAGE TREATMENT FACILITIES USING A WASTEWATER BASED EPIDEMIOLOGY APPROACH

ABSTRACT

Antibiotics and heavy metals are commonly found together in the sewage system, posing serious threats to human and environmental safety. Prolonged, albeit minimal, exposure to antibiotics and heavy metals as environmental pollutants have been associated with the emergence of antibiotic resistance. On the other side, sewage treatment plants (STPs) have been linked to the spread of antibiotic resistance. Despite the fact that antibiotic resistance is on the rise worldwide, no environmental study has ever been conducted in Malaysia on the prevalence of antibiotics and heavy metals in wastewater. Therefore, the purpose of this study was to analyse the presence and the fate of four (4) selected last-resort antibiotics (Colistin, Polymyxin B, Meropenem, and Vancomycin) with the additional five (5) selected heavy metals (Copper, Cadmium, Nickel, Zinc, and Iron) in wastewater samples from 18 different sewage treatment plants (STP) in the northern part of Malaysia. For this study, solid phase extraction–liquid chromatography-mass spectrometry (SPE–LCMS) was developed to determine the four target antibiotics in wastewater with satisfactory sensitivity (limits of detection below 10 ng/L), accuracy, and precision. Antibiotic levels were below 1 µg/L with below-average clearance in STPs, with effluent concentrations sometimes greater than influent. Meropenem and polymyxin B were the most prevalent antibiotics detected, followed by colistin and vancomycin. As for the analysis of the heavy metal, it was determined by using the atomic absorption spectrometric (AAS), with the limit of quantification (LOQ) for Cd, Cu, Ni, Fe, and Zn was set at 0.01, 0.02, 0.05, 0.1 and 0.5 (mg/L), respectively. The mean concentrations of heavy metals in the wastewater samples were found to be in the following order (unit of mg/L): Fe > Ni > Zn > Cu > Cd (influent), and Fe > Ni > Zn > Cd > Cu (effluent). Aside from their occurrence and elimination, this study also evaluate the exposure of the antibiotics and heavy metals in urban populations by using a wastewater-based epidemiology (WBE) approach. Based on the WBE finding, it is projected that polymyxin B and vancomycin were the most prevalent antibiotic been exposed within the urban population in Penang and Kedah, whereas iron was the most prevalent heavy metal been exposed. In conclusion, this study has shed some light on the occurrence, fate, and estimated exposure of certain antibiotics and heavy metals within the urban population of northern Malaysia.

CHAPTER 1

INTRODUCTION

1.1 Overview

Antibiotics are a type of medicine that can help fight off bacterial infections in the body. Antibiotic work by either eliminating the bacteria or reducing their growth, thereby giving the body's immune system a better opportunity to combat the infection. The use of antibiotics has been in existence for more than eight decades and has had a great impact on the medical field (World Health Organization, 2020). Numerous reports have indicated that antibiotics have decreased the severity of contagious diseases and saved a great number of lives (World Health Organization, 2020; Calhoun et al., 2023). To make antibiotics, scientists use a process called fermentation, which involves growing bacteria or fungi in large tanks under very specific conditions (Clardy et al., 2009; Isaiah, 2020). Once the microorganisms have produced the antibiotics, they are isolated and purified to create the final product. All the types of antibiotics have their own special way of functioning and range of effectiveness. Some of the antibiotics, like beta-lactams, disrupt the bacterial cell wall synthesis. Others, such as macrolides, work by inhibiting bacterial protein synthesis, while fluoroquinolones target bacterial DNA synthesis (Calhoun et al., 2023).

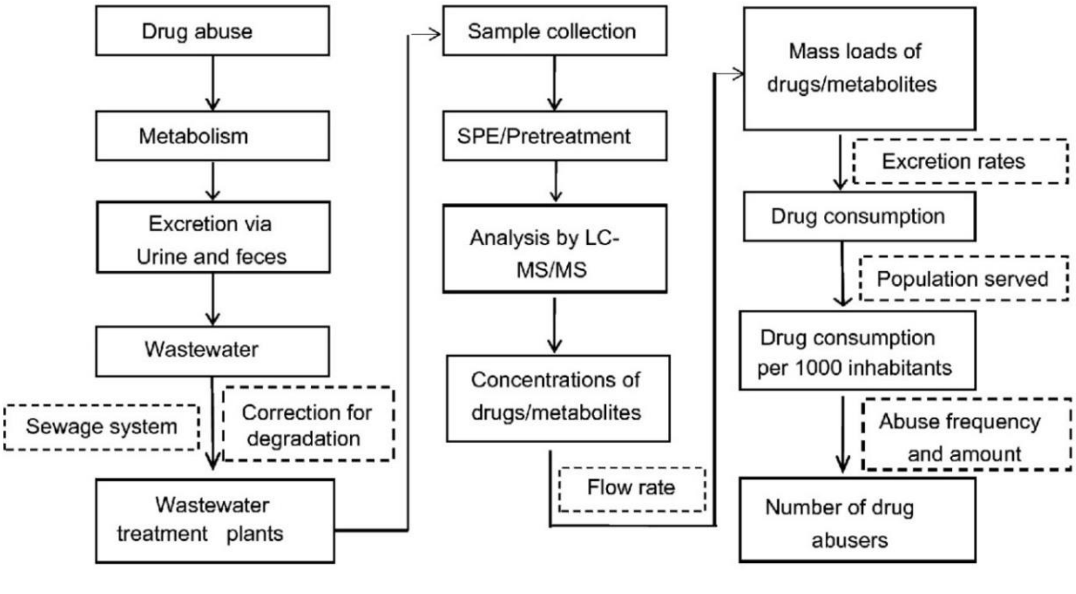
Nowadays, tons of antibiotics are dispensed and consumed yearly in human and veterinary medicine for treatment and prevention. In recent years, pharmaceuticals and related products, particularly antibiotics, have become emerging chemicals for environmental concern (Puckowski et al., 2016). It was reported that thousands of chemical substances have been utilized in medical and veterinary settings (Centers for Disease Control and Prevention, 2021), whereby these chemicals can be found freely in the environment. Out of these chemicals, the occurrence of antibiotics in terrestrial and aquatic organisms has become a main concern due to its potential to induce antimicrobial resistance in bacterial strains during their stay in the environment (Bengtsson-Palme and Larsson, 2015). To date, antibiotics have not undergone extensive testing for the environmental fate and consequences compared to other micro-pollutants (Jones et al., 2001), which contribute to their widespread distribution in the environment. In consequences, no permissible limit for the prevalence of antibiotics has ever been set by a responsible agency.

Antibiotics and their metabolites typically enter the aquatic environment through wastewater, usually through human intake and excretion (Kraemer et al., 2019). Numerous studies have shown the insufficient removal of pharmaceutical products, such as antibiotics, during the sewage treatment process (Ahmed et al., 2015; Lien et al., 2016; Subedi et al., 2017). Antibiotic features, such as high water solubility and limited degradability, have increased the likelihood of antibiotics passing through filtration and entering groundwater and drinking water (Mahmood et al., 2019). Through bioaccumulation and biomagnification, their presence at a certain concentration as the parent compound or metabolites may be sufficient to cause hazardous consequences in aquatic or terrestrial environments. Studies have been conducted with limited data on antibiotics and metabolites in the aquatic environment in various countries such as Sweden (Lindberg et al., 2005), United States (Batt et al., 2006; Kim and Carlson, 2007), Germany (Sacher et al., 2001), United Kingdom (Ashton et al., 2004), Italy (Calamari et al., 2003), Japan (Yasojima et al., 2006), Vietnam (Le and Munekage, 2004), China (Xu et al., 2007) and Hong Kong (Gulkowska et al., 2008). Data on antibiotic presence in wastewater in Asian countries, particularly Southeast Asia and Malaysia, is limited. Furthermore, no study to date has investigated antibiotic presence in the wastewater system in the Malaysian region

According to Malaysian National Antimicrobial Guideline 2019, tons of antibiotics are used yearly. Currently, the nationwide monitoring of antibiotic usage from all hospitals and wards has monitored 18 antibiotics from six classes (Ministry of Health (MOH), 2019). Some of them are the last-resort antibiotics (final antibiotic option when all other antibiotics have failed to treat an infection.), such as polymyxin and carbapenem. The latest survey reported an increment in antibiotic usage among the patient in the country, whereby the total antibiotic use had increased by 7.3% in 2018 from the previous year's data. Compared to 2017, there is an upward trend in the utilization of certain antibiotics: penicillin with β-lactamase inhibitor combination at 89.8%, glycopeptide at 88.5%, fluoroquinolones at 17.4%, and cephalosporins at 3.4%. There is evidence that the aquatic environment is a source and conduit for the propagation of antimicrobial resistance; therefore, their presence within the aquatic environment can induce the emergence of resistance microbial in humans and animals (Michael et al., 2013). If no appropriate action been taken, like limiting the use of "last-resort" antibiotics, antibiotic resistance will become a huge problem in hospitals within the next ten years or even sooner, before a new type of antibiotic can be developed.

Aside from antibiotic, heavy metals found in environmental reservoirs (municipal wastewater, agricultural runoff and industrial and pharmaceutical waste) were strongly linked to the emergence of antibiotic resistance (Braoudaki and Hilton, 2004; SCENIHR, 2009; Andersson and Hughes, 2012; Gillings et al., 2015; Dickinson et al., 2019; Komijani et al., 2021). Two main mechanisms, co-resistance and cross-resistance, have been identified as driving the link between antibiotic resistance and metal resistance in microorganisms (Baker-Austin et al., 2006; Knapp et al., 2017). The finding was supported by a few monitoring report that stated that antibiotic resistance is still increasing despite the efforts to minimise and control the antibiotic usage. Thus many researcher conclude that we can no longer assumed that emergence of antibiotic resistance only occurs when antibiotics are being used incorrectly or excessively. Few studies had considered that resistance might occur not only as a result of antibiotic-induced selective pressure, but also as a result of pollutants that enhance the transmission of genetic elements through cross-resistance and co-resistance mechanisms (Ashbolt et al., 2013; Baker-Austin et al., 2006; Berg et al., 2010; Perry and Wright, 2013). In line with the antibiotic study, no study has been carried out to investigate the occurrence of heavy metals in the wastewater system of any region in Malaysia.

More recently, Wastewater-Based Epidemiology (WBE) has been extensively used to monitor the use of pharmaceutical drugs, including antibiotics, based on the detection of excreted drug and their metabolites in wastewater at the population level (Kankaanpää et al., 2016). The principle is that each of the drugs consumed will eventually be drained into the treatment of sewage in the original molecule or metabolic products. Through the processing of wastewater, the study of drug residues or the metabolites in wastewater, and the back-calculation of drug intake, considering the flow of wastewater, the persistence of drug-target residues in wastewater, and drug/metabolite excretion , it is possible to profile drug usage at population level in a larger picture (Kim and Oh, 2020). Figure 1.1 shows the WBE approach and its flow.



Schematic diagram of wastewater-based epidemiology

Figure 1.1: Wastewater-Based Epidemiology approach for estimating the drug used in a community (adopted from Ferreira Aldo Pacheco, 2020).

There is an evident and pressing battlefront to continue working for the production of new antibiotics; unfortunately, the of new antibiotic discovery has declined dramatically due to the lack of incentives for generating new antibiotics. As a result, it is crucial to take every possible step to reduce the burden of antibiotic resistance. Antibiotics study in wastewater, especially in domestic environments, may provide early information on antibiotic occurrence and elimination.

1.2 Problem statement

Although antibiotics and heavy metals have been identified as posing potential environmental and human health risks, there remains a lack of comprehensive study into their presence and fate within the environment. The proliferation of antibiotic-resistant bacteria in aquatic ecosystems as a result of the occurrence of antibiotics and heavy metals has been recognised as a significant apprehension for mankind (Bengtsson-Palme and Larsson, 2015; Komijani et al., 2021). The Director of the Centres for Disease Control and Prevention (CDC) has released a warning about the possibility of antibiotic-resistant bacteria posing a significant health risk, characterised as a potential "nightmare" and "catastrophic threat" (McKenna, 2013). Therefore, this study would like to assess the population's exposure to selected antibiotics and heavy metals and gain fresh insights into their status as pollutants and contaminants in the sewage treatment plant (STP) in northern Malaysia.

1.3 Scope of the study

This study may mainly focus on two crucial scopes: evaluating the selected antibiotics and heavy metals concentration in the wastewater sample from the domestic sewage treatment plant (STP) and estimating their exposure to the population in the northern

Malaysia region. In order to determine the concentration of antibiotics and heavy metals, the liquid chromatography-mass spectrometric (LCMS) and Atomic absorption spectrometric (AAS) approaches were used. Colistin, Polymyxin B, Vancomycin, and Meropenem were the antibiotics chosen for this study, together with some heavy metals such as Copper, Cadmium, Nickel, Zinc, and Iron. A sampling of the wastewater was done for consecutive weeks to determine the average antibiotic/heavy metal level and their trend throughout the study period.

The population's exposure to antibiotics and heavy metals was obtained through the Wastewater Based Epidemiology (WBE) approach. The approach involves determining the concentrations of selected antibiotics and heavy metals in wastewater and back calculating the antibiotic and heavy metals mass loads associated with the investigated population. The metabolisms and excretion patterns of each antibiotic and heavy metal were determined in the calculation to estimate the exposure level per day to the population.

1.4 Objective of the study

This study aims to achieve the following general and specific objectives.

1.4.1 General objective

The general objective of this study is to assess the level of selected antibiotics (Colistin, Polymyxin B, Vancomyxin and Meropenem) and heavy metals (Iron, Copper, Cadmium, Zinc and Nickel) exposure in northern Malaysia sewage treatment facilities using a wastewater-based epidemiology (WBE) approach.

1.4.2 Specific objectives

1. To determine the concentrations of selected antibiotics in influents and effluents samples from various sewage treatment facilities in Penang and Sungai Petani district of Kedah.
2. To determine the concentrations of selected heavy metals in influents and effluents samples from various sewage treatment facilities in Penang and Sungai Petani district of Kedah.
3. To estimate the selected antibiotics and heavy metals population exposure in Penang and Sungai Petani district of Kedah using the wastewater-based epidemiology approach.

1.5 Hypothesis of the study

1. The concentrations of selected antibiotics (Colistin, Polymyxin B, Vancomyxin and Meropenem) from various sewage treatment facilities in Penang and Sungai Petani district of Kedah are within/exceeding the permissible limit set by the regulations.
2. The concentrations of heavy metals (Iron, Copper, Cadmium, Zinc and Nickel)) from various sewage treatment facilities in Penang and Sungai Petani district of Kedah are within/exceeding the permissible limit set by the regulations.
3. The exposure of the population to selected antibiotics and heavy metals is estimated for the first time in Malaysia to indicate their consumption in areas adjacent to sewage treatment facilities in Penang and the Sungai Petani district of Kedah.

CHAPTER 2

LITERATURE REVIEW

2.1 Antibiotics contamination in the environment

2.1.1 Occurrence of antibiotics in the aquatic environment

Antibiotics have been used extensively to support human and veterinary medicine and agriculture for decades. In past years, the occurrence and fate of antibiotics in the ecosystem have piqued the interest of scientists all around the world (Li et al., 2014; Wei et al., 2014). Please be noted that all the reports on the presence of antibiotics from the previous studies had used almost similar experimental approach and study design as has been adapted in this study. Currently, antibiotics have commonly found in urban wastewater (H. Zhang et al., 2013; Pan et al., 2014; Wang et al., 2018), surface water (Peng et al., 2011; Valcárcel et al., 2011; López-Serna et al., 2013), groundwater (López-Serna et al., 2013; Dhahir et al., 2019; Lu et al., 2019; Mahmood et al., 2019), soils, and sediments (Martínez-Carballo et al., 2007; Lapen et al., 2008; Silva et al., 2011; Na et al., 2013). While their concentration is low, antibiotics and their metabolites are now known as emerging toxins because they can contribute to the long-term growth, transfer, maintenance, and spread of antibiotic-resistant bacteria and resistant genes, posing long-term hazards to human and ecological health (Boxall et al., 2003; M. Silvia Díaz-Cruz et al., 2003; Golet et al., 2003; Göbel et al., 2005; Watkinson et al., 2007; Viswanathan, 2014; Shao et al., 2018). The continuous exposure to sub-toxin quantities of antibiotics could alter microbial ecology, encourage the development and spread of antibiotic resistance, cause harmful implications toward aquatic animals, and may have unanticipated impacts on human health via the food chain (Halling-Sørensen et al., 1998; Thiele-Bruhn, 2003). In the past years, the method of analysis and removal efficiencies of pharmaceuticals product, such as antibiotics, were developed by various technologies (Fatta-Kassinos et al., 2011). The information on antibiotic contamination in the environment below were compiled by two researchers recently (Gothwal and Shashidhar, 2015).

2.1.1.1 Sewage treatment plant

Generally, STPs are the last point for antibiotics that can be treated in sewage systems. Sadly, none of them intended to remove any pharmaceutical product, such as antibiotics, and as a result, they became the primary anthropogenic sources of the antibiotics present. The antibiotic build-up was well documented in sewage sludge from STPs in several countries, including the United States, China, Canada, Spain, and Sweden (Lindberg et al., 2006; Carballa et al., 2007; Kümmerer, 2009; Chang et al., 2010; MacLeod and Wong, 2010; Massey et al., 2010; Zuccato et al., 2010; Fatta-Kassinos et al., 2011; Jelic et al., 2011; Hedgespeth et al., 2012; Li et al., 2013a; Li et al., 2013; Zhou et al., 2013).

For the past years, China has conducted numerous research on the prevalence of antibiotics in STPs. Shanxi Province's household sludge had recorded amounts of quinolones as high as 29,647 mg/kg, as determined by an assessment of 45 STPs in 23 town in China (Li et al., 2013a). A previous study conducted in Beijing reported mean concentrations of quinolones, sulfonamides, and macrolides were found in Beijing’s wastewater reclamation plant are around 4,916 ng/L, 2,916 ng/L, and 365 ng/L, respectively (Li et al., 2013b). In the meantime, one study had observed a few antibiotics from eleven classes can be detected in Guangdong, South China, and the treatment plants only had removal efficiency of around 21 to 100 percent (Zhou et al., 2013).

2.1.1.2 Tap water

The widespread use of antibiotics has resulted in the contamination of water supplies that were previously thought to be safe for human consumption. For example, a study conducted in Madrid, Spain, found that pharmaceutical residues, including macrolides, erythromycin, and clarithromycin, were present in the city's tap water (Valcárcel et al., 2011). In other studies, four different fluoroquinolones (enrofloxacin, lomefloxacin, norfloxacin, and ciprofloxacin) were found inside the cities of Guangzhou’s (1.0 to 679.7 ng/L) and Macao’s (2.0 to 37 ng/L) tap water (Yiruhan et al., 2010).

2.1.1.3 Lakes, rivers and streams

Few antibiotics like ciprofloxacin (3.0 ng/L), clarithromycin (235.0 ng/L), erythromycin (320.5 ng/L), metronidazole (1195.5 ng/L), norfloxacin (10.0 ng/L), ofloxacin (179.0 ng/L), sulfamethoxazole (326.0 ng/L), tetracycline (23.0 ng/L), and trimethoprim (424.0 ng/L) have all been detected in the Spanish rivers Jarma, Manzanares, Guadarrama, Henares, Tagus (Valcárcel et al., 2011), and the Ebro and the Llobregat (López-Serna et al., 2013). Norfloxacin (5770.0 ng/g), ofloxacin (1290.0 ng/g), ciprofloxacin (653.0 ng/g), and oxytetracycline (652.0 ng/g) were also been detected in four rivers in China (Peng et al., 2008; Peng et al., 2009; Peng et al., 2011).

2.1.1.4 Sea water

Although coastal regions are recognized for their ecological significance, little research has been done to assess the prevalence of antibiotic contamination in ocean water. Generally, seawater antibiotic concentrations were much lower than those in river water and sewage treatment plant sludge (Gevao et al., 2022; Mirzaie et al., 2022). Direct sewage discharge and river confluences are believed to be the main contributors to antibiotic contamination in the ocean. Antibiotics have been detected in Beibu Gulf, the Dalian Coast, Bohai Bay, the Yellow Sea offshore, and the Bohai Sea itself (Zou et al., 2011; Zheng et al., 2012; Na et al., 2013; R. Zhang et al., 2013). With average concentrations between 0.5 to 6.30 ng/L, erythromycin-H_2O, sulfamethoxazole, and trimethoprim were more prevalent in the Beibu gulf (Zheng et al., 2012). The presence of erythromycin, sulfamethoxazole, and trimethoprim in the range of 0.10 to 16.6 ng/L has been reported from the coast of the Yellow Sea and the Bohai Sea (R. Zhang et al., 2013). An average of 2.11 to 9.23 ng/L of tetracycline was also detected in seawater and 1.42 to 71.32 mg/kg sulphonamides in the sediment of Dalian province, China (Na et al., 2013).

2.1.1.5 Aquifers

Human actions have made it possible for antibiotics to get into urban aquifers. Even though soil slows the movement of contaminants into groundwater, it is hard to lessen their effects. Sources of groundwater recharge include natural bank filtration, the seepage of wastewater and water supply pipes, and rain. Antibiotics and other chemicals like hormones, pharmaceuticals, personal care items, pesticides, and industrial compounds have been found in groundwater at far lower amounts than in rivers and sewage treatment plants (Barnes et al., 2008; Hu et al., 2010; Cabeza et al., 2012; Jurado et al., 2012; Lapworth et al., 2012; López-Serna et al., 2013). An investigation had found 72 pharmaceutical products and their metabolites in the groundwater of rural and urban areas beneath the city of Barcelona (López-Serna et al., 2013). Ciprofloxacin was found in the highest concentration, at a mean of 323.75 ng/L, in another study. This was attributed to infiltration from agriculture and/or poorly treated water (Cabeza et al., 2012). Whereas, in Catalonia, Spain, researchers found 18 different sulfonamides in concentrations ranging from 0.01 to 3460.57 ng/L in the region's groundwater, which they believed originated from the livestock veterinary practice in the area (García-Galán et al., 2010; Jurado et al., 2012). Sulfamethoxazole was the most commonly found veterinary and human antibiotic in 2000 groundwater samples from 18 states in the United States (Barnes et al., 2008). Interestingly, one study reported that antibiotic levels in groundwater around pig and cattle farms were equivalent to the drinking water of the surrounding area (Bartelt-Hunt et al., 2011).

2.1.1.6 Soils and sediments

Most antibiotics will enter the environment via sludge and manure; when manure is dumped repeatedly, the antibiotics will build up in the soil. Antibiotics, as they are well-known, are produced naturally by microorganisms that make their homes in soil and sediment and through a biosynthetic process that results in antibiotics. One study conducted in Korea, where a few of the antibiotics were identified such as macrolides, sulfonamides and tetracyclines in cattle (0.07 to 0.14 mg/kg, 0.49 mg/kg and 1.65 mg/kg), pig (1.05 to 2.1 mg/kg, 8.44 mg/kg and 16.56 mg/kg) and poultry (0.62 to 1.24 mg/kg, 1.39 mg/kg and 15.62 mg/kg) manures (Kim et al., 2011). Antibiotics have been documented in manure, soil, and sediments by several other investigations employing a similar methodology (Martínez-Carballo et al., 2007; Lapen et al., 2008; Silva et al., 2011; Li et al., 2012; X. Li et al., 2013; Na et al., 2013).

Antibiotics can also be transferred via direct use as pesticides on vegetables, fruits, and ornamental plants, as well as indirect use via fish farming, surface water floods, dumping of industrial waste, and direct use in livestock (Kim et al., 2011). One comparative study found that organic (manure-applied) vegetable fields contained more antibiotics than conventional (chemical fertilizer-applied) vegetable fields (Karcı and Balcıoğlu, 2009).

2.1.1.7 Animals and plants

Antibiotics could enter biota due to their presence in surface water, groundwater, ocean, soil, and sludge, which will be absorbed by plants, aquatic plants, and animals (Kümmerer, 2009a; Hu et al., 2010; Eggen et al., 2011; Dong et al., 2012; Li et al., 2012; Li et al., 2013; Na et al., 2013). A study conducted in winter season was reported that celery leaves had accumulated ofloxacin (1.7 to 3.6 mg/kg), pefloxacin (1.1 mg/kg), and lincomycin (5.0 to 20.0 mg/kg); radish leaves had accumulated sulfadoxine (0.2 to 0.6 mg/kg), sulfachloropyridazine (0.1 to 0.5 mg/kg), chloramphenicol (8.0 to 30.0 mg/kg), and sulfamethoxazole (0.9 to 2.7 mg/kg); coriander leaves had accumulated oxytetracycline (78.0 to 330.0 mg/kg), tetracycline (1.9 to 5.6 mg/kg), and chlortetracycline (92.0 to 481.0 mg/kg) (Hu et al., 2010)**.** Quinolones were found at the highest amounts among aquatic plants (8.37-6532.0 mg/kg) in a study at Baiyangdian Lake in northern China, with quinolones levels in the range of 17.8-167.0 mg/kg and macrolide level up to 182.0 mg/kg also were detected in aquatic organisms and birds (Li et al., 2012).

2.1.2 Fate of antibiotics in the aquatic environment

In general, the degradation of antibiotics (including colistin, polymyxin B, vancomycin, and meropenem) can occur in aquatic environments through various mechanisms, such as hydrolysis, oxidation, and photolysis, and will affect the antibiotics' stability, effectiveness, and pharmacokinetics. (Yang et al., 2021). Hydrolysis is a process where the interaction between water and antibiotics results in the breakdown of antibiotic molecules into smaller components (Meliá et al., 2015; He et al., 2020). The process of oxidation can lead to the generation of diverse oxidation byproducts across all four antibiotics (Wang and Zhuan, 2020; Li et al., 2023). The process of photolysis, characterized by degradation upon exposure to sunlight, has the potential to take place in surface waters (Timm et al., 2019; Cuerda-Correa et al., 2020). The degradation rate and extent may be subject to environmental factors and coexisting substances, necessitating additional investigation to enhance comprehension of their destiny and probable repercussions on water quality and environmental well-being.

The presence of antibiotics in STPs makes the antibiotics undergo some chemical, biological and mechanical processes. Generally, antibiotic degradation ultimately depends on their physicochemical characteristics. The efficiency of removing the antibiotics in conventional wastewater treatment varies significantly because the original purpose of the conventional biological treatment design for the treatment plant is not to cope with the emerging pollutants such as antibiotics. However, it has been observed that current conventional activated sludge treatment methods are less effective than membrane bioreactor systems, which have marginally greater efficacy. Therefore, a few antibiotics, such as b-lactams, macrolides, tetracyclines, fluoroquinolones, and sulfonamides, have been studied to improve the removal process through biological treatment, advanced oxidation processes, and activated carbon adsorption treatment. Despite the success of the study, operational and maintenance expenses will rise (Le-Minh et al., 2010; Michael et al., 2013).

The occurrence of antibiotics in the aquatic environment is primarily from STP discharges. During their presence in the aquatic environment, the antibiotics may form a complex with the suspended matter. Then it can gather up on sediments and be reintroduced into the water column. Certain antibiotics with low adsorption affinity will remain within the water column, whereas the remaining will be stored in sediments of aquatic bodies. Antibiotics with poor sorbing capability in the soil are usually leaked into the surface runoff and groundwater. In contrast, antibiotics with a strong sorbing capability will accumulate in soil and sediment (M.Silvia Díaz-Cruz et al., 2003). In addition, the water partitioning coefficient is an important factor in affecting the photodegradation, adsorption, and biodegradation of antibiotics.

The environmental soil system is much more complicated when compared to the aquatic system due to the buffering ability of the soil to attenuate the contaminant's toxicity easily. Nevertheless, it only offers excellent accumulation but is very poor in mobility. This low mobility in the soil environment causes a long exposure to the contaminants and may increase the effects of the contaminant toxicity. Antibiotic persistence in sediment or soil is primarily determined by its adsorption capacity, photostability, of breakdown, and water leaching. It was reported that sorption, photodegradation, biodegradation, and oxidation were fundamental processes in eliminating antibiotics from the environment (Knapp et al., 2005; Li and Zhang, 2010).

2.2 Antibiotic resistance

The term "antibiotic resistance" is referred to the phenomenon in which bacteria become immune to or develop defences against the antibiotic treatment that was previously successful. As a consequence to the rise of resistant bacteria toward antibiotics, the past 80 years of global usage of antibiotics have unfortunately led the way to an increase in the severity of global health issues. Since antibiotics are essential in the medical treatment of bacterial infections, the resistance toward antibiotics leads to serious problems resulting in tens of thousands (10.00) of deaths per year that are expected to worsen dramatically over the next few years (O’Neill, 2014). Thus, the shortcoming of effective antibiotics would significantly impede numerous current medical operations, including surgery, cancer therapy, and newborn care.

Antibiotics are not only used in the clinical setting. In addition, their usage in agriculture (Hoelzer et al., 2017) and aquaculture (Done et al., 2015). The massive usage of antibiotics has led to the emergence of antibiotic-resistant bacteria. For example, a penicillin-inactivating enzyme that was found prior to the clinical drug usage (Abraham and Chain, 1940), its resistant strains were found to will evolve for each new antibiotic introduced after then (Davies and Davies, 2010). There is a kind of situation where numerous kinds of pathogens exhibit multiresistant capability, and often any; infectious diseases caused by these multiresistant pathogens are normally incurable. The growing issue regarding antibiotic resistance has been seen as a serious danger to global health (World Health Organization, 2014) since antibiotics have been fundamental to contemporary medicine, and their loss would be catastrophic.

It is suggested that misuse and overuse of antibiotics in livestock and people are likely one of the leading causes of the global problem of antibiotic resistance. Although there is a lack of information on the role the environment plays in providing resistance novel genes and transmitting mechanisms for both resistant bacteria and resistance genes (Ashbolt et al., 2013; Bengtsson-Palme et al., 2014), but it is highly probable since the environment had been proved to accommodate several unidentified antibiotic resistance genes (ARG) that could get enhanced and transmitted to any infectious agent. Probably, many ARGs have experienced this in the past (Davies and Davies, 2010).

Antibiotic resistance, both directly and indirectly through co- or cross-resistance, has been demonstrate to increase with exposure to biocides and heavy metals (Braoudaki and Hilton, 2004; SCENIHR, 2009; Andersson and Hughes, 2012; Singer et al., 2016). Considering that antimicrobials are being released into nature as a result of human activities, the possibility that antibiotics, biocides, and metals will exert selective pressure on environmental microorganisms, hence fostering the creation of antibiotic-resistant bacteria cannot be neglected.

2.2.1 Emergence of antibiotic resistance bacteria

The capability of bacteria to become resistant to antibiotics is one of the natural phenomena, and resistance strains are inevitable toward antibiotic treatment. To date, bacteria have developed a defence mechanism that allows them to withstand the effects of any antibiotic now on the market (Souli et al., 2008; Pattnaik et al., 2019). It is possible that a lack of antibiotics with novel mechanisms will return the globe to a state more or less the same as the pre-antibiotic era, in which non-curable bacterial diseases were more prevalent. Unfortunately, this is the situation in many regions worldwide (Lagamayo, 2008; Boucher et al., 2009; Lepape et al., 2009; Kumarasamy et al., 2010; Lepape et al., 2020). Generally, it is easier for the resistance to emerge than to lose it (Andersson and Hughes, 2010; Johnsen et al., 2009; Sundqvist et al., 2010). Thus, antibiotic treatment failure has extremely serious effects in hospitals, where frail patients are treated for the most acute and occasionally fatal illnesses.

Antimicrobial resistance can be intrinsic or acquired. The intrinsically resistant microbe is resistant to certain antimicrobials due to innate characteristics. Additionally, microorganisms can develop immunity by acquiring resistance genes from others or through mutation. Antibiotics may have the capability to impose selective pressure on both normal microbial flora and the targeted bacterium. The eradication of antibiotic-susceptible microorganisms promotes the proliferation of antibiotic-resistant germs, which could lead to an increase in the frequency of pathogens with resistance variations and an ecological shift characterized by a rise in the proportion of natural microbes. This will result in the spread of more genes for antibiotic resistance into the environment, leading to antimicrobial resistance (Livermore, 2003; Davies and Davies, 2010; Martinez, 2014).

Antibiotic resistance development, which may limit therapeutic potentials against human and animal infections, is the most pressing concern about the discharge of antibiotics into the environment. The same species' microorganism will inherit the resistance ability through cell division (vertical gene transfer), a primary resistance. In contrast, secondary resistance will develop when there is contact between a microbe and the antibiotic (Kümmerer, 2009a).

The transfer of plasmid-mediated resistance between microorganisms, including the transfer of extrachromosomal genetic material between different species by conjugation, is known as horizontal gene transfer (Kümmerer, 2009a). Horizontal gene transfer will allow the antibiotic resistance gene to spread rapidly among the microorganism and adapt to the environmental selection pressures due to the presence of antimicrobials in the environment. There are three fundamental mechanisms for horizontal gene transfer to occur: (i) Conjugation,

(ii) Transduction, and (iii) Transformation (Cytryn, 2013; von Wintersdorff et al., 2016). Through conjugation, exchanging DNA by transmitting the genetic component like plasmid among the microorganism. Transduction is the exchange of DNA via viral-mediated, usual bacteriophages of the microorganism; meanwhile, the absorption of bare DNA is called transformation (von Wintersdorff et al., 2016). According to recent information, the estimated number of bacterial cells on Earth exceeds a quintillion (Microbiology by numbers, 2011; Kallmeyer et al., 2012). The horizontal gene transfer is expected to occur more frequently within species, including gene transfers and mutations.

Few studies have shown that antibiotic resistance can also increase over time when exposed compared to other antibacterial substances such as biocides and heavy metals (Braoudaki and Hilton, 2004; SCENIHR, 2009; Andersson and Hughes, 2012; Singer et al., 2016). This exposure will allow the heavy metal or biocide to co-select antibiotic resistance. Three fundamental mechanisms for the co-selection of antibiotic resistance are co- and cross-resistance and co-expression/co-regulation. When co-resistance occurs, the antibiotic, biocide, or heavy metal resistance genes are on the same genetic element, such as a plasmid. The term "cross-resistance" refers to the fact that a single gene (or mechanism) can confer resistance to many antibiotics, biocides, or heavy metals. In contrast, co-expression or co-regulation mechanism regulates one regulatory gene in other resistance genes. Many antibiotic, biocide and heavy metal resistant bacteria have already been identified (Pal et al., 2015). The resistance to erythromycin and copper (Amachawadi et al., 2011), silver and fluoroquinolones (Fang et al., 2016), and mercury and several antibiotics (Drace et al., 2018) are a few of the examples that have been reported. Co-selection makes reversing antibiotic resistance much more difficult, as other compounds hold the selection pressure even when the antibiotic is removed (Enne et al., 2004)

2.2.2 Environmental role to the existence of antibiotic-resistance bacteria

There is a growing consensus that human and animal antibiotic usage and misuse are major contributors to the global problem of antibiotic resistance, where the environmental factor may contribute to the occurrence of the problem (Ashbolt et al., 2013). The presence and availability of a greater number of environmental bacterium compared to human infections make it possible for the environment to act like a reservoir for ARGs that can be passed to other microbial agents (D'Costa et al., 2006; Wright, 2010; Finley et al., 2013). Some ARGs that are causing problems in healthcare now are likely passed on by non-pathogenic microorganisms found in the environment (Bonomo and Szabo, 2006). The occurrence of the resistance genes in bacterium is a natural process based on the genetic material that has survived in the wild for a very long period (D'Costa et al., 2011). Antibiotics are a part of the ecosystem since many of the antibiotics used in medicine come from the environment, specifically soil microorganisms; therefore, it is likely that resistance genes developed long before human antibiotic usage. It is quite probable that the environment contains unidentified antibiotic resistance genes that could be transmitted to infections and subsequently enriched (Bengtsson-Palme et al., 2018). The environmental factor likely contributes to the transfer of resistant genes among the hosts. Thus, the environment might be the origin of some opportunistic pathogens (Berg et al., 2005).

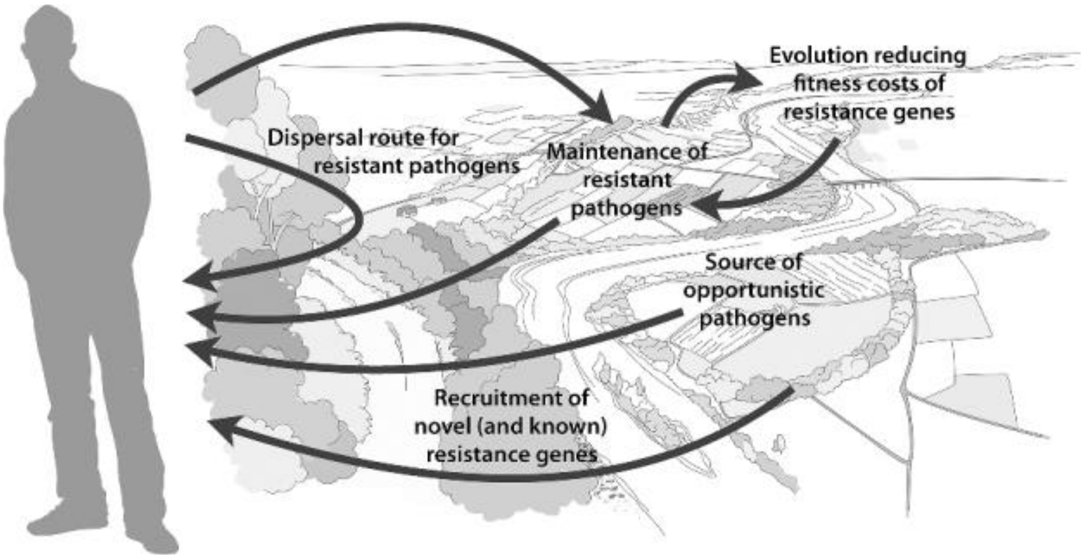


Figure 2.1. Influence of the environment on the emergence and spread of antibiotic-resistant microorganisms (adopted from Östman, 2018).

2.2.3 Dissemination of resistant genes and resistant bacteria

Bacteria can acquire the antibiotic-resistant gene through horizontal gene transfer (HGT) or mutation of a gene. HGT enables resistant genes to rapidly disseminate among all bacteria and respond to environmental antimicrobial selection pressure. Generally, HGT occurs by conjugation, transduction, and transformation, which is crucial for developing resistant bacteria (von Wintersdorff et al., 2016). Conjugation is a process where DNA is transmitted between bacteria via a plasmid, while transformation covers the reception of the DNA, and transduction involves the exchange of DNA by bacteriophages (von Wintersdorff et al., 2016). When the total of bacteria within the surround is uncountable, many believe HGT and mutation can occur between species.

One study reported that the acquired resistance gene had been shown to incur a fitness cost, like a slower growth . In general, with high fitness costs and no antimicrobial selection pressure, the mutation will most likely not become fixed in the population (Andersson and Hughes, 2010). However, compensatory mutations frequently occur, with the availability of low-cost or free-cost ARGs making it extremely hard for reverse resistance to happen, even when the selective pressure is eliminated Andersson and Hughes, 2010). Additionally, there are no barriers preventing the mixing of gene pools from diverse ecosystems with gene pools from more therapeutically relevant contexts (Aminov and Mackie, 2007; Aminov, 2011).

2.2.4 Past resistant activity toward last-resort antibiotics

The term "last resort antibiotic" refers to a class of antibiotics that are employed in the treatment of severe bacterial infections that have become resistance to conventional antibiotics. The prudent and meticulous usage of this particular antibiotic is imperative in order to mitigate the progression of antibiotic resistance and safeguard its efficacy for the management of critical infections in situations where alternative treatments are unavailable (World Health Organization, 2014).

Since the microorganism's ability to evade and adapt to antibiotics' therapeutic effects is inexorable, this issue has triggered a warning alarm from a few crucial personages. It is very alarming to hear that Thomas Frieden, Head of the U.S. Centers for Disease Control and Prevention (CDC), and Sally Davies, Chief Medical Officer of the United Kingdom, issued an alert regarding antibiotic-resistant bacteria as a potential health "nightmare" and "catastrophic threat" (McKenna, 2013). Sally Davies also has described antibiotic-resistant bacteria as a risk as serious as terrorism (Nature, 2013). The growing awareness of antibiotic resistance created by the World Health Organization and the E.U., especially in the global and regional reports on antibiotic surveillance, can be witnessed throughout the years (World Health Organization, 2011; World Health Organization, 2014).

A study has detected a significant amount of resistance genes toward clinically effective antibiotics, such as sulfonamides, trimethoprim, and tetracyclines, in rivers fed with drainage effluents from farms where antibiotics were used and the sewage treatment plants

(Rowe et al., 2016). India and Bangladesh have recorded massive amounts of antibiotic resistance enzymes such as NDM-1 and CTX-M-15, which impart resistance to most β-lactams and cephalosporins, which were found in the environment and tap water (Walsh et al., 2011; Rashid et al., 2015; Toleman et al., 2015). The consequences of this escalating preponderance of resistance genes in the environment and consequently in human pathogens cannot be ignored, however momentarily, the end of antibiotics as effective therapeutic agents in the battle against bacterial infections (Laxminarayan et al., 2013; Liu et al., 2016)

The discovery of antibiotics regularly has decreased the incidence and mortality of many previously lethal diseases, including sepsis, meningitis, measles, gangrene, dysentery, food-borne diarrhea, salmonellosis, and pneumonia (World Health Organization, 2014). The increased life expectancy, improved quality of life, and increased wealth were concrete benefits of antibiotic use; however, antibiotic resistance remains a challenge to these gains. One explanation for increased bacterial resistance is the growing usage of uncontrolled antibiotics in clinical and veterinary medicines (Laxminarayan et al., 2013; Liu et al., 2016). Antibiotics such as carbapenems, colistin, and tigecycline are currently used alone or in combination as a reserve antibiotic to combat lethal bacterial infections (Osei Sekyere et al., 2016). Unfortunately, recent studies have shown that resistance to these last-resort antibiotics has risen frequently (Sekyere et al., 2016).

2.2.5.1 Resistance to Colistin

Colistin is a polymixin antibiotic (polymixin E), a product of *Bacillus colistinus* (Koyama et al., 1950). Polymyxin was discovered in 1947 (Ainsworth et al., 1947; Benedict and Langlykke, 1947), while Koyama and his colleagues isolated 'colistin' in 1950 (Koyama et al., 1950). In the 1940s, five polymixins (A through E) were isolated from the soil bacteria Bacillus *polymyxa* (Ainsworth et al., 1947; Benedict and Langlykke, 1947); only polymixin B (PMB) and polymixin E have been identified as having considerable antibacterial action (colistin) (Landman et al., 2008). However, in the early 1980s, colistin and polymyxin B intravenous formulations were eventually banned in most parts of the world due to the high incidence of nephrotoxicity been reported (Ryan et al., 1969; Brown et al., 1970; Koch-Weser et al., 1970). In the past, intravenous use of colistin for the treatment of lung infections attributable to multidrug-resistant (MDR), gram-negative bacteria cystic fibrosis patient, was consequently prohibited (Conway et al., 1997; Ledson et al., 1998; Cunningham et al., 2001).

In past decades, the absence of innovative antibiotics to treat drug-resistant infections, particularly those caused by Gram-negative organisms, has prompted authorities to reassess colistin as the treatment of choice (Helen W. Boucher et al., 2009). The medical practitioner is employing Colistin against P. *aeruginosa* caused bacteremia and ventilator-associated pneumonia (VAP) (Landman et al., 2008; Nation and Li, 2009). According to a few reports, colistin is utilized as inhaled colistimethate sodium (CMS) to treat VAP in intensive care unit patients (Michalopoulos et al., 2008; Falagas et al., 2009). Colistin has proven successful when no other medication is available for multi-resistant bacteria in patients with severe burns (Cho et al., 2012). Recent reports indicate that intraventricular administration of colistin is used to treat ventriculitis caused by MDR A. *baumannii* (Dalgic et al., 2009; López-Álvarez et al., 2009).

Colistin resistance cases reported were surprisingly low, possibly due to its rare application. Nevertheless, some Gram-negative bacterial species have recently been identified as resistant to this antibiotic. A report from Ko et al. in 2007 described the large proportion of A. *baumannii* subgroups II and III strains in Korea that are resistant to colistin/polymyxin. The heteroresistance toward colistin in MDR A. *baumannii* was reported in about 23% to 100% of clinical isolates in the past few years (Li et al., 2006; Hawley et al., 2008; Yau et al., 2009). There were studies reported that the occurrence of heteroresistance to colistin has also been discovered in several species, including P. *aeruginosa* (Bergen et al., 2011) and K. *pneumoniae* (Poudyal et al., 2008; Meletis et al., 2011). P. *aeruginosa's* resistance to colistin is becoming a