ISOLATION OF GRAM NEGATIVE ORGANISMS FROM SELECTED ENVIRONMENTAL HOTSPOTS IN IKPOBA OKHA L.G.A, BENIN CITY, NIGERIA.

BY

Foluke Mercy OLANIYAN (MISS)

LSC2209689

DEPARTMENT OF MICROBIOLOGY,

FACULTY OF LIFE SCIENCES,

UNIVERSITY OF BENIN,

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A PROJECT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY. IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF SCIENCE (B.SC.) DEGREE IN MICROBIOLOGY.

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CERTIFICATION

We certify that this project work was carried out by **OLANIYAN, FOLUKE MERCY** with Matric No; **LSC2209689** in partial fulfillment of the requirement for the award of Bachelor of Science (B.Sc.) Degree in Microbiology.

MRS. O. B . AGBONWANETEN **DATE**

(Project Supervisor)

Professor E. O. IGBINOSA **DATE**

(Head of Department)

DEDICATION

This project is dedicated to Almighty God that sustained me till this time. To my parents, Mr. I.S. Olaniyan and Mrs. R.B. Olaniyan, thank you for your love, support, and guidance, Your patience, understanding, and sacrifices have made this journey possible. Your encouragement and support mean the world to me.

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ABSTRACT

This study investigated the presence of pathogenic and antibiotic-resistant bacteria in wastewater collected from selected environmental hotspots within Ikpoba-Okha Local Government Area, Benin City, Edo State, Nigeria. Wastewater samples were aseptically collected from Ramat Park and Ramat Market, sites characterized by intense commercial activity and poor sanitation. Samples were serially diluted and cultured on selective and differential media including MacConkey and EMB agar. Bacterial isolates were identified based on colony morphology, Gram staining, and biochemical tests. Total heterotrophic bacterial counts ranged from 3×10^4 to 9.2×10^5 CFU/mL, indicating high microbial contamination. Predominant isolates included *Escherichia coli*, *Pseudomonas* species, and *Klebsiella* species, suggesting fecal pollution and environmental contamination. Antibiotic susceptibility testing revealed high resistance to β-lactam antibiotics (such as Augmentin, Ceftazidime, Ceftriaxone, and Cefuroxime), while isolates showed greater susceptibility to fluoroquinolones (such as Ofloxacin and Ciprofloxacin) and aminoglycosides (such as Gentamicin and Streptomycin). These findings indicate the presence of opportunistic pathogens and multidrug-resistant bacteria in wastewater environments, posing potential public health risks. The study emphasizes the urgent need for improved wastewater management, environmental sanitation, and continuous microbial surveillance to prevent the spread of waterborne diseases and antimicrobial resistance within the community.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Microorganisms that have been isolated from a sample and found to be responsible for disease in humans, animals, or plants are known as pathogenic isolates. These isolates may be harmful germs such as bacteria, viruses, fungus, or other microbes. Globally, bacterial infections such *Mycobacterium tuberculosis, Staphylococcus aureus, and Escherichia coli (E. coli)* are major sources of morbidity and mortality (Todar, 2020). Human health is also seriously threatened by viral diseases, such as the Ebola virus, influenza virus, and HIV virus (Fields *et al.,* 2013).

In immunocompromised people, fungi like *Aspergillus fumigatus* and *Candida albicans* can produce infections that are potentially fatal (Dagenais and Keller, 2009). In order to effectively treat and manage illnesses, pathogenic isolates aid in disease diagnosis and the identification of the causal agent (Forbes *et al.,* 2018).

Faecal pollution from point and non-point sources has resulted in the presence of human enteric pathogens in environmental waters, including *Salmonella spp.* (Savichtcheva *et al.,* 2007), *Cryptosporidium spp.* (Hörman *et al.,* 2004), enteric viruses (Haramoto *et al.,* 2005), and *Escherichia coli* O157:H (Ibekwe and Grieve, 2003). Water businesses have long employed fecal indicator bacteria, such as *enterococci* and *E. coli,* to evaluate the microbiological quality of drinking and environmental waters due to the difficulties involved in trying to identify every potential pathogen in a water supply. Recent research, however, indicates that there may not be a strong correlation between fecal indicator bacteria and their capacity to reliably identify the presence of pathogens, particularly enteric viruses and protozoa (Hörman *et al.,* 2004; McQuaig *et al.,* 2006). According to certain reports, fecal indicator bacteria like *E. coli* can exist in the environment even when there is no fecal pollution, and they can develop again if the right conditions are met (Desmarais *et al.,* 2002; Power *et al.,* 2005). Numerous *E. Coli* strains with one or more virulence genes may be found in the feces of humans, domestic animals, and wild animals (Ishii *et al.,* 2007). Pathogenic *E. coli* strains with these virulence genes may also enter environmental waters through runoff from agricultural areas and sewage overflows. Increased disease outbreaks and resulting fatalities have been linked to surface water pollution with pathogenic strains of *Escherichia coli* (Feldman *et al.,* 2002; Olsen *et al.,* 2002).

Environmental waters frequently contain comparatively modest quantities of waterborne diseases. As a result, pathogen detection requires some kind of filtration and proliferation. After the sample has been filtered, bacteria that have been retained on the filters can be found by cultivating them in or on selective media. Additional procedures are required as confirmation testing, including genetic techniques, biochemical tests, and serological assays (Echeverria *et al.,* 1991, 1992). Numerous water sources tainted with human waste can serve as infection sources, according to epidemiological studies of *shigellosis* (Rosenberg *et al.,* 1976; Egoz *et al.,* 1991; CDC, 1996; Pergram *et al.,* 1998; Alamanos *et al.,* 2000; Faruque *et al.,* 2002; Simchen *et al.,* 1991; Islam *et al.,* 1993). *Shigella spp.* infections are frequently contracted by swimming in or drinking tainted water. Global wastewater surveillance of SARS-CoV-2 was established following its detection in sewage and fecal material early in the COVID-19 pandemic (Wang *et al.,* 2020; Medema *et al.,* 2020; Ahmed *et al.,* 2020). Wastewater-based epidemiology (WBE) for SARSCoV-2 has been carried out at over 4,000 sampling locations across more than 70 countries as of April 2023 (Naughton *et al.,* 2021). WBE explicitly refers to connecting wastewater data to the health data, whereas wastewater surveillance refers to the routine testing of wastewater to gather health-related data.inhabitants (Larsen *et al.,* 2021).

With a more varied microbial community (Dueholm *et al.,* 2022) and more molecular inhibitors than surface water (Gibson *et al.,* 2012), wastewater is a very different matrix from surface water. This implies that in order to detect target infections, technique modifications are probably required. Many groups are employing pre-analytical (i.e., sampling, matrix concentration, and nucleic-acid extraction) procedures developed for SARS-CoV-2 monitoring because there is a lack of published and peer-reviewed research validating methods for bacterial WBE. However, the morphology of bacteria and viruses is fundamentally different, and distinct factors dictate their fate and movement within a wastewater system. A review by Chahal *et al.* (Chahal *et al.,* 2016) states that the size, content, and growth stage of the particles are the primary factors that influence bacterial-particle interactions. bacteria.

Numerous pathogenic species, such as bacteria, viruses, and protozoa, have been found to be present in wastewater. If these bacteria are not properly controlled, they can present serious health problems. The necessity for reliable techniques of pathogen detection and separation is highlighted by the fact that they mostly come from untreated sewage, industrial discharges, and agricultural runoff (Albert *et al.,* 2021). The first step in the procedure is sample collection, which is essential to getting representative wastewater samples. The characteristics and microbiological communities of the wastewater system are reflected in the samples that are gathered thanks to appropriate sampling techniques. A thorough picture of the microbial community can be obtained by gathering samples from multiple locations, including influent, effluent, and various stages of treatment (Holzweber *et al.,* 2019). Samples must be properly kept after collection, frequently by chilling or the use of chemical preservation agents, to stop microbial populations from changing before analysis. To separate harmful microbes from the various microbial consortia found in wastewater, isolation techniques are crucial. In this process, the utilization of selective culture media is crucial. *Salmonella spp.* and *Escherichia coli* are two examples of Gram-negative bacteria that can be preferentially isolated using MacConkey agar (Zhao *et al.,* 2021). Recovery from pathogens can be improved by enrichment cultures. Inoculating samples with Rappaport Vassiliadis broth, for instance, can encourage *Salmonella* development, improving detection in the presence of competing microbes (Yarza and Parvez, 2020). Although they might not always provide a conclusive identification, biochemical tests such as catalase and oxidase assays aid in the differentiation of different microbial species (Griffiths, 2017). Molecular techniques, including sequencing and polymerase chain reaction (PCR), have grown in significance because of their sensitivity and specificity, which enable precise identification of harmful pathogens (Albert *et al.,* 2021).

Isolating harmful bacteria from wastewater has significant ramifications. Pathogens play a major role in waterborne illnesses, which can have serious health consequences for the general public, particularly in places with poorly treated wastewater (Martínez *et al.,* 2019). The threat that antibiotic resistance poses to public health can be exacerbated by wastewater acting as a reservoir for resistant bacteria (Martínez *et al.,* 2019).

1.2. AIM OF THE STUDY

In order to comprehend the microbiological landscape of these important urban interfaces, the main goal of this study is to isolate and detect bacterial species from certain environmental hotspots surrounding Ikpoba Okha Local Government Area, Benin City.

1.3. OBJECTIVES OF THE STUDY

i. Identifying and characterizing a few environmental hotspots in the Ikpoba Okha Local Government Area, together with a thorough explanation of their nature and perceived environmental impact, was one of the specific goals.

ii. to count the heterotrophic bacterial counts of particular indicator bacteria (such as Escherichia coli, fecal coliforms, and total coliforms) in the samples that were gathered.

iii. to ascertain the distribution and frequency of various bacterial genera and species in the chosen environmental hotspots.

iv. to use selective and differential microbiological media to separate and obtain pure cultures of unique bacterial colonies from environmental materials.

v. to evaluate the prevalence of antibiotic-resistant strains by determining the antibiotic susceptibility patterns of a chosen group of bacterial isolates against a panel of widely used antibiotics.

CHAPTER TWO

LITERATURE REVIEW

2.1 Environmental hotspots

According to Myers *et al.,* (2000), environmental hotspots are regions of great ecological significance that are in danger because of either natural or human-caused reasons. Both the environment and human health may be at danger from wastewater collection systems, which can be major environmental hotspots (Kjeldsen *et al.,* 2002). Heavy rains, broken equipment, or obstructions can cause sewer overflows, which dump raw wastewater into the environment (Butler and Schütze, 2005). It can contaminate groundwater and surface water, endangering human health and aquatic life (Carr, 2001). Waterborne illnesses like cholera and typhoid fever can result from exposure to tainted water. Implementing appropriate wastewater collection system design and management is crucial to reducing these risks (Ashley *et al.,* 2005).

Due to a variety of operations, including food preparation and sales, cleaning, and sanitation, markets can be major sources of wastewater generation (Qasim, 1999). High concentrations of organic debris, nutrients, and pathogens may be present in the wastewater produced by these activities (Tchobanoglous *et al.,* 2003). For example, effluent from fish markets may contain significant concentrations of organic pollutants, such as scales, blood, and fish excrement (Islam *et al.,* 2016). Regular maintenance, wastewater treatment, and appropriate drainage systems can help achieve this (Metcalf and Eddy, 2014). To manage wastewater and lessen their effects on the environment, several markets have adopted sustainable techniques such the use of biological treatment systems or artificial wetlands (Vymazal, 2011). If correctly managed, market wastewater can be a useful resource. For instance, wastewater can be cleaned and repurposed for non-potable uses like toilet flushing and irrigation (Angelakis *et al.,* 2018). Effective wastewater management techniques can help markets reduce their environmental impact and help create a more sustainable future. Several tactics, such as cutting back on waste production, putting recycling programs in place, and encouraging sustainable consumer habits, might lessen the negative effects of markets on the environment (Jackson, 2011). Markets can also embrace eco-friendly practices including utilizing green infrastructure, conserving water, and utilizing renewable energy sources (Kibert, 2016). By boosting local economies, promoting eco-friendly goods, and encouraging community involvement, markets can also be extremely important in advancing sustainable development (Seyfang, 2006). Markets can help create a more resilient and healthy environment for coming generations by placing a higher priority on environmental sustainability. Ecosystems, human health, and the economy are all significantly impacted by polluted water bodies. Waterborne illnesses including cholera and typhoid fever can spread as a result of water contamination (Lee, 2018). Aquatic biodiversity can drastically drop as a result of pollution, upsetting ecosystems (Dudgeon, 2019).

Human health can suffer greatly from waterborne illnesses like cholera, typhoid fever, and dysentery, especially in places with inadequate sanitation and hygiene. In order to stop the development of waterborne illnesses, Lee highlights the necessity of appropriate water treatment and management (Lee, 2018). Significant drops in aquatic biodiversity brought on by pollution can disturb ecosystems and result in the loss of ecosystem services. In order to preserve aquatic habitats and biodiversity, Dudgeon emphasizes the necessity of conservation initiatives (Dudgeon, 2019). Developmental delays, cognitive impairment, and other neurological issues can be brought on by these toxins. In order to prevent brain injury, Grandjean emphasizes the necessity of stringent rules and restrictions on pollution (Grandjean, 2018). Exposure to certain chemicals, such as trichloroethylene and benzene, through contaminated water can raise the risk of developing cancer. In order to lower the risk of cancer, Freeman highlights the necessity of appropriate water treatment and management (Freeman, 2019). Certain pollutants can impact fetal development, lower fertility, and raise the chance of birth abnormalities. Swan emphasizes the necessity of reducing exposure to contaminants and conducting additional study on the impact of water pollution on reproductive health (Swan, 2019). Important functions like habitat provision, nitrogen cycling, and water filtering are offered by aquatic ecosystems. In order to preserve aquatic ecosystems and uphold their health, Power emphasizes the necessity of conservation initiatives (Power, 2018).

Reduced crop yields and higher water treatment costs are only two examples of the major economic effects that water pollution can have on agriculture. In order to lessen the negative economic effects of water pollution, Molden highlights the necessity of sustainable water management techniques (Molden, 2019). Algal blooms, decreased water quality, and biodiversity loss are all consequences of eutrophication. Carpenter highlights the necessity of taking action to stop eutrophication and lessen nutrient contamination (Carpenter, 2018).

Water pollution is just one of the environmental effects of waste disposal sites. Additionally, leachate can contaminate soil, which can impact ecosystem balance and plant growth (Mor et al., 2006). Numerous health issues, including as cancer and neurological damage, can result from exposure to harmful chemicals in leachate (Vrijheid et al., 2014). Effective leachate treatment, such as chemical or biological methods, can also reduce the dangers of pollution to the environment and negative health effects. (Renou and others, 2008).

2.2 Sources of wastewater

Water contamination can be classified as either "point source" or "non-point source." While pollutants from a single source, like industrial water emissions, fall under the first category, pollutants from multiple sources, such as mining activities, which include radioactive waste, municipal sewage, including urban development, and industrial and agricultural wastes, including pesticides and chemical fertilizers, fall under the second category (Bai *et al.,* 2021). Waterways include rainwater, agricultural runoff, animal farm water, industrial effluents, and domestic sewage. Wastewater is categorized as either "black water" or "grey water" depending on its source. Black water originates from kitchen sinks and toilets, while grey water is made up of waste from bathing water (Rakesh *et al.,* 2020). Furthermore, chemicals were separated into two main priority substance groups by the "Water Framework Directive." While the second list, known as the "black list," contains hazardous priority compounds that are believed to be persistent, extremely poisonous, or to cause bio-accumulation, the first category, known as the "grey list," gathers various organic and inorganic matter important compounds that pose a significant environmental concern. Unfortunately, commercial activities involving the printing, processing, and preparation of foods, beverages, and supplementary goods generate and contaminate industrial/commercial effluents, and 200 to 500 gallons of wastewater are usually produced for each category. Usually, the presence of pesticides causes neurological issues; for instance, when mothers are contaminated with mercurial pollution, mercurial salts induce cognitive issues in their offspring (Patnaik and Padhy, 2015).

2.3 Associated problems of wastewater

In water bodies, a wide range of pathogens from animal feces are commonly observed, including intestinal worms, bacteria, fungi, and viruses (Wells and Whiteford, 2022). Depending on the natural level of toxicity, certain algal strains can overgrow in eutrophic waters with nitrates and phosphates, especially from agricultural runoffs. This overgrowth may result in water blooms that produce algal toxins as death factors, which can be slow-death factors (or fast-death factors) that normally cause health problems in small amounts. Both people and the general flora and fauna in the food chain are affected by their toxins (Fazal-Ur-Rehman, 2019). Furthermore, a number of ailments are known to be caused by the presence of cyanobacteria, or pathogenic microorganisms, in drinking water. As it is, water contamination can result in hepatitis, lung infections, diarrhea, encephalitis, stomach cramps, pains, vomiting, gastroenteritis, and poisoning episodes that affect the digestive system and/or any internal organs. Certain chlorinated solvents can harm DNA, cause cancer, and harm the liver. Numerous refractory substances can lead to endocrine disruptions, cancer, poor immunological function, decreased fertility or infertility, stopped sexual development, reproductive and endocrine harm, and more (Moshrefi *et al.,* 2021).

Additionally, the presence of pesticides usually results in neurological issues; for instance, when mothers are contaminated with mercurial pollution, mercurial salts induce cognitive issues in their offspring (Patnaik and Padhy, 2015). However, 1.2–2.7 million people die from malaria each year, which is spread by mosquitoes and is facilitated by contaminated stagnant water (Rohr *et al.,* 2019). Furthermore, bathing in tainted beach water can result in a number of less serious health issues, including cholera, hepatitis, typhoid fever, endocrine damage, rashes, earaches, dracunculiasis, pink eyes, diarrhea, respiratory tract infections, kidney damage, and ulcers. In fact, water-borne illnesses can occasionally be extremely dangerous to human life and death.

2.3.1 Arsenicosis

Drinking water containing high levels of arsenic can cause arsenicosis, a chronic illness. A drinking water arsenic limit of 0.01 mg/l is recommended by the WHO (Adeloju *et al.,* 2021). The observed general health implications include skin cancer, bladder cancer, lung disorders, renal damage to the legs' blood vessels, and likely reproductive problems, diabetes, and high blood pressure (Adeloju *et al.,* 2021). Depending on how you were exposed, arsenic poisoning can often induce mild symptoms including fatigue, headaches, confusion, and severe diarrhea, as well as long-term health issues. A person may have symptoms within 30 minutes of consuming arsenic (Adeloju *et al.,* 2021). Data from the field indicates that 90 million Indians are at risk from high groundwater levels. For instance, West Bengal had 28 million, Bihar had 21 million, Uttar Pradesh had 15 million, Assam had 8.6 million, and Punjab had 6 million, making them the five Indian states most at risk (Mukherjee *et al.,* 2021). This may result in death or a coma. Long-term exposure to arsenic has been associated with a number of diseases, including cancer, diabetes, liver illness, nervous system problems like hearing loss and limb loss of sensation, and digestive problems. Trace levels of arsenic have been found in groundwater. The world's leading cause of arsenic poisoning is drinking groundwater containing high levels of the metal, which may quickly kill a person if consumed in sufficient quantities (Briffa *et al.,* 2020; Shaji *et al.,* 2021).

2.3.2 Cholera

The most common cause of cholera is *Vibrio sp.* contamination of water. Hard, dirty, and polluted water can harbor a variety of dangerous bacteria that can cause cholera and other ailments (Fazal-Ur-Rehman, 2019). Its symptoms include rapid diarrhea, severe dehydration, stomach ulcers, and sometimes even death. The primary causes of cholera are eating hard water tainted with cholera-causing germs and the bacteria present in contaminated water. Swimming in dirty, tainted water can also result in cholera (Legros, 2018).

2.3.3 Diarrhea

Increased frequency of bowel movements or more loose stool are signs of diarrhea (Prüss-Ustün *et al.,* 2019). To keep food liquid throughout regular digestion, the pancreas, stomach, gallbladder, and upper small intestine all secrete large amounts of water. Severe dehydration may require hospitalization because undigested food travels in liquid form to the colon and lower small intestine, where *Salmonella typhi* toxins predominate and blood mineral contents drastically drop, ultimately leading to the body's collapse (Fazal-Ur-Rehman, 2019).

2.3.4 Dracunculiasis

The guinea worm, *Dracunculus medinensis,* is the cause of dracunculiasis, a serious parasitic disease that can only be contracted by drinking stagnant water contaminated by water feces. Even though dracunculiasis seldom causes mortality, those who get it lose function for weeks because, within 48 hours, painful blisters appear on the skin's surface anywhere on the body, particularly on the feet and legs. People in isolated, rural, and impoverished areas are most impacted (Mutono *et al.,* 2020).

2.3.5 Endocrine Damage

Diabetes, excessive thirst, excessive appetite, frequent urination, weight loss or gain, joint discomfort, and swollen hands or feet are all signs of endocrine damage (Fazal-Ur-Rehman, 2019). Any bacterial infection can cause endocrine damage. Hormone replacement treatment can occasionally be successful in treating a variety of endocrine disorders. In fact, to avoid endocrine damage, a healthy lifestyle, frequent exercise, a balanced diet, and routine blood tests are advised (Fazal-Ur-Rehman, 2019).

2.3.6 Hepatitis

There are five different varieties of infectious viral hepatitis: A, B, C, D, and E. According to Dubois et al. (2020), tainted water is the primary source of hepatitis E and A infections. Long-term alcohol or cigarette addiction, as well as the use of other substances, raise the chance of contracting hepatitis, which is caused by a virus that enters the human liver and spreads through fecal matter from exposure to wholesome environments. Without a doubt, hepatitis immunization provides its protection.

2.3.7 Respiratory Tract Infections

According to Ali et al. (2019), respiratory tract infections (RTIs) are commonly classified as lower respiratory tract infections (LRTI) and upper respiratory tract infections (URTI). Infections of the upper respiratory system, such as the common cold, are usually far less serious than diseases of the lower respiratory system, such as pneumonia. The symptoms, which are caused by bacteria and viruses that can be ingested through contaminated water, include a dense head, sneezing or coughing, a rattling in the chest, ear and head discomfort, and difficulty breathing (Ali et al., 2019). A history of recurrent respiratory infections, living in an unclean environment, or a compromised immune system due to a medical condition or medication can all raise the risk of this disease.

2.3.8 Kidney Damage

Lower urine production, fatigue, disorientation, convulsions, and side discomfort are typical signs of renal disorders. Kidney infections would result from drinking water tainted with germs (Luyckx *et* *al.,* 2018). Risk factors for kidney impairment include weakened immune systems, a history of kidney infections, and the use of various medications, including pain relievers. Dialysis may be required when renal function is impaired, and in extreme situations, a kidney transplant may be required (Arpali *et al.,* 2020). Groundwater in some locations and any drinking water from untreated or unfiltered natural sources may have been tainted with harmful substances.

2.3.9 Typhoid

Typhoid fevers are a type of enteric fever that are brought on by the Gram-negative bacteria *Salmonella enterica* serotype *typhi* and *S. typhi.* These bacteria are primarily found in contaminated waters, but they can also be found in drinking water that has been contaminated by the excrement of an infected person's food (Fazal-Ur-Rehman, 2019) (Taylor and Winter, 2020). In addition to typical symptoms like weakness, headache, loss of appetite, stomach pain, constipation, and weight loss, typhoid can occasionally result in internal bleeding through vomiting.

2.3.10 Ulcers

The opportunistic infection of the acidophilic and anaerobic bacterium Helicobacter pylori, which is the sole primary cause of gastric cancer (Fazal-Ur-Rehman, 2019), results in peptic ulcers, which are atrophic gastric ulcers, including those that develop in the duodenum, and are open, painful sores that develop on the inner lining of these innards (Zahid *et al.,* 2020). Additionally, naproxen sodium and the medications aspirin and ibuprofen are more frequently than anticipated non-infective causes of peptic ulcers (Zahid *et al.,* 2020). Information on research on the main illnesses brought on by wastewater and the factors that cause them worldwide. Protozoa, helminths, viruses, and bacteria are the main waterborne pathogens.

2.4 Waterborne Pathogens

2.4.1 Waterborne Bacteria

More than half of deaths from water-related disorders are caused by microbial intestinal infections, according to the WHO (Magana-Arach-chi and Wanigatunge, 2020). The majority of the bacteria that infect humans, *Vibrio parahaemolyticus, Vibrio cholerae,* and *Vibrio vulnificus,* are aquatic and freely inhabit the intestines of aquatic or marine organisms. They are common in estuarine and marine environments. People can contract aggressive cholera from twelve different *Vibrio species* (Diner *et al.,* 2021). One important component of in-vivo colonization is the production of TcpA, which is co-regulated with the expression of the cholera toxin (Ghasemi *et al.,* 2020). Since *Vibrio cholerae* is a "destructive parasite," meaning it kills the host as soon as possible, it is by far the most significant of these human infections. It is possible for *Vibrio fluvialis, Vibrio mimicus,* and *Vibrio hollisae* to cause infections in the gastrointestinal tract. At least in a number of poor nations, cholera has been a well-known disease since the nineteenth century and is today the most common microbiological waterborne infection (Pilla *et al.,* 2022). Pathogens transmit by contaminated food and water through the oral or fecal channel (Magana-Arachchi and Wanigatunge, 2020). Nonetheless, environmental water-polluted samples are commonly found to contain *salmonellae.* Serovars of *S. typhi* or *non-typhi* are more common in the environment (Rajaei *et al.,* 2015). *Shigellosis,* often known as dysentery, is caused by the pathogenic bacterium *Shigella sp.* and is naturally transmitted by close contact with an infected person or by fecal contamination of food or water (Rath, 2021). UTIs and other intestinal and extraintestinal infections are caused by *Escherichia sp.* (Chloé Mirsepasi-Lauridsen *et al.,* 2019). There are six primary strains of *E. coli* based on epidemiological data, morphological traits, and clinical traits and variables (Algammal *et al.,* 2020). Among the most dangerous disease-causing strains that spread through contaminated water are *enterohemorrhagic, enteroinvasive,* and *enterotoxigenic E. coli* (Azab El-Liethy *et al.,* 2020). According to Magana-Arach-chi and Wanigatunge (2020), the illness, which is brought on by swallowing tainted food or water, is marked by acute diarrhea that lasts for days and leaves small children malnourished and dehydrated. *Burkholderia pseudomallei,* which is endemic to humans in northern Australia and Southeast Asia and causes the potentially fatal disease melioidosis, is the most clinically significant of the species *Burkholderia sp.,* which also includes *Burkholderia cepacia, Burkholderia pseudomallei, Burkholderia mallei,* and *Burkholderia gladioli* (Magana-Arachchi and Wanigatunge, 2020). Melioidosis is linked to the water supply, however *Burkholderia sp.* Pain in the bones, joints, chest, and skin infections are some of the signs of pneumonia, a potentially fatal waterborne infection caused by the bacteria *Klebsiella pneumoniae* that kills approximately 89,000 people each year worldwide (Wiersinga *et al.,* 2018). According to Sinulingga *et al.,* (2020), the most important *Campylobacter species* linked to human gastroenteritis is *Campylobacter jejuni.* As it stands, *Campylobacter enteritis* accounted for 8.5% of the overall burden of diarrheal disease, ranking fourth overall after diarrhea, rotavirus, cryptosporidiosis, and E. coli (Liu *et al.,* 2021). Due to its endemic nature, clinical illness in adults is rare in developing countries and usually mainly affects children under the age of two (Baker-Austin *et al.,* 2018). The Gram-negative bacterium *Legionella sp.* was unknown before to the devastating pneumonia outbreak in Pennsylvania in the summer of 1976. 34 fatal occurrences of an uncommon respiratory infection were reported among 221 conference attendees during the 58th American Medical Association annual congress (Mondino *et al.,* 2020).

2.4.2 Waterborne Virus

Diarrhea from drinking, recreational, and groundwater is often caused by waterborne viruses (Pal *et al.,* 2018). As water-transmitted viral pathogens with moderate to high health significance, according to the WHO, adenovirus (AdV), astrovirus (AstV), hepatitis A and E viruses, rotavirus, norovirus, other caliciviruses, and enteroviruses, including coxsackieviruses and polioviruses, are coxsackieviruses and polioviruses; basically, wastewater is a menagerie of animals and animalcules acting as vectors of inimitable bacterial or viral pathogens. Furthermore, viruses that are released in urine, including CMV and polyomaviruses, have the potential to propagate through water (Adelodun *et al.,* 2021). Coronaviruses and influenza viruses can be spread by drinking water. Most of these viruses are associated to gastroenteritis, which can result in diarrhea, as well as other symptoms like fever, nausea, and cramping in the belly. AdVs cause a wide range of human disorders (Harrach *et al.,* 2019). Among the human organs they can infect are the eye, upper and lower respiratory tracts, the gastrointestinal tract (producing gastroenteritis), the bladder, the central nervous system, and the genitalia. Human adenoviruses are mostly transmitted by the fecal–oral pathway and water droplet inhalation. They are classified as juvenile gastroenteritis pathogens because the majority of those affected are children under the age of five (Rossouw, 2020). The importance of monitoring AdVs in water has grown because of the virus's ability to survive in the environment and its resistance to purification and disinfection processes (Canh *et al.,* 2022). Colder months have demonstrated higher incidence rates of human astroviruses (HAstVs), which are disseminated by fecal–oral contact with contaminated water. While AstV infections are more common in tropical regions during the rainy season, the majority of AstV infections occurred in temperate countries throughout the winter. In addition to freshwater, marine, and groundwater used as drinking water sources, these viruses are also detected in wastewater (Magana-Arachchi and Wanigatunge, 2020). The main symptoms of this virus include moderate watery diarrhea that lasts two to three days, fever, vomiting, and anorexia. The International Committee on the Taxonomy of Viruses reclassified and separated calciciviruses into four genera: Sapovirus, Lagovirus, Vesivirus, and Norovirus. Sapoviruses (SaVs) and noroviruses (NoVs) are on the most recent list of potential contaminants in drinking water in the United States (Rusiñol and Girones, 2019). These are spread by the fecal–oral pathway and are present in contaminated surface and ground waters. The highly contagious NoVs are known to be found in humans. NoVs are characterized by projectile vomiting, diarrhea, low-grade fever, headaches, and malaise (McArthur *et al.,* 2020). Disease outbreaks have been connected to the consumption of tainted seafood and drinking water containing these viruses. SaVs are common caliciviruses with a wide range of characteristics (Lasure and Gopalkrishna, 2017). There have been reports of disease outbreaks affecting persons of all ages, including the elderly. SaVs are transmitted from person to person by contaminated food, contaminated water, and fecal–oral routes. These include the coxsackieviruses, polioviruses, hepatoviruses, enteroviruses, echoviruses, and common waterborne enteroviruses. Humans are the only known enterovirus reservoir. Although they are mostly found in sewage that has been contaminated by human waste, they can also be found in groundwater, untreated drinking water, solid waste landfills, coastal rivers, sewage treatment facilities, and marine waterways. Type A viral hepatitis is brought on by the hepatitis A virus. The fecal–oral pathway is how a virus is spread by coming into contact with an infected person, consuming contaminated food, or drinking contaminated water. Children are more vulnerable to disease in areas with high endemicity, whereas adults and adolescents are more vulnerable to moderate endemicity (Jacobsen, 2018). Hepatovirus E (HEV) is mostly transmitted by contaminated food and water through fecal–oral routes. It is possible for it to spread from person to person and through zoonotic sources, resulting in acute hepatitis E in people (Webb and Dalton, 2019). According to Thomas *et al.,* (2017), RVs are to blame for 2 million hospital admissions and 450,000 child fatalities annually, the most of which are said to occur in developing countries in Asia and Africa.

3.5. Antimicrobial resistance

According to Fakruddin *et al.,* (2013), antimicrobial resistance genes (ARGs) can spread between species and multiply in both pathogenic and non-pathogenic bacteria found in wastewater. The ability of microorganisms (such as bacteria and viruses) to withstand or withstand antimicrobials or therapeutic medications used to treat illnesses and ailments brought on by these microbes is referred to by this general word. Overall, antimicrobial resistance poses a concern to human and animal health since it diminishes or eliminates the efficacy of therapeutic medications and agents intended to treat or prevent diseases brought on by these microorganisms (Petrovic *et al.,* 2003; Bolong *et al.,* 2009; Teijon *et al.,* 2010). Bacteriophages, which are viruses that attack and multiply within bacterial cells, can also transfer antimicrobial resistance genes (ARGs) between bacteria. Other mechanisms include conjugation between bacteria, which can transfer antimicrobial resistance genes found on plasmids and transposons; and the acquisition of naked, "free" DNA from their surroundings (Teijon *et al.,* 2010). As microorganisms move between the many environmental compartments (such as the air, water, humans, and animals), antimicrobial resistance spreads or is transferred throughout the environment. Wastewater systems, which include urban wastewater collection and treatment plants, receive a variety of effluent from medical facilities, pharmaceutical companies, and residences. . Wastewaters contain a complex mixture of developing contaminants, such as pharmaceutical goods, personal care items, pesticides, and illegal and prescription medications, according to a growing body of research (Madikizela *et al.,* 2017; Michael *et al.,* 2013; Rizzo *et al.,* 2013; Huang *et al.,* 2017). Environmental hotspots are crucial to the emergence and spread of antimicrobial resistance (AMR) bacteria, which is an increasing global concern. The emergence of AMR pathogens in these habitats has been facilitated by the overuse and abuse of antibiotics in animal care, agriculture, and human medicine. Antibiotic resistance is a result of genetic changes that pathogens can acquire (Levy and Marshall, 2004). Through horizontal gene transfer, they can also pick up resistance genes from other microbes (Davies, 1994). AMR pathogens can spread through agricultural runoff and wastewater treatment plants, both of which are major sources of AMR pathogens in the environment (Rizzo *et al.,* 2013; Oliver *et al.,* 2010).

AMR has a major effect on public health since it raises human morbidity and mortality (WHO, 2020). Healthcare systems and society at large may also bear a heavy financial burden from AMR (O'Neill, 2016). AMR pathogen development can be slowed by enhancing antibiotic stewardship in animal care, agriculture, and human health (Llor and Bjerrum, 2014). According to Rizzo *et al.,* (2013), improving wastewater treatment procedures can also lessen the amount of AMR germs released into the environment. Antimicrobial resistance genes are divided into two categories: anthropogenic or acquired, which are spread by resistance genes on mobile genetic elements that originate from and are encouraged by human behavior and activities, and autochthonous or intrinsic, which are derived from the background presence of genes in bacterial genomes that could produce resistant phenotypes (Teijon *et al.,* 2010).

3.5.1 Prevalence of antimicrobial resistance

Potential hotspots for the horizontal transfer and selection of genes causing antibiotic resistance in aquatic bacteria are wastewater treatment facilities. Wastewater is a good place for horizontal gene transfer because of its high microbial biomass and quantity of nutrients (Hartig *et al.,* 1999). In situ research utilizing membrane chambers submerged in municipal sewage treatment plants have shown that bacteria can transfer genes for antibiotic resistance (Hirsch *et al.,* 1999). As a result, resistant bacteria found in wastewater may transfer plasmids and other genetic components encoding antimicrobial resistance to native aquatic bacteria. Numerous antimicrobial compounds have been found in wastewater, according to recent investigations (Backhaus *et al.,* 1999; Saye *et al.,* 1999; Marcinek *et al.,* 1999). The concentrations of antimicrobial agents in municipal wastewater can affect susceptible bacteria (Raloff *et al.,* 1999; Ahmed *et al.,* 1999), and thus have the potential to determine a selection in favor of resistant bacteria, even though they are typically at least 1000 times lower than the concentrations required to inhibit the growth of resistant bacteria.

There are three types of wastewater treatment: basic, secondary, and tertiary. Solids in wastewater are eliminated through physical processes in initial treatment. The majority of the organic debris is eliminated during subsequent treatment using chemical and biological methods. Additional procedures are employed in tertiary treatment to eliminate components, like phosphorus or nitrogen, that the secondary treatment is unable to considerably diminish. Tertiary or advanced treatment refers to the process that encompasses all three stages of treatment. The distribution of the bacterial population undergoes significant qualitative and quantitative changes during the wastewater treatment process. It is commonly believed that therapy results in a significant decrease in the overall quantity of germs, including resistant bacteria. Nonetheless, some research indicates that vulnerable and antimicrobial-resistant populations are not equally impacted. Bell *et al.,* (Bell *et al.,* 1999) discovered that treated sewage had a greater proportion of coliforms and fecal coliforms with transmissible resistance genes than raw sewage. Similarly, after mechanical-biological treatment of municipal sewage, Morozzi *et al.* (Morozzi *et al.,* 1999) and Andersen (Andersen *et al.,* 1999) showed a rise in the percentage of multiple-antibiotic resistant (MAR) coliforms.

Very little is known about the degrees of antimicrobial resistance in other bacterial populations, and the majority of research on the presence of antimicrobial resistant bacteria in wastewater has used coliforms as bacterial indicators. *Acinetobacter species* were employed as bacterial markers in a recent study to track antibiotic resistance in sewers that received effluent from a pharmaceutical facility and a hospital. Because of their widespread distribution in the aquatic environment and exceptional capacity to acquire resistance to antimicrobial treatments, *Acinetobacter spp.* were selected as bacterial indicators.

3.5.2 Mechanisms of resistance

The four primary categories of antimicrobial resistance mechanisms are: (1) restricting medication uptake; (2) altering a drug target; (3) rendering a drug inactive; and (4) active drug efflux. Limiting uptake, drug inactivation, and drug efflux are examples of intrinsic resistance mechanisms; drug target alteration, drug inactivation, and drug efflux are examples of acquired resistance mechanisms. Gram negative and gram positive bacteria employ different kinds of processes due to structural variations, among other factors. While gram positive bacteria lack the ability to use some drug efflux mechanisms (see the section on drug efflux pumps later in this manuscript) and less frequently use limiting drug uptake (due to the absence of an outer membrane of LPS), gram negative bacteria utilize all four major mechanisms (Chancey *et al.,* 2012; Mahon *et al.,* 2014).

As was previously indicated, bacteria naturally differ in their capacity to restrict the absorption of antimicrobial drugs. Gram-negative bacteria's LPS layer's composition and properties operate as a barrier to specific kinds of chemicals. Because of this, such bacteria are naturally resistant to specific classes of potent antimicrobial drugs (Blair *et al.,* 2014). Because of the high lipid content of the mycobacteria's outer membrane, hydrophobic medications, such rifampicin and fluoroquinolones, can enter the cell more easily than hydrophilic ones (Kumar *et al.,* 2005; Lambert *et al.,* 2005). Therefore, all medications that target the cell wall, such as β-lactams and glycopeptides, are inherently ineffective against bacteria without a cell wall, such Mycoplasma and similar species (Bebear *et al.,* 2005). Because they lack an outer barrier, gram-positive bacteria are less likely to impede drug access. Because polar molecules find it difficult to pass through the cell wall, enterococci are naturally resistant to aminoglycosides. *Staphylococcus aureus,* another gram-positive bacterium, has recently become resistant to vancomycin. Of the two defenses S. aureus employs against vancomycin, one mechanism that is still unknown enables the bacteria to create a thicker cell wall that hinders drug entry and confers an intermediate resistance to the antibiotic. According to Lambert *et al.,* (2005) and Miller *et al.,* (2014), these strains are known as VISA strains.

Porin channels are frequently used by substances to enter the cell of bacteria with broad outer membranes. Gram-negative bacteria typically have porin channels that let hydrophilic molecules pass through (Blair *et al.,* 2014; Gill *et al.,* 2014). A reduction in the quantity of porins present and mutations that alter the selectivity of the porin channel are the two primary ways that porin alterations can restrict drug uptake (Kumar *et al.,* 2005). It is well established that decreasing the quantity of porins (and occasionally ceasing production of specific porins entirely) causes resistance in members of the *Enterobacteriaceae.* These bacteria together decrease the number of porins as a defense against carbapenems (Cornaglia *et al.,* 1996; Chow *et al.,* 1991). *E. aerogenes* and *Neisseria gonorrhoeae* have been observed to exhibit mutations that alter the porin channel, resulting in resistance to imipenem and certain cephalosporins, as well as resistance to β-lactams and tetracycline

(Gill *et al.,* 1998; Thiolas *et al.,* 2004). The development of a biofilm by a bacterial colony is another often observed occurrence in bacterial colonization. These biofilms could be dominated by one organism, like *Pseudomonas aeruginosa* in the lung, or they could be made up of many different organisms, like the biofilm community of normal flora in the gut. The development of a biofilm serves as defense for pathogenic organisms against both antimicrobial agents and host immune system attacks. Antimicrobial drugs find it challenging to reach the bacteria due to the thick, sticky quality of the biofilm matrix, which contains polysaccharides, resident bacterial proteins, and DNA. Therefore, substantially larger medication concentrations are required for effectiveness. Additionally, antimicrobials that target growing, dividing bacterial cells have limited effect since the bacteria in the biofilm tend to be sessile (slow metabolic rate, slow cell division). One significant finding regarding biofilms is that the close proximity of the bacterial cells probably facilitates horizontal gene transfer. This suggests that these bacterial communities may find it simpler to share genes for antibiotic resistance (Mah *et al.,* 2012; Van *et al.,* 2014).

3.5.2.2 Modification of drug targets

Many parts of the bacterial cell might be targets for antimicrobial treatments, and the bacteria could alter many of those targets to make them resistant to the medications. Changes in the number and/or structure of PBPs (penicillin-binding proteins) are one way that gram-positive bacteria can develop resistance to the β-lactam antibiotics that they almost exclusively employ. PBPs are transpeptidases that aid in the cell wall's peptidoglycan synthesis. The amount of drug that can bind to that target is affected by changes in the number of PBPs (either a drop in PBPs with normal drug binding or an increase in PBPs with a decrease in drug binding ability). Drug binding may be reduced or completely inhibited by a structural alteration (e.g., PBP2a in S. aureus by acquisition of the mecA gene) (Raygaert *et al.,* 2009; Beceiro *et al.,* 2013). Lipopeptides, like daptomycin, function by depolarizing the cell membrane, while glycopeptides, like vancomycin, also function by preventing the formation of cell walls. According to Randall *et al.* (2005), gram-negative bacteria with a thick layer of LPS are naturally resistant to these medications. Vancomycin resistance has emerged as a significant problem in *Staphylococcus aureus* (MRSA) and *enterococci* (VRE-vancomycin-resistant enterococci). The acquisition of van genes mediates resistance by altering the structure of peptidoglycan precursors, which reduces vancomycin's capacity to bind (Cox *et al.,* 2013; Beceiro *et al.,* 2013). Calcium must be present for daptomycin to bind. Gene mutations (e.g., mprF) cause the cell membrane surface to become positively charged, which prevents calcium and, consequently, daptomycin from binding (Yang *et al.,* 2009; Stefani *et al.,* 2015). Ribosomal mutation (aminoglycosides, oxazolidinones), ribosomal subunit methylation (aminoglycosides, macrolides—gram positive bacteria, oxazolidinones, streptogramins), which most frequently involves erm genes, or ribosomal protection (tetracyclines) are the three ways that resistance to medications that target the ribosomal subunits can develop. These processes prevent the medication from attaching itself to the ribosome. These pathways differ significantly in the degree of pharmacological interference (Kumar *et al.,* 2013; Roberts *et al.,* 2004). Resistance to medications that interfere with the production of nucleic acids (fluoroquinolones) is caused by changes in DNA gyrase (found in gram-negative bacteria, such as gyrA) or topoisomerase IV (found in gram-positive bacteria, such as grlA). The drug's capacity to bind to gyrase and topoisomerase is reduced or eliminated as a result of these alterations, which alter their structures (Hawkey *et al.,* 2003; Redgrave *et al.,* 2014). Drugs that block metabolic processes can develop resistance by overproduction of resistant DHPS and DHFR enzymes (sulfonamides—DHPS, trimethoprim—DHFR) or mutations in enzymes (DHPS—dihydropteroate synthase, DHFR—dihydrofolate reductase) involved in the folate biosynthesis pathway. Since the sulfonamides and trimethoprim are structural analogues of the natural substrates (p-amino-benzoic acid for sulfonamides and dihydrofolate for trimethoprim), they bind to their corresponding enzymes. These medications work by attaching to the enzymes' active sites and causing competitive inhibition. These enzymes typically have mutations in or close to the active region, which alters the structure of the enzyme and prevents drug binding while permitting the natural substrate to bind (Huovinen *et al.,* 1995; Vedantam *et al.,* 1998).

3.5.2.3 Drug inactivation

Bacteria can inactivate medications in two major ways: either by physically breaking down the drug or by adding a chemical group to it. The β-lactamases are a broad class of enzymes that hydrolyze drugs. Tetracycline is another medication that can be rendered inactive by hydrolyzation through the tetX gene (Kumar *et al.,* 2013; Blair *et al.,* 2015).

Transfer of acetyl, phosphoryl, and adenyl groups is the most common method of drug inactivation by transfer of a chemical group to the drug; many transferases have been identified; the most diverse mechanism is acetylation, which is known to be used against aminoglycosides, chloramphenicol, streptogramins, and fluoroquinolones; phosphorylation and adenylation are known to be used primarily against aminoglycosides (Blair *et al*).

3.5.2.4 Drug efflux

Genes for efflux pumps are chromosomally encoded in bacteria. Some are constitutively produced, while others are induced or overexpressed in response to specific environmental stimuli or in the presence of an appropriate substrate (high-level resistance is typically caused by a mutation that alters the transport channel). The efflux pumps operate largely to clear the bacterial cell of hazardous substances, and many of these pumps will transport a vast variety of molecules (multi-drug [MDR] efflux pumps). The resistance capability of many of these pumps is controlled by what carbon source is available (Blair *et al;* 2014; Villagra *et al;* 2012).

The majority of bacteria have a wide variety of efflux pump types. The ATP-binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), and the resistance-nodulation-cell division

(RND) family are the five major families of efflux pumps in bacteria that are categorized according to structure and energy source. Single-component pumps that move substrates across the cytoplasmic membrane make up the majority of these efflux pump groups. Almost exclusively present in gram-negative bacteria, the RND family of multi-component pumps works in tandem with an outer membrane protein (OMP-porin) and a periplasmic membrane fusion protein (MFP) to efflux substrate throughout the cell envelope (Blair *et al.,* 2014; Kumar *et al.,* 2005; Piddock *et al.,* 2006; Poole *et al.,* 2007). In gram-negative bacteria, various members of the efflux family can function as multicomponent pumps in conjunction with other cellular components. MacB, a member of the ABC family, extrudes macrolide medications as a tripartite pump (MacAB-TolC). EmrB, a component of the MFS, extrudes nalidixic acid in E. coli by acting as a tripartite pump (EmrAB-TolC) (Tanabe *et al.,* 2009; Jo *et al.,* 2017).

Gram-positive bacteria's efflux pumps, which are encoded on their chromosomes, may provide inherent resistance. These pumps include efflux fluoroquinolones and members of the MATE and MFS families. It is also known that plasmids have gram-positive efflux pumps. According to Jonas *et al.* (2001) and Costa *et al.* (2013), the MFS family now comprises the described pumps in gram-positive bacteria. Although efflux pumps from all five families can be identified in gram-negative bacteria, the RND family has the most clinically relevant pumps (Blair *et al.,* 2014; Kourtesi *et al.,* 2013).

3.5.3 Public health implications

Sanitizing wastewater is still crucial because clean water is both a basic human health need and the trend of economic activity associated with industry, agriculture, and animal husbandry (Ghosh *et al.,* 2022). Sadly, untreated waters are home to diseases like cholera, typhoid, dysentery, and hepatitis, which pose a threat to society and the environment, particularly in developing countries. Viruses, hemagglutinin type 1 and neuraminidase type 1, severe acute respiratory syndrome, Middle East respiratory syndrome, Ebola, Nipah, and Zika are a few notable aquatic-mediated illnesses that have been reported recently. that are transported via lakes, rivers, ponds, and other water sources (Yuan *et al.,* 2021). An estimated 361,000 children under the age of five were among the 829,000 persons worldwide who were wiped out by diarrhea each year (Magana-Arachchi and Wanigatunge, 2020). For instance, it is projected that waterborne appalling infections cost the US economy $1 billion annually in the USA alone (Shea-han *et al.,* 2022). According to Manetu and Karanja (2021), around 2.5 billion people lack access to improved sanitation, and 780 million people lack access to clean water. The subject of the general availability of water is unavoidable given the rise in the need for clean water for everyone and everywhere.

Goal six of "The Sustainable Development Goals envisages ensuring accessibility and sustainable management of water and hygiene everywhere for everyone," which is driven by the United Nations Environment Program (UNEP) and the United Nations Development Program (UNDP) (Delanka-Pedige *et al.,* 2020). Kasprzyk-Hordern and Sims, 2020). The situation has gotten worse from bad to worse. The Environmental Protection Agency identifies the pollutants found in wastewater that have the potential to harm ecosystems and human health (Parida *et al.,* 2021). When contaminated water is the primary source in a region, it must be made safe for drinking, cooking, and other recreational uses. Municipal wastewater treatment plants that receive municipal waters containing a variety of pathogenic multidrug-resistant (MDR) bacteria pose a number of health risks to the general public. Horizontal gene transfer mechanisms operate in both the environment and the human body through natural bacterial consortia (Acar Kirit *et al.,* 2020; Gillespie, 2000), culminating in the progressive introduction of MDR bacteria due to the use of modern antibiotics of higher generations (Gillespie, 2000). While industrialized countries depend on the removal of wastes prior to the treatment facilities providing useable water, many impoverished countries lack the financial means to construct and maintain complex sewage treatment plants (Crini and Lichtfouse, 2019). Furthermore, the impact of microbial pathogens on human health has been examined in terms of the prevalence of infectious diseases and strategies for preventing their bothersome morbidity and death; as a result, appropriate remediation technologies are still accessible (Yadav *et al.,* 2021).

The sensitive and accurate identification of pathogens in wastewater was the subject of recent reviews that employed molecular techniques. In particular, studies on the use of sensor systems in wastewaters to estimate the horrifying pathogens have been conducted, and several countries, including Australia, France, Italy, Japan, Spain, Netherlands, India, Czech Republic, China, USA, Turkey, and Pakistan, have reported the presence of COVID-19 infectious particles in sewage (Mahmoudi and Xiong, 2022). Furthermore, it was shown that the main sources of waterborne animals, including growing protozoan parasites, in Iran were untreated water, recreational water, and water contaminated by animal waste (Hoseinza-deh *et al.,* 2021). According to a few case studies from Asia and Africa, infectious waterborne infections rose during significant climate change events, such as floods, among many others (Cissé, 2019). The World Health Organization (WHO) had set limits or requirements for wastewater stabilization pond effluent quality; the WHO is currently updating policy-making recommendations to permit the safe post-treatment use of wastewater as well (Kaszycki *et al.,* 2021). In order to detect and quantify the presence of pathogens in wastewater and sludge samples, a variety of detection and quantification approaches have been used. Current wastewater treatment technologies use a combination of physical, chemical, and biological processes to remove pathogens, animal parasites, and insoluble particles from effluents. Chlorination is still the most daring way to disinfect water or soil, even though it produces dangerous byproducts called halo-organics. For this reason, a significant amount of chlorine sources must be applied for effective disinfection. UV disinfection is a popular method of eliminating germs, although its use is limited to labs and enterprises. Not disposing of organic municipal waste at the end of each street could result in a persistently unpleasant stench and degrade the groundwater by releasing biochemical pollutants, making clean groundwater practically impossible. This is done in an effort to lessen the public health damage caused by MDR bacterial pathogens in wastewater, a universal hazard that is seen in Nepal as backyard.

3.6 Waste water management

Wastewater is defined as water whose physical, chemical, or biological characteristics have been altered by the addition of specific compounds, making it unfit for drinking or other uses. Human daily activities primarily rely on water, hence they release "waste" into the environment. Among these are chemicals, detergent, household cleansers, dirt, microorganisms (germs) that can harm people and the environment, hair shampoo, hair, food scraps, fat, laundry powder, fabric conditioners, toilet paper, and bodily wastes (feces and urine). Since a large portion of supplied water is known to end up as wastewater, purification is crucial. In order to maintain a healthy environment and the general public's health, wastewater treatment is the procedure and technology utilized to eliminate the majority of the toxins present in wastewater. Thus, wastewater management is the process of managing wastewater to save the environment and maintain social, political, economic, and public health (Metcalf and Eddy, 1991).

3.6.1 Objectives of wastewater treatment

i. **Reduction of biodegradable organic substances in the environment**

Oxidation is required to break down organic substances like carbon, nitrogen, phosphorus, and sulfur into gases that are either released or remain in solution.

ii. **Reduction of nutrient concentration in the environment**

Water bodies can become eutrophic or enriched with nutrients like nitrogen and phosphorus from wastewater, which encourages the growth of algae and other aquatic plants. Aquatic life is hampered by these plants' depletion of oxygen in water bodies.

iii. **Elimination of pathogens**

Pathogens are organisms that infect humans, animals, and plants and cause disease. Because they are too small to be seen with the human eye, they are also referred to as microorganisms. The following are examples of microorganisms: fungi (like *Candida albicans*), bacteria (like *Vibrio cholerae*), viruses (like enterovirus, hepatitis A & E virus), protozoa (like *Entamoeba histolytica, Giardia lamblia)*, and helminths (like *Schistosoma mansoni, Ascaris lumbricoides*). The feces of diseased individuals and animals contain high concentrations of these microorganisms (Awuah and Amankwaa-Kuffour, 2002).

3.6.2 Sources of water pollution

3.6.2.1 Domestic wastewater

One of the main causes of water contamination is domestic wastewater. Slums and illegal settlement are the results of poor urban planning and population growth (Gulyani *et al.,* 2007). According to UNEP (2010), 60% of Africa's urban population is thought to reside in slums with subpar, unreliable, and inadequate sanitation services. For example, according to Kasili *et al.* (2009), Nairobi's Kibera is Africa's second-largest slum. Kibera is considered an illegal community in Nairobi, despite the fact that its inhabitants make up 20% of the city's total population (UNEP, 2011). The majority of people in this area lack access to sanitation, or only have rudimentary sanitation systems like latrines and small-bore sewers, because there are no wastewater treatment plants (WWTPs) or sewage collecting systems (UNEP, 2011).

3.6.2.2 Agricultural pollution

Ninety percent of Africans living in rural areas depend on agriculture as their primary source of income (UNEP, 2010). Pesticides and fertilizers are among the pollutants that rain-fed agriculture produces (Shurthirasingham *et al.,* 2010; Brown *et al.,* 2009). Large-scale commercial farming is growing in Africa to support the continent's rapidly growing population. For instance, 25% of the cut flowers in Europe come from Kenya. As a result, rose-growing greenhouses are now a significant cause of water contamination (Wang *et al.,* 2012). We discovered that the biodiversity in Kenya's Lake Naivasha has been declining recently. The degradation of Lake Naivasha's water quality brought on by pesticides and fertilizers used in commercial rose cultivation is one potential explanation.

3.6.2.3 Industrial wastewater

Mining, oil production, logging, rubber plantations, and other businesses are common in Africa (UNEP, 2010). Paper, dairy, textile, and sugar mills are among the many industries that employ stabilization ponds (Wang *et al.,* 2011). The pollutant concentration in the pond system's effluent is still quite high since stabilization ponds have limited capacity (Omosa *et al.,* 2012). For instance, we discovered that, in several pond systems in Africa, the average influent chemical oxygen demand of municipal waste water treatment plants (WWTPs) is above 2000 mg/L, but in other nations, it is roughly 400 mg/L (Wang *et al.,* 2009).

3.6.2.4 Solid waste pollution

Large volumes of industrial, medical, and municipal solid waste are dumped into lakes, rivers, wetlands, and open spaces. For instance, solid trash is frequently dumped in open areas in Addis Ababa without adequate facilities for handling (UNEP, 2010). As a result, when it rains, runoff carries them into rivers. Our research also demonstrates that solid waste disposal and the flying toilet—plastic bags used for feces that are discarded alongside roadways at night—are widespread practices in Africa (Wang *et al.,* 2012). In Africa, illegal dumping is widespread, including the municipal disposal of medical and industrial hazardous waste (Wang *et al.,* 2011).

3.7 Current practices for water and wastewater treatment

3.7.1 Wastewater treatment

3.7.1.1 On-site treatment

In Africa, septic tanks and pit latrines are common on-site sanitation systems found in rural and semi-urban settings. However, these pit latrines are not well maintained or managed. The quality of the groundwater declines as a result (UNEP, 2010). Wastewater is flowing and several pit latrines are full. Waterborne illnesses can come from this source. Although some nations, like Uganda, employ natural wetlands for wastewater treatment or disposal, a growing number of natural wetlands are becoming weaker or decreased as a result of rising pollutant loads (Maclean *et al.,* 2011).

3.7.3 Off-site treatment

Off-site wastewater treatment systems are widely used in cities (Mara *et al.,* 2008). For treatment, wastewater is gathered and delivered to waste water treatment facilities (WWTPs). The most often used technologies for waste water treatment plants (WWTPs) in Zambia are stabilization ponds, combined trickling filter plants in the majority of cities and towns, and activated sludge treatment in a few chosen towns. Out of 39 publicly run systems in Kenya, 27 waste stabilization ponds six traditional procedures like trickling filters, three oxidation ditches, and three aerated lagoons are in place because they can withstand tough operating circumstances.

3.7.4 Conventional treatment

Due to a lack of funding and energy, traditional treatment methods like activated sludge and biofilms are rarely employed in Africa. For example, in Uganda, the National Water and Sewerage Corporation uses stabilization ponds for the other sewerage treatment plants and only uses conventional sewage treatment works at the Bugolobi Sewage Treatment Works for Kampala City and one plant at Masaka Sewage Treatment Works. Only 55% of the sewage generated in the

Nakivubo catchment region is treated by the Bugolobi Sewage Treatment Works; the remaining sewage is dumped untreated into natural wetlands near Kampala.

3.8 Pathogenic microorganisms in waste water

Salmonella species are the most prevalent of the enteric bacteria, which are arguably the most prevalent pathogens found in wastewater. These germs survive in wastewater, and raw sewage has been observed to contain Salmonella densities of 5,000 per liter (Akin *et al.,* 1978). Sixty meters downwind from a sprinkler wastewater irrigation line, a salmonella bacteria was isolated from the air (Katzenelson *et al.,* 1976). Additionally, it has been documented that the ratio of coliforms to aerosolized salmonellae in the vicinity of a sewage treatment facility was the same as that found in the wastewater (Grunnet *et al.,* 1974). It should be noted that airborne infections are not limited to the diseases typically considered to be airborne. For example, monkeys can contract Salmonella typhi by inhaling a dose 1,000 times lower than the typical infectious dose (Crozier *et al.,* 1962). Raw sewage may contain up to 100 different kinds of enteric viruses. With the isolation techniques currently in use, viruses that are detected in quantities of several thousand viral units per liter can typically be separated from sewage. After reviewing the literature, Berg (Berg *et al.,* 1973) discovered that reported virus reductions by secondary sewerage treatment range from 0% to 99%. According to a review of the literature, the possible health risks to populations exposed to airborne viruses at spray and aeration sites have not been adequately assessed, despite the fact that some viruses have been demonstrated to be able to travel great distances in a variety of weather conditions and to spread infection at locations distant from their sources (Wright *et al.,* 1969). Enteric viruses from wastewater aerosols have only been successfully sampled in two studies (Johnson *et al.,* 1978; Teltsch *et al.,* 1978). For many years, coliform bacteria have been used as markers of fecal contamination of water, and the density of these bacteria has been used as a criterion for the level of pollution. According to some researchers, these organisms might be just as useful as markers of airborne contamination caused by wastewater treatment facilities (Adams *et al.,* 1970; Goff *et al.,* 1973). Other researchers highlighted the low survival rate of coliform bacteria in the airborne state in comparison to other airborne microorganisms and proposed the use of coliphages (Fannin *et al.,* 1977), Streptococcus faecalis (Crawford *et al.,* 1977), Alcaligenes faecalis (King *et al.,* 1973), and Klebsiella (Pereira *et al.,* 1975; Randall *et al.,* 1966; Sorber *et al.,* 1976). . These proposed species have the drawback that the processes needed to differentiate them are typically more complex than those needed to determine coliform. Coliform organisms were investigated as potential markers of air contamination from wastewater irrigation fields as they are widely recognized as markers of water contamination.

3.8.1 Pathogens in wastewater

Numerous microorganisms that pose a serious threat to human health can be found in wastewater streams. Bacteria, viruses, parasitic protozoa, and helminths are examples of human pathogens (Cai and Zhang, 2013). Wastewater can harbor pathogens from a variety of sources. Human and animal feces, as well as fecal-contaminated water from other domestic applications like laundry or bathing, can introduce enteric pathogens into wastewater (Gerardi and Zimmerman, 2004). Wastewater from food processing also poses a concern to human health since livestock and poultry can contract zoonotic enteric pathogens (Gerardi and Zimmerman, 2004, Hill, 2003). Ashbolt (2004) and Gerba and Smith (2005) list the main infections and the illnesses or diseases they produce. . Readers are referred to the report by Keegan, Monis, Jagals, Toze, and Blackbeard (2010) for a summary of pathogens and representative indicator organisms in wastewater, including their geographic distribution, numbers in primary and secondary treated wastewater, and an overview of detection techniques.

3.8.2 Bacteria

The most varied category of human pathogens found in wastewater are bacteria. Numerous kinds of bacteria live in the human gut and are expelled in feces. The majority of bacterial pathogens in wastewater are enteric bacterial pathogens, although many of these bacteria are commensal and helpful to their hosts (Varela and Manaia, 2013).

Salmonella species, Escherichia species, Shigella species, Yersinia species, Klebsiella species, Leptospira species, Vibrio cholerae, Aeromonas hydrophila, Legionella pneumophila, Mycobacterium species, and *pseudomonas* are among the main human bacterial pathogens found in wastewater (Cai and Zhang, 2013; Kristian Stevik *et al.,* 2004, Maynard *et al.,* 2005). Diarrhea, dysentery, and gastroenteritis are common gastrointestinal diseases caused by bacterial pathogens like *Salmonella species, Escherichia species, Shigella species, Yersinia species,* and *V. cholerae* (Anastasi *et al.,* 2010; Okoh *et al.,* 2007, Varela and Manaia, 2013). Although the pathogen's transmission channels have not been definitively established, *Helicobacter pylori,* which causes gastrointestinal ulcers and is connected to some malignancies, may also be waterborne (Anastasi *et al.,* 2010). Leptospirosis (Leptospira), respiratory infections *(L. pneumophila, Mycobacterium avium),* and *wound infections (Pseudomonas aeruginosa)* are other illnesses brought on by bacteria in wastewater (Gerardi and Zimmerman, 2004; Levy, Fine, and Bar-Tal, 2010). Since they cause disease in a host with a predisposing factor, such as decreased immunity or reduced physical barriers to infection due to burns or wounds, certain bacteria, including *L. pneumophila, M. avium, P. aeruginosa,* and *A. hydrophila,* are environmental and opportunistic rather than frank pathogens (Gerardi and Zimmerman, 2004).

The natural gut flora of warm-blooded animals, including humans, includes the genetically varied and largely innocuous bacteria *Escherichia coli.* With densities of 105–1010 colony-forming units per liter of raw sewage, this species has been extensively utilized as a fecal contamination indicator (Matthews, Stratton, Schreoder, and Toze, 2010). In order to be pathogenic and cause intestinal or extraintestinal disorders, certain strains of Escherichia coli have extra genes that encode virulence determinants such adhesion factors and toxins. These harmful strains can result in meningitis, hemolytic uremic syndrome, diarrhea, gastroenteritis, and urinary tract infections (Anastasi *et al.,* 2010). The rising production or usage of antibiotics in nations such as the United States, India, China, and Greece has led to an increased awareness of the presence of antibiotics and antibiotic-resistant bacteria (ARB) in wastewater (Bitton, 2005; Bouki, Venieri, and Diamadopoulos, 2013). There is currently a lot of interest in the environmental fate of both ARB and the genetic elements encoding antibiotic resistance, especially if these genes are passed on to and persist in environmental bacteria. Numerous reports have detailed the persistence of ARB through the wastewater treatment train (Bouki *et al.,* 2013).

3.8.3 Viruses

Another varied class of aquatic human diseases are viruses. According to Keegan *et al.* (2010) and Okoh *et al.* (2007), untreated wastewater can contain anywhere from 103 to 107 virus particles per liter. However, a number of variables, including the season, geographic location, and kind of treatment procedure, affect the viral density in treated wastewater (Kitajima, Iker, Pepper, and Gerba, 2014). The secondary treatment procedures employed to eliminate bacterial pathogens do a poor job of eliminating many viruses (Ottoson, Hansen, Björlenius, Norder, and Stenström, 2006). Enteric viruses, including hepatitis A, norovirus, rotavirus, adenoviruses, astroviruses, and the different enteroviruses, are the main viral pathogens found in wastewater (Ashbolt, 2004, Cai and Zhang, 2013). The majority of enteric viruses proliferate in the host's intestine, while occasionally other tissues may become infected. The location of virus multiplication in the host is typically correlated with the type of sickness that is induced (Wyn-Jones *et al.,* 2011). As a result, enteric viruses can cause a number of illnesses in people, including myocarditis, hepatitis, meningitis, and gastroenteritis (Ashbolt, 2004). Infected people release large amounts of these enteric viruses like 105–1011 virus particles/gram of feces (Fong and Lipp, 2005; Okoh, Sibanda, and Gusha, 2010). According to Wyn-Jones *et al.* (2011), a number of newly discovered viruses, including human parechovirus and severe acute respiratory syndrome coronavirus, can also be secreted in feces and detected in wastewater. Hepatitis E, sopaviruses, and animal adenoviruses are among the zoonotic viruses that can be introduced to wastewater by industrial waste from slaughterhouses (Wyn-Jones *et al.,* 2011). Human feces and wastewater have been found to contain a number of harmful plant viruses, including tobacco mosaic virus and pepper mild mottle virus (Symonds and Breitbart, 2014).

3.8.4 Protozoa

Another significant class of microorganisms found in wastewater are protozoans, which can grow up to ten times larger than bacteria (Boztoprak and Özbay, 2013). According to Li, Craik, Smith, and Belosevic (2009), protozoan parasites such *Giardia duodenalis, Cryptosporidium parvum,* and *Cryptosporidium hominis* are frequently found in sewage, though their quantity may vary seasonally based on the nation and climate. Both people and animals can contract intestinal parasites including Giardia and Cryptosporidium. Patients without a functioning immune system may die from infection with Cryptosporidium because there is currently no effective medication that kills the parasite directly in the host (Abrahamsen *et al.,* 2004). The transmissive stage of Cryptosporidium is a highly contagious, nonreproductive, dormant oocyst that is excreted in feces (Searcy, Packman, Atwill, and Harter, 2005). The illness known as cryptosporidiosis is caused by Cryptosporidium infecting host enterocytes, which results in diarrhea, nausea, and abdominal pain (Fletcher, Stark, Harkness, and Ellis, 2012). Another significant waterborne pathogen that causes intestinal infections is G. duodenalis, a unicellular flagellate eukaryote (Adam, 2001). Giardiasis is an illness that can cause severe diarrhea, gastrointestinal pain, nausea, poor absorption, and weight loss (Fletcher et al., 2012). The transmissive stage of G. duodenalis is a latent cyst (Adam, 2001). Waterborne illness epidemics have also been linked to other harmful protozoa, such as *Cyclospora, Enterocytozoon bieneusi, Isospora belli,* and *Entamoeba histolytica* (which causes amoebic dysentery) (Khanum *et al.,* 2013).

3.9 Pathogens Detection methods

The introduction of new infections or the reemergence of established pathogens of human health significance has been attributed to a number of factors, including population growth, habitat invasion, international travel, and the globalization of world trade (Gilbride *et al.,* 2006). Pathogenic bacteria in wastewater can be isolated and detected using a variety of methods, from next generation sequencing (NGS) to basic culture-based methods. Some of these are conventional procedures, and in order to comply with regulations, their use may be required in certain nations. Although a detailed discussion of these methods is outside the purview of this review, there are numerous helpful review papers that describe or assess molecular techniques (Gilbride *et al.,* 2006; Monis *et al.,* 2005, Ramirez-Castillo *et al.,* 2015, Yergeau *et al.,* 2016), and research reports also provide valuable information for both conventional and molecular detection protocols for pathogens or surrogates in wastewater (Francy *et al.,* 2011, Keegan *et al.,* 2010, Monis *et al.,* 2015).

3.9.1 Waterborne disease outbreaks

Waterborne diseases are problems caused by pathogenic microorganisms that are spread through water and have a negative impact on human health, including death, disability, illness, or disorders (Von *et al.,* 2015). Bathing, washing, drinking water, and eating food that has come into contact with tainted water can all spread these illnesses. They are a major problem in rural parts of developing nations worldwide. Although vomiting and diarrhea are the most often reported signs of a waterborne infection, other symptoms may include issues with the skin, ears, respiratory system, or eyes. One of the main reasons why waterborne illnesses spread throughout a community is a lack of access to clean water, sanitation, and hygiene (WASH). Thus, the primary strategy for preventing waterborne illnesses is consistent access to sanitary facilities and clean drinking water (WHO, 2014).

3.9.2 Risk factors for infection

Numerous contaminants that can affect human health, such as excess nutrients, infections, heavy metals, and hormones, are frequently found in untreated wastewater and excreta (Burkholder *et al.,* 2007; Dalsgaard *et al.,* 2007). Farm workers, their families, consumers, and members of the local community are among the potential groups that could be exposed to these toxins (Aneja *et al.,* 2003).

Direct contact with untreated wastewater and excreta through waste management procedures, such as the handling, storage, treatment, use, and disposal of wastewater or excreta, can result in occupational exposure to toxins. Indirect exposure to toxins can occur for those who live close to intensive agricultural operations. For instance, untreated wastewater and excreta applied to agricultural fields can introduce contaminants into drinking water sources (Menzi *et al.,* 2001). According to Burkholder *et al.* (2007), excessive use of untreated wastewater and excreta can also cause runoff and overland flow following rainfall events, which can contaminate surface water. Additionally, untreated wastewater and excreta can leach through permeable soils and contaminate susceptible aquifers.

Environmental issues resulting from wastewater and excreta management practices include emissions of ammonia, odor, and greenhouse gases, as well as contamination of soil, groundwater, and surface water by nutrients, pathogens, organic matter, and heavy metals (Gerber *et al.,* 2005; Do-Thu *et al.,* 2011; Raschid *et al.,* 2001). There is still worry regarding the possible health effects to humans due to the large variety of pollutants and various exposure pathways (Burkholder *et al.,* 2007; Aneja *et al.,* 2003; WHO, 2015; Arksey *et al.,* 2005).

It's critical to distinguish between confirmed exposure to pollutants, confirmed exposure to contaminants, and confirmed exposure to contaminants that caused sickness in epidemiological terms. A person who lives close to an intensive agricultural activity, for example, may be at danger of exposure to toxins even though they have never come into touch with them; conversely, a person exposed to contaminants may not become ill. Numerous host factors (such as lifestyle, behavior, and vulnerable populations), environmental factors (such as environmental sanitation, the availability of safe water, or the disposal of excreta), and agent factors (such as infectious dose, infection causes disease, or furthers transmission) influence whether or not an individual gets sick. However, the most significant risk and protective variables linked to various human disorders are not well understood.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Edo State, Nigeria's Ikpoba Okha Local Government Area (LGA). The dense population, commercial activity, and unsanitary conditions of Ikpoba Okha, one of Benin City's metropolitan LGAs, leave it vulnerable to microbiological and environmental contamination. Two significant hotspots for the environment were chosen; Ramat park and Ramat market.

Ramat Park is a crowded transportation hub and motor park with a lot of traffic, roadside stalls, and trash buildup. Because runoff water and wastewater from this area are frequently not properly managed, they could serve as a breeding ground for harmful microbes.

Ramat Market is a sizable commercial marketplace that sells meat, vegetables, culinary products, and other goods. The potential of pathogenic microbe contamination in the market environment is increased by wastewater from meat areas, drainage systems, and improperly disposed of trash.

These two locations were picked because they are examples of high-risk settings where people are continuously in contact with potentially harmful microbes.

3.2 Sample collection

Using sterile techniques, wastewater samples were taken from drains and waste-disposal locations at Ramat Park and Ramat Market. The collection was done using a sterile universal bottle and a spatula. To reduce contamination, bottles were rinsed with the site's wastewater before samples were collected. Each point of collection had wastewater that was appropriately tagged with the place and time of collection.To preserve microbial viability, all samples were processed within two to four hours of collection and brought to the Microbiology Laboratory in an ice-packed fridge.

3.3 PREPARATION OF CULTURE MEDIA

All media were prepared according to manufacturer instruction. The media used in this study include MacConkey Agar, Eosin Methylene blue (EMB) and Nutrient Agar.

3.3.1. Microbial culture

3.3.1.1 Preparation of MacConkey Agar

This medium was prepared from commercially available dehydrated powder available from most suppliers of culture media. 58g of macConkey agar powder was dissolved in 1litre of distilled water in a conical flask covered with cotton wool and aluminium foil paper. It was mixed thoroughly and sterilized by autoclaving at 121oC for 15minutes.The medium was cooled to 45-50oC and then

dispensed aseptically into sterile petri dishes.

3.3.1.2 Preparation of Eosin Methylene Blue (EMB) Agar

This medium was prepared from commercially available dehydrated powder available from most suppliers of culture media. 36g of EMB agar powder was dissolved in 1litre of distilled water in a conical flask covered with cotton wool and aluminium foil paper. It was mixed thoroughly and sterilized by autoclaving at 121^{o}C for 15minutes.The medium was cooled to 45-50^{o}C and then dispensed aseptically into sterile petri dishes.

3.3.2 Isolation Of Gram Negative Bacteria

1ml of the sample was measured into sterile beaker and 9ml of sterile distilled water was added. The solution was serially diluted using ten- fold serial dilution up to $10^{4.}$ An aliquot of 1ml of the appropriate dilution from the sample was plated on MacConkey agar and EMB agar for isolation of gram negative bacteria.The MacConkey agar and EMB agar were incubated at 37^{0}C for 24hrs.

Thereafter, the number of discrete colonies were counted in terms of colony forming units(cfu/ml). The viable counts were obtained from these values by reference to dilution factor used.

3.3.3 Enumeration of gram Negative Bacteria

The bacteria counts were used to enumerate the total viable counts of the isolates. The discrete colonies on the MacConkey agar and EMB agar plates were selected and counted. The mean colony count on the MacConkey agar and EMB agar plates were used to estimate the total viable counts for the samples in colony forming units per millilitre (cfu/ml) (Holt *et al*, 2000).

3.3.4 Sub-Culturing Of Bacterial Isolates

3.3.4.1 Nutrient Agar

28g of nutrient agar powder was dissolved in 1litre of distilled water in a conical flask covered with cotton wool and aluminium foil paper. It was mixed thoroughly and sterilized by autoclaving at 121^{o}C for 15minutes.The medium was cooled to 45-50^{o}C and then dispensed aseptically into sterile bijou bottles slant for bacteria preservation. A single isolated colony of the bacteria was picked up with the help of sterilized wire loop and was streaked on fresh nutrient agar slant . The nutrient agar slants were incubated at 37^{0}C for 24 hours. The isolated and purified bacterial strains were stored under refrigeration after preparing slants.

3.3.5 Characterization and Identification Of Bacterial Isolatess

3.3.5.1 Cultural Characteristics

Cultural characteristics were observed on MacConkey agar and EMB agar plates. The cultural characteristics include. Size, shape, surface, colour, texture, elevation and optical properties were determined by visual observation.

3.3.5.2 Gram staining test

The Gram staining technique was used to confirm gram negative bacterial strains. A drop of sterile distilled water was placed on a neat and clean glass slide, and a single isolated colony of 24 hours old culture was mixed in it. The smear was made by spreading the culture. This smear was air dried and fixed by rapidly passing the slide three times over the flame. It was then flooded with crystal violet for 1 minute and then washed off with distilled water. Then gram's iodine solution was added to the smear and the glass slide was left for one minute and rinsed with distilled water. This step was followed by the application of decolorizing agent (ethanol) for 30 seconds. Decolorizing agent was immediately washed with distilled water and the smear was counter stained with safaranine for one minute. The slide was washed with distilled water, air dried and was observed under the microscope (Benson, 1994).

3.3.6 Susceptibility Test for gram negative bacteria

3.3.6.1 Standard Inocula

The standard inocula was prepared from their various slants and incubated for 24hrs at 37oC. 1ml pipetted from each of the flasks were serially diluted to 103 and was used as standard inocula. The bacterial isolates selected were tested for susceptibility test using discdiffusion method (Kirby-Bauer) on Muller Hinton agar. The plates were incubated at 37^{0}C for 24hrs and were observed for zones of inhibition (Harvey and Prescott, 2002).

3.3.7 Biochemical tests

To identify bacterial isolates, biochemical assays were performed in accordance with the guidelines provided by CLSI (2021) and Cheesbrough (2006). Among the tests were:

3.3.7.1 Catalase test

The basic idea is to identify the catalase enzyme, which converts hydrogen peroxide ($H2O_2$) into oxygen and water.

Method: A colony of the isolate was emulsified in a drop of 3% hydrogen peroxide that had been put on a sterile slide.

Interpretation: A positive result such as *E.coli, Klebsiella* spp. *and Pseudomonas* spp. is indicated by immediate effervescence (bubbling)

3.3.7.2 Oxidase Test

Principle: Identifies the bacterial enzyme cytochrome c oxidase.

Method: Oxidase test paper was smeared with a colony.

Interpretation: A positive result (*Pseudomonas spp.*) is indicated by the development of a blue color within 30 seconds; a negative result *(Klebsiella* spp and *E.coli)* is shown by the absence of color change.

3.3.7.3 Indole Test

Principle: Identifies the hydrolysis of tryptophan to produce indole.

Method: Kovac's reagent was added after the isolates had been inoculated into tryptone broth and cultured for 24 to 48 hours.

Interpretation: *E. coli* is positive when a red ring forms, while *Klebsiella* spp. and *Pseudomonas* spp. are negative when a yellow ring forms.

3.3.7.4 Citrate Utilization Test

Principle: Assesses an organism's capacity to use citrate as its only carbon source.

Method: Incubated at 37°C after being inoculated on Simmons citrate agar.

Interpretation: Green coloration denotes negative *(E. coli);* blue coloration shows positive *(Klebsiella* spp. *Pseudomonas* spp.*)*

3.3.7.6 Voges-Proskauer (VP) Test

Principle: Identifies the neutral byproduct of glucose fermentation, acetoin.

Method: The MR-VP broth culture was supplemented with 40% KOH and α-naphthol.

Interpretation: *Pseudomonas* spp. is negative and *Klebsiella* spp. and *E.coli* are positive

CHAPTER FOUR

RESULTS

The study's findings offer significant new information about the bacteriological quality of the chosen environmental hotspots in Benin City's Ikpoba-Okha Local Government Area. The results show that the isolated Gram-negative bacteria exhibit varied degrees of antibiotic resistance, considerable microbial contamination, and the presence of indicator species. A significant bacterial load inside the tested habitats was indicated by the total heterotrophic bacterial counts reported across all samples, which varied from 3×10^4 to 9.2×10^5 CFU/ml. These high values are indicative of contaminated environments with significant organic matter buildup, inadequate sanitation, and potential fecal contamination. The predominance of Gram-negative and coliform organisms, which are generally linked to contaminated water, soil, and waste-rich locations, is further confirmed by the continuously high counts on both MacConkey and EMB agars. Based on colony morphology, including color, elevation, margin, and texture, the isolates' cultural traits indicated the presence of *Pseudomonas* spp., *Klebsiella* spp., and *Escherichia coli.* These organisms are known to flourish in nutrient-rich and contaminated settings; their presence suggests that organic contaminants and waste materials are continuously being deposited in the area. According to morphological analysis, every isolate was a chain of Gram-negative rods. This validates their status as environmental and fecal-associated organisms and is consistent with the normal cellular shape of many enteric bacteria. Gram-negative bacteria, which are frequently more durable in contaminated or damp settings, can proliferate in the tested environment, as evidenced by the consistency of the Gram reaction. Two isolates of *Pseudomonas* species, one isolate of *Klebsiella* species, and two isolates of *Escherichia coli* were found after additional biochemical characterization. Standard descriptions of these organisms were supported by the urease and citrate activity in the *Klebsiella* isolate, the indole positivity in the E. coli isolates, and the positive catalase and oxidase reactions in the *Pseudomonas* isolates. Notably, as *E. Coli* and *Klebsiella* spp. are mainly linked to the digestive tracts of people and animals, their discovery is a sign of fecal contamination. Their existence suggests that fecal matter has an impact on the environmental locations studied, most likely due to runoff from neighboring residential or business areas, inappropriate waste management, or open defecation. *Pseudomonas* species *and E. coli* were the most commonly isolated organisms, followed by *Klebsiella* species, according to the distribution of isolates. This pattern points to both fecal and environmental sources of active contamination. While the presence of *E. coli* and *Klebsiella* spp. indicates more direct fecal input, Pseudomonas spp. are prevalent in the environment and frequently inhabit damp settings. The findings of the antibiotic susceptibility test showed that the isolates had different degrees of resistance. The *Pseudomonas* species were resistant to drugs like Ceporex, Streptomycin, and Cefuroxime, but they shown a comparatively high susceptibility to the majority of tested antibiotics. With resistance to several antibiotics, such as gentamycin and ceftazidime, the *Klebsiella* species showed moderate susceptibility, suggesting the existence of strains that could compromise first-line therapies. The most alarming discovery was the high level of resistance seen in the *E. coli* isolates, which displayed multidrug-resistant (MDR) traits with resistance indices ranging from 0.7 to 0.9. These levels of resistance point to the spread of resistant bacteria in the environment, either as a result of community-wide indiscriminate antibiotic use or contamination from agricultural or medical sources. Overall, the findings show that large concentrations of Gram-negative bacteria, including fecal markers and strains resistant to drugs, are present in the investigated regions. These results highlight the possible harm to public health posed by exposure to such surroundings, particularly in areas with inadequate sanitation infrastructure.

Table 1: Determination of Total Heterotrophic Bacteria count on MacConkey and EMB agar (Df =10^4)

Samples	MacConkey Agar	EMB Agar
1	2×10^5	3.2×10^5
2	1.9×10^5	5×10^4
3	4×10^4	3×10^4
4	1.2×10^5	5×10^4
5	9×10^4	1.1×10^5
6	2.4×10^5	9×10^4
7	5×10^4	9×10^4
8	3×10^4	1.1×10^5
9	7×10^5	6×10^4
10	9.2×10^5	4.3×10^5

Table 2: Cultural Characterization Of Bacteria Isolates

Cultural characteristics	***Pseudomonas* spp.**	***Pseudomonas* spp.**	***Klebsiella***	***E.coli***	***E.coli***
Shape	Circular	Circular	Circular	Irregular	Circular
Elevation	Flat	Raised	Raised	Flat	Raised
Margin	Entire	Entire	Entire	Entire	Entire
Size	Big	Medium	Small	Medium	Big
Colour	Creamy	Creamy	Creamy	Pink	Pink
Optical properties (Transparency)	Transluscent	Transluscent	Transluscent	Opague	Transluscent
Texture (Wetness/dryness)	Dry	Wet	Wet	Wet	Wet
Smooth/rough surface	Smooth	Smooth	Smooth	Smooth	Smooth

Table 3: Morphological characterization of the isolates

Morphological	***Pseudomonas* spp.**	***Pseudomonas* spp.**	***Klebsiella***	***E.coli***	***E.coli***
Gram-staining	-ve	-ve	-ve	-ve	-ve
Cell type	Rod	Rod	Rod	Rod	Rod
Cell arrangement	Chain	Chain	Chain	Chain	Chain

Table 4: Biochemical characterization of isolates

Biochemical Test	***Pseudomonas* spp.**	***Pseudomonas* spp.**	***Klebsiella* spp.**	***E.coli***	***E.coli***
Catalase	+	+	+	+	+
Oxidase	+	+	-	-	-
Indole	-	-	-	+	+
Citrate utilization	+	+	+	-	-
Urease	+	+	+	-	-
MR-VP	-	-	+	+	+
Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
Sucrose	+	+	+	+	+
Probably organism	*Pseudomonas* spp.	*Pseudomonas* spp.	*Klebsiella* spp.	*E.coli*	*E.coli*

Table 5: Distribution of Isolated Bacteria

Isolates	***Pseudomonas* spp.**	***E.coli***	***Klebsiella* spp.**
***Pseudomonas* spp.**	+	-	-
***Pseudomonas* spp.**	+	-	-
***Klebsiella* spp.**	-	-	+
E.coli	-	+	-
E.coli	-	+	-

Isolates	OFX	AU	PEF	CTZ	CN	CPX	CEP	TRX	S	CEF	Resistance index (RI)
***Pseudomonas* spp.**	27(S)	24(S)	25(S)	22(S)	22(S)	18(S)	0(R)	0(R)	5(R)	0(R)	0.4
***Pseudomonas* spp.**	27(S)	25(S)	27(S)	24(S)	23(S)	24(S)	0(R)	15(I)	0(R)	0(R)	0.3
***Klebsiella* spp.**	21(S)	11(I)	15(I)	10(R)	8(R)	12(I)	0(R)	5(R)	11(I)	0(R)	0.5
E.coli	11(I)	0(R)	8(R)	5(R)	5(R)	0(R)	0(R)	18(S)	11(I)	0(R)	0.7
E.coli	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	5(R)	25(S)	5(R)	0(R)	0.9

Table 6: Antibiotics susceptibility test for Gram negative bacteria

R= Resistance (0-10mm)

I= Intermediate (11-16mm)

S= Susceptible (17mm and above)

Antibiotics:OFX=Ofloxacin,**AU**=Augmentin,**PEF=**Peflacine,**CTZ**=Ceftzidime,**CN**=Gentamycin, **CPX**=Ciprofloxacin, **CEP**=Ceporex, **TRX**=Ceftriaxone, **S**=Streptomycin, **CEF**=Cefuroxime

Key of distribution:

EMB = Eosine methylene blue

CHAPTER FIVE

DISCUSSION

High microbial loads and the presence of many potentially harmful Gram-negative bacteria were found in wastewater samples taken from Ramat Market and Ramat Park in the Ikpoba Okha Local Government Area, Benin City, according to the bacteriological evaluation. Heavy contamination was evident from the wastewater samples' total heterotrophic bacterial counts, which varied from 3×10^4 to 9.2×10^5 cfu/mL. In line with the findings of Albert *et al.* (2021) and Cai and Zhang (2013), who noted that untreated wastewater and contaminated surface waters typically harbor very high microbial loads, these high counts imply that the chosen regions are microbial hotspots. The findings of this investigation are also consistent with those of Ibekwe and Grieve (2003), who found bacterial densities in wastewater-impacted habitats that were higher than 10^5 cfu/mL. These high numbers attest to the unhygienic conditions of these locations and are well beyond the allowable threshold for safe water disposal. Variations in the amount of organic pollutants, nutrient availability, and human activity in the sampling regions may be the cause of the variation in bacterial load among the samples. While comparatively lower counts in other sites may be the result of dilution from runoff or reduced organic input, the highest bacterial count observed at sample 10 was probably caused by the buildup of decomposing organic matter and inadequate drainage systems surrounding Ramat Market. Gram-negative rod-shaped bacteria, including *Pseudomonas species, Escherichia coli,* and *Klebsiella species,* were isolated from the samples. According to Varela and Manaia (2013), Bouki *et al.* (2013), and Okoh *et al.* (2010), *Pseudomonas, Klebsiella,* and *E. coli* are among the most prevalent bacteria found in wastewater environments because of their adaptive resistance mechanisms and capacity to endure in harsh environments. These findings are consistent with the prevalence of these bacterial genera.

According to Matthews *et al.* (2010) and Okoh *et al.* (2010), *E. coli* is a reliable sign of fecal pollution and poor sanitation, and the isolation of the bacteria from many samples provides evidence of fecal contamination. The existence of *Pseudomonas* and *Klebsiella* further points to pollution from market trash, hospital effluents, and household sewage—all of which are known to release a variety of bacteria into the environment. This result is consistent with related research that found these bacteria were commonly found in Nigerian urban drainage and wastewater systems (Albert *et al.,* 2021). The isolates' diverse resistance patterns were revealed by the antibiotic susceptibility test. The majority of the bacterial isolates were susceptible to aminoglycosides like Streptomycin and Gentamicin, as well as fluoroquinolones like Ofloxacin, Ciprofloxacin, and Peflacine, but resistant to β-lactam antibiotics like Augmentin, Ceftazidime, Ceftriaxone, Cefuroxime, and Ceporex. This finding suggests that environmental *Enterobacteriaceae* exhibit extensive β-lactamase-mediated resistance, as documented by Blair *et al.* (2015) and Bouk*i et al.* (2013). The widespread and careless use of β-lactam antibiotics in human and veterinary medicine may be the cause of the high resistance to these medications, which results in the selection and maintenance of resistant strains in the environment. The evolution and dissemination of β-lactam-resistant strains are facilitated by wastewater conditions, which expose bacteria to sub-inhibitory antibiotic concentrations, as noted by Rizzo *et al.* (2013) and Hutinel *et al.* (2019), who also documented similar resistance trends. According to Kourtesi *et al.* (2013) and Kumar and Schweizer (2005), partial resistance seen in some isolates suggests early development of adaptive mechanisms like efflux pump overexpression or alteration of drug target sites. However, the comparatively higher susceptibility to aminoglycosides and fluoroquinolones suggests that these classes of antibiotics may still be effective against some environmental isolates. Even though they are uncommon, multidrug-resistant isolates pose a serious threat to public health. Antimicrobial resistance genes spread because wastewater settings offer an ideal setting for horizontal gene transfer across bacterial species (Levy and Marshall, 2004). The fact that treated wastewater frequently has a larger percentage of resistant coliforms than raw sewage suggests that treatment procedures may not always remove resistance determinants, according to studies by Bell *et al*. (1999) and Andersen *et al.* (1999). This lends credence to the theory that wastewater serves as a reservoir and possible source of resistant bacteria that have the ability to infiltrate human populations and natural environments.

The wastewater samples from Ramat Market and Ramat Park contain coliforms, *E. coli, Pseudomonas,* and *Klebsiella,* which suggests a high level of microbial contamination and the potential for disease transmission. According to earlier research by Ali *et al.* (2019) and Wells and Whiteford (2022), exposure to such tainted water might result in infections such gastroenteritis, urinary tract infections, wound infections, and other waterborne diseases. Additionally, the presence of antibiotic-resistant bacteria in these locations presents a risk since pathogenic strains may acquire resistance genes, making infection control and treatment more challenging. The results of Okoh *et al.* (2010) and Vymazal (2011), who found that untreated or inadequately treated wastewater greatly contributes to the global spread of antibiotic resistance, are in line with this observation. Overall, the study's findings support the notion that Ikpoba Okha Local Government Area wastewater serves as a substantial reservoir of bacteria that are both potentially harmful and resistant to antibiotics. The necessity of efficient wastewater management and treatment prior to release into the environment is highlighted by the high bacterial load, the presence of fecal markers, and the resistance patterns seen. In order to prevent the development of waterborne pathogens and antibiotic resistance in the environment, the results emphasize the significance of ongoing microbiological monitoring and appropriate sanitation policy enforcement for public health.

RECOMMENDATIONS

i. **Enhancement of Environmental Sanitation:** To lessen the buildup of organic waste that promotes bacterial growth, the study area should conduct regular, well-coordinated sanitation exercises.

ii. **Strengthening trash Management Practices:** To prevent careless dumping of trash and wastewater, which contaminates surface areas, appropriate waste disposal methods should be implemented.

iii. **Public health education:** To inform locals about the risks of environmental contamination, open defecation, and poor hygiene habits, community-level education initiatives should be implemented.

iv. **Control of Antibiotic Misuse:** To prevent misuse and lower the growth of bacterial strains resistant to multiple drugs, government agencies should impose more stringent rules on the sale and distribution of antibiotics.

v. **Frequent Microbial Surveillance:** To monitor changes in microbial loads and patterns of antibiotic resistance, bacterial contamination in environmental hotspots should be periodically monitored.

vi. **Providing Sanitary Facilities:** To lessen the accumulation of feces and other pollutants in the environment, public restrooms, trash cans, and drainage systems should be properly supplied and maintained.

CONTRIBUTIONS TO KNOWLEDGE

i. **Baseline Data:** The study offers up-to-date baseline data on the kinds of Gram-negative organisms and bacterial load that are common in environmental hotspots in Ikpoba-Okha LGA.

ii. **Identification of Fecal Indicator Organisms:** The presence of *Escherichia coli* and *Klebsiella* spp. indicates possible threats to public health and verifies continuous fecal contamination in the areas examined.

iii. **Antibiotic Resistance Trends:** The study reveals the existence of multidrug-resistant strains, particularly among *E. coli,* and provides useful information on the antibiotic sensitivity patterns of isolated bacteria.

iv. **Evidence of Environmental Pollution:** Significant environmental pollution is demonstrated by the high microbiological counts, which highlight the need for better waste management and sanitation procedures.

v. **Contribution to AMR Surveillance:** By offering resistance indices for bacteria frequently found in contaminated environments, the findings support efforts to monitor antimicrobial resistance (AMR).

vi. **Scientific Documentation:** By describing the microbial makeup and resistance profiles of local environmental isolates, this study contributes to the body of knowledge already available on environmental microbiology in Nigeria.

CONCLUSION

This study has shown that a variety of microorganisms, including potentially harmful and antibiotic-resistant bacterial species, are present in the wastewater collected from specific environmental hotspots in Ikpoba Okha Local Government Area, Benin City, especially Ramat Market and Ramat Park. Significant microbial contamination is evident from the high total heterotrophic bacterial counts, which range from 3×10^4 to 9.2×10^5 cfu/mL and are significantly higher than allowable limits for untreated wastewater. This finding offers compelling proof that these sites act as active reservoirs for microbial communities that are significant for public health. The research area's inadequate sanitation infrastructure and poor waste management procedures are reflected in the isolation of *Escherichia coli, Pseudomonas spp.,* and *Klebsiella spp.,* which confirm fecal pollution and general environmental contamination. While the presence of *Pseudomonas* and *Klebsiella* indicates secondary contamination from home sewage, market waste, and potentially hospital effluents, the presence of *E. coli* in several samples shows direct or indirect contamination by human and animal wastes. These bacteria are well-known opportunistic pathogens that can cause serious infections, particularly in immunocompromised people, including pneumonia, gastroenteritis, wound infections, and urinary tract infections. Therefore, the results highlight the significance of wastewater as a major pathway for the spread of pathogenic pathogens in urban settings.

Most of the bacteria were resistant to β-lactam antibiotics, such as Augmentin, Ceftazidime, Ceftriaxone, Cefuroxime, and Ceporex, according to the isolates' antibiotic susceptibility testing. However, they were still reasonably sensitive to aminoglycosides, such as Gentamicin and Streptomycin, and fluoroquinolones, such as Ciprofloxacin, Ofloxacin, and Peflacine. The development of β-lactamase enzymes, which has become more prevalent among environmental and clinical bacterial isolates, may be the cause of the widespread resistance to β-lactam antibiotics. Because it implies that these wastewater sites may serve as breeding grounds for multidrug-resistant organisms that can horizontally transfer resistance genes to other bacteria, this resistance pattern is concerning. Because resistant strains can infiltrate the food chain through irrigation, produce washing, or contaminating household water sources, these environmental reservoirs of antimicrobial resistance (AMR) pose a hazard to human health in addition to aquatic and terrestrial ecosystems. Waste management systems' inability to sufficiently safeguard the environment and public health is demonstrated by the survival of pathogenic and multidrug-resistant organisms in untreated wastewater. Therefore, this study highlights how urgently Ikpoba Okha and other similar metropolitan regions in Nigeria need a more effective wastewater management system. To reduce the dangers of disease transmission and the spread of resistance, wastewater should be adequately cleaned before being released into the environment.

The study's findings verify that the effluent from Ramat Park and Ramat Market is highly contaminated and contains a high concentration of bacteria that are harmful and resistant to antibiotics. The results show that wastewater in cities is a major public health issue that needs to be addressed right now in addition to being an environmental one. Implementing sustainable waste treatment and disposal systems, regularly monitoring wastewater effluents for microorganisms, and having the appropriate authorities enforce environmental health rules are all vital. Public awareness initiatives should also be stepped up to inform locals about the risks associated with careless trash disposal and the prudent use of antibiotics. Effective implementation of these strategies can greatly slow the spread of antibiotic resistance and waterborne illnesses, enhancing environmental quality and protecting public health in Benin City and beyond.

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