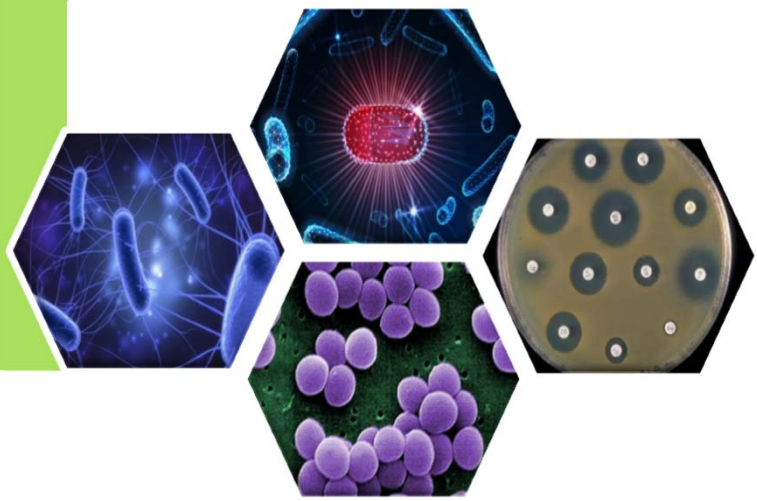
Multidrug Resistant Bacteria in Mekong River, Tonle Sap, Bassac River and Sewage Water



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ABSTRACT

Cambodia has ample supply of water, mainly from the Mekong River, Tonle Sap River, Bassac River and other tributaries that is vital sources for national economic in many sectors, such as agriculture, industrial, environmental protection and daily life. Due to rapid population growth, urbanization, industrialization, climate change, and economic development, Cambodia's water resources are facing increasing pressure. Water pollution is becoming major concerns for the Cambodian authorities and development partners. According to these concerns on public's health, small research related to Multidrug Resistant Bacteria have been conducted, which aimed to (i) detect pathogenic multidrug resistance bacteria in Mekong River, Tonle Sap and Bassac River and sewage water and (ii) disseminate project finding to public and relevant stakeholders. To achieve the objectives, 6 locations of water sampling sites have been randomly chosen including 4 public water and 2 sewage water sampling sites in 3 provinces and 1 municipality. All samples have been analyzed 4 physicochemical and 3 microbiological parameters. The six most common antibiotics have been used for multidrug-resistant bacteria (MDR) analysis. As results, the isolate showed high resistance to Ampicillin and Trimethoprim while Gentamicin indicates the lowest resistance among the six antibiotics. Interestingly, some isolates of the *E. coli*, *Staphylococcus* and *Salmonella* found resistant to Trimethoprim while some isolates of the *E. coli* and *Salmonella* indicate high resistance to Ampicillin. This prevalence of resistant may be explained by the fact that these two antibiotics (Ampicillin and Trimethoprim) have been widely used for therapeutic purpose against bacterial infections in humans and animals in Cambodia. For correlations, *E. coli* has a strong and positive relationship with *Salmonella* ($P < 0.001$; $R = 0.72$), while EC had a moderate positive relationship with pH ($P = 0.016$; $R = 0.4$) and strong positive relationship with *Staphylococcus* ($P = 0.002$; $R = 0.62$). Which means that when EC increases, the present of *Staphylococcus* and pH may also be higher, and vice versa. For other parameters, there were no relationship at all, and although one parameter increases, that does not affect the others. After understanding of the findings of the study some recommendations have been raised as following: (1) safely manage all antibiotic residual waste, both liquid and solid form should be strictly practiced; (2) installing treatment facilities at main pollution sources, especially pharmaceutical manufacturers, hospital, health center, etc. must be applied; (3) ensure safe consumption of antibiotic in all sectors. Further study need to be conducted for more detail on the identification of resistant colonies—pathogenic or nonpathogenic.

Keywords: Antibiotics; Resistant; Susceptible; Multidrug resistant bacteria

ABBREVIATIONS AND SYMBOLS

ARB	Antibiotic Resistant Bacteria
BSR	Bassac River
CFU	Colony Forming Unit
CDC	Centers for Disease Control and Prevention conservatively
CLSI	Clinical and Laboratory Standards Institute
DO	Dissolve Oxygen
EC	Electrical Conductivity
EPA	Environmental Protection Agency
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
MDR	Multidrug Resistant
MR	Mekong River
PBS	Phosphate Buffer Saline
pH	Potential Hydrogen
SI	First Sampling
SII	Second Sampling
TSL	Tonle Sap Lake
TSR	Tonle Sap River
WW	Wastewater
WHO	World Health Organization

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1. INTRODUCTION

1.1. Background

Water, a substance composed of the chemical elements hydrogen and oxygen and existing in gaseous, liquid, and solid states. It is one of the most plentiful and essential of compounds. A tasteless and odorless liquid at room temperature, it has the important ability to dissolve many other substances. Indeed, the versatility of water as a solvent is vital to living organisms. Life is believed to have originated in the aqueous solutions of the world's oceans, and living organisms depend on aqueous solutions, such as blood and digestive juices, for biological processes. The water on the surface of Earth is found mainly in its oceans (97.25 %) and polar ice caps and glaciers (2.05 %), with the balance in freshwater lakes, rivers, and groundwater. As Earth's population grows and the demand for fresh water increases, water purification and recycling become increasingly important. Interestingly, the purity requirements of water for industrial use often exceed those for human consumption (Zumdahl, 2024).

Cambodia has ample supply of water, mainly from the Mekong River, Tonle Sap River, Bassac River and other tributaries. Rivers and streams, lakes, aquifers and marine water are important sources for national economic development in many sectors, such as agriculture, manufacturing and small-scale industries, hydropower, navigation, tourism, environmental protection and daily life. In general, surface water in Cambodia meets the national ambient water quality standards. However, water quality has been under threat in some areas, especially during the dry season due to the inflow of untreated effluents into public water bodies from urban activities, agricultural run-off, navigation, etc.

Cambodia's water resources are facing increasing pressure due to rapid population growth, urbanization, industrialization, climate change, agriculture, and economic development. Water supply and wastewater treatment are becoming major concerns for the Cambodian authorities and development partners. Even though discharging untreated and low-quality treated wastewater carries environmental and health-related risks, it remains a common practice in Cambodia because the systems required to treat wastewater are insufficiently developed.

The water pollution is a significant impact on water resources, causing seasonal instability of surface water and ground water, as well as increasing water quality issues. Contaminated water and poor sanitation are linked to transmission of diseases such as cholera, diarrhea, dysentery, hepatitis A, typhoid and polio (World Health Organization, 2023). Anyway, present of bacteria in our environment, including Minnesota's surface waters and groundwater, can be harmful to human health. Drinking water with disease-causing bacteria, viruses, or parasites (collectively called

pathogens) can make you sick. It is not practical to test drinking water for every type of pathogen, but it is simple to test drinking water for coliform bacteria. The presence of coliform bacteria can indicate there may be harmful pathogens in the water (Department of Health, 2023). The presence of coliform bacteria, specifically *E. coli* (a type of coliform bacteria), in drinking water suggests the water may contain pathogens that can cause diarrhea, vomiting, cramps, nausea, headaches, fever, fatigue, and even death sometimes.

Antibiotics have been developed in the 1940s, and since then, these drugs have saved millions of lives. However, almost simultaneously with their discovery, it was observed that bacteria were capable of developing mechanisms of resistance to antibiotics (Butler and Paterson, 2020). Notwithstanding, the use of antibiotics has been neglected, being used massively over the years in both human and veterinary medicine leading to the increase of antimicrobial resistance (AMR) (Huijbers, Flach and Larsson, 2019). AMR is a natural evolutionary process since genes encoding resistance mechanisms are naturally present in nature. However, the overuse of antibiotics introduced a selective pressure that inevitably selected and favored the development and evolution of resistance (Davies, 2010).

Antimicrobial resistance in the environmental dimension is one the greatest challenges and emerging threats. The presence of resistant bacteria and resistance genes in the environment, especially in aquatic systems, has been a matter of growing concern in the past decade. Monitoring the presence of antimicrobial resistance species, in this particular case, *Staphylococcus* spp., in natural water environments could lead to a better understanding of the epidemiology of staphylococci infections. Thus, the investigation of natural waters as a potential reservoir and vehicle for transmission of these bacteria is imperative. Only a few studies have investigated the prevalence, antimicrobial resistance and genetic lineages of staphylococci in natural waters. Those studies reported a high diversity of staphylococci species and lineages in surface waters. Methicillin-resistant S. aureus (MRSA) were relatively prevalent in surface waters and, as expected, often presenting a multidrug-resistant profile. There was a high diversity of S. aureus lineages in surface waters. The presence of S. aureus CC8 and CC5 suggests a human origin. Among the coagulase-negative staphylococci, the most frequently found in natural waters was *S. warneri* and *S. epidermidis*. These studies are extremely important to estimate the contribution of the aquatic environment in the spread of pathogenic bacteria (Vanessa Silva, 2020).

Most cases of salmonella come from ingesting food or water contaminated with feces. Undercooked meat, egg products, fruits, and vegetables can also carry the disease. Most people do not develop complications, but children, pregnant women, older adults, and people with weakened immune systems are most at risk (Lifewater, 2019).

1.2. Objective

The objective of this research is:

- To detect pathogenic multidrug resistance bacteria in Mekong River, Tonle Sap and Bassac River and sewage water and
- To disseminate project finding to public and relevant stakeholders.

1.3. Scope and Limitation

In this research, there are 6 locations of water sampling have identified, such as 4 public waters sampling and 2 wastewater sampling. The sampling located in Kandal province (1 public water and 1 wastewater), Kampong Chhnang province (1 public water), Kampong Cham (1 public water) and Phnom Penh (1 public waters and 1 wastewaters). All sample collection for experiment in laboratory. The procedures of this work will be performed under limitation as shown below:

- There are performed 2 times as replication of water sampling.
- Samples will also be analyzed: physicochemical analysis (pH, temperature, DO, and EC).
- Samples will also be analysed: Microbiological analysis (*E. coli*, *Staphylococcus* and *Salmonella*).

Multidrug-Resistant Bacteria (MDR): 6 antibiotics with different concentrations were used such as Ampicillin, Cefpodoxime, Ciprofloxacin, Gentamicin, Trimethoprim, and Erythromycin.

2. LITERATURE REVIEW

2.1. Water sources in Cambodia

2.1.1. General feature

Cambodia is governed by the alternating between wet season and dry season in a year. The wet season is characterized by the southwest monsoon from May to October with heavy rainfall, and the dry season is characterized by the northeast monsoon from November to February with dry and cool. Cambodia is highly depended on renewable freshwater in term of inland fisheries to support as a main protein resource, rural livelihoods, and economic development through industrial and agricultural sector (FAO, 2003).

Cambodia rich in water resources where most the resources pass through The Mekong River that flow through Cambodia about 500 Km before entering the Mekong Delta (MD). Territory of Cambodia about 86% is within Mekong River Basin including of catchment of Bassac River, the Tonle Sap River, and the Great Lake and its tributaries (Sagara, 2021). The Mekong Delta (MD) extends from Kratie town, Cambodia to the East Sea in Viet Nam where comprises of the mainstream Mekong River and its adjacent flood plains and wetlands including the Tonle Sap Lake (TSL). The MD in Cambodia splits into Mekong and Bassac rivers and split downstream into nine smaller channels with total area 8.1% of the Mekong Basin which 29,200 Km^2 lie within Cambodia, and the remaining 39,400 Km^2 constitute of Vietnam. Cambodia MD is home to approximately 6.6 million people live and covers parts of Cambodian such as Takeo, Kandal, Prey Veng and Svay Rieng provinces (Mak *et al*., 2011).

The river basins in Cambodia are grouped into River Basin Groups (RBGs) based on their hydrological characteristics (Sagara, 2021):

- The Tonle Sap RBG is in the northwestern part of the country and covers about 45% of the territory, including the Great Lake, and the Tonle Sap River and each of its tributary catchments.
- The upper Mekong RBG is the upper part of the Mekong River, from the border of Cambodia and Lao P.D.R to about 20 Km downstream of Kratie.
- The MD RBG covers the Mekong River from downstream of Kratie to the border of Cambodia and Viet Nam. Most of the area is on the Mekong River floodplain, and most rivers and affected by the backwater effects of the Mekong River during the flood season and tidal effects during the dry season.

- The coastal RBG is in the southwestern part of the country and is confined by the Gulf of Thailand in the Southwest and the Elephant and Cardamom Mountain chain in the northeast.
- The three rivers namely the Se Kong, Se San, and Sre Pok known as 3S RBG is in the northeastern part of the country.

Figure 2.1. River Basin Groups in Cambodia (Ministry of Water Resources and Meteorology)

2.1.2. Tonle Sap Lake

Tonle Sap Lake (TSL) is described as one of the most productive freshwater ecosystems in the word, and it is symbolically called "the heart of Cambodia" and known as one of the most important natural resources in Cambodia. The TSL watershed extend approximately 85 786 Km^2 which is notified as the largest permanent freshwater body in South-East Asia (Kummu *et al*., 2008). Water surface area changes seasonally between 3000 Km^2 in dry season to more than 15000 Km^2 in wet season (ADB, 2005). TSL exchange water with Mekong River through Tonle Sap River in the distance 120 Km long and flow direct into the TSL. Consequently, the lake surface area is a very special and unique in hydrology, water quality, biodiversity, and productivity (Campbell *et al*., 2006). It mainly has a remarkable natural phenomenon wherein its flow is reversible twice a year (FAO, 2009). In July till the end of September (raining season), the Tonle

Sap Lake is filled with water flowing from northward-flowing Mekong River with having 14 meters deep and expands surface area around 10,000 Km^2. In early October to May (dry season), its flow direction changes from Tonle Sap Lake into the Mekong River via Tonle Sap, and it's shrink in size to 3,000 Km^2 with an average depth of only two meters.

The majority of people living in and around TSL, Cambodia are highly dependent on water and natural resources. On account of these, it is disputable that it is cultivating land and rice, and providing fishery sources and aquatic resources to local communities in the five provinces such as Siem Reap, Battambang, Pursat, Kampong Chhnang and Kampong Thom (Hap *et al*., 2006). The region surrounding TSL and floodplain provide numerous ecosystems service to animal and plant species and people in that area. It is the greatest fresh water lake of accounting for more than 75% of Cambodia's inland fish catch and 60% of the country's protein needs (Burnett *et al*., 2013).

2.1.3. Tone Sap River

Tonle Sap is a short river which connects Tonle Sap Lake to Mekong River, which lies 120 Km across Phnom Penh, Kandal and Kampong Chhnang province (Olson and Morton, 2018). This incredible natural phenomenon leads to the river's increase in depth from 2 meters to 10 meters. To put it simply with its height capacity and tributaries, it contributes 62% of the total water supply in the surrounding communities (Open Development, 2016). During the wet season, flooding in delta downstream of Phnom Penh causes water levels in Mekong to raise higher. This lead of reverse flow of Tonle Sap through pushed back upstream of Mekong River toward the Great Lake.

Generally, Cambodian residents living along the Tonle Sap River downstream of Phnom Penh rely on the river water as the main source of water supply. Meanwhile, rapid growth of the population, industrial development, and agriculture in Cambodia lately; whereas many food industries and factories have been located on the bank of the Tonle Sap River. These activities were realized as the distributor in water pollution (Chanpiwat and Sthiannopkao, 2014).

2.1.4. Mekong River

Mekong River Basin includes the Mekong River and its network of tributaries and drains within six countries namely Cambodia, China, Lao PDR, Myanmar, Thailand and Vietnam. Tonle Sap Lake and the Mekong River are best described as being the two largest and most significant hydrological features. During the wet season, both Tonle Sap and the Mekong increase in water quantity and flood the surrounding wetland (WEPA, 2019).

2.1.5. Surface Water

Surface water is any body of water found on the Earth's surface, including both the saltwater in the ocean and the freshwater in rivers, streams, and lakes. It is an essential natural resource used for many purposes, such as drinking water, irrigation, agricultural and industrials. Surface water is water on the surface of the planet that can be contrasted with groundwater and atmospheric water. Surface water can become polluted in any form, which dissolved and suspended solids are present in most surface waters. Surface water pollution occurs when hazardous substances flow into contact and either dissolve or physically mix with the water. Likewise, rapidly population growth, human activities and economic growth also play a major role in surface water pollution (National Geographic Society, 2022).

2.2. MALDI-TOF

Several studies have been carried out by using alternative techniques for accurate identification of foodborne pathogens. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been introduced in the last decade as an excellent tool in different research laboratories for detection and discrimination of various types of microorganism like bacteria and fungi. MALDI-TOF MS protein fingerprinting is one of the most powerful techniques that has been considered fast and accurate identification of pathogens. This technology is characterized by its simple procedures and shortened analysis time (Elbehiry *et al*., 2017). MALDI-TOF MS is used for identifying bacterial species such as *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, and other various pathogens approved by Food and Drug Administration (FDA) (Dieckmann *et al*., 2011 and Josten *et al*., 2013).

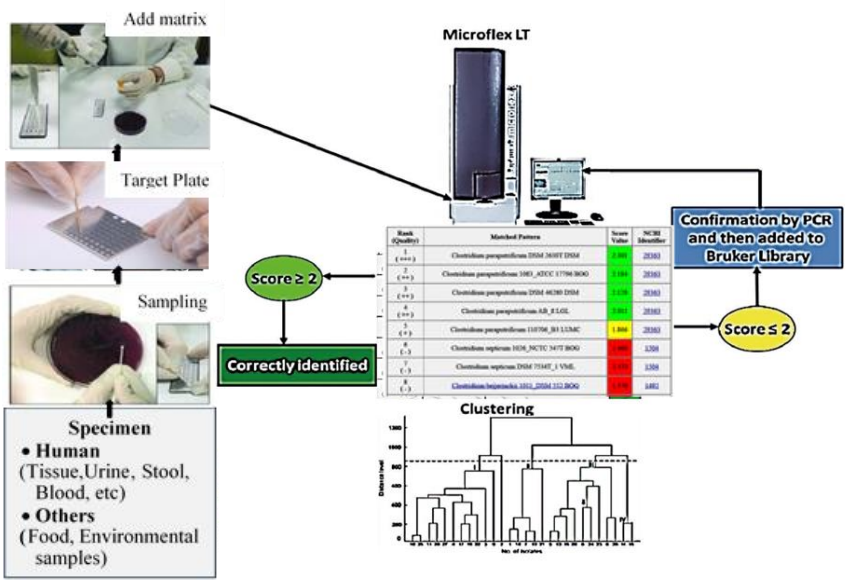

Figure 2.2. Bacteria identification by using MALDI-TOF (Angelakis et al., 2014 and Jang & Kim, 2018)

2.3. Definition of Surface Water

Surface water is any body of water above ground, including streams, rivers, lakes, wetlands, reservoirs, oceans, and creeks. It participates in the hydrologic cycle, or water cycle. Precipitation and water runoff feed bodies of surface water. On the other hand, Water seeps deep into the ground is called groundwater.

Surface water and groundwater are reservoirs that can feed into each other. While surface water can seep underground to become groundwater, groundwater can resurface on land to replenish surface water. Springs are formed in these locations. There are three types of surface water: perennial, ephemeral, and man-made. Perennial, or permanent, surface water persists throughout the year and is replenished with groundwater when there is little precipitation. Ephemeral, or semi-permanent, surface water exists for only part of the year. Ephemeral surface water includes small creeks, lagoons, and water holes. Man-made surface water is found in artificial structures, such as dams and constructed wetlands (National Geographic Society, 2022).

Since surface water is more easily accessible than groundwater, it is relied on for many human uses. It is a crucial source of drinking water and is used for the irrigation of farmland. Based on National Institute of Statistics, Ministry of Planning, in 2002, there are 28.2% of all water used in Cambodia came from surface water, for example, spring, river stream, lake/pond and rain. Wetlands with surface water are also vital habitats for aquatic plants and wildlife.

2.3.1. Surface Water Characteristics

There are three types of surface water characteristics, including physical, chemical and biological characteristics, are described.

2.3.1.1. Physical Characteristics

Table 2.1. The physical water parameters characteristics

Essential parameters	Characteristics
Turbidity	measured by a turbidity rod or by a turbidity meter, in mg/l.
Colour	determined by an instrument is known as tintometer, in TCU. For public supplies, the colour number on cobalt scale should not exceed 20 and should be preferably less than 10.
Taste and Oudour	measured by a term called odour intensity, which is related with the threshold odour or threshold odour number. For public supplies, the water should generally free from odour, i.e. the threshold number should be 1 and should never exceed 3.

pH	is an indicator of the hydrogen ion concentration of aqueous solutions.
Temperature	for potable water, temperature of about 10 ^{0}C is desirable. It should not be more than 25 ^{0}C.
Dissolved Oxygen (DO)	is the amount of oxygen that is present in the water. It is measured in milligrams per liter (mg/l).
Specific Conductivity or Electricity Conductivity	the total amount of dissolved salts present in water can be easily estimated by measuring the specific conductivity of water.

2.3.2. Chemical Characteristics

2.3.2.1. Total Solids and Suspended Solids

Total solids (suspended solids + dissolved solids) can be acquired by evaporating a sample of water and weighing the dry residue left and weighing the residue left on the filter paper. The suspended solid can be found by filtering the water sample. Total permissible amount of solids in water is generally limited to 500 ppm.

2.3.2.2. Potential Hydrogen—pH

Table 2.2. The pH formula description

pH formula	Item	Result
$pH = -\log\left[H^+\right] = \log\left[\frac{1}{H^+}\right]$	If H^+ concentration increases, pH decreases	acidic
	If H^+ concentration decreases, pH increases	alkaline
$\left[H^+\right]\left[OH^-\right] = 10^{-14}$	If the pH of water is more than 7	alkaline
pH + pOH = 14	If the pH of water is less than 7	acidic

The alkalinity is occurred by the presence of bicarbonate of calcium and magnesium or by the carbonates of hydroxides of sodium, potassium, calcium and magnesium. Furthermore, it is not all of the compounds that cause alkalinity also generate hardness. The pH value of water can be measured rapidly and automatically with the assist of a Potentiometer which can also be measured by indicators as given below:

Table 2.3. The pH values

Indicator	pH range of indicator dye	Original	Final color produced
Methyl orange	2.8 – 4.4	Red	Yellow
Methyl red	4.4 – 6.2	Red	Yellow

Phenol red	6.8 – 8.4	Yellow	Red
Phenolphthalein	8.6 – 10.3	Yellow	Red

Permissible pH value for public supplies may range around 6.6 to 8.4. The lower value of pH might cause incrustation, sediment deposits, difficulty in chlorination (Mishra, G, 2018).

2.3.2.3. Hardness

Water hardness is the traditional measure of the capacity of water to react with soap. Hard water requiring considerably more soap to produce a lather. It often produces a noticeable deposit of precipitate (such as insoluble metals, soaps or salts) in containers, including bathtub ring. Hard water caused by a variety of dissolved polyvalent metallic ions, predominantly Ca^{+} and Mg^{+}, although other cation (e.g. Al, Ba, Fe, Mn, Sr and Zn) also contribute. Likewise, Carbonate hardness = Total hardness or Alkalinity (whichever is less) non-carbonate hardness = Total hardness – Alkalinity. By the way, the nature of hardness in water are from sedimentary rocks, seepage and runoff from soils.

Table 2.4. The hardness values

Range	Describe
0-60 mg/l	Soft water
61-120 mg/l	Moderately hard water
121-180 mg/l	Hard water
>181 mg/l	Very hard water

- One French degree of hardness is equal to 10 mg/l of $CaCO_3$.
- One British degree of hardness is equal to a hardness of 14.25 mg/l.
- Underground water is generally harder than surface water.
- The prescribed hardness limit for public supplies ranges between 75 to 115 ppm.

2.3.2.4. Chloride Content

The chloride content of treated water to be supplied to the public should not exceed a value of about 250 ppm. It can be measured by titrating the water with standard silver nitrate solution using potassium chromate as indicator.

2.3.2.5. Nitrogen Content

There are one or more reasons that nitrogen present as below:

- Free ammonia: It indicates very first stage of decomposition of organic matter. It should not exceed 0.15 mg/l

- Albuminous or Organic Matter: It indicates the quantity of nitrogen present in water before the decomposition of organic molten has started. It should not exceed 0.3 mg/l
- Nitrites: Not fully oxidized organic matter in water.
- Nitrates: It indicates fully oxidized organic matter in water (representing old pollution).
- Nitrites are highly dangerous and therefore the permissible number of nitrites in water should be nil.
- Ammonia nitrogen + organic nitrogen = kjeldahl nitrogen
- Nitrates in water are not harmful. However, the presence of too much of nitrates in water might adversely influence the health of infants occurring a disease called methemoglobinemia commonly called blue baby disease.
- The nitrate concentration in domestic water supplies is limited to 45 mg/l.

2.3.2.6. Metal and other chemical substances in water

Table 2.5. Metal and other chemical substances in water

Items	Standard	Result
Iron	> 0.3 ppm	cause discolouration of clothes
Manganese	> 0.05 ppm	
Copper	> 1.3 ppm	effects human lungs and other respiratory organs
Sulphate	> 250 ppm	
	> 1.5 ppm	
Fluoride	> 1.5 ppm	causing spotting and discolouration of teeth (a disease called
	0.8 – 1 ppm	cause dental cavity (tooth decay)

2.3.2.7. Dissolved gases

Oxygen gas is generally absorbed by water from the atmosphere but it being consumed by unstable organic matter for their oxidation. Hence, if the oxygen present in water is found to be less than its saturation level, it indicates presence of organic matter and consequently making the waters suspicious (Mishra, G, 2018).

2.3.2.8. Biological Oxygen Demand (BOD)

BOD is known as biological oxygen demand (BOD). It is not practically possible to determine ultimate oxygen demand. Hence, BOD5 of water at 20^{o}C is generally considered as the standard demand. BOD5 = Loss of oxygen in mg/l x dilution factor. For safe drinking water, BOD must be zero.

2.3.2.9. Chemical Oxygen Demand (COD)

COD determines the quantity of oxygen required to oxidize the organic matter present in water body under specific conditions of oxidizing agent, temperature, and time. It is an essential water quality parameter that provides an index to assess the effect discharged wastewater will have on the receiving environment (Arora, P, 2017).

2.3.3. Biological Characteristics

The biological characteristics of a water body refer to a variety of living organisms that can be found in water, such as microscopic viruses, bacteria and protozoans; as well as phytoplankton (microscopic algae), zooplankton (tiny water animals), insects, worms, large plants and fish. Viruses and bacteria are a vital disease causing to human. They can be present and transported in water. Many of these pathogens can entry the water system into sewage (human and animal waste). In case, fecal coliforms are selected to identify and measure water pollution with sewage. It is known to live in the intestines of mammals and not dangerous in themselves. The presence of fecal coliforms in water indicates that sewage is present and that other disease-causing organisms may also be in the water (Arora, P, 2017).

Table 2.6. Biological water parameters characteristic

Type	Characteristic
Bacteria	Salmonella, typhus, cholera, shigella
Protozoa	Amoeba, cryptosporidium, giardia
Viruses	Polio, hepatitis A, meningitis, encephalitis
Feacal Matter	Total Coliform and Feacal Coliform

2.4. Wastewater

Water, because of its properties as a solvent and its capacity to transport particles, incorporates in itself various impurities that characterize the water quality (Sperling, 2007). Water quality is a result of natural phenomena and the acts of human beings.

Natural conditions: even with the catchment area preserved in its natural condition, the surface water quality is affected by run off and infiltration resulting from rainfall. The impact of these is dependent on the contact of the water with particles, substances and impurities in the soil. Therefore, the incorporation of suspended solids (e.g. soil particles) or dissolved solids (e.g. ions originating from the dissolution of rocks) occurs even when the catchment area is totally preserved in its natural condition (e.g. occupation of the land with woods and forests). In this case, the soil protection and composition have a great influence.

Interference of human beings: The production of waste from human activities is unavoidable. A significant part of this waste will end up as wastewater. The quantity and quality of wastewater is determined by many factors (Mogens Henze, Yves Comeau, 2008). Not all human or industries produce the same amount of waste. The amount and type of waste produce in household is influenced by the behavior, lifestyle and standard of living of the inhabitants as well as the technical and juridical framework by which people are surrounded. In households most waste will end up as solid and liquid waste, and there are significant possibilities for changing the amounts and composition of the two waste streams generated. For industry similar considerations apply.

In case of domestic sewage, it contains approximately 99.9% water (Sperling, 2007). The remaining part includes organic and inorganic, suspended and dissolved solids, together with microorganisms. It is because of this 0.1% that water pollution takes place and the wastewater needs to be treated. The composition of the wastewater is a function of the uses to which the water was submitted. These uses, and the form with which they were exercised, vary with climate, social and economic situation and population habits. In the design of a WWTP, there is normally no interest in determining the various compounds that make up wastewater. This is due, not only to the difficulty in undertaking the various laboratory tests, but also to the fact that the results themselves cannot be directly utilized as elements in design and operation. Therefore, many times it is preferable to utilize indirect parameters that represent the character or the polluting potential of the wastewater in question. These parameters define the quality of the sewage, and can be divided into three categories: physical, chemical and biological parameters as mention in table below.

2.4.1. Wastewater Characteristics

2.4.1.1. Physical Characteristic

Temperature: Due to more biological activity, wastewater will have a higher temperature. However, the temperature of the receiving waterbody must not increase by more than 2°C or 3°C, since greater increases in temperature may affect the population balance and also reduce the solubility of oxygen, thereby threatening the survival of some forms of aquatic wildlife.

Color: Fresh sewage is normally brown and yellowish in color but over time becomes black in color which was influenced by the presence of organic matter, chemicals, and waste products it carries from various sources.

Odor: Wastewater that includes sewage typically develops a strong odor which mostly caused by the decomposition of the organic matter that emits volatile amines, diamines and sometimes ammonia. In wastewater that has become septic, the characteristic odor of hydrogen

sulphide may also develop. Odors are very important in relation to the public perception and acceptance of any waste treatment plant. Although relatively harmless, they may affect people by inducing stress and nausea.

For measurement or estimation of odor, a test panel is exposed to odors diluted with clean air. The number of dilutions needed to reduce the odor intensity to its detectable limit is called as the detectable threshold odor concentration. The technique for determination of the threshold odor can be found in the Standard Methods (1989), but as it is complicated and subject to errors due to adaptation of the test subjects, subjectivity and sample modification, it is seldom used.

Turbidity: Due to suspended solids in wastewater, wastewater will have a higher turbidity, or cloudiness. Solids may be present either in dissolved or suspended form. Suspended solids are of primary concern since they are objectionable on several grounds: those that settle can do so in the wastewater ducts, reducing their capacity; or if they settle in the receiving waterbody they may affect the bottom- dwelling flora and the food chain; if they float, the light dig enters from the surface is reduced, and those that remain suspended reduce the amount of light that enters the water thereby affecting wildlife.

The suspended solids are measured by passing a well-mixed sample through a fiberglass filter. The weight of suspended solids can be calculated by weight differences between the filter alone and the weight plus retained solids after drying.

Soluble solids can be measured by gravimetric after evaporation of the filtrate of a sample of known volume, but are generally not checked even though in effluents with a low degree of contamination they can be significant.

2.4.1.2. Chemical Characteristic of Wastewater

Wastewater contains different chemicals in various forms as mentioned below.

Biochemical Oxygen Demand (BOD): The main ecological effect of organic pollution in a water body is the decrease in the level of dissolved oxygen. Similarly, in sewage treatment using aerobic processes, the adequate supply of oxygen is essential, so that the metabolic processes of the microorganisms can lead to the stabilization of the organic matter. The basic idea is then to infer the “strength” of the pollution potential of a wastewater by the measurement of the oxygen consumption that it would cause. The organic content of the wastewater can be estimated in several ways. The most common are the oxygen demand methods, although organic carbon measurement may also be used.

Chemical Oxygen Demand (COD): Measures the consumption of oxygen occurring as a result of the chemical oxidation of the organic matter. The value obtained is, therefore, an indirect indication of the level of organic matter present. The main difference with the BOD test is clearly found in the nomenclature of both tests. The BOD relates itself with the biochemical oxidation of the organic matter, undertaken entirely by microorganisms. The COD corresponds to the chemical oxidation of the organic matter, obtained through a strong oxidant (Potassium dichromate) in an acid medium.

Total Organic Carbon (TOC): In this test the organic carbon is directly measured, in an instrumental test, and not indirectly through the determination of the oxygen consumed, like the three tests above. The TOC test measures all the carbon released in the form of CO_2. To guarantee that the carbon being measured is really organic carbon, the inorganic forms of carbon (like CO_2, HCO_3^- etc.) must be removed before the analysis or be corrected when calculated (Eckenfelder, 1980). The TOC test has been mostly used so far in research or in detailed evaluations of the characteristics of the liquid,

Nitrogen: In its cycle in the biosphere, nitrogen alternates between various forms and oxidation states, resulting from various biochemical processes. Nitrogen is a component of great importance in terms of generation and control of the water pollution. The determination of the prevailing form of nitrogen in a water body can provide indications about the stage of pollution caused by an upstream discharge of sewage. If the pollution is recent, nitrogen is basically in the form of organic nitrogen or ammonia and, if not recent, in the form of nitrate (nitrite concentrations are normally low).

In raw domestic sewage, the predominant forms are *organic nitrogen* and *ammonia*. Organic nitrogen corresponds to Amina groups. Ammonia is mainly derived from urea, which is rapidly hydrolyzed and rarely found in raw sewage. These two, together, are determined in the laboratory by the Kjeldahl method, leading to the *Total Kjeldahl Nitrogen* (TKN).

Phosphorous: Total phosphorus in domestic sewage is present in the form of phosphates, according to the following distribution:

- inorganic (polyphosphates and orthophosphates): main source from detergents and other household chemical products
- organic (bound to organic compounds): physiological origin

Phosphorus is an essential nutrient for the growth of the microorganisms responsible for the stabilization of organic matter. Usually, domestic sewage has sufficient levels of phosphorus, but a lack may occur in some industrial wastewaters. Phosphorus is also an essential nutrient for

the growth of algae, eventually leading, under certain conditions, to the eutrophication of lakes and reservoirs.

2.4.1.3. Biological Characteristic of Wastewater

Pathogenic Organisms: Most of these organisms play various essential roles, mainly related to the transformation of the constituents in the biogeochemical cycles. Biological wastewater treatment also relies on these organisms. Another important aspect in terms of the biological quality of a water or wastewater is that related to the disease transmission by pathogenic organisms. The major groups of pathogenic organisms are: (a) bacteria, (b) viruses, (c) protozoans and (d) helminths. The number of pathogens present in the sewage of a certain community varies substantially and depends on: (a) socio-economic status of the population; (b) health requirements; (c) geographic region; (d) presence of agroindustry; (e) type of treatment to which the sewage was submitted.

The detection of pathogenic organisms, mainly bacteria, protozoans and viruses, in a sample of water is difficult, because of their low concentrations. In this sense, the final concentration of pathogens per unit volume in a water body may be considerably low, making detection through laboratory examination highly difficult. This obstacle is overcome through the search for indicator organisms of fecal contamination. These organisms are predominantly non-pathogenic, but they give a satisfactory indication of whether the water is contaminated by human or animal feces, and, therefore, of its potential to transmit diseases. The organisms most commonly used with this objective are bacteria of the coliform group. The indicators of fecal contamination most commonly used are:

- Total Coliforms (TC)
- Fecal Coliforms (FC) or Thermotolerant Coliforms
- *Escherichia coli* (EC)

Bacteriophages: For the indication of the presence of viruses, bacteriophages may be representative, owing to their similarities with the enteric human viruses. Bacteriophages are specific viruses that infect bacteria, for example the coliphages, which infect *E. coli*. Coliphages are not present in high numbers in fresh human or animal feces, but may be abundant in sewage, owing to their fast reproduction rate resulting from the attack to bacterial cells (Mendonça, 2013). Their significance is as indicators of sewage contamination and, because of their greater persistence compared with bacterial indicators, as additional indicators of treatment efficiency or for groundwater protection.

Helminth Eggs: For helminths, there are no substituting indicators, and helminth eggs are determined directly in laboratory tests. However, the eggs of nematodes, such as *Ascaris, Trichuris, Necator americanus and Ancilostoma duodenale* may be used as indicators of other helminths (cestodes, trematodes and other nematodes), which are removed in water and wastewater treatment by the same mechanism (e.g., sedimentation), being thus indicators of treatment efficiency. Helminth eggs are an important parameter when assessing the use of water or treated wastewater for irrigation, in which workers may have direct contact with contaminated water and consumers may eat the irrigated vegetable uncooked or unpeeled. Helminth eggs may be removed by physical operations, such as sedimentation, which takes place, for instance, in stabilization ponds. Eggs may be viable or non-viable, and viability may be altered by specific disinfection processes.

Table 2.7. Biological characteristic of wastewater and diseases

Organism	Disease	Causal agent	Symptoms / manifestation
Bacteria	Bacillary dysentery (shigellosis)	*Shigella dysenteriae*	Severe diarrhea
	Campylobacter enteritis	*Campylobacter jejuni*, *Campylobacter coli*	Diarrhea, abdominal pain, malaise, fever, nausea, vomiting
	Cholera	*Vibrio cholerae*	Extremely heavy diarrhea, dehydration, high death rate
	Gastroenteritis	*Escherichia coli* – enteropathogenic	Diarrhea
	Leptospirosis	*Leptospira* – various species	Jaundice, fever
	Paratyphoid fever	*Salmonella* – various species	Fever, diarrhea, malaise, headache, spleen enlargement, involvement of lymphoid tissues and intestines
	Salmonella	*Salmonella* – various species	Fever, nausea, diarrhea
	Typhoid fever	*Salmonella typhi*	High fever, diarrhea, ulceration of small intestine
Protozoan	Amoebic dysentery	*Entamoeba histolytica*	Prolonged diarrhea with bleeding, abscesses of the liver and small intestine

	Giardiasis	*Giardia lamblia*	Mild to severe diarrhea, nausea, indigestion, flatulence
	Cryptosporidiosis	*Cryptosporidium*	Diarrhea
	Balantidiasis	*Balantidium coli*	Diarrhea, dysentery
Viruses	Infectious hepatitis	Hepatitis A virus	Jaundice, fever
	Respiratory disease	Adenovirus – various types	Respiratory illness
	Gastroenteritis	Enterovirus, Norwalk, rotavirus, etc. – various species	Mild to strong diarrhea, vomiting
	Meningitis	Enterovirus	Fever, vomiting, neck stiffness
	Poliomyelitis (infantile paralysis)	*Poliomyelitis virus*	Paralysis, atrophy
Helminths	Ascariasis	*Ascaris lumbricoides*	Pulmonary manifestations, nutritional deficiency, obstruction of bowel or another organ
	Trichuriasis	*Trichuris trichiura*	Diarrhea, bloody mucoid stools, rectal prolapse

The generalization of typical industrial wastewater characteristics is difficult because of their wide variability from time to time and from industry to industry. Industrial effluents, depending on the type of the industrial process, can contain in greater or lesser degrees, the various pollutants described in domestic sewage (suspended solids, biodegradable organic matter, nitrogen, phosphorus and pathogenic organisms). The present section covers other pollutants, which are not usually found in typical domestic sewage, but which can be of concern in industrial or municipal wastewaters containing a fraction of industrial effluents.

2.4.1.4. Metals

In the present context, the main implications of metals are:

- Toxicity to human beings and other forms of plant or animal life, as a result of the discharge or disposal of wastewaters to receiving water bodies or land.
- Inhibition to the microorganisms responsible for the biological treatment of wastewater.

In spite of being widely used, the expression “heavy metal” does not have a sole definition, varying from branch to branch of science. From the environmental point of view of this book, heavy metals can be understood as those that, under certain concentrations and exposure time,

offer risks to human health and the environment, impairing the activity of living organisms, including those responsible for the biological treatment of wastewater.

The main chemical elements that fit into this category are: Ag, As, Cd, Co, Cr, Cu, Hg, Ni, Pb, Sb, Se and Zn. These elements may be naturally found in soils or waters in variable concentrations, but lower than those ones considered toxic to different living organisms. Among these, As, Co, Cr, Cu, Se and Zn are essential to organisms in certain small quantities, while others have no function in biological metabolism, being toxic to plants and animals. Most living organisms need only few metals, and in very small doses, characterizing the concept of micronutrients, as zinc, magnesium, cobalt and iron. These metals become toxic and dangerous to human health when they exceed certain concentration thresholds. As for lead, mercury and cadmium, these are metals that do not exist naturally in any organism. They do not perform any nutritional or biochemical function in microorganisms, plants or animals. That is, the presence of these metals in living organisms is harmful at any concentration.

In human beings, metals can produce several effects, resulting from their action on molecules, cells, tissues, organs and even the whole system. Besides, the presence of a metal might restrict the absorption of other nutrients essential to the activity of the organism. Metals, because they cannot be metabolized, remain in the organism and carry out their toxic effects, combining with one or more reactive groups, which may be indispensable for normal physiological functions. Depending on the material involved and on the intensity of the intoxication, the effect may range from a topic skin manifestation, pulmonary membrane or digestive tract, to mutagenic, teratogenic or carcinogenic effects, and even death. It is important to emphasize that synergistic effects also need to be taken into account. In most cases, synergistic effects might be far greater than the mere sum of the individual effects.

Although in general metals may be poisonous to plants and animals under the low concentrations in which they may be present in domestic wastewater, no chronic toxicity problems associated with their disposal have been reported. On the other hand, the same could not be said for industrial wastewaters and the resulting sludge (in which metals are concentrated). In wastewater treatment, limitations associated with metals are mainly related to the inhibition of, or toxicity to, microorganism growth and the incorporation of metals in the sludge. For a certain metal, the maximum allowable load needs to be determined, such that there are no problems with microorganism inhibition, deterioration of effluent quality and impairment to agricultural use of the sludge.

The discharge of a particular industrial wastewater into the public sewerage system will have a variable impact in the WWTP, depending on the dilution factor, the content and type of

pollutant, and the treatment process employed. To analyses the impact, it is interesting to perform simulations and to apply a safety factor to the calculated limits. In this way, decisions may be made regarding the acceptance of the effluents into the system, and finally at the WWTP. If the estimated loads are lower than the acceptable limits, the discharge may be accepted. Conversely, if the limits are exceeded, pre-treatment may be required, or no further admissions to the public systems may be accepted. A check must be made on the system to verify whether the biological process is being inhibited, or whether the treated effluent and the sludge to be reused are outside the limits established by the environmental agency. The control must be centered on the industrial discharges, since domestic sewage may not be prevented to be discharged to the public network system.

2.4.1.5. Toxic and Dangerous Organic Compounds

Toxic and dangerous organic compounds, even though they usually do not represent a concern in domestic sewage, may be of concern in municipal wastewaters that receive industrial effluents (Sousa, 2018). When wastewaters containing toxic organic compounds are disposed of in the receiving water body without adequate treatment, severe damage may occur, both to the aquatic life and to human beings, who use it as a source of water supply. Most of these compounds are very slowly biodegraded, persisting in the environment for a long period. These compounds are able to penetrate the food chain and, even if they are not detectable in the receiving body, they may be present in large quantities in the higher trophic levels, owing to their bioaccumulation characteristics. Another important fact is that, although some compounds do not pose serious health damages when ingested, their metabolites may be more toxic than the original products. Besides, since wastewaters have a complex composition and normally contain more than one organic pollutant, synergistic effects may take place (the combined effect may be higher than the sum of the individually exerted effects).

Several dangerous pollutants are volatile because of their low solubility, low molecular weight and high vapor pressure. Therefore, they may be transferred to the atmosphere in open units in the WWTP, such as aeration tanks, equalization tanks and clarifiers, and also pumping stations. If adequate control means are not taken, their volatilization represents a potential health risk to the population and workers who are frequently exposed to it. The structural integrity of the sewerage collection system is also affected, because many compounds are corrosive, inflammable and explosive (methanol, methyl-ethyl ketone, hexane, benzene, among others).

Other pollutants are adsorbed and concentrated in the biological flocs in the treatment process, and might cause inhibition to sludge digestion or generate sludge with dangerous

characteristics which, if not adequately disposed of, could contaminate groundwater. In some cases, the toxic pollutants are present in such low concentrations that are not able to inhibit the biological process, but also are very hard to be removed. Consequently, the treatment plant effluent may still contain these pollutants and, when discharged into the receiving body, may cause damages to the aquatic life and human beings.

2.5. Selective Media

Culturing bacteria we need medium, different bacteria require different types of medium. For example, Chomocult® Coliform Agar is used for *E. coli* and total coliform, Xylose Lysine Deoxycholate Agar is used for *Salmonella* and *Shigella*, Mannitol Salt Agar is used for *Staphylococcus epidermidis* and *Staphylococcus aureus*, Sakazakii DHL Agar and Cetrimide Agar is used for *K. pneumoniae* and *Pseudomonas aeruginosa* respectively. We can distinguish the different bacteria according to its colors showing on the agar. For instance, violet color is counting as *E. coli*, pink color is considered as total coliform, *K. pneumoniae* and *S. epidernisdis*, yellow color is represented to *P. aeruginosa* and *S. aureus*, red with black in the center is *Salmonella* while red to pink color is *Shigella*. In this study selective media plays an important role in the identification and isolation of pathogenic microorganisms from environmental sample (Takeshi Kawanishi, 2011). Selective media only allows certain types of organisms to grow and inhibits the other organisms. It can select the microorganism in many ways. For instance, selective of microorganism can be accomplished by adding antibiotics, salts or specific inhibitors (Marion Bonnet, 2019). To put it simply, Sakazakii DHL Agar, stands for Sakazakii Deoxycholate-Hydrogen sulphide-Lactose Agar, a selective media which is used for the detection and isolation of pathogenic *Enterobacteriaceae* from all type of specimens in water samples (HIMEDIA, 2023). It can be supplemented with penicillin, and tetracycline using to be select for gram-negative such as *klebsiella pneumoniae* and *proteus mirabilis.* In order to study the bacteria of interest, 3 types of agar namely Chromocult® agar, Manitol Salt agar and Sakazakii DHL Agar take into account.

2.5.1. Chromocult® Coliform Agar

Chromocult® Coliform Agar is a selective and differential chromogenic culture medium and usually it is chosen for the enumeration of *E. coli,* coliform as well as non-coliform bacteria culturing within 24 hours (Group, 2023). It has the ability to easily distinguish these bacteria by counting of *E. coli* and coliform bacteria by color. The combined action of peptone, pyruvate and sorbitol allow rapid colony growth in this phosphate buffered medium, which also permits simple recovery of sublethal thermally injured coliforms. Sodium chloride provides the correct osmotic environment necessary for growth. The different of colony's color was the result and counting. For detection of Coliforms and *E. coli* was used traditional culture media used, it often contained

ox bile or bile salts to inhibit Gram-positive bacteria. These strong inhibitors; however, may limit the growth of the targeted microorganisms if they were already sub lethally damaged. Therefore, in Chromocult® Coliform Agar, Tergitol-7 was added to inhibit any Gram-positive organisms and some non-enteric bacteria (Group, 2023).

2.5.2. Mannitol Salt Agar

Mannitol Salt Agar was a selective medium for the isolation of pathogenic *Staphylococci* from clinical and non-clinical samples. This media is used to cultivate pathogenic *Staphylococci* because it is a species which have an ability to grow on high salt media. Mannitol Salt Agar was also discovered to select *S. aureus*, which is in yellow. Mannitol Salt Agar contains beef extract and proteose peptone, which makes it very nutritious as they provide essential growth factors and trace nutrients such as nitrogen, vitamins, minerals and amino acids essential for growth. The compositions in this agar is a high concentration of sodium chloride 7.5% (75 g/L) to complete inhibition of bacterial organisms other than *Staphylococci*. Mannitol (10 g/L) for fermentation, which indicated by a change in the phenol red (25 mg/L) indicator, agar (15 g/L) is used as a solidifying agent. Peptones (5 g/L) and yeast extract (1 g/L) that supply essential growth factors, including nitrogen, carbon, sulfur and trace nutrients (Arya, 2023).

2.5.3. Sakazakii DHL Agar

Sakazakii DHL Agar is in short of Sakazakii Deoxycholate-Hydrogen sulphide-Lactose Agar. Sakazakii DHL Agar is used for the detection and isolation of pathogenic *Enterobacteriacae* from all types of specimens. Sodium Deoxycholate, a composition in the medium, is a selective agent and inhibits gram-positive bacteria and also prevents swarming growth of Proteus species. While lactose and sucrose are fermentable carbohydrate source. Sulphur is released from thiosulphate or other sulphur-containing compounds in the form of sulphide. The H_2S thus produced is detected by ferric ammonium citrate to form insoluble heavy metal sulphides that appear as a black precipitate (HiMedia Laboratories, 2023). In this study Sakazakii DHL Agar is a selective and differential medium used for the isolation and identification of *K. pneumoniae.*

2.6. Pathogenic Bacteria

2.6.1. *Escherichia coli* (*E. coli*)

Escherichia coli which belongs to Class Gammaproteobacteria, Family: Enterobacteriaceae are Gram-negative bacteria that is one among natural intestinal microbial community of human and animals (Health Canada, 2022). They are facultatively anaerobic, motile or non-motile rod-shaped bacteria that can grow over a broad temperature range (7–45°C) with an optimal growth temperature of 37°C (Health Canada, 2022). *E. coli* have a size of 2.0-0.5 µm in diameter. *E. coli* is a natural and essential part of the bacterial flora in the gut of humans and animals (Magana-Archchi, D.N., & Wanigatunge, R. P , 2020). Pathogenic E. coli are broadly categorized into

functional groups based on the mechanisms with which they interact with their target cells and cause symptoms (Health Canada, 2022). Different types can bind to, invade, or cause structural alterations of cells and produce specific types of toxins (Health Canada, 2022). The scientific classification of *Escherichia coli* has been shown in table 2.8 below.

Table 2.8. Scientific classification of *E.coli*

Domain	Bacteria
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	Escherichia
Species	Escherichia coli

There are six major groups of *pathogenic E. coli* that cause gastrointestinal infections: enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) and diffuse adherent *E. coli* (DAEC) (Health Canada, 2022). Among them, enterotoxigenic *E. coli* O148, enterohemorrhagic E. coli O157, and enteroinvasive *E. coli* O124 serotypes are major disease-causing organisms and can be transmitted through contaminated water (Magana-Archchi, D.N., & Wanigatunge, R. P , 2020). Enterotoxigenic *E. coli* (ETEC) serotypes can cause infantile gastroenteritis. Disease is caused due to consumption of ETEC-contaminated food or water and is characterized by profuse watery diarrhea continuing for several days leading to dehydration and malnutrition in young children (Magana-Archchi, D.N., & Wanigatunge, R. P , 2020).

EHEC is a typically food-born pathogen causing hemorrhagic colitis or hemolytic-uremic syndrome (HUS) (Kaper, J.B.; Nataro, J.P.; Mobley, H.L., 2004). Typical EHEC strains produce Shiga-like toxins (named Shiga toxin producing E. coli, STEC) (Bilinski, *et al*., 2012). Shiga toxin-producing E. coli O157:H7 is an enterohemorrhagic bacterial strain that is an important food and a waterborne pathogen that causes diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans (Atif, M., Wasey, A., & Salen, P., 2023). EPEC strains cause diarrhea primarily in children, particularly under conditions of poor hygiene, as well as in animals (Kaper, J.B.; Nataro, J.P.; Mobley, H.L., 2004). ETEC are the most common pathogens causing travelers' diarrhea with mild to severe watery diarrhea in humans of all ages (Qadri, F.; Svennerholm, A.M.; Faruque, A.S.; Sack, R.B., 2005,) EAEC strains are associated with persistent diarrhea in humans, and have been recognized as the cause of several outbreaks of diarrheal disease worldwide. EAEC, frequently found in the gut of asymptomatic humans, is the second foremost cause of travelers'

diarrhea worldwide. EAEC is frequently associated with diarrhea in children in developing countries and in HIV-infected patients (Weintraub, 2007) and (Nataro, Steiner, & Guerrant, 1998) . DAEC causes diarrhea particularly in children (Servin, 2005) . EIEC is frequently cause of watery diarrhea and occasionally dysentery in both children and adults (Kaper, J.B.; Nataro, J.P.; Mobley, H.L., 2004). EIEC strains are closely related to Shigella spp. The E. coli pathogenic types have been shown in Table 2.9 below (Allocati, Masulli, & Alexeyev, 2013)

Table 2.9. *E. coli* pathogenic types with demonstrative diseases and symptoms

Pathotype (acronym)	Diseases	Symptoms	Virulence
Enteric E.coli			
EnteroPathogenic *E. coli* (EPEC)	Diarrhoea in children	Watery diarrhoea and vomiting	Bfp, Intimin, LEE
EnteroHaemorrhagic *E. coli* (EHEC)	Haemorrhagic colitis, HUS	Bloody diarrhoea	Shiga toxins, Intimin, Bfp
EnteroToxigenic *E. coli* (ETEC)	Traveler's diarrhoea	Watery diarrhoea and vomiting	Heat-labile and sheat-stable toxins, CFAs
EnteroAggregative *E. coli* (EAEC)	Diarrhoea in children	Diarrhoea with mucus and vomiting	AAFs, cytotoxins
Diffusely Adherent *E. coli* (DAEC)	Acute diarrhoea in children	Watery diarrhoea, recurring UTI	Daa, AIDA
EnteroInvasive *E. coli* (EIEC)	Shigellosis-like	Watery diarrhoea; dysentery	Shiga toxin, hemolysin, Cellular invasion, Ipa
Adherent Invasive *E. coli* (AIEC)	Associated with Crohn disease	Persistent intestinal inflammation	Type 1 fimbriae, Cellular invasio
Extraintestinal E. coli (ExPEC)			
UroPathogenic *E. coli* (UPEC)	Lower UTI and systemic infections	Cystitis, pyelonephritis	Type 1 and P fimbriae; AAFs, hemolysin
Neonatal Meningitis *E. coli* (NMEC)	Neonatal meningitis	Acute meningitis, sepsi	S fimbrie; K1 capsule
Avian Pathogenic *E. coli* (APEC)	Probable source of food-borne disease	-	Type 1 and P fimbriae; K1 capsule

Bfp: Bundle-forming pili; LEE: Locus for enterocyte effacement; HUS: haemolytic-uraemic syndrome; CFA: colonization factor antigen; AAF: aggregative adherence fimbria; Daa: diffuse adhesin; AIDA: adhesin involved in diffuse adherence; Ipa: Invasion plasmid antigen.

2.6.2. *Salmonella*

Salmonella are Gram-negative rod-shaped bacteria (see in Table 2.10) with a facultative metabolism, that is able to grow in any condition of the presence and absence of oxygen (John A Crump & John Wain, 2017). Salmonella is one among members of Enterobacteriaceae family and their species are about 2-3 x 0.4-0.6 μm in diameter (Oladapo O. Oludairo, 2022). *Salmonella* was first identified in 1800s and the organism was cultured in 1888 by Salmon and Smith (Oladapo O. Oludairo, 2022). The bacteria can be transmitted to human through ingested food and water (Oladapo O. Oludairo, 2022). There are more than 2600 serotypes of *Salmonella* had been reported by Kauffmann-Le Minor, 1600 of which belong to the subspecies *enterica* and over 200 serotypes were able to cause diseases to humans (Oladapo O. Oludairo, 2022). *Salmonella* can also cause health affect to humans through animals—poultry, cattle and swine and the organisms are typically transmitted either via contaminated food of animals' origin, food or water contaminated by animal feces or sometime through direct contact with animals and their environment (John A Crump & John Wain, 2017). *Salmonellae* have also been found in shellfish, fresh fruits, and many vegetables and they can be carried by birds, and be carried asymptomatically in many warm-blooded animals including pigs, dogs, and even elephants (Mumy, 2014).

Table 2.10. Scientific classification and Taxonomic ranks of Salmonella

Domain	Bacteria
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gamma proteobacteria
Order	Enterobacteriales
Family	*Enterobacteriaceae*
Genus	*Salmonella*
Species	*Salmonella* spp

Kauffmann-White scheme classifies *Salmonella* base on the three major antigenic determinants composed of flagellar H antigens, somatic O antigens and virulence (Vi) capsular K antigens. This was adopted by the International Association of Microbiologists in 1934 (Pui, 2011). Various O antigens were aggregated by antibodies specific to group Salmonellae into 6 serogroups: A, B, C1, C2, D and E, for instance; *S.* Paratyphi A, B, C and *S.* Typhi express O antigens of serogroups A, B, C1 and D, respectively (Pui, 2011). More than 99% of *Salmonella* strains causing human infections belong to *Salmonella enterica* subspecies *enterica* (Pui, 2011).

O antigens are lipopolysaccharide (LPS) of outer bacterial membrane and they are heat stable and resistant to alcohol and dilute acids and further classification of serotypes is based on the antigenicity of the flagellar H antigens which are highly specific for *Salmonella* (Pui, 2011). H antigens are heat-labile proteins associated with the peritrichous flagella (Pui, 2011). According to the Centers for Disease Control and Prevention (CDC), there are only two species within the genus *Salmonella: Salmonella enterica* and *Salmonella bongori* (Mumy, 2014). *Salmonellae* are able to multiply under various environmental conditions outside the living hosts and they do not need sodium chloride for growth, however; they can grow in the presence of 0.4 to 4%. Most *Salmonella* serotypes grow at temperature range from 5 to 47 ^{o}C with optimum temperature of 35 to 37 ^{o}C but some can grow at temperature as low as 2 to 4 ^{o}C or as high as 54 ^{o}C (Pui, 2011). *Salmonellae* are sensitive to heat and normally killed at temperature of 70 ^{o}C or above. They can grow in pH range from 4 to 9 with the optimum between 6.5 and 7.5 (Pui, 2011).

Table 2.11. Kauffmann-White scheme's *Salmonella* species, subspecies, serotypes and their usual habitats (Oladapo O. Oludairo, 2022)

Salmonella species and subspecies	**No of serotypes within subspecies**	**Usual habitat**
S. enterica subspecies *enterica* (I)	1,454	Warm-blooded animals
S. enterica subspecies *salamae* (II)	489	Environment /Cold-blooded animals
S. enterica subspecies *arizonae* (IIIa)	94	Environment /Cold-blooded animals
S. enterica subspecies *diarizonae* (IIIb)	324	Environment /Cold-blooded animals
S. enterica subspecies *houtenae* (IV)	70	Environment /Cold-blooded animals
S. enterica subspecies *indica* (VI)	12	Environment /Cold-blooded animals
S. bongori (V)	20	Environment /Cold-blooded animals
Total	**2463**	

Table 2.12. The nomenclature of Salmonella used at the CDC (Oladapo O. Oludairo, 2022)

Position of taxonomy	Nomenclature
Genus (in italics)	– Salmonella
Species (in italics)	– *enterica* (subspecies I, II, IIIa, IIIb, IV and VI) – *bongori* (formerly subspecies V)
Serotype (Word not italicized, first letter capitalized)	– The name of a serotype should be preceded by the word "serotype" or "ser.", the first time it's mentioned in a text

	– Subspecies I serotypes are named, those in subspecies II to IV, VI and *S. bongori* are designated by antigenic formulae – Subspecies II, IV, VI and *S. bongori* members retain their names if named before 1966

CDC- Centers for Disease Control and Prevention

Table 2.13. The nomenclature of Salmonella in recent literature reflecting location of isolation (Oladapo O. Oludairo, 2022)

Complete name	CDC designation	Older designation
S. enterica subsp. *enterica* ser. Typhi	*Salmonella* ser. Typhi	*Salmonella typhi*
S. enterica subsp. *enterica* ser. Typhimurium	*S.* ser. Typhimurium	*Salmonella typhimurium*
S. enterica subsp. *salamae* ser. Greenside	*S. ser. Greenside*	*S.* II 50:z:e,n,x, *S. greenside*
S. enterica subsp. *arizonae* ser. 18:z4,z23:-	*S.* IIIa 18:z4,z23:-	*Arizona hinshawii*" ser. 7a,7b:1,2,5:-
S. enterica subsp. *diarizonae* ser. 60:k:z	*S. IIIb 60:k:z*	"*A. hinshawii*" ser. 24:29:31
S. enterica subsp. *houtenae* ser. Marina	*S. ser. Marina*	*S.* IV 48:g,z51:-, *S. marina*
S. bongori ser. Brookfield	*S. ser. Brookfield*	S. V 66:z41:-, *S. brookfield*
S. enterica subsp. *indica* ser. Srinagar	*S. ser. Srinagar*	*S.* VI 11:b:e,n,x, *S. srinagar*

2.6.3. Staphylococcus epidermidis (S. epidermisdis)

Staphylococcus epidermidis (S. epidermisdis) is a Gram-positive, coagulase-negative caucus, facultative anaerobes, non-spore-forming, and spherical shape. Usually, it can be found on the human skin and nasal cavity. Microscopic organisms within the genus *Staphylococcus* are pathogens of the human including *S. aureus, S. epidermidis, Staphylococcus epidermidis* and *Staphylococcus aureus* are currently considered two of the most important pathogens in nosocomial infections associated with catheters and other medical implants and are also the main contaminants of medical instruments *saprophytic, S. haemolyticus* and other warm-blooded animals (Mehta *et al.*, 2009). *S. epidermisdis* and *S. aureus* are currently considered two of the most important pathogens in infections associated with catheters and other medical implants and are also the main contaminants of medical instruments (Chessa *et al.*, 2016). *S. epidermisdis* rarely

effects to human, but it is very difficult to treat when effected because its genes are resistant to many types of antibiotics.

Moreover, *S. epidermidis* can cause chronic disease by the damage the immune system. It is a serious burden for the public health system. In specific, *Staphylococcus epidermidis* and *Staphylococcus aureus* are currently considered two of the most important pathogens in infections associated with catheters and other medical implants and are also the main contaminants of medical instruments *S. epidermidis* symbolize the foremost common source of contamination on indwelling medical devices. This likely stems from the fact that *S. epidermidis* could be a permanent and omnipresent colonizer of the human skin, and the coming about the high likelihood of device contamination during insertion. In spite of the fact that *S. epidermidis* diseases as it were once in a while create into life-threatening illnesses, their recurrence, and the reality that they are amazingly troublesome to treat, interprets to a genuine burden for the public health system (Otto, 2009). Among the coagulase-negative staphylococci (CNS), *S. epidermidis* has been the primary frequently isolated species and the foremost common species account for disease (Gara *et al.*, 2015).

Table 2.14. Taxonomy classification of *S. epidermidis*

Domain	**Bacteria**
Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	*Stapylococcaceae*
Genus	*Staphylococcus*
Species	*Staphylococcus epidermidis*

2.6.4. *Staphylococcus aureus (S. aureus)*

Staphylococcus aureus (S. aureus) is an aerobic or anaerobic, and Gram-positive coccus with non-motile, non-spore-forming, catalase-positive and coagulase-positive. The genus *Staphylococcus* contains at least 15 different species (WHO, 2006). In the genus of *Staphylococcus* not only *S. aureus* but *S. epidermidis* and *S. saprophyticus* also can cause disease to human being. Although *S. aureus* can produce disease to human through two different mechanisms. One is based on the ability of the organisms to multiply and spread widely in tissues, and the other is based on the ability of the organisms to produce extracellular enzymes and toxins (WHO, 2006). *S. aureus* is widespread in the environment but is usually found on the skin and mucous membranes of animals. Staphylococci are occasionally detected in the gastrointestinal tract and can be detected in sewage water. *S. aureus* can be released by human contact into water environments such as

swimming pools, spa pools and other recreational waters. It has also been detected in drinking-water supplies. Hand contact is by far the most common route of transmission. The consumption of foods containing. *S. aureus* toxins can lead to enterotoxin food poisoning within a few hours (WHO, 2006). The symptom gastrointestinal disease after consumption is vomiting, diarrhea, fever, abdominal cramps and loss of fluids within a few hours. It can grow at pH 4.2 to 9.3 and in a salt concentration of up to 15%.

Table 2.15. Taxonomy classification of *S. aureus*

Domain	Bacteria
Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	*Stapylococcaceae*
Genus	*Staphylococcus*
Species	*Staphylococcus aureus*

2.7. Antibiotics

2.7.1. Definition of Antibiotic

There are many authors who gave antibiotic definitions, which are identical to each other. One interesting example of antibiotic definition is that antibiotics are literally the chemical substance that can kill or inhibit the growth of microorganism (Guardabassi and Dalsgaard, 2002). Another point of view, it said that the word "antibiotic" stems from two classical Greek words (Walsh and Wencewicz, 2015) anti, which means against and bio, which refers to life. Antibiotics are, therefore, in principle against life. In microbiology, most people know it as antimicrobial drug. It is widely used in human medicine, veterinary, agriculture production, and food processing in the world (Princess and Kaster, 2018). To conclude, antibiotics are drugs that are utilized for fighting disease and saving lives of both humans and non-humans.

2.7.2. History and Discovery of Antibiotic

Antibiotics have changed dramatically in many respects. It has predominantly survived countless lives; thus, its discovery was a turning point in human history and has been a productive academic research topic until recently in industry 4.0 era (Davies and Davies, 2010).

As a result of the evolution of the antibiotic penicillin by Alexander Fleming, antibiotics were introduced in 1928 (Karkman *et al*., 2015) . However, regardless of the studies of Dorothy Davies, the complete structure of simple molecule was revealed in 1949 by the X-ray crystallography. Several years before 1940, bacterial Penicillin was surprisingly identified by two

members of the penicillin discovery team. On top of that, the discovery in antimicrobial infection therapy of synthetic sulphonamides was revolutionized in 1935 (Gangle, 2005). Furthermore, streptomycin, the treatment for tuberculosis (TB), was introduced in 1944 (Davies , 2010). With respect to the introduction of antimicrobial agents, it assisted to decrease mortality rates. For instance, the subcutaneous use of sulphanilamide caused reduction of acute meningococcal meningitis from 70 to 90% to nearly 10% (Powers, 2004). Also, penicillin was widely used in World War II and saved millions of live ever since.

Since the intensive work on antimicrobial agents in the 19th century, research found that there were more than 20 new classes of antibiotics discovered; mainly, they are either natural or semi-synthetic (Li, 2009) **(**Table 2.16**).**

Table 2.16. Commonly classes of antibiotic used and examples of each class (Li, 2009)

Antibiotic class	Example of class member
Beta-Lactams	Penicillin (ampicillin), cephalosporin (Cefpodoxime), carbapenem (meropenem), monobactam (aztreonam)
Aminoglycosides	Kanamycin, gentamicin, streptomycin, spectinomycin
Glycopeptides	Vancomycin, teicoplanin
Tetracyclines	Tetracycline, minocycline, tigecycline
Macrolides	Erythromycin, azithromycin
Lincosamides	Clindamycin, clindamycin
Streptogramins	Synercid
Oxazolidinones	Linezolid
Phenicols	Chloramphenicol
Quinolones	Ciprofloxacin, norfloxacin
Lipopeptides	Daptomycin

Since antibiotic resistance exists today and it is a grave problem in various nations, it is useful to understand the history and development of antibiotics shown in Figure 2.3. It leads us to better comprehension about antibiotics and to identify the problem of antibiotic resistance.

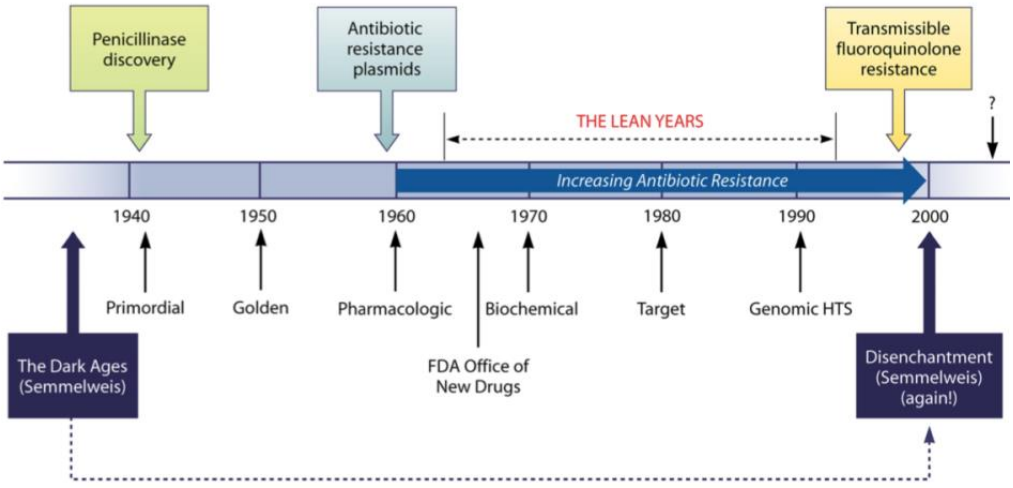

Figure 2.3. Events in the age of antibiotics

2.7.3. Ampicillin ($C_{16}H_{19}N_3O_4S$)

Ampicillin is a member of penicillin beta-lactam antibiotic (Davies and Davies, 2010). It was discovered in 1958 and marketed to treat bacterial infection caused by gram-positive organisms (Simon and Miller, 1986). Also, it is the effective drug for treatment of infection causing by *E. coli, P. mirabilis, enterococci, Shigella, S. typhosa and other Salmonella, nonpenicillinase-producing N. gononhoeae, H. influenzae.* The activity of bactericidal of Ampicillin is mediated by binding to specific penicillin-binding proteins (PBSs) and resulted from inhibition of cell wall synthesis (DrugBank, 2018).

2.7.4. Cefpodoxime ($C_{15}H_{17}N_5O_6S_2$)

Cefpodoxime is a third generation of cephalosporin with a broad spectrum of antibacterial activity. It has in vitro activity against many common Gram-positive and Gram-negative bacteria, including those that are frequently associated with common paediatric infections, such as acute otitis media, pharyngitis and lower respiratory tract infections e.g. bronchitis and pneumonia. The drug is active against penicillinsusceptible strains of *Streptococcus pneumoniae* and has modest activity against strains that are intermediately susceptible to penicillin. Cefpodoxime shows good in vitro activity against *S. pyogenes* and *S. agalactiae* but only modest activity against *Staphylococcus aureus* (methicillin-susceptible strains). The drug also has good in vitro activity against a number of Gram-negative pathogens, such as *Haemophilus influenzae, Moraxella catarrhalis* and Enterobacteriaceae (*Escherichia coli, Klebsiella pneumoniae*, *Proteus mirabilis*).

2.7.5. Erythromycin ($C_{37}H_{67}NO_{13}$)

Erythromycin, belong to the class of medicines known as macrolide antibiotics, is derived from a strain of Streptomyces erythreus, an actinomycete. Erythromycin is most active against gram-positive bacteria, including most strains of penicillin-resistant staphylococci. The gram-negative coccobacilli show varying degrees of sensitivity; most strains of Hernophilus influenzae, Bordetella pertussis, as well as species of Neisseria, are susceptible to Erythromycin. Macrolides inhibit the synthesis of protein. They impair the elongation cycle of the peptidyl chain through binding to the 50S subunit of the ribosome which result in inhibiting of peptide bond formation and protein synthesis within the bacterial cell. The activity is bacteriostatic or bactericidal depending on concentration.

2.7.6. Gentamicin ($C_{60}H_{125}N_{15}O_{25}S$)

Gentamicin is an aminoglycoside antibiotic naturally synthesized by *Micromonospora,* a Gram-positive genus of bacteria widely found in water and soil. Gentamicin is used for the treatment of several gram-negative infections making it a good option to enhance the probability of successful treatment in bacterial septicemia, meningitis, urinary tract infections, gastrointestinal

tract infections, and soft tissue infections. This antibiotic is useful against a wide variety of bacteria, especially the therapeutic responses to members of the Enterobacteriaceae family (e.g., Escherichia coli, Klebsiella pneumoniae, Serratia spp., and Enterobacter spp.), Pseudomonas aeruginosa, and some strains of Neisseria, Moraxella, and Haemophilus genera by binding to the 16s rRNA at the 30s ribosomal subunit, disturbing mRNA translation and, thus, interrupts bacterial protein synthesis.

2.7.7. Trimethoprim ($C_{14}H_{18}N_4O_3$)

Trimethoprim is an antimicrobial agent which to be active againt a wide range of gram-positive bacteria **(***Staphylococcus* species) and gram-negative bacteria **(***Enterobacter* species, *Escherichia coli, Klebsiella pneumonia, Proteus mirabilis).* Trimethoprim is a folic acid antagonist that blocks the production of tetrahydrofolic acid from dihydrofolic acid by binding to and reversibly inhibiting the required enzyme, dihydrofolate reductase. This binding is very much stronger for the bacterial enzyme than for the corresponding mammalian enzyme. Thus, trimethoprim selectively interferes with bacterial biosynthesis of nucleic acids and proteins. It exerts a bactericidal effect in vitro in the presence of methionine, glycine and a purine and this allows the inhibition of DNA synthesis without inhibition of protein synthesis. This result in bacterial cell elongation without division and leads to cell death.

2.7.8. Ciprofloxacin ($C_{17}H_{18}FN_3O_3$)

Ciprofloxacin is descripted as an antimicrobial drug agent of fluoroquinolones class (Krcmery, 2013). The ciprofloxacin product was approved by FDA in 1987 (FDA, 2004). It has treated more than 250 million patients in the globe after this approval (Sharma *et al*., 2010). It has a wide range of activity to against gram-negative and gram-positive microorganism including *Staphylococcus saprophyticus, Enterococcus faecalis, Escherichia coli Pseudomonas aeruginosa, Klebsiella pneumoniae*, so on and so forth (FDA, 2004). Ciprofloxacin is used for treating the infections include urinary tract infections, respiratory tract infections, skin and skin structure infections, bone and joint infections, infectious diarrhea, and kidney infections in children (Drug Bank, 2018). It produces its mechanism of action through inhibition of bacterial DNA gyrase and topoisomerase which are required for bacterial DNA replication, transcription, repair, and recombination (Drug Bank, 2018). Therefore, the mechanism of action of ciprofloxacin is inhibition of DNA synthesis.

2.7.9. Mechanisms of Action of Antibiotic Resistance

Antibiotics either destroy or slow down the growth of bacteria. If it destroys bacteria, it is bactericidal. If it slows down the bacteria, it is called bacteriostatic. Within bacterial inhibition, there are three main mechanisms, which include inhibition of protein synthesis, cell wall synthesis, and DNA synthesis (Kohanski *et al*., 2010).

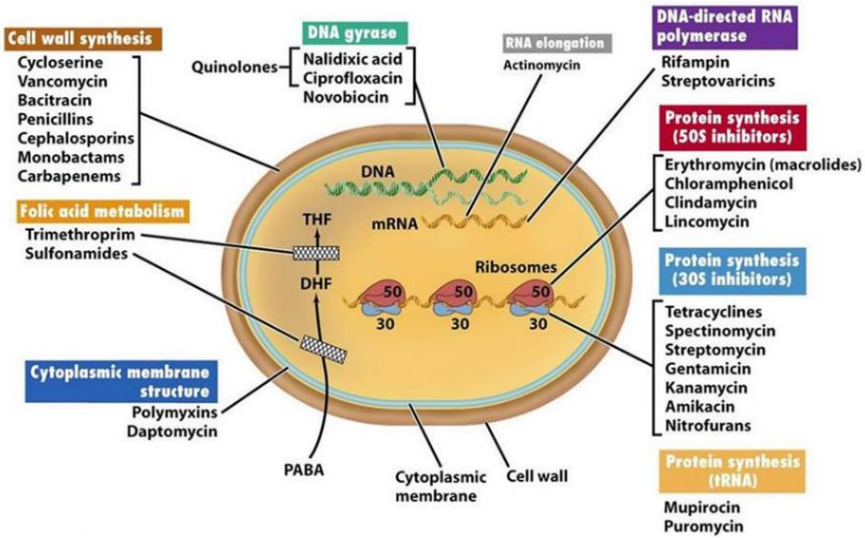

Figure 2.4. Mechanisms of action of antibiotics (IJAMBR, 2016)

2.7.9.1. Inhibition of Cell Wall Synthesis

In General, a cell of bacterial forms by a cell wall, cell membrane, and nucleus. The cell wall is the outer part of the bacteria containing peptidoglycan layer which is made up of cross-linked polymers. This peptidoglycan is mainly responsible for all the mechanisms of bacteria including resistivity, virulence factors include shaping of that bacteria. Cell wall synthesis inhibitors are the most used antibiotics for treating Gram-negative as well as Gram-positive infections.

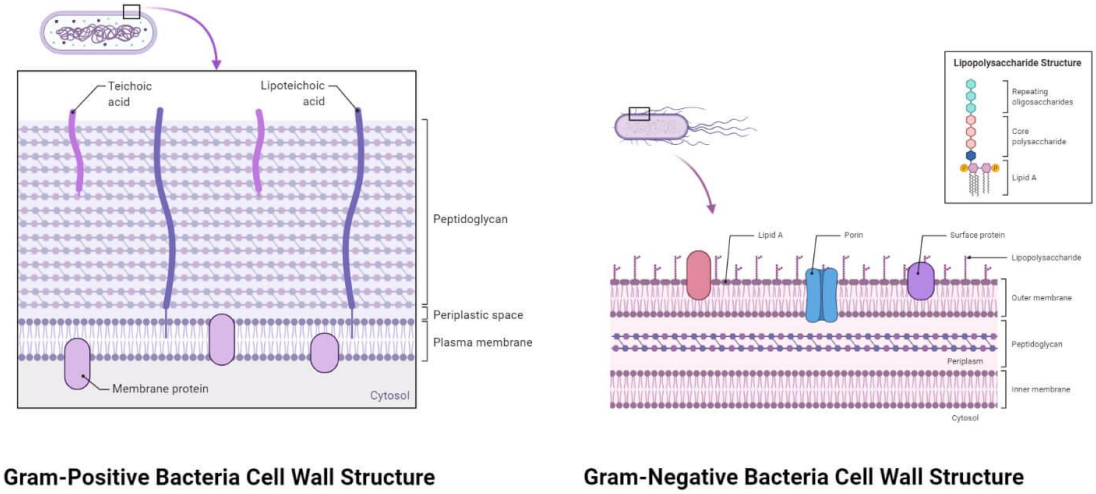

Figure 2.5. Inhibition of cell wall synthesis

2.7.9.2. Inhibition of Protein Synthesis

The inhibition of protein synthesis occurs at the ribosome level, working at different stages of prokaryotic mRNA translation into proteins (Figure 2.6). They are highly selective to 70S ribosomes in prokaryotic cells since eukaryotic cells have a different ribosomal size, sequence, structure, and ratio of protein to RNA. Translation in prokaryotes involves the assembly of a small subunit (30S) and a large subunit (50S) to form a ribosome that binds mRNA to read the nucleotide sequence. tRNA binds to the A, P, and E sites during the initiation, elongation, and termination

stages to translate the polypeptide sequence. By targeting different stages of mRNA translation, antibiotics can be swapped if resistance develops.

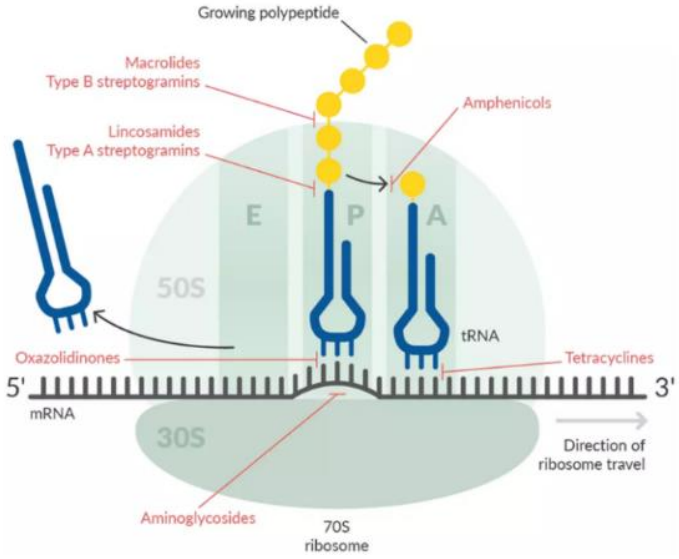

Figure 2.6. Antibiotics interfere with multiple stages of protein synthesis

2.7.9.3. Inhibition of DNA Synthesis

The synthesis of DNA occurs in chromosome which requires substrates, primers, templates, and enzymes. Some drugs, included antibiotic, were used to treat diseases by inhibiting DNA synthesis in different ways. The antibiotics affect the DNA synthesis through its perturbation on the template function of DNA and its inhibition of enzymes associated with DNA replication and transcription. Examples of those that inhibit DNA replication include the quinolones, coumermycins and novobiocin. The quinolones selectively inhibit DNA gyrase (aka topoisomerase II) by binding to the A subunit of the enzyme at exposed single strand ends of the cut DNA chain. Hence, DNA gyrase becomes unable to reseal the DNA with the end result that the chromosome becomes highly fragmented.

2.8. Antibiotic-Resistant Bacteria (ARB)

Antibiotic-resistant bacteria is a term used to illustrate the ability of bacteria to resist the effectiveness of an antibiotic, but the bacteria are not killed and their growth does not stop (CDC, 2013). It is universally accepted that the development of antimicrobial drug around the globe led to historically notable advancement in public health. On account of the effectiveness of antibiotic and mitigation of bacterial health risk, the prevalence of antibiotic use has increased in the 20th century (Kaiser, 2016). However, with misuse and overuse, the rate of antibiotic resistance in pathogenic bacteria has been increasing significantly over the last several decades (Jones *et al.*, 2008). As a result, the occurrence of antibiotic resistance has been investigated as a critical issue in public health by the US Centre for Disease Control (CDC), World Health Organization (WHO), and various nations.

Bacteria resistance to one or more antibiotic may present direct and indirect risks to human health (Blaak *et al*., 2011). Direct risk refers to the exposure of pathogenic bacteria that are resistant to antibiotic relevant for treatment of infection. As this is directly caused by pathogens, it will be more harmful and difficult to treat. Indirect risks are related to the exposure of harmless bacteria that carry antibiotic resistance. These bacteria can colonize intestines or skin without causing disease, and pass their resistance genes to other bacteria that inhibit these tissues.

The predominant role of human activities in the generation of environmental reservoirs of antibiotic resistance cannot be disputed (Davies and Davies, 2010) (Figure 2.6). Antibiotic resistant bacteria are emitted into environment; mainly, it disembogues with faeces of humans and animals that are treated with antibiotics (Jones *et al*., 2005). These bacteria exist in soil and surface water through the discharge of untreated or partially treated sewage and wastewater treatment. People are high at being risk when they are being contaminated because of gene transfer to pathogenic bacteria that may be later infect humans.

Since antibiotic resistance has been found in many organisms which involves pathogens, antibiotic resistance has become a global threat to public health (WHO, 2014). These pathogenic organisms are becoming increasingly more common and their infections are difficult to treat.

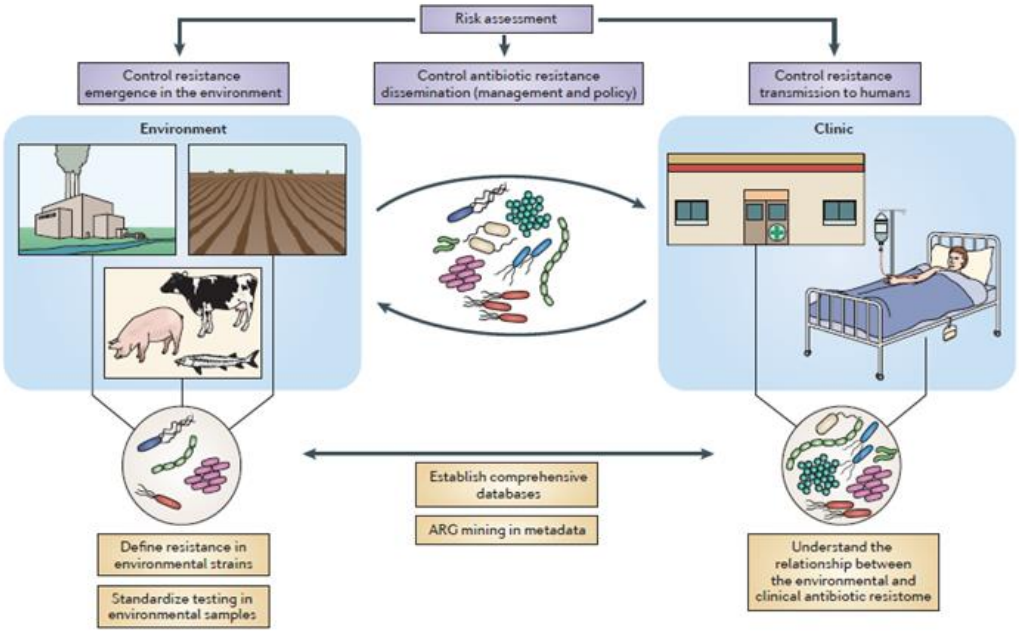

Figure 2.7. Antibiotic-Resistant Bacteria (ARB)

3. METHODOLOGY

3.1. Study Sites

The study focused on surface water and wastewater. Surface water samples were taken from Tonle Sap, Bassac River and Upper Mekong River where most urbanized areas with a dense of populations, building, houses, hospitals and schools were located. Surface water samples were collected at four points. The first sampling site is in Upper Mekong River near the Kizona Bridge, Kampong Cham city. The second sampling sites located in Tonle Sap River, Kampong Chhnang Tourist Port, Kampong Chhnang city. The third sampling site is in Phnom Penh Tourist Port, Phnom Penh city and the fourth sampling site is at Takhmao Ferry, Kandal Province. As for the wastewater, samples were collected from two points. The first sampling site located in Stung Chrov, Kandal Province, which finally end up in Bassac River. Lastly, the second sampling site for wastewater is at Kilometer No 11 village, Phnom Penh city, where the wastewater channel finally end up in Tonle Sap River.

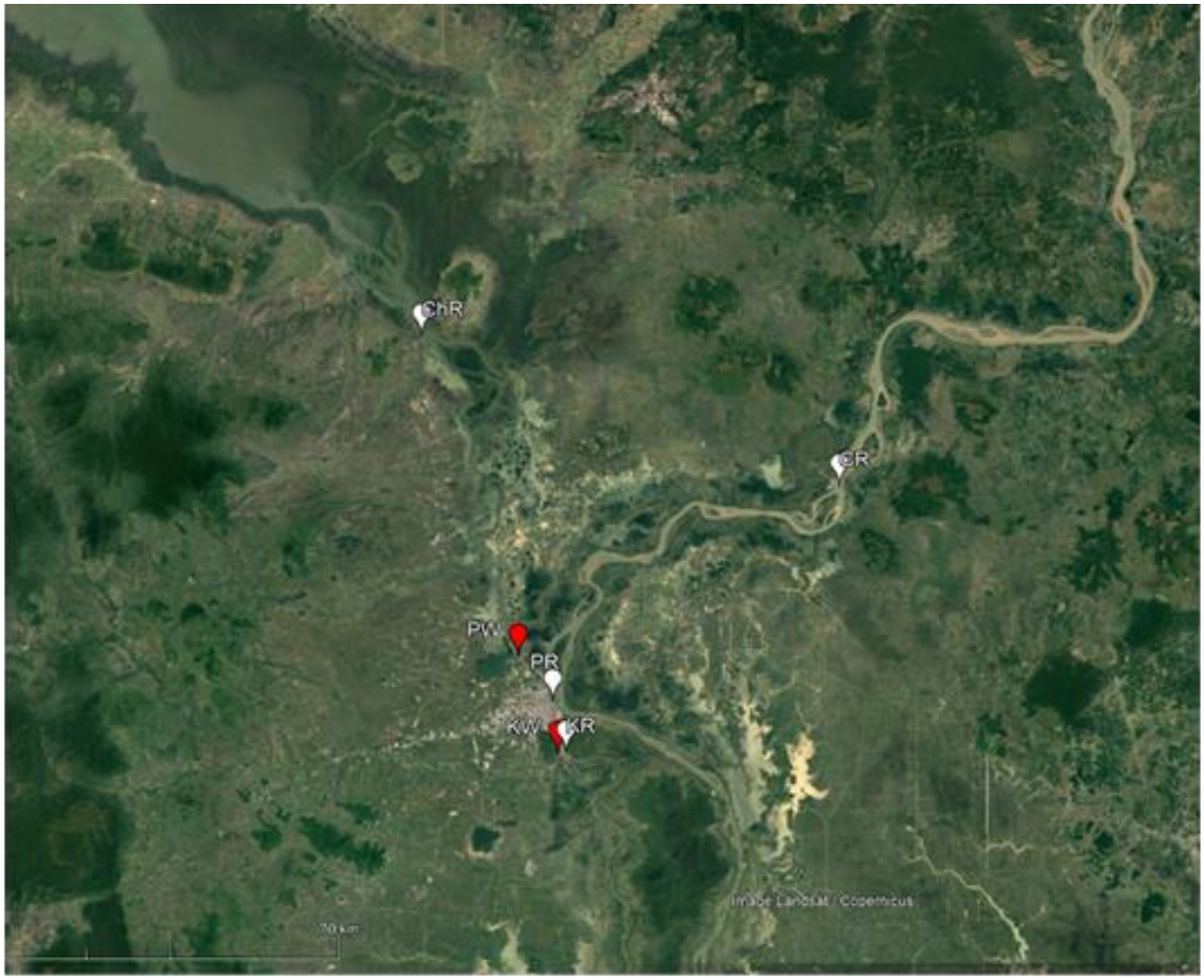

Figure 3.1. Sampling sites (Google Earth, 2023)

3.2. Sample Collection

A total of 12 samples, in which 8 and 4 samples are taken from surface water and wastewater respectively, were collected in this study in order to investigate the occurrence of multidrug resistance bacteria. Surface water and wastewater samples were collected and coordinated by Google Map in July and September, 2023. The water samples were kept in sterile plastic bottles of 1000 ml. After sampling, the samples were stored in an ice box and transported immediately to ITC laboratory to analysing.

Table 3.1. Detail of sampling sites

Sample	Sample Site	Code	Capital/Province	Coordinate	
				X	Y
Surface water	Phnom Penh Tourist Port	PR	Phnom Penh	492092	1279636
	Takhmao Ferry	KR	Kandal	494793	1268847
	Kampong Chhnang Tourist Port	ChR	Kampong Chhnang	465197	1356353
	Kizona Bridge	CR	Kampong Cham	550697	1324782
Wastewater	Km 11	PW	Phnom Penh	485029	1289025
	Stung Chrov	KW	Kandal	493109	1268635

3.3. Physicochemical Analysis

In this study we conducted four main physicochemical parameters including temperature, pH, Electrical Conductivity (EC) and Dissolved Oxygen (DO). Horiba U-50 multi-parameter water quality checker have been used to analyze the four parameters.

Figure 3.2. Horiba U-50 multi-parameter water quality checker

3.4. Microbiological Analysis Process

3.4.1. Sample Preparation

Once the sample arrived Environmental Microbiology laboratory at ITC, the water samples are prepared based on colony cultured technique. Two main techniques were applied: plating and filtration technique (see Table 3.2).

Table 3.2. Division of sample microbial culture methods based on sample types.

Location	Sample Type	Microbial culture techniques		
		E. coli	***Salmonella* spp.**	***Staphylococcus* spp.**
Phnom Penh	Surface water	0.1mL plating technique	10 mL filtration technique	0.1mL plating technique
	Wastewater	0.1mL plating technique	5 mL filtration technique	0.1mL plating technique
Kandal	Surface water	0.1mL plating technique	10 mL filtration technique	0.1mL plating technique
	Wastewater	10 mL filtration technique	5 mL filtration technique	0.1mL plating technique
Kampong Chhnang	Surface water	5 mL filtration technique	10 mL filtration technique	0.1mL plating technique
Kampong Cham	Surface water	0.1mL plating technique	10 mL filtration technique	0.1mL plating technique

According to Table 3.2, both 5mL and 10mL filtration technique followed the same procedures. 5mL or 10mL of water were poured into metal sample container that attached with cellulose nitrate filter paper (47mm, ∅=0.45µm, Sartorius, Germany). Figure 3.3 illustrated the procedure for water filtration technique. After filtered, the filter papers were placed on the prepared agar media for culture the microbial.

Figure 3.3. Procedure for microbial culture by using filtration method

3.4.2. Medium Preparation

The following media agar plates were used in recovering the total bacterial population. In order to study the bacteria of interest, 3 types of agar namely Chromocult agar, Manitol Salt agar and Sakazakii DHL Agar plates were prepared.

3.4.2.1. Chromocult Agar

Chromocult Agar is a nutrient media essential for *E. coli* and Total coliforms growth. It was prepared by dissolving 26.5g of powder in 1 liter in distilled water, mixing and heating gently to receive a complete dissolved solution. Prepared antibiotics were added into the medium solution while the medium temperature was around 40 to 50 ^{o}C by stirring to help distributed them evenly. The medium was then poured out into plastic petri dishes for cultivation bacteria. Be aware of that all plates were flipped after solidifying. Flipping the plates prevents possible contamination from water droplets that condenses onto the lid due to the heat from the medium.

3.4.2.2. Sakazakii DHL

Sakazakii DHL Agar is used to indicate the present of *Salmonella.* spp. It was made by dissolving 64 g of powder in 1 liter in distillated water, mixing and heating to achieve a dissolved solution. The medium was then autoclaved at 121 ^{o}C for 15 minutes and kept it until the temperature decreased to 50 ^{o}C. Other steps were done the same as Chromocult Agar.

3.4.2.3. Mannitol Salt

Mannitol Salt Agar is used to indicate the present of *Staphylococcus.* spp. It was made by dissolving 64 g of powder in 1 liter in distillated water, mixing and heating to achieve a dissolved solution. The medium was then autoclaved at 121 ^{o}C for 15 minutes and kept it until the temperature decreased to 50 ^{o}C. Other steps were done the same as Chromocult Agar.

3.4.2.4. MHA Medium

Mueller Hinton Agar (MHA) was developed in 1941. MHA contains beef extract, casein**,** starch and agar**.** Beef extract and casein provide nitrogen, vitamins, carbon, amino acids, sulphur and other essential nutrients. Starch help with absorption of any toxic metabolites and hydrolyzation of yields dextrose, which serves as a source of energy while agar is play a role as the solidifying agent. MHA is more commonly used for the routine susceptibility testing of non-fastidious microorganism by the Kirby-Bauer disk diffusion technique. MHA is known as a non-selective medium that almost all organisms plated on here will grow and also a loose agar that enable a better diffusion of the antibiotics than most other plates which mean it allows a truer zone of inhibition. The MHA medium is prepared as following:

- Suspend 38 g of MHA in 1 liter of distilled water
- Heat with frequent agitation and boil for one minute to completely dissolve the medium

– Autoclave at 121°C for 15 minutes. Cool to room temperature
– Pour MHA into sterile petri dishes on a level, horizontal surface to give uniform depth
– Allow to cool to room temperature
– Check for the final pH 7.3 ± 0.1 at 25ºC
– Store the plates at 2-8 ºC

3.4.3. Cultivation Technique

For spreading technique, the 0.1 mL of samples were spread on prepared medium plates using glass spreader.

3.4.3.1. Bacterial Isolation

The isolation was performed to purify the colony in order to use in the next steps. The isolates were cultured on agar plate containing same concentration of each antibiotic the same as platting count method. After counting, 4 different colonies from the original agar plate were selected based on its morphology. Those colonies were taken to isolate on the new selective medium. All streaking plates were kept in the incubator at the temperature of 37 ºC for 24h.

Table 3.3. Color of colonies appear on agars

Target bacteria	Chromocult coliform	DHL agar	Mannitol Salt	Incubation time
E. coli	Violet			24h
Salmonella		Black		24h
Staphylococcus			Golden yellow/white with yellow zone	24h

E-Coli isolation on Chromocult agar

Salmonella isolation on Sakazakii DHL

Staphylococcus isolation on Mannitol salt agar

Figure 3.4. Color of colonies appear on agars

3.4.3.2. *E. coli* Colony Culture and Isolations

One of the pure colonies from isolated agar plates were inoculated into flacon tube 15ml which contained 2ml of LB broth agar. After inoculating, it was incubated in shaker incubator at 35±2 ºC for 12h to 24h following by 150 rpm. Finally, the flacon tubes were checked for turbidity purpose with corresponding to bacterial cell count in the LB broth agar.

All isolates are stored in tubes containing Luria-Bertani (LB) broth with 30% v/v glycerol at -80°C for further analysis (Scarano *et al*., 2018).

3.4.3.3. *Salmonella spp.* Colony Culture and Isolations

The presence of *Salmonella* spp. was detected and isolated on Xylose, Lactose, Dextrose agar (XLD agar, Oxoid, UK). Media preparation procedure followed the manufacturer's instruction and incubated at 37ºC for 24h. The presumptive colony has blacked color surrounded by red zone. (See Table 3.3)

3.4.3.4. *Staphylococcus spp.* Colony Culture and Isolations

The presence of *Staphylococcus* spp. was detected and isolated on Mannitol Salted Agar (MSA agar, Hemedia, India) supplemented with emulsion egg yolk. The incubations were at 37ºC for 24h and presumptive bacteria has golden yellow/white colonies surrounded by yellow zone. (See Table 3.3)

3.4.3.5. Overnight Culture

One of the pure colonies from isolated agar plates were inoculated into flacon tube 15ml which contained 2ml of LB broth agar. After inoculating, it was incubated in shaker incubator at 35±2 ºC for 12h to 24h following by 150 rpm. Finally, the flacon tubes were checked for turbidity purpose with corresponding to bacterial cell count in the LB broth agar. All isolates are stored in tubes containing Luria-Bertani (LB) broth with 30% v/v glycerol at -80°C for further analysis (Scarano *et al.*, 2018).

3.4.4. Microbiological Analysis

3.4.4.1. Antibiotic Susceptibility Test (AST)

Bacteria were considered to be resistant to at least three or more antibiotic classes. In this study, the susceptibility to antibiotics of three pathogenic strains were assessed using the disk diffusion technique as described by Clinical and Laboratory Standard Institute (CLSI). It was performed on Mueller-Hinton agar (MHA) which is considered as the best medium used for

routine susceptibility testing. The overnight culture was washed by adding PBS and conducting centrifugation to remove the organic matters. The overnight culture tubes were checked for turbidity against a pre-prepared 0.5 McFarland.To obtain uniform growth, cotton swabs were used to dip into the suspension of inoculum. The culture suspension is spread onto Muller Hinton agar (~4 mm depth corresponding to 25-30mL) in three directions using sterile swabs (~3 rotations of 60◦ angle). After that, agar plates were allowed to dry for approximately 3 to 5 minutes before applying the blank disc and antibiotics. There were 6 antibiotic discs known as Ampicillin, Cefpodoxime, Erythromycin, Gentamicin, Trimethoprim, and Ciprofloxacin were applied on MHA agar using sterilized forceps. It was then incubated at 28°C ± 2 for 24 h to 28 h (CLSI 2015). The result interpretation is based on diameter. The diameters of the inhibition zones measured to determine resistance, intermediate resistance, or susceptibility of each isolate to the antibiotics according to CLSI standards. Table 3.4 showed the list of used antibiotics dish for each bacterial species.

Table 3.4. List of antibiotic drugs and diameter zone (intermediate resistant diameter) in mm

Antibiotic	**Class of Antibiotic**	***Staphylococcus* spp.**	***E. coli***	***Salmonella* spp.**
Ampicillin (10 µg)	Penicillins	-	≤13 (14-16)	≤13 (14-16)
Cefpodoxime (10 µg)	Cephalosporins (Third generation)	-	≤17 (18-20)	≤17 (18-20)
Ciprofloxacin (5 µg)	Fluoroquinolone	≤15 (16-20)	≤21 (22-25)	≤21 (22-25)
Erythromycin (15 µg)	Macrolide	≤13 (14-22)	-	-
Gentamicin (10 µg)	Aminoglycoside	≤12 (13-14)	≤12 (13-14)	≤12 (13-14)
Trimethoprim (1.25 µg)	Sulfonamides	≤10 (11-15)	≤10 (11-15)	≤10 (11-15)

3.4.4.2. Bacterial identification

MALDI-TOF techniques aims to identify the specific species of target bacteria such as *Staphylococci* and *Salmonella* that were isolated from the samples. This confirmation is essential for accurate bacterial specie identification for the further determination of antibiotic susceptibility test which requires high certainty of isolate identity the colonies species of isolated were determined by MALDI-TOF MS analysis. With the two target bacteria species (*Staphylococcus* spp. and *Salmonella* spp.), the pure colonies of the presumptive isolation which the freshly grown are recommended to use for direct transfer method analyzed by MALDI-TOF MS biotyper. For direct transfer method, sterilized wood toothpick was used to pick a small amount of single colony from the agar plates then smeared onto full of spot on MALDI target plate as thin film. Sample on

spot was overlay with 1 µl of martrix solution -cyano-4-hydroxycinnamic acid (HCCA), (50% acetonitrile (AN), 2.5% trifluoroacetic acid (TFA) and water 47.5% with martrix HCCA) and allow drying at room temperature prior to analysis using MALDI-TOF biotyper. The reference strains were used as controls in this study for MALDI-TOF biotyper analysis. Bacteria Test Standard Escherichia coli. (BTS) was used as reference strain for the calibration and instrument parameter optimization following to manufacturer's instruction (Bruker Daltonik Germany) (Viver *et al*., 2015 & Panda *et al*., 2014). BTS 1 µl can be laid on MALDI plate let it dry and then overlay with 1 µl of HCCA. It can be used at the first last spot on MALDI and the score value of BTS should be more than 2 to be used as the standard identify comparison.

Data processing for automated data analysis, the generated peak lists derived from the bacteria MALDI-TOF profile mass spectra were compared with each entry of the MALDI-TOF biotyper database, using the standard parameters of the pattern-matching algorithms. The result of pattern-matching process was expressed as log (score) values, computed by comparison of peak list for an unknown isolate with the reference main spectral pattern (MSP) in the database. The log (score) value ranged from 0 to 3, a log (score) value ≥1.7 is indicative of a close relationship (i.e., at the genus level) and a log (score) value ≥2.0 is the set threshold for a match at the species level. The highest log (score) of a match against the score in the database was used for species identification (Panda *et al*., 2014). The results based on log (score) value <1.7 were rated as being unidentifiable by the software.

4. RESULTS AND DISCUSION

4.1. Physicochemical Characteristics of Water

4.1.1. Temperature

Figure 4.1 shows about temperature condition of freshwater along main rivers—Tonle Sap river, Bassac river, Mekong river and in sewage from two different natural purification wetland at the northern and southern part in Phnom Penh. Sampling points have been identified in three different provinces—Kampong Cham (Kizona Bridge, Upper Mekong river), Kampong Chhnang (Tourist Port, Tonle Sap river), Kandal (Takhmao Port, Bassac river), Km 11 (sewage from northern part of Phnom Penh), and Stung Chrov (sewage from southern part of Phnom Penh, Kandal province).

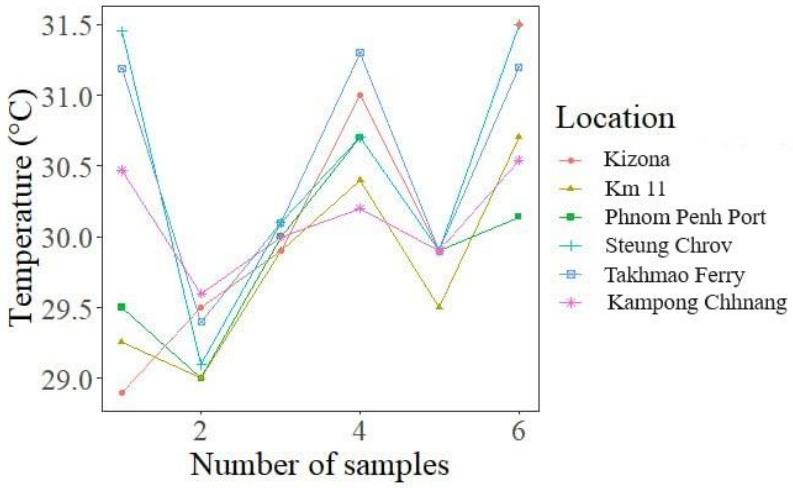

Figure 4.1. Temperature condition of freshwater and sewage.

According to Figure 4.1, the results show that temperature of freshwater and sewage in the designated sampling sites are in between 28 to 31 ºC. The mentioned temperature characteristic provides suitable and comfortable condition for the growth of bacteria—*E. coli* which can grow in abroad range of temperature from 7 to 45 ºC with the specific optimum growth at 37 ºC (Health Canada, 2022). The temperature also fits to the growth of most *Salmonella* serotypes which they can survive within range of 5 to 47 ºC with optimum temperature of 35 to 37 ºC, but some can grow at temperature as low as 2 to 4 ºC or as high as 54 ºC. Furthermore, *Salmonellae* are sensitive to heat and normally killed at temperature of 70 ºC or above (Pui, 2011). *Staphylococci* are really similar to *Escherichia coli* and *Salmonellae* in terms of growth within the above-mentioned water temperature, and they are able to grow within range of 6.5 to 46ºC (Onyango, *et al*., 2012). Meanwhile, the optimal temperature is from 30 to 37ºC and they can survive at extremes of 6.5ºC and 46ºC for limited periods of time (Onyango, *et a*l., 2012). Within this characteristic, it can be concluded that the temperature of river water and sewage along the designated sampling sites

provides suitable growth condition for those three bacteria—*E. coli*, *Salmonellae* and *Staphylococci*.

4.1.2. Electrical Conductivity (EC)

The results in Figure 4.2 show about the concentration of electrical conductivity in both freshwater and sewage at main river sources and stream in Cambodia. According to the results shown in this Figure 4.2, the concentration of electrical conductivity is in the range between 0 to 200 mS/cm in all sampling sites. Among those sampling points, there are three sampling points—Tourist Port (Tonle Sap river, Kampong Chhnang), Phnom Penh Port (Tonle Sap, Phnom Penh), and Km 11 (Sewage, Phnom Penh) that are sometimes presence in very low concentration while other locations—Kizona Bridge (Upper Mekong river, Kampong Cham), Stung Chrov (Sewage, Kandal), and Takhmao Port (Bassac river, Kandal) are as high as around 200 mS/cm. There are 5 physical parameters highly significant correlation with *E. coli* include pH, Temperature, Time, Turbidity, and DO while 2 parameters—EC and Dissolved solid are not significant correlated (Siti. N. Shamsudin, 2016). With this relationship, even EC level is low or high, it doesn't affect to the growth of *E. coli* and its survival.

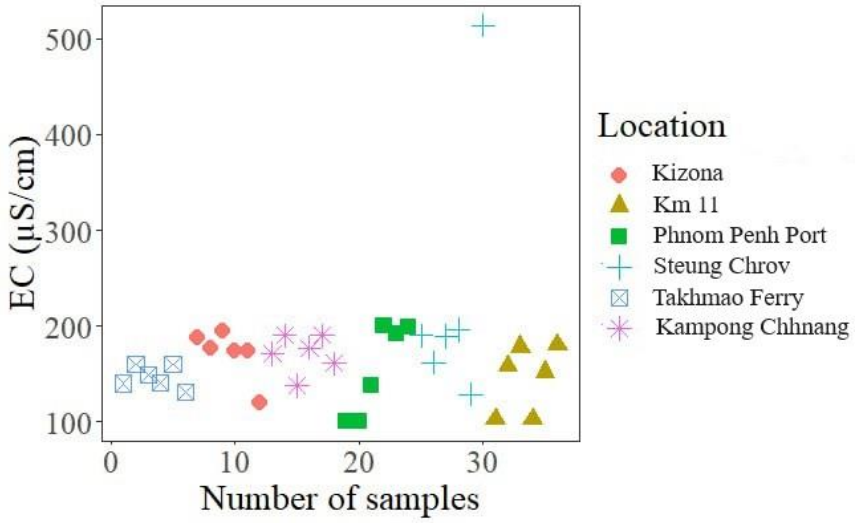

Figure 4.2. Concentration of Electrical Conductivities

4.1.3. Potential Hydrogen (pH)

Potential Hydrogen (pH) has been selected in this study as one among other physical parameters identifies in freshwater environment and sewage in six different locations. As the result, Figure 4.3 shows that in almost all location, pH level is in between 6.5 to 8.5 in which the range complies with Cambodia national standard stated in Sub decree No.103 and only few samples from two sampling sites (Km11 and Phnom Penh port) that pH level is around 5 (MoE, 2021). Within this pH range, it does not affect to the growth of bacteria specifically *E. coli*,

Salmonella and *Staphylococcus. E. coli* can survive in the range of pH 5 and pH 7 however with the increase of acidity or alkalinity status, it may decrease the survival rate of *E. coli* and its growth limit is at approximately a pH of 4 (Fritz Petersen & Jason A. Hubbart, 2020). While *Salmonella* can grow in pH range from 4 to 9 with the optimum between 6.5 and 7.5 (Pui, 2011). For *Staphylococcus*, growth condition is from pH 4.2 to 9.3 and in a salt concentration of up to 15%. In short, pH characteristic in all sampling sites still provides good condition for bacteria to grow in their living habitats.

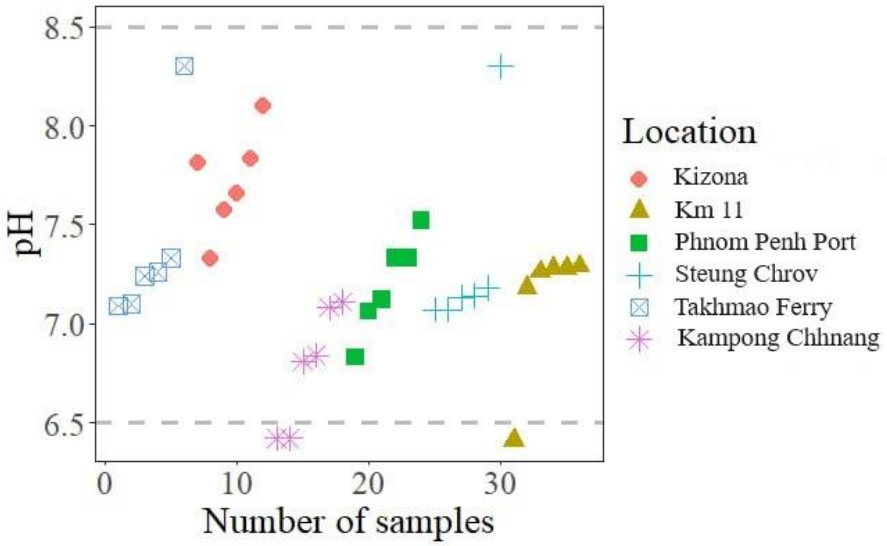

Figure 4.3. pH level identify in freshwater and sewage

4.1.4. Dissolved Oxygen (DO)

Dissolved oxygen (DO) is the fundamental parameter supports lives including micro-organisms particularly in aquatic environment and they need DO to breathe and decompose organic matters (USEPA, n.d). DO has been observed in six different locations in three provinces and one city. For river water, sample were collected from Mekong river (Kampong Cham province), Tonle Sap river (Tourist Port—Phsakrom), Tonle Bassac river (Takhmao Port) and two sewage samples: (1) Km 11 (Phnom Penh City) and (2) Stung Chrov (Takhmao Town). As the result, Figure 4.4 shows about DO concentration at all sampling site in main rivers present in the range between 3.8 and 7.5 mg/l. At the same time, DO level for two sites along sewage canal both at the southern and northern part of Phnom Penh is range from 0.3 to 4 mg/l. DO concentration in all sampling sites provide general condition for *E. coli* to grow and does not highly affect to growing processes while the primary habitat is in anaerobic environment and secondary habitat varies between aerobic and anaerobic (Fritz Petersen & Jason A. Hubbart, 2020). Even anaerobic environment is the primary habitat, both *E. coli* and *salmonella* multiplied massively in fecal matter outside host being growth higher in aerobic condition (Teresa Guerrero, Sonia Zapata & Gabriel Trueba, 2019). *Staphylococcus* also can grow in both aerobic and anaerobic habitats like

E. coli and *Salmonella* does. However, the greater condition for better growth is in aerobic state (Negash Belay & Avraham Rasooly, 2001). Through these results, it can be concluded that the studied bacteria—*E. coli*, *Salmonella* and *Staphylococcus* can live and grow in both anaerobic and aerobic habitat, but may grow better in aerobic environment that correspond with food abundant.

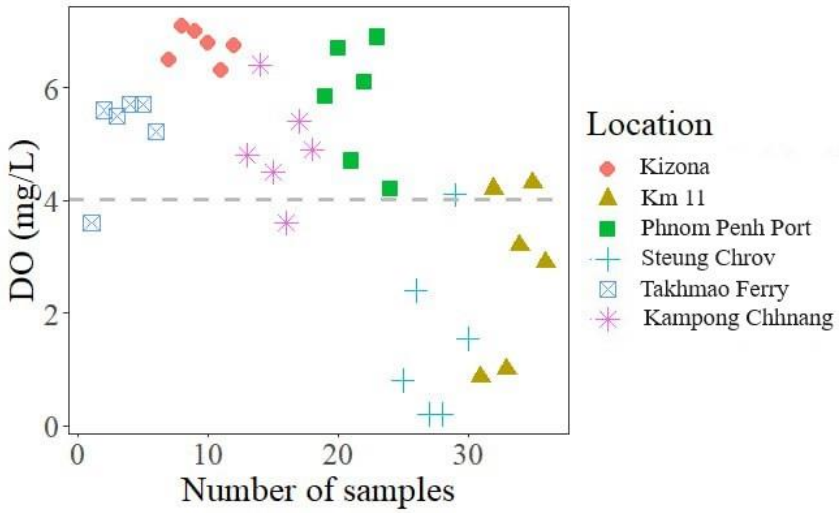

Figure 4.4. Concentration of Dissolved Oxygen (DO)

4.2. Biological Characteristics of Water

In figure 4.5 shown the number of bacteria in the diverse of six surface water samples which sampling from fresh water and sewage. The three differences bacteria namely *Escherichia coli* (*E. coli*), *Salmonella* spp., and *Staphylococcus* spp. were enumerated and detected from the water samples though culture and isolation technique. *E. coli* has been enumerated range from 0.08×10^2 to 5×10^2 CFU/ml where it was found with the highest concentration of *E. coli* in sewage sampling from Km 11 in Phnom Penh and followed by sewage sampling from Steung Chrov in Takmao city, Kandal province. The value of bacteria populations varied and counted at 0.07×10^3 to 4.1×10^3 CFU/ml for *Staphylococcus* spp. in water sample. Sewage water at Km 11 was found with the highest contaminated, and it was decline to a lower contaminate in the water sample from tourist port (Tonle Sap) in Kampong Chhnang province. The enumeration of *Salmonella* spp. concentration was greatly low if compare to the other two bacteria species isolated from every water sample with the range of total colony from 0.02×10^1 to 1.4×10^1 CFU/ml. Sewage sample obtained from Km 11 in Phnom Penh was highly contaminated of *Salmonella* spp. if compare the other water sources. This result shown a comparative water quality obtained from different water sources with assessment of microbiological contamination and essential for bacteria species identification. The presence of these three bacteria species including *E. coli*, *Salmonella* spp., and *Staphylococcus* spp. with the high concentration in sewage water specifically from Km 11. This sampling site is considered as a short transport into Tamok natural wetlands where is a major

receiver of household waste from the southern part of Phnom Penh. In addition, there are many households are living along this stream (stream that collected sewage), and they discharged untreated domestic waste/no storage tank directly into the stream. They are identified as a key contaminant to water source and infect to human health with associated gastrointestinal disease, vomiting, fever, abdominal cramps and skin problem (WHO, 2006) and (Kaper, J.B.; Nataro, J.P.; Mobley, H.L., 2004). It is confirmed with many studies that those bacteria species are widespread in the environment which can be detected in sewage water (Coburn *et al*., 2007). Some study also illustrated that environmental waters are not natural habitat for bacteria and their presence in this milieu is result of fecal pollution. Most common species found in environmental waters are *E. durans*, *E. faecalis*, *E. faecium* and *E. hirae*, and less commonly, *E. avium*, *E. cecorum*, *E. columbae* and *E. gallinarum*. However, pristine waters in Finland have been reported to contain *E. casseliflavus* (Wilson, 2005 and Švec, P.; Devriese, L.A., 2009).

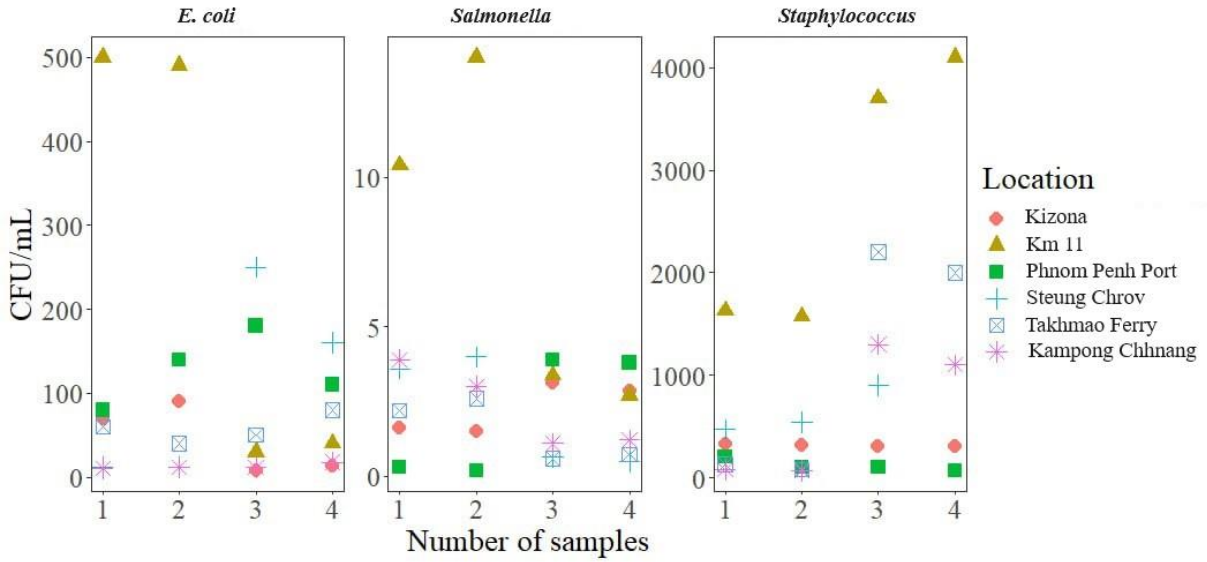

Figure 4.5 Total coliform concentration of water

4.3. Correlations and Regressions

4.3.1. Physical Correlations

Four physical properties of wastewater collected from six different sources, Phnom Penh Port, KM 11, Takhao, Steung Chrov, Kizona, and Kampong Chhnang, were checked whether they had correlation or not (Table 4.1). Table 4.1 shows that among the four physical properties: pH, DO, EC, and Temperature, only EC had a correlation with pH, and that relationship was positively moderate (P = 0.016; R = 0.40). This means that when EC increases, so does pH. However, other parameters had no relationship at all. In that case, when one parameter fluctuates, it does not affect the others.

Table 4.1. Correlation among physical properties

Parameter	pH	DO (mg/L)	EC (µs/L)	Temperature (°C)
pH				
DO DO (mg/L)	0.2			
EC (µs/L)	0.4*	-0.24		
Temperature (°C)	0.21	0.14	0.16	

Note: Asterisk "*" means significant difference at 0.05, while no asterisk denotes non-significance.

4.3.2. Biological Correlations

Three types of bacteria that might be present in wastewater collected from six different locations, as specified in Table 4.1, were studied to detect their correlation (Table 4.2). The three bacteria include *E. coli*, *Staphylococcus*, and *Salmonella*, while only *E. coli* and *Salmonella* had a strong and positive relationship ($P < 0.001$; $R = 0.72$). This means that in case *E. coli* increases, so does *Salmonella* and vice versa. Despite that, *Staphylococcus* had no relationship with *E. coli* and *Salmonella* at all. Although those two parameters change, they will not affect the content of *Staphylococcus*.

Table 4.2. Correlation among biological properties

Parameter	*E. coli*	*Staphylococcus*	*Salmonella*
E. coli			
Staphylococcus	0.11		
Salmonella	0.72**	0.12	

Note: Asterisk "****" means significant difference at 0.001, while no asterisk denotes non-significance. All these biological properties are set in CFU/mL.

4.3.3. Physical and Biological Correlations

After checking correlation among physical and biological properties individually in Tables 4.1 and 4.2, these two types of properties were also studied to detect their correlation (Table 4.3). The findings show that *E. coli* has a strong and positive relationship with *Salmonella* ($P < 0.001$; $R = 0.72$), which was the same as Table 4.2, and EC had a moderate positive relationship with pH ($P = 0.016$; $R = 0.4$), as presented in Table 4.1. However, it can also be found that EC and *Staphylococcus* had a strong and positive relationship ($P = 0.002$; $R = 0.62$), which means that when EC increases, the present of *Staphylococcus* may also be higher, and vice versa. For other parameters, there were no relationship at all, and although one parameter increases, that does not affect the others.

Table 4.3. Correlation between physical and biological properties

Parameter	*E. coli*	*Staphylococcus*	*Salmonella*	pH	DO	EC	Temperature
E. coli							
Staphylococcus	0.11						
Salmonella	0.72***	0.12					
pH	0.16	0.18	0.15				
DO	0.32	-0.13	0.15	0.37			
EC	-0.04	0.62**	-0.14	0.40*	-0.29		
Temperature	0.06	0.25	0.055	0.18	0.059	0.19	

Note: Asterisks "*", "**", and "****" means significant difference at 0.05, 0.01, and 0.001. Without asterisks denote non-significance.

4.3.4. Simple Linear Regressions

Simple linear regressions were analyzed between *E. coli* and *Salmonella*; *Staphylococcus* and *Salmonella*; and *Staphylococcus* and *E. coli*, respectively (Table 4.4). The result indicates that only the linear regression analysis performed for *E. coli* and *Salmonella* was very highly significant ($P < 0.001$), while there was no significant difference between *Staphylococcus* and *Salmonella*. The same result was seen between *Staphylococcus* and *E. coli*. Therefore, these two linear regression analyses could not be used to make prediction at all. However, in case of *E. coli* and *Salmonella*, if *Salmonella* increases by one CFU/L, *E. coli* may increase 31 times, which is so high, so proper prior wastewater treatment should be applied to reduce those bacteria to a level that meets the national standard.

Table 4.4. Simple linear regression between *E. coli* and *Salmonella*; *Staphylococcus* and *Salmonella*; *Staphylococcus* and *E. coli*

Dependent variable	Predicator	Estimate	Std. Error	t value	Pr(>|t|)
E. coli	Intercept	9.62	27.40	0.35	0.731ns
	Salmonella	31.10	6.367	4.88	< 0.001***
Staphylococcus	Intercept	819.66	323.69	2.53	0.019*
	Salmonella	41.99	75.21	0.56	0.582ns
Staphylococcus	Intercept	855.57	293.94	2.91	0.008**
	E. coli	0.87	1.75	0.50	0.622ns

Note: Asterisks "*", "**", and "****" means significant difference at 0.05, 0.01, and 0.001, while "ns" denotes non-significance. All these biological properties are set in CFU/mL.

4.4. Susceptibility Test of Isolates

The analysis is done for resistance to antibiotics of the three pathogenic bacteria. 140 colonies are tested for resistance to individual antibiotics. The resistance is determined by the diameter of the inhibition zone, which recorded in millimetre. Based on CSLI standard, level of

resistance R (resistant), I (intermediate) and S (susceptible) are determined (Appendix1, Appendix2 and Appendix3). The number of isolates expressing its resistance to antimicrobial agents by sampling site are displayed in Table 4.5. In overall, the isolate show high resistance to Ampicillin and Trimethoprim while Gentamicin indicates the lowest resistance among the six antibiotics. Interestingly, some isolates of the *E. coli*, *Staphylococcus* and *Salmonella* found resistant to Trimethoprim while some isolates of the *E. coli* and *Salmonella* indicate high resistant to Ampicillin. This prevalence of resistant may be explained by the fact that these two antibiotics (Ampicillin and Trimethoprim) have been widely used for therapeutic purpose against bacterial infections in humans and animals in Cambodia. And another reason is that a highly antibiotic contaminated environment can promote antibiotic resistance.

Table 4.5. Number of isolate bacteria expressing resistance to antibiotics

Bacteria	Sampling Site	Number of Isolate	Ampicillin	Cefpodoxime	Ciprofloxacin	Gentamicin	Trimethoprim	Erythromycin
E. coli	Phnom Penh Port	8	6	8	3	8	4	-
	Km 11	8	0	0	5	0	0	-
	Takhmao Ferry	8	6	4	1	0	2	-
	Steung Chrov	8	4	4	4	0	4	-
	Kizona	8	4	4	2	0	4	-
	Kompong Chhnang	8	5	2	2	0	4	-
Salmonella	Phnom Penh Port	8	0	0	0	0	0	0
	Km 11	4	4	0	0	0	4	4
	Takhmao Ferry	8	0	0	0	0	0	0
	Steung Chrov	8	0	0	0	0	0	0
	Kizona	8	6	0	0	0	0	6
	Kompong Chhnang	8	1	0	0	0	0	1
Staphylococcus	Phnom Penh Port	8	-	-	0	0	8	0
	Km 11	8	-	-	0	0	0	0
	Takhmao Ferry	8	-	-	0	0	0	2
	Steung Chrov	8	-	-	0	0	0	0
	Kizona	8	-	-	0	0	3	0
	Kompong Chhnang	8	-	-	0	0	1	0
		140	36	22	17	8	34	13

4.4.1. *E. coli* Susceptibility Test of Isolates

There are total of 48 isolates of *E. coli* taken from 12 samples to test for its susceptibility to 5 individual antibiotics know as Ampicillin, Cefpodoxime, Ciprofloxacin, Gentamicin and Trimethoprim. The column chart in Figure 4.6 demonstrates the percentage of isolate responses by sampling sites and antibiotics. Ciprofloxacin presented resistance to some *E. coli* isolates from all sampling sites. A significant proportion (75%) of *E. coli* isolates expressed resistance to Ampicillin, with rates of resistance at each individual sampling site dipped consistently between 0% to 75%. This high resistance prevalence could be induced by long term Ampicillin exposure and the level residue. Followed by Cefpodoxime, the highest resistance rate is 100% with the rate of resistance of individual sites vary from 0% to 100%. Interestingly, Ampicillin, Cefpodoxime, Gentamicin and Trimethoprim are susceptible for isolates taken Km 11.

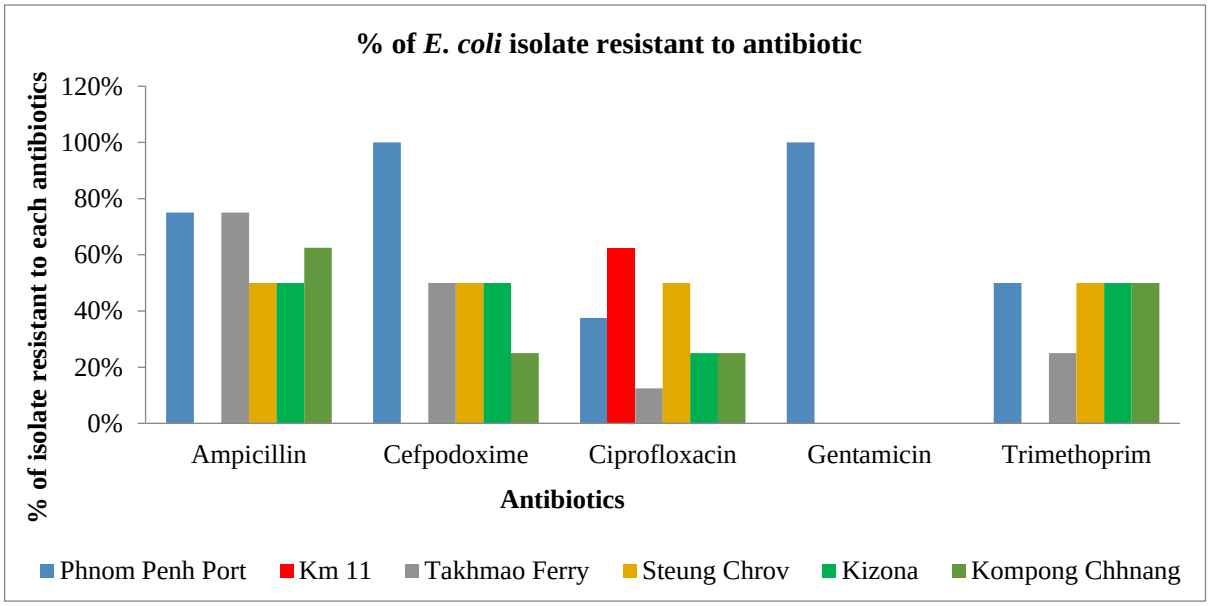

Figure 4.6. Percentage of *E. coli* resistant to each antibiotics

4.4.2. *Salmonella* Susceptibility Test of Isolates

Salmonella susceptibility test of isolates is done by taking a total of 44 colonies from 6 sampling sites pairing with 5 antimicrobial agents as follow: Ampicillin, Cefpodoxime, Ciprofloxacin, Gentamicin and Trimethoprim.

4.4.2.1. Intermediate Resistant of *Salmonella* Isolates

Figure 4.7 illustrates the percentage of *Salmonella* isolates responding to each individual antibiotic used in the study. With the 5 antibiotics, there are 2 antibiotics present intermediate resistant (IR) to *Salmonella* isolates, among which Cefpodoxime shows its significant intermediate resistance. At Phnom Penh Port, the isolates expressed its resistance to Cefpodoxime with the

resistance rate of 100%. At Takhmao Ferry and Kampong Chhnang Port, the IR rate is at 13% and 50% while this rate is at 13% and 38% respectively.

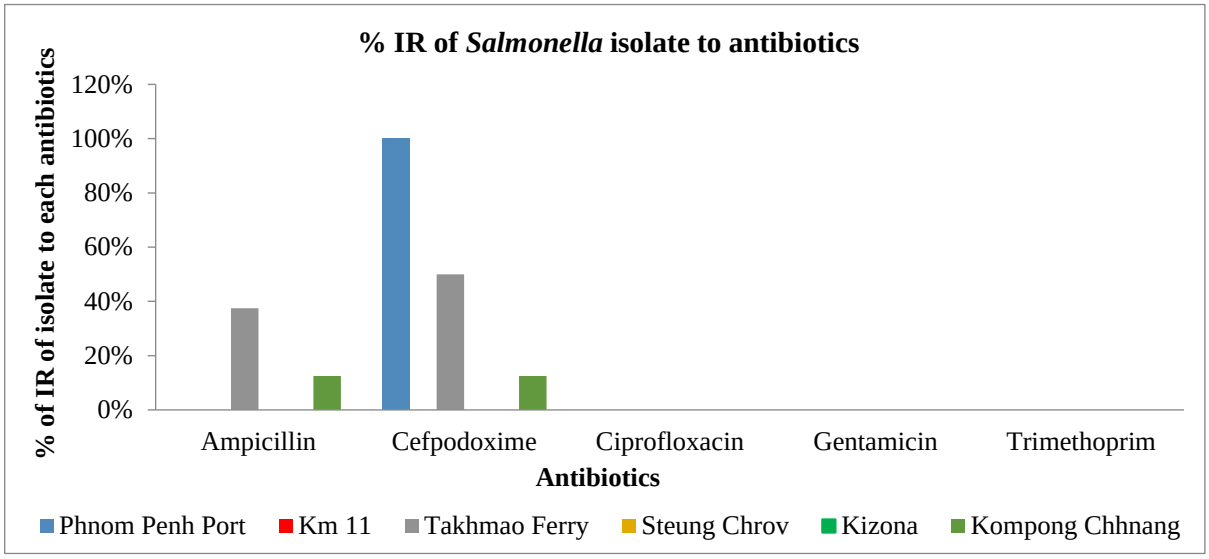

Figure 4.7. Percentage of *Salmonella* intermediate resistant to each antibiotics

4.4.2.2. Resistant of *Salmonella* Isolates

Figure 4.8 demonstrates the percentage of *Salmonella* isolates expressing resistance to individual antimicrobial agents by sampling site. Among the 5 antibiotics, there are 2 antibiotics present resistance to *Salmonella* isolates in which Ampicillin shows its significant resistance. At Kizona, the isolates expressed its resistance to Ampicillin with the resistance rate of 75%. At Km11 and Kampong Chhnang Port, the resistant rate is at 50% and 13% respectively. Only isolates from Km11 are resistant to Trimethoprim (50%).

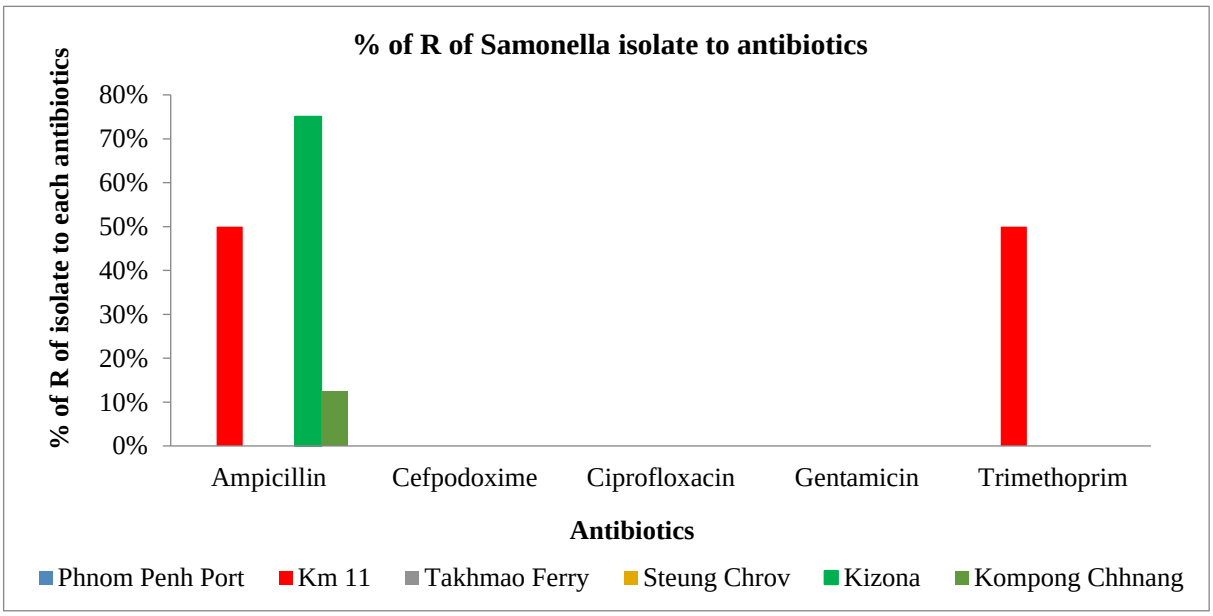

Figure 4.8. Percentage of *Salmonella* resistant to each antibiotics

4.4.3. *Staphylococcus* Susceptibility Test of Isolate

To perform the susceptibly test, a total of 48 isolates are selected and paired with 4 antibiotics known as Ciprofloxacin, Erythromycin, Gentamicin and Trimethoprim. And the results have shown as below.

4.4.3.1. *Staphylococcus* Intermediate Resistant of Isolate

Figure 4.9 denotes the percentage of antibiotic intermediate resistant of isolated *Staphylococcus* to four antibiotics as listed above. The isolates are significantly intermediate resistant to Trimethoprim. The isolates taken from Km11, Kizona and Kampong Chhnang Port have its intermediate resistant level of 63% while the level is at 38% and 50% for Stung Chrov and Takhmao Ferry respectively. Along with Trimethoprim, Erythromycin indicate its intermediate resistant in low level, with the level vary 13% to 30%, in all sampling sites. The finding of this study was is in line with the previous study of (Magiorakos *et al*., 2011) saying that the pathogenic bacteria are developing its resistance to multiple antimicrobial agents. However, no intermediate resistance found with Ciprofloxacin and Gentamicin in this study.

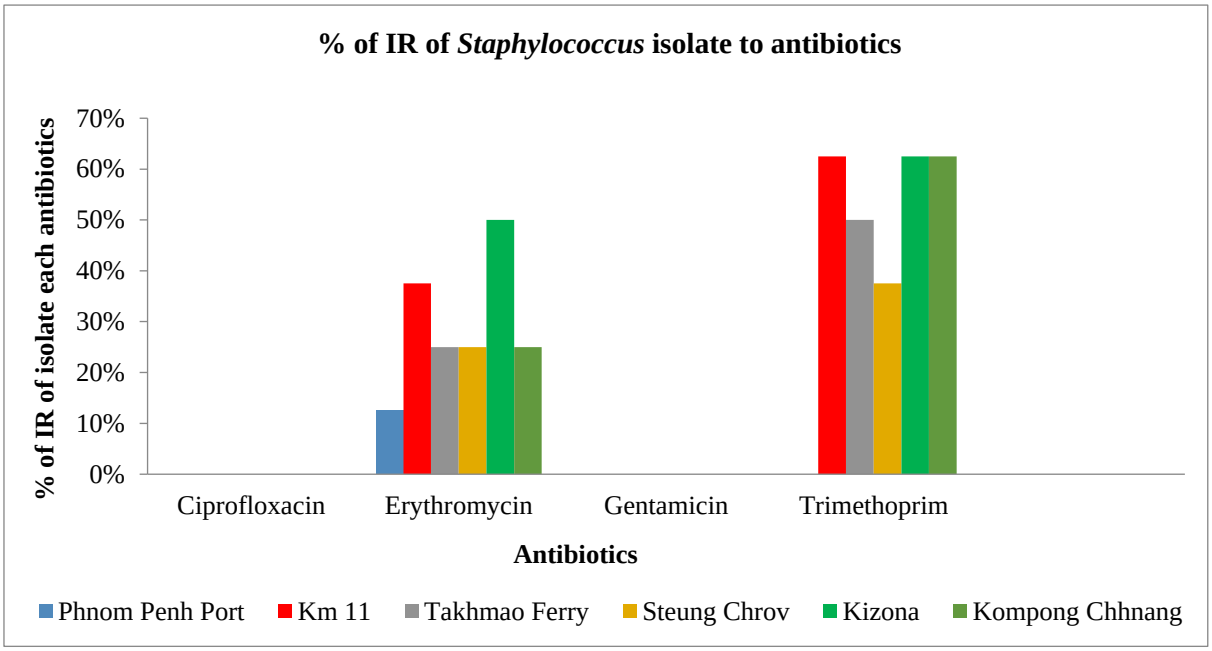

Figure 4.9. Percentage of *Staphylococcus* Intermediate resistant to each antibiotics

4.4.3.2. Staphylococcus Resistant of Isolates

Figure 4.10 demonstrates the percentage of isolates expressing resistance to individual antimicrobial agents by sampling site. With the 4 antimicrobial agents, there are 2 antibiotics that appears resistant to *Staphylococcus* isolates, among which Trimethoprim shows its significant resistance. At Phnom Penh Port, the isolates expressed its resistance to Trimethoprim with the resistance rate of 100%. At Kampong Chhnang Port and Kizona, the resistance rate is at 13% and

38% respectively. In addition, at Ta Khmao Ferry, the isolates denote low resistant to Erythromycin (25%). Interestingly, the isolates express it is intermediate resistant and resistant to Erythromycin and Trimethoprim while showing susceptibility to Ciprofloxacin and Gentamicin.

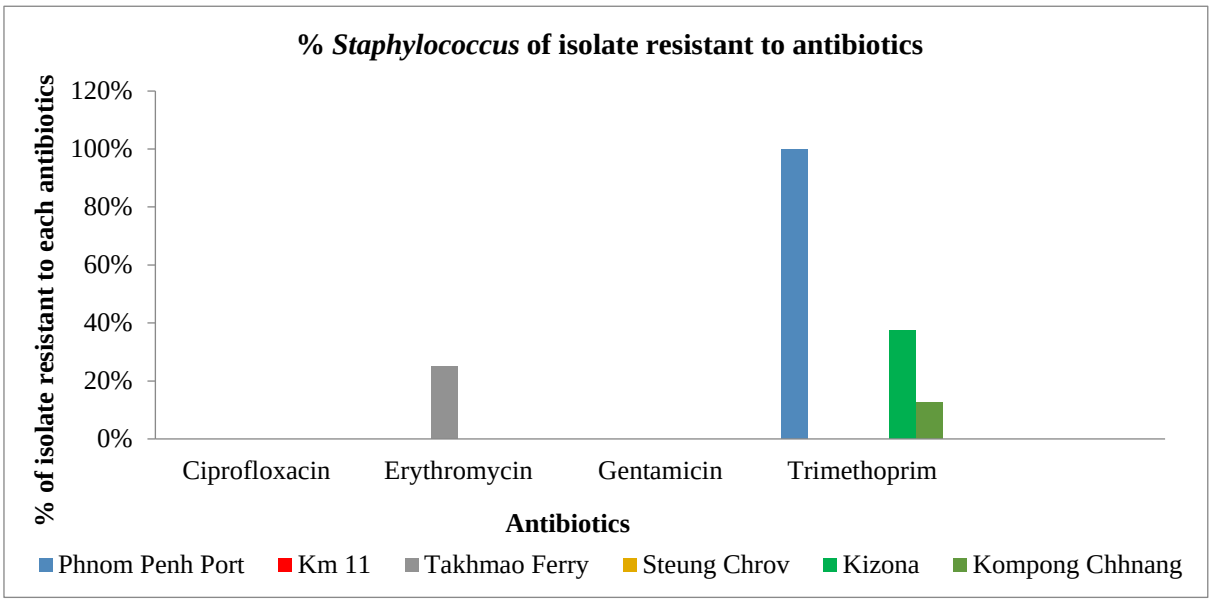

Figure 4.10. Percentage of *Staphylococcus* resistant to each antibiotic

4.5. Multi-drug Resistance

The analysis of 48, 44 and 48 isolates of *E. coli, Salmonella* and *Staphylococcus* respectively is done for multi-drug resistance. Figure 4.11 indicate the percentage of isolate with the number of antibiotics to which they resist. In this study, *E. coli* is considered to be multi-drug resistant as the colonies resist to more than 3 antimicrobial agents (n ≥3). The total number of 12 colonies (equal to 25%) taken from Phnom Penh (3), Takhmao Ferry (3), Kizona (3), Steung Chrov (2) and Kompong Chhnang (1) is resistant to 3 antimicrobial agents. In addition, 2 colonies (equal to 4%) taken from Steung Chrov sampling site are resistant to 4 antimicrobial agents.

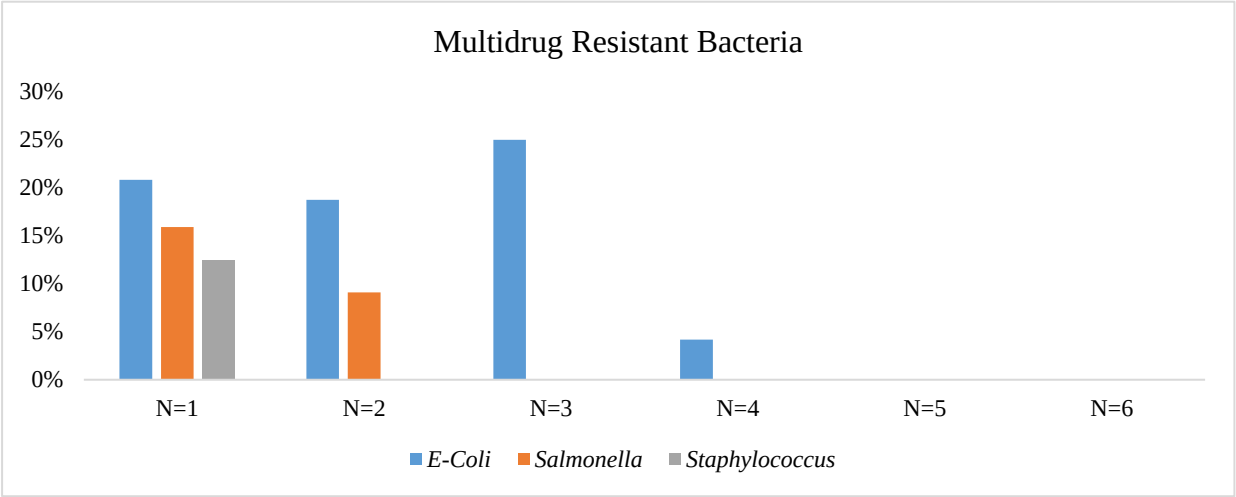

Figure 4.11. Percentage of isolate with resistance to n antibiotics

4.6. Bacteria Identification

https://www.gideononline.com/blogs/citrobacter-freundii/

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

In this study, 6 water sampling locations was randomly selected, located in Phnom Penh (1 sample of surface and 1 sample of sewage water), Kandal province (1 sample of surface and 1 sample of sewage water), Kampong Cham province (1 sample of surface water), and Kampong Chhnang province (1 sample of surface water) to detect multidrug resistance bacteria in Mekong River, Tonle Sap and Bassac River and sewage water. Four physicochemical parameters and three biological parameters were analyzed including pH, Dissolve Oxygen (DO), Temperature and Electricity Conductivity (EC) and *E. coli*, *Staphylococcus*, and *Salmonella*, respectively. According to the result, Physical parameters of water samples in all sampling sites are in the range that enable for bacteria growing. Mostly microbial contamination of 3 enumerated bacteria found higher concentration in sewage water than fresh water. Moreover, there is only *E. coli* has strong positive relation with *Salmonella*, while EC has strong and moderated positive relation with *Staphylococcus* and pH, respectively. In addition, the isolate showed high resistance to Ampicillin and Trimethoprim while Gentamicin indicates the lowest resistance among the six antibiotics. This prevalence of resistant may be explained by the fact that these two antibiotics (Ampicillin and Trimethoprim) have been widely used for therapeutic purpose against bacterial infections in humans and animals in study area of Cambodia.

5.2. Recommendation

In this research study, the amount of water sampling is still limited since there are performed only two times as replication. It should be more than twice due to data analysis and evaluation of water characteristics in surface and sewage water.

Furthermore, safely manage all antibiotic residual waste, both liquid and solid form should be strictly practiced; installing treatment facilities at main pollution sources, especially pharmaceutical manufacturers, hospital, health center, etc. must be applied; and ensure safe consumption of antibiotic in all sectors. Further study need to be conducted for more detail on the identification of resistant colonies—pathogenic or nonpathogenic. The capacity of municipal wastewater treatment plants should be adequate to treat sewage in the city/town. Sewage and industrial wastewater are not properly treated and prior to discharge directly to receiving sources/public water, must be terminate, especially pollution sources located along the water source, including streams, canals, rivers, etc. Moreover, capacity building and researches is more prevalent for government and stakeholder. So, develop strengthening and promoting the implementation of legal and institutional framework is needed. Cooperation, experience exchange and networking especially with the academic is needed.

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APPENDICES

Appendix 1. *E-Coli* Susceptibility Test

Zone Diameter			[R ≤ 13mm, 14mm≤IR≤16mm]		[R≤17mm; 18mm≤IR≤20mm]		[R≤21mm; 22≤IR<25mm]		[R≤12mm; 13mm≤IR≤14mm]		[R≤10mm; 11mm≤IR<15mm]
Antibiotic		Ampicillin	Ampicillin	Cefpodoxime	Cefpodoxime	Ciprofloxacin	Ciprofloxacin	Gentamicin	Gentamicin	Trimethoprim	Trimethoprim
1	Phnom Penh Port	24.79	S	26.83	S	31.04	S	23.89	S	22.51	S
2		23.02	S	30.64	S	34.25	S	19.57	S	19.83	S
3		6	R	22.61	S	26.83	S	20.11	S	22.05	S
4		6	R	20.13	S	23.29	IR	18.44	S	24.32	S
5		6	R	25.43	S	19.33	R	18.83	S	6	R
6		6	R	26.29	S	23.78	IR	18.99	S	6	R
7		6.02	R	26.69	S	19.34	R	20.25	S	6	R
8		6	R	26.12	S	17.14	R	21.4	S	6	R
	Number of Resistant colonies		6		8		3		8		4
1	Km 11	21.63	S	27.31	S	33.82	S	19.57	S	20.47	S
2		21.68	S	25.94	S	38.29	S	19.01	S	18.66	S
3		17.62	S	25.04	S	36.9	S	19.18	S	18.32	S
4		15.76	IR	20.29	S	33.67	S	20.37	S	18.38	S
5		20.42	S	27.28	S	39.46	S	19.09	S	20.95	S
6		21.87	S	28.03	S	37.62	S	18.95	S	20.9	S
7		19.81	S	25.34	S	40.32	S	17.68	S	17.73	S
8		19.52	S	23.29	S	37.52	S	17.31	S	17.32	S
	Number of Resistant colonies		0		0		0		0		0
1	Takhmao Ferry	19.3	S	23.93	S	42.49	S	19.18	S	22.3	S
2		17.65	S	23.22	S	39.07	S	20.33	S	20.64	S
3		6.59	R	6.56	R	25.12	S	18.05	S	21.98	S
4		6.06	R	6	R	20.23	R	20.23	S	21.7	S
5		6	R	11.8	R	24.55	IR	22.64	S	6	R
6		6	R	13.01	R	28.08	S	23.06	S	6	R

7		6	R	26.08	S	26.15	S	20.91	S	24.55	S
8		6	R	25.23	S	26.53	S	18.58	S	25.07	S
	Number of Resistant colonies		6		4		1		0		2
1	Steung Chrov	19.56	S	23.63	S	32.73	S	18.37	S	23.04	S
2		20.12	S	28.25	S	29.09	S	21.63	S	21.12	S
3		21.07	S	29.17	S	6.12	R	19.49	S	18.05	S
4		18.23	S	27.51	S	6	R	21.48	S	15.49	S
5		6	R	14.87	R	10.35	R	25.32	S	6	R
6		6	R	15.83	R	16.94	R	23.33	S	6	R
7		6	R	14.95	R	47.91	S	18.74	S	6	R
8		6	R	10.21	R	45.32	S	19.55	S	6	R
	Number of Resistant colonies		4		4		4		0		4
1	Kizona	6	R	6	R	25.38	S	25.39	S	20.47	S
2		6	R	6	R	25.86	S	25.58	S	24.06	S
3		6	R	6.02	R	20.05	R	20.86	S	19.69	S
4		6	R	6	R	20.61	R	20.85	S	22.57	S
5		20.9	S	29.27	S	39.82	S	20.31	S	6	R
6		21.83	S	29.52	S	39.55	S	21.1	S	6	R
7		21.28	S	29.49	S	43.65	S	21.89	S	6	R
8		20.7	S	29.58	S	40.14	S	21.52	S	6	R
	Number of Resistant colonies		4		4		2		0		4
1	Kompong Chhnang	23.57	S	32.73	S	26.84	S	17.82	S	21.59	S
2		19.29	S	34.88	S	25.42	S	20.44	S	18.94	S
3		6.37	R	6.4	R	29.02	S	19.73	S	19.33	S
4		6.16	R	6.37	R	28.63	S	20.53	S	19.2	S
5		13.62	IR	27.23	S	19.51	R	21.37	S	6	R
6		12.9	R	28.39	S	15.73	R	20.12	S	6	R
7		9.1	R	28.46	S	25.12	S	20.9	S	6	R
8		10.12	R	27.61	S	25.25	S	19.15	S	6	R
	Number of Resistant colonies		5		2		2		0		4

Appendix 2. *Salmonella* Susceptibility Test

Zone Diameter			[R ≤ 13mm, 14mm≤IR<16mm]		[R≤17mm; 18mm≤IR≤20mm]		[R≤21mm; 22≤IR<25mm]		[R≤12mm; 13mm≤IR≤14mm]		[R≤10mm; 11mm≤IR<15mm]
Antibiotic		Ampicillin	Ampicillin	Cefpodoxime	Cefpodoxime	Ciprofloxacin	Ciprofloxacin	Gentamicin	Gentamicin	Trimethoprim	Trimethoprim
1	Phnom Penh Port	17.18	S	19.25	IR	38.25	S	20.36	S	22.69	S
2		16.65	S	18.64	IR	38.55	S	20.75	S	22.94	S
3		17.5	S	19.1	IR	32.42	S	22.07	S	19.53	S
4		17.36	S	18.41	IR	31.52	S	20.67	S	19.77	S
5		20.01	S	19.77	IR	32.61	S	27.49	S	16.31	S
6		19.94	S	19.46	IR	35.24	S	24.14	S	21.95	S
7		19.69	S	18.17	IR	32.46	S	22.73	S	17.41	S
8		19.91	S	19.55	IR	34.06	S	23.72	S	19.45	S
	Number of Resistant colonies		0		0		0		0		0
1	Km 11	6	R	28.34	S	33.86	S	20.42	S	6	R
2		6	R	28.33	S	33.19	S	20.76	S	6	R
3		6	R	28.62	S	34.56	S	20.73	S	6	R
4		6	R	28.44	S	33.17	S	20.92	S	6	R
5											
6											
7											
8											
	Number of Resistant colonies		4		0		0		0		4
1	Takhmao Ferry	17.34	S	20.65	S	37.76	S	19.61	S	23.77	S
2		16.01	S	20.69	S	37.59	S	19.8	S	23.31	S
3		17.61	S	20.22	S	37.58	S	18.98	S	23.39	S
4		14.37	IR	21.06	S	37.69	S	19.16	S	23.29	S
5		13.46	IR	18.46	IR	37.28	S	22.09	S	23.69	S
6		15.97	IR	18.29	IR	37.59	S	22.65	S	23.75	S
7		16.23	S	19.19	IR	37.55	S	21.52	S	24.98	S
8		18	S	19.9	IR	38.5	S	23.84	S	18.8	S

	Number of Resistant colonies		0		0		0		0		0
1	Steung Chrov	21.83	S	21.5	S	34.02	S	20.25	S	20.81	S
2		20.79	S	21.82	S	32.36	S	21.18	S	22.56	S
3		22.34	S	21.9	S	33.85	S	21.38	S	20.25	S
4		22.22	S	21.94	S	33.15	S	22.38	S	22.63	S
5		22.85	S	21.44	S	34.52	S	18.17	S	21.26	S
6		23.02	S	22.99	S	36.69	S	18.93	S	23.33	S
7		25.77	S	24.44	S	32.65	S	19.03	S	21.07	S
8		25.6	S	24.4	S	32.06	S	22.38	S	22.63	S
	Number of Resistant colonies		0		0		0		0		0
1	Kizona	6	R	26.51	S	36.22	S	22.83	S	23.85	S
2		6	R	27.24	S	35.53	S	25.29	S	24.02	S
3		19.53	S	23.65	S	35.32	S	24.2	S	24	S
4		21.26	S	29.47	S	35.14	S	21.98	S	23.84	S
5		6	R	29.7	S	36.61	S	22.69	S	20.94	S
6		6	R	30.28	S	37.01	S	24.79	S	21.65	S
7		8.06	R	30.55	S	32.25	S	24.19	S	22.17	S
8		9.12	R	32.2	S	31.2	S	24.57	S	22.91	S
	Number of Resistant colonies		6		0		0		0		0
1	Kompong Chhnang	17.26	S	20.05	S	31.65	S	20.15	S	20.91	S
2		17.18	S	19.82	IR	30.71	S	20.58	S	23.4	S
3		18.56	S	21.48	S	32.03	S	20.55	S	20.95	S
4		17.36	S	20.76	S	31.4	S	20.86	S	23.12	S
5		16.42	S	30.39	S	37.55	S	22.8	S	22.32	S
6		18.81	S	32.31	S	36.61	S	21.89	S	23.47	S
7		12.83	R	29.68	S	38.14	S	23.51	S	23.94	S
8		13.37	IR	29.47	S	37.98	S	21.47	S	21.16	S
	Number of Resistant colonies		1		0		0		0		0

Appendix 3. *Staphylococcus* Susceptibility Test

Zone Diameter			[R≤15mm; 16≤IR<20mm]		[R≤13mm; 14mm≤IR<22mm]		[R≤12mm; 13≤IR<14mm]		[R≤10mm; 11mm≤IR<15mm]
Antibiotic		Ciprofloxacin	Ciprofloxacin	Erythromycin	Erythromycin	Gentamicin	Gentamicin	Trimethoprim	Trimethoprim
1	Phnom Penh Port	17.18	S	19.25	IR	38.25	S	20.36	S
2		16.65	S	18.64	IR	38.55	S	20.75	S
3		17.5	S	19.1	IR	32.42	S	22.07	S
4		17.36	S	18.41	IR	31.52	S	20.67	S
5		20.01	S	19.77	IR	32.61	S	27.49	S
6		19.94	S	19.46	IR	35.24	S	24.14	S
7		19.69	S	18.17	IR	32.46	S	22.73	S
8		19.91	S	19.55	IR	34.06	S	23.72	S
	Number of Resistant colonies		0		0		0		0
1	Km 11	6	R	28.34	S	33.86	S	20.42	S
2		6	R	28.33	S	33.19	S	20.76	S
3		6	R	28.62	S	34.56	S	20.73	S
4		6	R	28.44	S	33.17	S	20.92	S
5									
6									
7									
8									
	Number of Resistant colonies		4		0		0		0
1	Takhmao Ferry	17.34	S	20.65	S	37.76	S	19.61	S
2		16.01	S	20.69	S	37.59	S	19.8	S
3		17.61	S	20.22	S	37.58	S	18.98	S
4		14.37	IR	21.06	S	37.69	S	19.16	S
5		13.46	IR	18.46	IR	37.28	S	22.09	S
6		15.97	IR	18.29	IR	37.59	S	22.65	S
7		16.23	S	19.19	IR	37.55	S	21.52	S
8		18	S	19.9	IR	38.5	S	23.84	S
	Number of Resistant colonies		0		0		0		0
1	Steung Chrov	21.83	S	21.5	S	34.02	S	20.25	S
2		20.79	S	21.82	S	32.36	S	21.18	S
3		22.34	S	21.9	S	33.85	S	21.38	S

4		22.22	S	21.94	S	33.15	S	22.38	S
5		22.85	S	21.44	S	34.52	S	18.17	S
6		23.02	S	22.99	S	36.69	S	18.93	S
7		25.77	S	24.44	S	32.65	S	19.03	S
8		25.6	S	24.4	S	32.06	S	22.38	S
	Number of Resistant colonies		0		0		0		0
1	Kizona	6	R	26.51	S	36.22	S	22.83	S
2		6	R	27.24	S	35.53	S	25.29	S
3		19.53	S	23.65	S	35.32	S	24.2	S
4		21.26	S	29.47	S	35.14	S	21.98	S
5		6	R	29.7	S	36.61	S	22.69	S
6		6	R	30.28	S	37.01	S	24.79	S
7		8.06	R	30.55	S	32.25	S	24.19	S
8		9.12	R	32.2	S	31.2	S	24.57	S
	Number of Resistant colonies		6		0		0		0
1	Kompong Chhnang	17.26	S	20.05	S	31.65	S	20.15	S
2		17.18	S	19.82	IR	30.71	S	20.58	S
3		18.56	S	21.48	S	32.03	S	20.55	S
4		17.36	S	20.76	S	31.4	S	20.86	S
5		16.42	S	30.39	S	37.55	S	22.8	S
6		18.81	S	32.31	S	36.61	S	21.89	S
7		12.83	R	29.68	S	38.14	S	23.51	S
8		13.37	IR	29.47	S	37.98	S	21.47	S
	Number of Resistant colonies		1		0		0		0