ANTIBIOTIC PROFILE OF BACTERIA ISOLATED FROM WASTE WATER IN SELECTED PRIVATE HOSPITALS IN BENIN CITY,NIGERIA

BY

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BENIN CITY

OCTOBER,2025

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A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc. Hons) IN MICROBIOLOGY

OCTOBER, 2025

CERTIFICATION

This is to certify that this project work was carried out by **Jacinta Chiamaka CHUKWUMA,** in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City under my supervision.

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PROF. E.O IGBINOSA **DATE**

(Head of Department)

DEDICATION

This project work is dedicated to God and my family members for their unwavering support.

ACKNOWLEDGEMENTS

First and foremost, I give all glory to God Almighty for his grace, mercy, strength, wisdom and guidance through this academic journey and for the completion of this work.

Special thanks go to my project supervisor, **Dr. (Mrs) O.A. Ologbosere** whose invaluable guidance and mentorship saw to the beginning and conclusion of this research project. Her continuous support, encouragement, and remarks contributed greatly to the success of this work.

I wish to extend my sincere gratitude to the Head of Department, **Prof. E.O. Igbinosa** for his leadership and commitment to creating an enabling environment for learning and research within the department.

I also want to appreciate my course advisors, for their guidance and helpful advice from the start of my year 1 journey till my final year.

I am highly grateful to my parents, **Dr and Dr. (Mrs) Martins Chukwuma** and siblings for their unwavering support, love, and prayers every step of the way.

To my friends especially Marcel Isaac and coursemates, thank you for the moments of collaboration and motivation throughout this academic journey.

Lastly, to my senior colleagues and everyone who has helped at every step of the way, I could not have done this entire project without your guidance. God bless you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The discharge of untreated hospital wastewater poses a public health threat due to antibiotic-resistant bacteria and residual pharmaceuticals. This study evaluated bacterial isolates from wastewater in four private hospitals in Benin City, Nigeria, for three consecutive weeks. Bacteriological analysis revealed total viable counts ranging from 1.6×10^3 to 5.6×10^3 CFU/mL, with the highest at Mount Gilead Hospital. Six bacterial species were identified: *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Enterococcus* sp., and *Pseudomonas aeruginosa*. *E. coli* was the most frequent (27.3%), while *Enterococcus* sp. was the least (9.1%). Antibiotic testing showed significant resistance, especially to β-lactam antibiotics and cotrimoxazole. Fluoroquinolones and gentamicin were more effective. The Multiple Antibiotic Resistance indices ranged from 0.3 to 0.5, with *Enterococcus* sp. and *Pseudomonas aeruginosa* showing the highest resistance. The findings highlight the potential of private hospital wastewater to spread antibiotic-resistant bacteria, emphasizing the need for improved wastewater treatment and stricter environmental regulations.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Antibiotic resistance is one of the most pressing global health threats of the 21st century. The emergence and rapid spread of multidrug-resistant (MDR) bacteria has compromised the effectiveness of available antibiotics, threatening to return humanity to a "pre-antibiotic era" where common infections could once again become fatal (WHO, 2020; Murray *et al*., 2022). A key contributor to this crisis is the environmental dissemination of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), particularly through wastewater systems.

Hospital wastewater has been recognized as a significant reservoir of antibiotic-resistant bacteria (ARB) due to its complex composition (Hassoun-Kheir *et al*., 2020). Unlike domestic wastewater, hospital effluents contain high concentrations of antibiotics, disinfectants, chemical residues, and pathogenic microorganisms originating from patients, laboratories, surgical theaters, laundries, and other clinical activities (Michael *et al*., 2013; Rizzo *et al*., 2013; Fatimazahra *et al*., 2023). The coexistence of antibiotics and bacteria in such effluents creates an ideal environment for the selection, persistence, and horizontal transfer of resistance genes between commensal and pathogenic bacteria (Manaia, 2017; Hembach *et al*., 2019).

Globally, studies have shown that untreated hospital effluents frequently harbor clinically relevant bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus* spp. with high levels of resistance to β-lactams, fluoroquinolones, carbapenems, and aminoglycosides (Karkman *et al*., 2018; Fouz *et al*., 2020;

Endalamaw *et al*., 2024). These bacteria can persist even after conventional wastewater treatment processes, making hospital wastewater treatment plants (WWTPs) hotspots for AMR dissemination into natural ecosystems (Berendonk *et al*., 2015).

Furthermore, wastewater from hospitals contains high numbers of resistant bacteria and antibiotic residues at concentrations able to inhibit the growth of susceptible bacteria (Beyene andi Redaie, 2011). Accordingly, hospital wastewater could increase the numbers of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection pressure (Stalder *et al*., 2014). The release of resistant bacteria to the receiving environment can pose serious public health impacts through transmissible genes, by acting as vectors or reservoirs of resistance determinants (Keen andi Patrick, 2013; Nunez andi Moretton, 2007). The most common bacterial pathogens found in hospital wastewater include *Salmonella, Shigella, S. aureus, P. aeruginosa, Vibrio, Clostridium, Yersinia, Campylobacter, Leptospira,* and coliform groups consisting of *Serratia, Enterobacter, Klebsiella,* and *Escherichia coli* (Anitha andi Jayraaj, 2012; Pandey *et al*., 2011).

In low- and middle-income countries (LMICs), including Nigeria, the problem is more acute due to weak environmental regulations, inadequate wastewater treatment facilities, and limited surveillance systems (Odonkor andi Addo, 2018). Most hospitals, especially private facilities, discharge untreated or partially treated effluents directly into open drains, rivers, or municipal sewers. This indiscriminate disposal not only contaminates surface and groundwater sources but also facilitates the spread of resistant pathogens into the community, where they can cause infections that are increasingly difficult and costly to treat (Akter *et al*., 2020; Emmanuel *et al*., 2021).

Benin City, the capital of Edo State, is home to numerous private hospitals and clinics that provide healthcare to a growing urban population. While these facilities are vital for bridging gaps in healthcare access, their waste management practices are poorly documented. Unlike large tertiary hospitals that may have rudimentary effluent management systems, private hospitals often lack structured wastewater treatment facilities. Consequently, the direct discharge of their effluents into the environment may contribute significantly to the local burden of antimicrobial resistance. Given the widespread reliance on surface and groundwater for domestic use in surrounding communities, exposure to resistant pathogens from such effluents poses a serious public health risk (Serwecińska. 2020; Dias *et al*., 2021; Fatimazahra *et al*., 2023).

The global escalation of antimicrobial resistance has been fueled partly by environmental dissemination pathways, including the discharge of untreated hospital effluents (Berendonk *et al*., 2015; Karkman *et al*., 2018). In Nigeria, the problem is compounded by poor wastewater treatment infrastructure and weak enforcement of environmental health regulations (Odonkor andi Addo, 2018; Emmanuel *et al*., 2021). Private hospitals, which constitute a significant proportion of healthcare providers in Benin City, often lack adequate waste management systems. Their wastewater may contain high loads of multidrug-resistant bacteria that are capable of spreading resistance determinants to commensal and pathogenic microorganisms in the environment (Michael *et al*., 2013; Manaia, 2017).

Despite the growing recognition of hospital wastewater as a reservoir of ARB, limited studies have focused on private healthcare facilities in Nigeria, especially in Edo State (Chinweuba *et al*., 2021). Most research has concentrated on tertiary hospitals (Akter *et al*., 2020; Emmanuel *et al*., 2021), leaving a critical knowledge gap regarding the contribution of private hospitals to environmental antimicrobial resistance. Understanding the bacterial profile and antibiotic susceptibility of isolates from private hospital wastewater is therefore essential for assessing environmental risks, guiding policy interventions, and strengthening the One Health response to AMR (WHO, 2020; Murray *et al*., 2022).

1.2 Aim and Objectives

The main objective of this study was to determine the antibiotic profile of bacterial isolates from wastewater in selected private hospitals in Benin City, Nigeria.

The specific objectives of this study were to:

1. Determine the total viable count (TVC**)** of bacteria present in wastewater from selected private hospitals.
2. Isolate and identify bacterial species present in wastewater from selected private hospitals in Benin City.
3. Determine the antibiotic susceptibility patterns of the bacterial isolates using standard laboratory techniques.
4. Assess the prevalence of multidrug-resistant (MDR) strains among the bacterial isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Wastewater

Wastewater is broadly defined as water that has been adversely affected in quality by anthropogenic activities, rendering it unsuitable for immediate use without treatment. It contains a mixture of physical, chemical, and biological contaminants depending on its source (Metcalf andi Eddy, 2014). Hospital wastewater, in particular, is distinct from domestic or industrial effluents because it is enriched with pathogenic microorganisms, pharmaceutical residues, chemical disinfectants, heavy metals, and organic matter generated from various healthcare activities such as patient care, diagnostics, surgery, laboratories, and hospital laundries (Kümmerer, 2009; Emmanuel *et al*., 2021).

The unique composition of hospital wastewater makes it an important reservoir for pathogenic bacteria and a hotspot for the selection and dissemination of antimicrobial resistance. Studies have consistently reported high concentrations of clinically important bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus* spp. in hospital effluents (Fouz *et al*., 2020; Michael *et al*., 2013). These bacteria are often resistant to multiple antibiotics, reflecting both selective pressure from antibiotics present in the wastewater and the clinical exposure of patients who shed these organisms into the environment.

Antimicrobial resistance (AMR) is defined as the ability of a microorganism to survive or grow in the presence of antimicrobial agents that would normally inhibit or kill it (WHO, 2020). Within the context of wastewater, AMR arises primarily due to the persistence of antibiotic residues, which exert selective pressure on microbial populations, favoring resistant strains over susceptible ones (Manaia, 2017). The mechanisms of resistance acquisition include mutation, efflux pumps, enzymatic degradation (e.g., β-lactamases), and more critically, horizontal gene transfer (HGT) through plasmids, integrons, and transposons (Karkman *et al*., 2018; Berendonk *et al*., 2015). Thus, wastewater provides both the chemical drivers (antibiotic residues) and the biological platforms (dense microbial communities) that facilitate the evolution and spread of resistance.

The conceptualization of hospital wastewater as a "hotspot" for AMR dissemination aligns with the broader *One Health* framework, which emphasizes the interconnectedness of human, animal, and environmental health. The discharge of untreated or poorly treated hospital effluents into municipal drainage systems, rivers, or soils extends the reach of resistant bacteria beyond healthcare settings into the wider community (Collignon andi McEwen, 2019). This poses risks for community-acquired infections, agricultural contamination through irrigation with polluted water, and even re-introduction of resistant organisms into healthcare systems via colonized patients.

In low- and middle-income countries such as Nigeria, the situation is particularly concerning due to poor waste management infrastructure, limited surveillance of AMR in environmental samples, and weak enforcement of environmental regulations (Odonkor andi Addo, 2018; Chukwu *et al*., 2020). Private hospitals, which often lack wastewater treatment facilities, discharge effluents directly into open drains and natural water bodies, exacerbating the cycle of environmental contamination and human exposure. Therefore, understanding the composition of hospital wastewater and the antibiotic profiles of its bacterial inhabitants is essential for effective AMR monitoring and control strategies.

2.2. Composition of Hospital Wastewater

Hospital wastewater is a unique effluent stream that differs significantly from domestic wastewater due to its highly complex mixture of organic, chemical, biological, and pharmaceutical substances. It originates from different sections of healthcare facilities, including patient wards, surgical theaters, diagnostic laboratories, laundries, and pharmacies. Because of its composition, hospital wastewater is considered a critical environmental concern, as it contains higher concentrations of hazardous pollutants that can directly or indirectly affect public health and ecological balance (Kümmerer, 2009; Emmanuel *et al.*, 2005). The main components of hospital wastewater can be broadly discussed under organic matter, chemicals, pathogens, pharmaceuticals, and antibiotics.

2.2.1. Organic Matter

One of the key constituents of hospital wastewater is organic matter, which is introduced primarily through patient excreta, food residues, laboratory discharges, and body fluids such as blood and urine. The organic fraction typically consists of proteins, carbohydrates, lipids, and urea, which contribute to the high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) values of hospital effluents. This high organic load is particularly problematic because it provides a rich nutrient source for microbial growth, thereby supporting the survival and multiplication of both commensal and pathogenic microorganisms. Furthermore, the presence of organic substances encourages the development of biofilms in sewage networks, which not only shield microorganisms from disinfection efforts but also facilitate horizontal gene transfer among bacterial populations. This process contributes to the persistence of pathogens and enhances the risk of antimicrobial resistance development in hospital effluent environments (Verlicchi *et al*., 2010).

2.2.2. Chemicals

Another significant component of hospital wastewater is the presence of diverse chemicals that are employed in daily hospital activities. Cleaning, sterilization, disinfection, diagnosis, and treatment procedures all contribute to chemical contamination. Disinfectants and antiseptics such as chlorine, alcohols, phenolic compounds, and quaternary ammonium compounds are frequently discharged in wastewater, while heavy metals such as mercury, cadmium, chromium, silver, and lead often originate from laboratory reagents and certain medical devices. Radiological units also contribute through the release of contrast agents containing iodine and gadolinium, which are highly persistent and poorly biodegradable. In addition, detergents and surfactants from hospital laundries add to the chemical burden. The combined presence of these chemicals not only poses toxicity risks to aquatic ecosystems but also creates strong selective pressures on microbial communities. This selective pressure facilitates the adaptation and survival of resistant strains, which can later emerge as difficult-to-treat pathogens (Kümmerer, 2010; Pauwels andi Verstraete, 2006).

2.2.3. Pathogens

Hospital wastewater is also widely recognized as a reservoir for diverse pathogenic microorganisms that originate from patients, healthcare staff, and contaminated hospital environments. These pathogens are excreted in large quantities and find their way into hospital drains and sewage systems. Among the bacterial pathogens frequently isolated are *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Acinetobacter baumannii*. Viral contaminants such as noroviruses, hepatitis A virus, rotaviruses, and adenoviruses are also commonly found, while fungi such as *Candida* and *Aspergillus* species have been detected as well. In addition, protozoan parasites including *Giardia lamblia* and *Cryptosporidium* species contribute to the microbial load. A particularly concerning aspect of these pathogens is the high prevalence of multidrug-resistant bacteria, which are shed by patients undergoing treatment. These organisms are often discharged untreated into municipal sewage systems, thereby contributing to the wider environmental dissemination of resistant pathogens (Chitnis *et al*., 2004; Emmanuel *et al*., 2005).

2.2.4. Pharmaceuticals

Pharmaceutical residues represent another important component of hospital wastewater. Hospitals are centers of intensive pharmaceutical use, and a wide variety of drugs are excreted in unmetabolized or partially metabolized forms by patients. These include analgesics, anti-inflammatory drugs, anesthetics, cytotoxic agents, antibiotics, and hormones. Of particular concern are cytostatic agents used in chemotherapy, such as cyclophosphamide and ifosfamide, which are excreted unchanged and remain highly active in the aquatic environment. Many of these pharmaceutical compounds are resistant to biodegradation and can persist for long periods, leading to bioaccumulation and toxic effects on aquatic organisms. Their continuous discharge into wastewater streams contributes to environmental pollution and increases the chemical complexity of effluents, making treatment and safe disposal more challenging (Verlicchi *et al*., 2012).

2.2.5. Antibiotics

Perhaps the most critical component of hospital wastewater is the presence of antibiotics, which are extensively used for the prevention and treatment of infections in hospital settings. Various classes of antibiotics—including β-lactams, fluoroquinolones, aminoglycosides, macrolides, tetracyclines, and sulfonamides—are commonly reported in hospital effluents. These compounds are frequently excreted in active form and persist in wastewater systems, often at sub-inhibitory concentrations. While such concentrations may not be sufficient to kill bacteria outright, they create ideal conditions for the selection of resistant strains by exerting continuous evolutionary pressure. The persistence of antibiotics in wastewater further drives the proliferation of antimicrobial resistance genes, transforming hospital effluents into critical hotspots for antimicrobial resistance development (Kümmerer, 2009; Rodriguez-Mozaz *et al*., 2015).

2.3. Hospital Wastewater as a Reservoir for Pathogens and Resistance Genes

Hospital wastewater is increasingly recognized as a critical environmental reservoir for both pathogenic microorganisms and antimicrobial resistance genes (ARGs). Unlike ordinary municipal sewage, hospital effluents contain a unique combination of pathogens, residual antibiotics, disinfectants, and other chemical agents that create an ideal ecological niche where bacteria can survive, adapt, and exchange genetic material. This makes hospital wastewater not just a passive conduit of waste but an active hotspot for the persistence and dissemination of resistant pathogens in the environment.

One of the major concerns is that hospital wastewater serves as a reservoir for clinically significant pathogens. Research has shown that multidrug-resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), and carbapenem-resistant *Enterobacteriaceae* (CRE) are frequently discharged through hospital effluents (Rizzo *et al*., 2013). The ability of these pathogens to survive in wastewater systems is enhanced by the abundance of organic matter, nutrients, and the formation of biofilms within sewage pipelines, which provide a protective environment against harsh conditions. These pathogens, once released, can contaminate receiving water bodies and become a persistent source of infection and transmission in both hospital and community settings.

Beyond harboring pathogens, hospital wastewater is also a well-documented reservoir for resistance genes. It has been identified as a hotspot for horizontal gene transfer (HGT), a process through which bacteria can share genetic material via mechanisms such as conjugation, transformation, and transduction. Genes responsible for resistance to multiple classes of antibiotics, including *blaCTX-M*, *blaNDM*, *mecA*, *vanA*, *qnr*, and *sul1*, are commonly detected in hospital effluents (Karkman *et al*., 2018). The dissemination of these genes is often facilitated by mobile genetic elements (MGEs) such as plasmids, transposons, and integrons, which accelerate the spread of resistance traits among bacterial populations. Importantly, this genetic exchange is not limited to pathogenic bacteria alone but also occurs between pathogens and environmental commensals, effectively expanding the environmental reservoir of antimicrobial resistance.

The implications of this phenomenon for public health are profound. The discharge of untreated or partially treated hospital wastewater into municipal sewage systems and surface waters increases the risk of widespread dissemination of antimicrobial resistance. Resistant pathogens released into the environment can colonize humans, animals, and even agricultural systems, thereby undermining the effectiveness of antibiotic therapy. Moreover, resistance genes and resistant bacteria can persist in environmental compartments such as soil, water, and sediments, where they may eventually enter the food chain and pose additional health risks. Another important consequence is the possibility of nosocomial reintroduction, whereby contaminated or recycled water sources serve as pathways for resistant pathogens to re-enter healthcare and community environments, creating a cycle of persistence and reinfection.

Given these risks, global health authorities such as the World Health Organization (WHO) and the European Centre for Disease Prevention and Control (ECDC) have highlighted the need for targeted management of hospital wastewater. They emphasize that continuous surveillance, effective treatment of effluents, and integrated monitoring of antimicrobial resistance in environmental compartments must be adopted as part of the broader One Health approach**.** This strategy recognizes the interconnectedness of human, animal, and environmental health in addressing antimicrobial resistance and underscores the importance of hospital wastewater control as a frontline measure in combating the global AMR crisis (WHO, 2017).

2.4. Bacterial Contaminants in Hospital Wastewater

Hospital wastewater represents a unique and complex ecosystem that harbors a wide range of microbial contaminants, including pathogenic bacteria of clinical and environmental significance. The release of such effluents into the environment without adequate treatment poses serious public health concerns due to the potential dissemination of pathogens and antimicrobial resistance determinants (Kümmerer, 2009; Rizzo *et al.*, 2013). Several studies conducted in both developed and developing countries have consistently reported the presence of clinically important bacterial species in hospital wastewater, many of which are implicated in nosocomial and community-acquired infections.

2.4.1. Common Bacterial Genera and Species Isolated from Hospital Effluents

2.4.1.1. *Escherichia coli*

Escherichia coli is one of the most frequently isolated bacteria from hospital wastewater and is commonly used as an indicator of fecal contamination. Pathogenic strains such as enterohemorrhagic and extraintestinal pathogenic *E. coli* are of major concern due to their association with severe diarrheal diseases, urinary tract infections, and septicemia (Ibrahim *et al*., 2020). Hospital effluents serve as reservoirs for multidrug-resistant *E. coli* strains, including those producing extended-spectrum β-lactamases (ESBLs) and carbapenemases, which significantly limit treatment options (Kumar *et al*., 2019).

2.4.1.2. *Klebsiella pneumonia*

Klebsiella pneumoniae is another clinically relevant pathogen often detected in hospital wastewater. It is an opportunistic pathogen responsible for pneumonia, bloodstream infections, and urinary tract infections, especially in immunocompromised patients. Its presence in effluents is alarming due to its capacity to acquire resistance genes such as those encoding ESBLs and carbapenemases (e.g., NDM-1, KPC), which enable survival under antibiotic pressure (Pitout *et al*., 2015; Khan *et al*., 2020). Studies from sub-Saharan Africa, including Nigeria, have reported the frequent occurrence of multidrug-resistant *K. pneumoniae* in untreated hospital wastewater, highlighting the risks of environmental dissemination (Odonkor andi Addo, 2018).

2.4.1.3. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a ubiquitous opportunistic pathogen capable of thriving in moist environments, including hospital wastewater systems. It is well-known for causing wound infections, ventilator-associated pneumonia, and bacteremia in immunocompromised individuals. The organism's intrinsic resistance to multiple antibiotics, combined with its ability to form biofilms, enhances its persistence in wastewater environments (Oliver *et al*., 2015). Moreover, multidrug-resistant strains, including carbapenem-resistant *P. aeruginosa*, are increasingly being reported in hospital effluents, making it an environmental and clinical threat (Vaz-Moreira *et al*., 2014).

2.4.1.4. *Staphylococcus aureus*

Staphylococcus aureus, including methicillin-resistant *Staphylococcus aureus* (MRSA), has been identified in hospital wastewater and sewage systems. This pathogen is a major cause of skin and soft tissue infections, bacteremia, and endocarditis. The presence of MRSA in wastewater is concerning because it indicates the persistence of multidrug-resistant Gram-positive pathogens in the environment (Guardabassi *et al*., 2002). Hospital effluents can act as reservoirs for *S. aureus*, enabling the dissemination of resistance genes through horizontal gene transfer to other environmental bacteria (Hassoun *et al*., 2017).

2.4.1.5. *Enterococcus* spp.

Members of the genus *Enterococcus*, particularly *Enterococcus faecalis* and *Enterococcus faecium*, are common in hospital wastewater. These bacteria are indicators of fecal contamination and are important opportunistic pathogens associated with urinary tract infections, endocarditis, and intra-abdominal infections (Gilmore *et al*., 2013). Of particular concern is the emergence of vancomycin-resistant enterococci (VRE), which have been frequently reported in untreated wastewater. Their resilience to harsh environmental conditions and resistance to multiple antibiotics make them highly problematic in terms of environmental persistence and public health risk (Zaheer *et al*., 2020).

2.4.2. Mechanisms of Survival in Wastewater Environments

The bacterial isolates commonly found in hospital wastewater are significant due to their potential to cause disease in humans and animals. They are responsible for a wide range of infections, including urinary tract infections, gastrointestinal diseases, respiratory tract infections, septicemia, wound infections, and opportunistic infections in immunocompromised individuals (Ruppé *et al*., 2019). In addition, the high prevalence of antimicrobial resistance among these pathogens magnifies their pathogenic potential, as infections caused by resistant strains are often more difficult to treat, leading to increased morbidity, mortality, and healthcare costs (WHO, 2017). The ability of these bacterial contaminants to persist in wastewater is attributed to a combination of physiological, genetic, and ecological factors. Key survival mechanisms include:

1. **Antibiotic Resistance Genes (ARGs):** Hospital effluents are enriched with antibiotics, disinfectants, and heavy metals, which exert selective pressure that promotes the survival of resistant strains. Genes encoding ESBLs, carbapenemases, and vancomycin resistance are often carried on plasmids, integrons, and transposons, facilitating horizontal gene transfer among bacterial populations (Berendonk *et al*., 2015).
2. **Biofilm Formation:** Bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* form biofilms on wastewater pipelines and treatment plants, which protect them

from antimicrobial agents and harsh environmental conditions. Biofilms enhance persistence and act as reservoirs of resistant genes (Fux *et al*., 2005).

3. **Efflux Pumps and Intrinsic Resistance:** Some bacteria, notably *Pseudomonas aeruginosa* and *Enterococcus spp.*, possess efflux pumps and intrinsic resistance mechanisms that enable survival in toxic environments (Poole, 2007).
4. **Stress Response Mechanisms:** Many bacteria can withstand nutrient limitation, temperature fluctuations, and oxidative stress through specialized stress response genes. *E. coli* and *K. pneumoniae*, for instance, adapt quickly to wastewater conditions by modulating their metabolic pathways (Marti *et al*., 2013).
5. **Horizontal Gene Transfer (HGT):** The high microbial diversity and density in wastewater facilitate genetic exchanges through conjugation, transformation, and transduction. This enhances the adaptability and pathogenic potential of bacterial populations (Rizzo *et al*., 2013).

Hospital wastewater serves as a critical hotspot for clinically relevant bacteria such as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *Enterococcus spp.*. These pathogens not only persist in wastewater environments but also pose a risk of spreading antimicrobial res

2.5. Prevalence of Antibiotic-Resistant Bacteria in Wastewater

Wastewater, particularly from urban, hospital, and industrial sources, harbors a diverse array of ARB. A cross-sectional study along the Sutlej River in Pakistan found that *Escherichia coli* exhibited the highest resistance prevalence at 90%, followed by *Klebsiella pneumoniae* (58%), *Pseudomonas aeruginosa* (55%), and *Salmonella* spp. (53%) (Qureshi *et al*., 2020). These bacteria displayed resistance to multiple antibiotics, including ciprofloxacin, co-trimoxazole, ampicillin, and tetracycline, highlighting the presence of multidrug-resistant (MDR) strains. Similarly, global analyses of WWTPs across six continents revealed a core set of 20 ARGs consistently present in activated sludge samples, with *Escherichia*, *Desulfobacter*, *Citrobacter*, and *Pseudomonas* species identified as primary hosts (Parnanen *et al*., 2019).

Hospital effluents are particularly significant contributors to ARB in wastewater. A study from South Africa in the 1970s noted that 26% of coliform bacteria in hospital wastewater exhibited transferable resistance, compared to only 4% in municipal wastewater (Grabow *et al*., 1973). More recent studies confirm that hospital effluents contain high levels of ARGs, such as those conferring resistance to beta-lactams, carbapenems, tetracyclines, aminoglycosides, fluoroquinolones, sulfonamides, macrolides, vancomycin, and erythromycin (Hocquet *et al*., 2016). Industrial wastewater, particularly from pharmaceutical manufacturing, also introduces high concentrations of antibiotic residues, further promoting resistance. For example, effluent from an oxytetracycline-manufacturing plant in China contained bacterial strains with significantly higher minimum inhibitory concentrations (MICs) and multiple *tet* genes downstream of the discharge point (Li *et al*., 2010).

Wastewater from agricultural practices, including livestock production and aquaculture, further contributes to the ARB burden. Pig and chicken farms, where antibiotics are extensively used for disease prevention and growth promotion, show higher ARG diversity and abundance compared to cattle or fish farms (He *et al*., 2020). These findings underscore the role of anthropogenic activities in shaping the resistance profiles of bacteria in wastewater.

2.5.1. Antibiotic Resistance Genes in Wastewater

ARGs are genetic determinants that confer resistance to antibiotics, often located on mobile genetic elements (MGEs) such as plasmids, transposons, and integrons, facilitating horizontal gene transfer (HGT) among bacteria. Wastewater environments are hotspots for HGT due to high bacterial density and the presence of selective pressures like antibiotic residues and heavy metals. Common ARGs detected in WWTPs include those conferring resistance to sulfonamides (*sul1*, *sul3*), tetracyclines (*tetA*, *tetB*, *tetD*), aminoglycosides (*aadA*), and cephalosporins (*bla_CTX-M*) (Pazda *et al*., 2019). A study in European WWTPs found that the resistome mirrors clinical resistance patterns, with a north-to-south gradient of increasing ARG abundance, particularly for Gram-negative bacteria (Parnanen *et al*., 2019).

Metagenomic analyses have revealed that while most ARGs are reduced during wastewater treatment (often by over 98%), some genes, such as those associated with efflux pumps (*mexD*, *mexF*) and class 1 integrons, may be enriched in effluents (Quintela-Baluja *et al*., 2019). Efflux pump genes, which confer broad-spectrum resistance to multiple antibiotic classes, are particularly prevalent in activated sludge due to the diverse stressors (e.g., heavy metals, oxidative stress) present during treatment. The persistence of ARGs in effluents, even after treatment, indicates that WWTPs are significant conduits for releasing resistance genes into the environment (Rizzo *et al*., 2013).

2.5.2. Factors Influencing Antibiotic Resistance in Wastewater

Several factors contribute to the development and proliferation of AR in wastewater:

2.5.2.1. Antibiotic Residues

Antibiotics in wastewater, originating from human excretion, hospital effluents, and industrial discharges, exert selective pressure on bacterial populations. Concentrations ranging from ng/L to mg/L have been reported, with sub-inhibitory levels (e.g., 100–2500 ng/L for ciprofloxacin) shown to select for resistant strains (Gullberg *et al*., 2011). Pharmaceutical manufacturing effluents, such as those containing oxytetracycline or ciprofloxacin, are particularly potent in driving resistance (Larsson *et al*., 2007).

2.5.2.2. Heavy Metals and Other Pollutants

Heavy metals like zinc, cobalt, and copper form complexes with antibiotics, enhancing selective pressure and co-selecting for resistance. Other pollutants, such as microplastics and disinfectants (e.g., triclosan), also contribute to resistance development by stressing bacterial communities (Zhang *et al*., 2018).

2.5.2.3. Microbial Interactions

The high bacterial density in WWTPs, particularly in biofilms and activated sludge, facilitates HGT via conjugation, transformation, and transduction. Biofilms, described as ideal habitats for ARB, enable gene sharing among diverse species, including phylogenetically distant ones. For example, *Aeromonas* species in wastewater have been shown to propagate beta-lactam and quinolone resistance genes through HGT (Czekalski *et al*., 2015).

2.5.2.4. Operational Conditions

Treatment processes, such as aeration and temperature, influence ARG dynamics. Seasonal variations also affect resistance profiles, with influent wastewater showing higher ARG abundance in warmer months (Caucci *et al*., 2016). Additionally, the design of WWTPs (e.g., reactor size, treatment methods) impacts the reduction of ARB and ARGs, with larger reactors in low-antibiotic-consumption countries showing better outcomes (Bengtsson-Palme *et al*., 2016).

2.6 Environmental and Public Health Implications of Hospital Wastewater

Hospital wastewater is a complex mixture of chemical and biological contaminants, including antimicrobial residues, disinfectants, heavy metals, and diverse microbial populations. The discharge of such wastewater into the environment has raised significant concern due to its role as a reservoir and dissemination route for antibiotic-resistant bacteria (ARB) and antimicrobial resistance genes (ARGs). The implications are both environmental and public health–related, as untreated or poorly treated hospital effluents have the potential to spread resistance determinants across ecosystems, food chains, and human populations.

2.6.1. Dissemination of ARB into Surface and Groundwater Sources

One of the primary concerns with hospital wastewater is the release of ARB into surface and groundwater sources. Studies have shown that effluents from hospitals often contain multidrug-resistant pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterococcus* spp. (Kümmerer, 2009; Michael *et al*., 2013). These bacteria, when discharged into rivers, lakes, or groundwater, can survive and persist due to selective pressure from residual antibiotics in the wastewater. In many low- and middle-income countries, including Nigeria, hospital wastewater is often released untreated into municipal drainage systems, leading to contamination of community water sources (Akter *et al*., 2020). Such contamination not only reduces water quality but also facilitates the horizontal transfer of ARGs to native environmental bacteria, further amplifying the spread of resistance (Rizzo *et al*., 2013).

2.6.2. Potential for Community-Acquired Resistant Infections

The presence of antibiotics resistant bacteria (ARB) in hospital wastewater also increases the risk of community-acquired infections. Individuals relying on contaminated water sources for drinking, domestic use, or recreation are directly exposed to resistant bacteria. This is particularly concerning in areas with limited access to safe drinking water and inadequate sanitation. Resistant pathogens disseminated through wastewater can cause infections that are difficult to treat, resulting in increased morbidity and mortality (Varela *et al*., 2016). Furthermore, exposure to sub-inhibitory concentrations of antibiotics in the environment promotes the selection of resistance traits, making it possible for previously susceptible bacteria to become resistant (Baquero, Martínez, andi Cantón, 2008). Community-acquired infections caused by multidrug-resistant organisms such as extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* and carbapenem-resistant *Acinetobacter* spp. have already been documented in several regions, and hospital wastewater has been identified as an important contributing factor (Manaia *et al*., 2016).

2.6.3. Risk of Resistant Bacteria Entering Food Chains

Hospital wastewater also has implications for food safety. In many parts of Africa and Asia, untreated wastewater is commonly reused for irrigation of crops or in aquaculture systems due to water scarcity (Qadir *et al*., 2010). This practice inadvertently introduces ARB and ARGs into agricultural soils, vegetables, and fish farms, creating an important route of entry for resistant pathogens into the human food chain. Studies have shown that vegetables irrigated with untreated wastewater harbor high levels of resistant *E. coli* and *Salmonella* spp., posing direct risks to consumers (Bougnom andi Piddock, 2017). Similarly, aquaculture ponds receiving effluents contaminated with antibiotics or resistant bacteria become breeding grounds for resistant pathogens, which can be transmitted to humans through consumption of contaminated fish (Shah *et al*., 2012). This route of exposure is particularly concerning because it bridges environmental and clinical settings, allowing resistant pathogens to spread rapidly across populations.

2.6.4. Burden on Healthcare Systems

The environmental release of resistant bacteria through hospital wastewater translates into significant challenges for public health systems. Resistant infections are associated with prolonged hospital stays, higher treatment costs, and increased mortality rates (World Health Organization, 2014). For instance, infections caused by carbapenem-resistant *Klebsiella pneumoniae* or multidrug-resistant *Pseudomonas aeruginosa* are notoriously difficult to treat, often requiring last-resort antibiotics such as colistin, which themselves are associated with severe toxicity and emerging resistance (Tacconelli *et al*., 2018). The spread of resistance via environmental reservoirs increases the likelihood of hospital outbreaks and community transmission, thereby escalating the burden on healthcare systems that are already resource-constrained. In sub-Saharan Africa, where hospital infrastructure and infection control are often inadequate, the burden of antimicrobial resistance linked to environmental dissemination is particularly severe (Tadesse *et al*., 2017).

2.7. Previous Studies on Antibiotic Resistance in Hospital Wastewater

2.7.1. Global Studies on Bacterial Isolates and Antibiotic Resistance in Hospital Wastewater

Hospital wastewater serves as a significant reservoir for antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), posing a global public health threat due to the potential dissemination of resistance into the environment. Numerous studies worldwide have investigated the prevalence of bacterial isolates and their antibiotic resistance profiles in hospital wastewater. For instance, Hocquet *et al*. (2016) conducted a comprehensive study in France, analyzing wastewater from a tertiary hospital. They identified high prevalence of multidrug-resistant (MDR) bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*, with resistance to beta-lactams, aminoglycosides, and fluoroquinolones. The study highlighted the role of hospital wastewater in disseminating ARGs, particularly those encoding extended-spectrum beta-lactamases (ESBLs) and carbapenemases.

Similarly, a study by Diwan *et al*. (2010) in India examined hospital wastewater and detected significant concentrations of *Enterobacteriaceae* resistant to third-generation cephalosporins. The researchers attributed this to the overuse of antibiotics in clinical settings and inadequate wastewater treatment systems. In China, Wang *et al*. (2018) investigated the antibiotic resistance profiles of *Acinetobacter baumannii* in hospital wastewater, reporting high resistance to carbapenems and colistin, which are often considered last-resort antibiotics. Their findings underscored the global challenge of managing hospital wastewater to curb the spread of MDR pathogens.

In Europe, Paulshus *et al*. (2019) conducted a metagenomic analysis of hospital wastewater in Norway, revealing a diverse pool of ARGs, including those conferring resistance to methicillin, vancomycin, and tetracycline. The study emphasized the role of hospital wastewater as a hotspot for horizontal gene transfer, facilitating the spread of resistance among bacterial populations. Similarly, a study in Brazil by Chagas *et al*. (2011) identified *Staphylococcus aureus* and *Enterococcus* species in hospital wastewater, with significant resistance to methicillin and vancomycin, respectively. These studies collectively highlight the global burden of antibiotic resistance in hospital wastewater, driven by the selective pressure of antibiotic residues and the presence of pathogenic bacteria.

2.7.2. Studies Conducted in Nigeria and Sub-Saharan Africa

In Nigeria and Sub-Saharan Africa, research on antibiotic resistance in hospital wastewater is relatively limited compared to global studies, but emerging evidence points to a growing concern. A study by Fahrenfeld *et al*. (2013) in South Africa investigated the presence of ARGs in hospital wastewater, detecting high levels of genes conferring resistance to beta-lactams and tetracyclines. The study suggested that inadequate wastewater treatment infrastructure in many African hospitals exacerbates the dissemination of ARB into the environment.

Several studies across Nigeria have investigated the occurrence and resistance profiles of bacterial isolates in hospital wastewater, underscoring the public health risks associated with untreated effluent discharge.

In Maiduguri, at the State Specialist Hospital, Odu *et al*. (2022) examined the resistance profiles of bacteria isolated from hospital wastewater. The study revealed a wide range of bacterial species including *Enterobacter* spp., *Staphylococcus aureus*, *Pseudomonas* spp., *Proteus* spp., *Klebsiella* spp., *Salmonella* spp., *Vibrio cholerae*, *Morganella morganii*, and *Proteus vulgaris*. Alarmingly high resistance rates were reported, with 96.4% of isolates resistant to cloxacillin, 89.2% to augmentin, 88.9% to ampicillin, and 85.1% to tetracycline. Further analysis at the University of Maiduguri Teaching Hospital demonstrated that *Escherichia coli* exhibited 100% resistance to nalidixic acid, while ampicillin and cephalexin showed resistance levels of 88%. Similarly, resistance to augmentin (75%), reflacine (63%), tarivid, gentamicin, and septrin (50%) was also observed, though comparatively lower resistance was recorded against ciprofloxacin and streptomycin (20%).

In Osun State, Bolaji *et al*. (2011) conducted a study on hospital wastewater from private hospitals in Ede. The isolates included *Enterobacter aerogenes*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Klebsiella edwardsii*, *Proteus mirabilis*, *P. aeruginosa*, *Shigella* spp., and *Flavobacterium meningosepticum*. Resistance testing showed that 100% of the isolates were resistant to Septrin, chloramphenicol, amoxicillin, and streptomycin. In addition, 90% resistance was observed against pefloxacin and tarivid, 80% against ciprofloxacin, and 70% against gentamicin, further illustrating the widespread multidrug resistance among bacterial isolates in hospital effluent.

A more recent study in Ondo State focused on the Okitipupa State Specialist Hospital, where Agboola *et al*. (2023) assessed the distribution and antimicrobial susceptibility of *Vibrio* species in hospital wastewater. The research was significant in reporting the presence of *Vibrio fortis* for the first time in Nigerian hospital wastewater. Alongside *V. cholerae* and other *Vibrio* species, many of the isolates exhibited multiple antibiotic resistance, highlighting the risk of waterborne disease outbreaks exacerbated by antimicrobial resistance.

In Ibadan, Oyo State, Adenike and Ayansina (2022) reported the occurrence of extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria in hospital wastewater. Out of 408 isolates obtained, 54 were confirmed as ESBL producers. These isolates exhibited alarming resistance rates: ceftazidime (100%), cefotaxime (74%), azithromycin (59%), sulfamethoxazole-trimethoprim (65%), streptomycin (76%), oxacillin (50%), ciprofloxacin (39%), and cefepime (31%). Molecular analysis revealed the presence of resistance genes including *blaTEM-1* (85%), *blaSHV-2* (20%), *blaSHV-1* (6%), and *blaCTX-M* (7%), confirming the genetic drivers of resistance in hospital effluent bacteria.

Similarly, a study conducted in Akure, Ondo State, by Emoruwa and Omoya (2024) investigated the detection and survivability of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospital wastewater. The results indicated high bacterial counts in hospital effluent compared with negligible levels in control samples. The *S. aureus* isolates demonstrated resistance to ampiclox (62.5%), oxacillin (43.8%), zinnacef (62.5%), and amoxicillin (50%). Notably, the presence of the *mecA* gene was confirmed, and MRSA isolates were shown to survive under varying pH, temperature, and salinity conditions, suggesting a high potential for persistence and dissemination in the environment once discharged.

Collectively, these studies provide compelling evidence that hospital wastewater in Nigeria is a major reservoir for multidrug-resistant bacteria and resistance genes. The detection of clinically important pathogens such as ESBL-producing Enterobacteriaceae, MRSA, and multidrug-resistant *Vibrio* spp. highlights the urgent need for stricter wastewater management policies and effective treatment strategies to mitigate environmental contamination and protect public health.

2.8. Control and Mitigation Strategies of Hospital Wastewater

The growing recognition of hospital wastewater as a reservoir for antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) has necessitated the development of control and mitigation strategies to minimize its impact on public health and the environment. Effective interventions must integrate technological, clinical, policy-driven, and multidisciplinary approaches to ensure sustainable solutions.

2.8.1. Wastewater Treatment Technologies for Hospital Effluents

Conventional wastewater treatment plants (WWTPs) are often insufficient for the complete elimination of ARB and ARGs, as many standard biological and physicochemical methods are unable to degrade antibiotic residues or fully inactivate resistant organisms (Rizzo *et al*., 2013). To address this challenge, advanced treatment technologies have been proposed and implemented in various contexts. Membrane bioreactors (MBRs), for example, provide high removal efficiency for pathogens and micropollutants by combining biological degradation with physical filtration (Michael *et al*., 2013). Similarly, advanced oxidation processes (AOPs), such as ozonation, UV/H_2O_2 treatment, and photocatalysis, have shown promising results in degrading antibiotic compounds and reducing ARG abundance (Paulshus *et al*., 2019). Activated carbon adsorption and constructed wetlands are also emerging as eco-friendly approaches that enhance the removal of pharmaceutical residues and resistant bacteria (Verlicchi *et al*., 2015).

In Nigeria and other low- and middle-income countries (LMICs), many hospitals discharge untreated or partially treated effluents directly into municipal sewers or natural water bodies, increasing the risk of AMR dissemination (Osińska *et al*., 2019). Therefore, the adaptation of cost-effective decentralized treatment systems, such as waste stabilization ponds, constructed wetlands, and solar disinfection (SODIS), could serve as practical solutions to mitigate risks while strengthening local capacities.

2.8.2. Role of Antimicrobial Stewardship in Reducing Environmental Load

Hospital wastewater contamination with ARB and ARGs is directly linked to the clinical misuse and over-prescription of antibiotics. Antimicrobial stewardship programs (ASPs) aim to optimize antibiotic use by ensuring the right drug, dose, and duration are applied, thereby reducing the amount of residual antibiotics excreted into wastewater systems (Dyar *et al*., 2017). By decreasing unnecessary antibiotic prescriptions, ASPs not only reduce selective pressure within patients but also diminish the environmental release of active antibiotic compounds.

Furthermore, infection prevention and control (IPC) measures, such as hand hygiene, proper waste segregation, and disinfection practices, indirectly minimize the microbial load entering hospital effluents (WHO, 2019). Integrating ASPs with waste management protocols creates a dual benefit of safeguarding patient health while reducing ecological exposure to resistance determinants.

2.8.3. Policy and Regulatory Frameworks (WHO, Nigeria's AMR Action Plan)

International bodies such as the World Health Organization (WHO) have emphasized the critical importance of regulating hospital effluents in the global fight against antimicrobial resistance (WHO, 2017). WHO's Global Action Plan on AMR identifies wastewater management as a key environmental component of AMR containment. Similarly, the European Union has included hospital effluent management in its directives on water quality, pharmaceutical disposal, and environmental safety (Kümmerer *et al*., 2018).

In Nigeria, the National Action Plan on Antimicrobial Resistance (2017–2022) underscores the need for effective environmental surveillance and the development of guidelines for wastewater management in healthcare facilities (Federal Ministry of Health Nigeria, 2017). However, implementation has been slow due to infrastructural, financial, and technical constraints. Strengthening regulatory enforcement through collaboration between the Federal Ministry of Environment, the National Agency for Food and Drug Administration and Control (NAFDAC), and local governments is critical to ensuring compliance with wastewater treatment standards.

2.8.4. The One Health Approach to Wastewater Management and AMR Containment

The complexity of AMR dissemination through hospital wastewater necessitates the adoption of the One Health approach, which recognizes the interconnectedness of human, animal, and environmental health. Hospital effluents containing ARB and ARGs may contaminate rivers used for aquaculture, fields irrigated with untreated wastewater, or groundwater accessed by communities, thereby bridging the gap between clinical and environmental reservoirs of resistance (Larsson *et al*., 2018).

A One Health framework advocates for cross-sectoral collaboration between healthcare providers, veterinarians, environmental scientists, and policymakers to implement holistic solutions. For example, joint monitoring programs could track AMR trends across hospital effluents, livestock farms, and community water sources. Similarly, integrating wastewater management into national AMR surveillance systems would provide a more accurate picture of environmental risks and guide interventions.

In Nigeria, operationalizing One Health for wastewater management requires coordinated investment in laboratory infrastructure, environmental microbiology research, and public awareness campaigns. By linking stewardship, wastewater treatment, and policy enforcement under the One Health umbrella, sustainable containment of AMR in hospital effluents can be achieved.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Benin City, Edo State, Nigeria. Benin City is the capital of Edo State and is characterized by a tropical rainforest climate with high humidity and annual rainfall ranging between 1,500 mm and 2,500 mm. The city hosts several public and private healthcare facilities that generate various forms of biomedical waste, including liquid effluents discharged into surrounding drains and water bodies. Four selected private hospitals within the city were used for this study. These hospitals were purposively chosen based on their patient load, wastewater disposal systems, and accessibility.

3.2 Study Design

A cross-sectional experimental study design was employed. Wastewater samples were collected from four private hospitals once weekly for three consecutive weeks, making a total of 12 wastewater samples. The samples were subjected to bacteriological analysis, enumeration, isolation, and identification, followed by antibiotic susceptibility testing of bacterial isolates.

3.3 Sample Collection

Wastewater samples were collected directly from the main effluent discharge points of the hospitals using sterile 1.5 L plastic containers. Before collection, containers were pre-sterilized with 70% ethanol and rinsed thoroughly with sterile distilled water. Approximately 500 mL of wastewater was collected per sample. Each container was tightly sealed, labeled with the date, time, and location of collection, and immediately transported in an ice-packed cooler to the Microbiology Laboratory, University of Benin, for analysis within 4 hours of collection.

3.4 Sterilization of Materials

Materials such as Petri-dishes, pipette, glass containers (conical flask, round bottom flask) and bottles were washed, drained and dried. They were wrapped with aluminum foil and sterilized in a hot-air oven at 160^{o}C for an hour. They were allowed to cool after sterilization before usage. An aseptic working environment was achieved with the use of Bunsen burner flame and disinfection of work surfaces with alcohol.

3.4.1 Preparation and Sterilization of media

Materials used include; Glass wares such as test tubes, beakers, conical flasks, Petri-dishes, McCartney bottles, Sterile cotton swabs, Sterile gloves, Normal saline, Sterile sampling containers, stirring glass rod and measuring cylinder. Media and Biochemical test reagents and Gram's staining kit. All glassware which include MacCartney bottles, Petri dishes, test tubes, conical flasks, measuring cylinders and pipettes, were sterilized at 160 ^{o}C for 1 hr in a hot-air-oven before use. The media used in this study were sterilized at 121 ^{o}C for 15 min in an autoclave. Agar media, agar slant and biochemical reagents were prepared freshly and refrigerated at 3-4 ^{o}C. Aseptic conditions were ensured during inoculation and subculturing.

3.4.1.1 Preparation of Nutrient agar

Twenty eight grams (28 g) of nutrient agar was dissolved in 1000 ml of distilled water in a conical flask corked with cotton wool and foil paper and allowed to dissolve in 1000 ml of distilled water in a conical flask. The medium will be the placed in an autoclave to sterilize it for 15 minutes at 121 °C. After sterilization, the flask will be allowed to cool (Cheesbrough, 2006;).

3.4.1.2. Preparation of Citrate agar

Twenty-four point twenty-eight (24.28) grams of agar was dissolved in 1000 ml distilled water and heated to boiling, to dissolve the medium completely. It was then mixed properly and distributed in conical flasks. The medium was sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 mins and then left to cool before dispension on sterile Petri dishes (Cheesbrough, 2006; Harrigan and McCance, 2014).

3.4.1.3 Preparation of Triple Sugar Iron agar

Sixty-four-point six (64.6) g of powder was dissolved in 1L of distilled water and then heated to properly dissolve the mixture. The mixture was autoclaved to sterilize the agar before it is dispensed into tubes and sterilized again at 121 °C for 15 mins. The agar was then left to solidify with short slant and good butts (Cheesbrough, 2006).

3.5. Enumeration and Isolation of Bacterial Isolates

Upon arriving at the laboratory, each hospital wastewater sample was subjected to serial dilution followed by the pour plate method for isolating bacterial microorganisms. This method was employed to ensure accurate colony counts and to minimize overcrowding on the plates.

For each sample, 1 ml of hospital wastewater was aseptically transferred into a sterile test tube containing 9 ml of sterile normal saline. The mixture was vortexed thoroughly to ensure proper homogenization. A series of tenfold dilutions were prepared by transferring 1 ml of the initial solution into a second test tube containing 9 ml of sterile saline, thereby producing a 10^{-1} dilution. This process was repeated to create further dilutions (10^{-2}, 10^{-3}, and 10^{-4}).

The pour plate method was then used to isolate microorganisms from the diluted samples. For each dilution, the following steps were carried out: 1 ml of each dilution (from 10^{-1} to 10^{-4}) was pipetted aseptically into sterile Petri dishes. Approximately 15–20 ml of molten Nutrient Agar (cooled to about 45°C) was poured into each Petri dish and gently swirled to ensure even distribution of the inoculum. The plates were incubated in an inverted position at 37°C for 24–48 hours to allow for the growth of bacterial colonies.

After the incubation period, distinct colonies were counted using a colony counter. The number of colony-forming units (CFUs) per milliliter of hospital wastewater was calculated based on the dilution factor using the formula:

$$\frac{cfu}{ml} = \frac{number\ of\ colonies\ x\ dilution\ fold/series}{volume\ of\ inoculum}$$

(Willey *et al.*, 2008).

3.6 Phenotypic Identification of Bacteria from Samples

Following successful pour plate technique, isolation and culture was made from a single colony and characterized using cultural, morphological and biochemical methods using the Bergey's manual. Several tests such as Gram reaction, catalase, urease, indole, oxidase, sugar fermentation, citrate utilization, respective reaction on triple sugar iron agar tests were carried out to presumptively identify bacterial isolates (Holt *et al*., 1994).

3.6.1. Morphology identification

The morphological identity of each bacteria isolate was obtained by Gram staining so as to know the gram reaction, cell morphology and arrangement by viewing under the microscope. The Gram stain procedure is as follows:

A smear of the bacteria isolate was made on grease free slide and heat fix by passing over flame. The smear was flooded with crystal violet which is the primary stain for 1min then washed with distilled water. Subsequently the slides were flooded with Lugol's iodine solution for 30sec and then washed off with distilled water. 95% alcohol was used for decolorization for 10sec and immediately washed off with distilled water. Finally, the smear was counter stained with saffranin for 1min and washed off. The slides were allowed to air dry before observing under the microscope using an oil immersion objective lens of ×100 magnifications to view the slides.

3.7 Biochemical identification

Biochemical test was carried out so as to help in the identification of the bacteria isolates as phenotypic (cultural) characteristics is not sufficient. The various biochemical test carried out are shown below;

3.7.1 Oxidase test

This is mainly used to differentiate between *pseudomonas* from other gram-negative rod bacteria. Oxidase test was carried out to identify bacteria species that will produce cytochtome oxidase enzyme. *Staphylococcus aureus* and *Escherichia coli* which are gram positive and gram negative respectively were employed as control. A piece of filter paper using sterilizes wire loop 2-3 drops of freshly prepared oxidase reagent (1% aqueous tetramethyl-3-phenyl nediamine dichloride) was added. A positive oxidase test is indicated by purple colouration within 10 seconds (MacFaddin, 2000; Cheesbrough, 2006).

3.7.2 Indole production test

An indole test was carried out to demonstrate the ability of certain bacteria that can decompose amino acid tryptophane to indole. The indole production test is essential in identifying the

Enterobacteriaceae family that breaks down the amino acid tryptophan by releasing indole in the presence of intracellular enzymes called "tryptophanase." Several drops of Kovac'sindole reagent were placed on a filter paper. A portion of a pure isolated colony was smeared onto the reagent-saturated area of the filter paper. It was allowed and examined to observe for colour development within 2 - 3 minutes. In this spot test**,** indole combined with the reagents in the filter paper matrix to produce a blue-to-blue-green colour change on the bacterial smear and advers**e** reactions remained colourless or light pink (Holt *et al.,* 1994; Cappuccino and Sherman, 2014).

3.7.3 Citrate utilization test

The citrate utilization test is a part of the test used to differentiate organisms on their ability to utilize citrate as the primary energy source. A citrate test was performed to differentiate members of Enterobacteriaceae capable of fermenting citrate in the presence of the enzyme citrate. Simon's citrate agar contained citrate as significant energy and was prepared for inoculation on Petri dishes. Well-prepared and sterilized citrate agar plates were inoculated from the pure isolated culture by streaking the surface with a sterilized loop. The plates were then incubated at 37°C for 24 hours. There were changes in colour due to bacterial growth of the organisms on the medium due to citrate metabolism, which gave a positive citrate test. The shift in pH turns the bromothymol blue indicator in the medium from green to blue (positive result). A negative test was demonstrated with no growth, no colour change, or the colour of the medium remains green (MacFaddin, 2000; Cheesbrough, 2006).

3.7.4 Catalase test

This is a test to detect the presence or absence of catalase enzyme. The catalase enzyme catalyses the breakdowns of hydrogen peroxide to release free oxygen gas and the formation of water. A few drops of freshly prepared 3% hydrogen peroxide were added onto the bacterial isolates smeared on a slide. The production of gas bubble indicated catalase enzyme positive (Holt *et al.*, 1994; Cheesbrough, 2006).

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

3.7.5. Triple sugar iron (TSI) agar test

The Triple Sugar Iron (TSI) test is an ability to test an organism's capability to ferment sugars and to produce hydrogen sulphide (H_2S) or gas (O_2), or both. The test was used primarily to differentiate members of the *Enterobacteriaceae* family based on their sugar fermentation patterns and from other Gram-negative rods. An agar slant prepared of a TSI agar was used in carrying out this test in a sterile test tube at a slanted angle. The slanted medium was inoculated with TSA pure culture using a straight inoculation needle by stabbing first through the center to the bottom of the tube and streaking the agar slant's surface. After inoculations, the test tubes were covered with foil paper and left at an ambient temperature of 36°C to incubate for 24 hours. Reactions on test tubes were examined, and sugar fermentations were indicated by the production of H_2S, gas and a change in colours from red (alkaline) to yellow (acid). When an alkaline/acid (red top/yellow bottom) slant reaction appeared, it only indicated dextrose (glucose) fermentation. When an acid/acid (yellow top/yellow bottom) slant reaction appeared, it showed the fermentation of dextrose, lactose and/or sucrose. The appearance of an alkaline/alkaline (red top/red bottom) slant reaction represented the absence of sugar fermentation. The blackening of the medium in the slant indicated H_2S production. Bubbles, cracks, or bottom-raised space in the slanted agar indicated gas production (formation of CO_2 and H_2) (Fawole and Oso, 2007).

3.8. Antibiotic susceptibility test

The antibiotic susceptibility profiles of the bacterial isolates were determined using the Kirby–Bauer disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI, 2020). Freshly cultured bacterial isolates (18–24 hours old) were used for the assay. Each isolate was first inoculated into sterile nutrient broth and incubated at 37 °C for 18–24 hours to obtain actively growing cultures. The turbidity of the bacterial suspension was then adjusted to correspond to 1.5×10^8 CFU/ml, equivalent to 0.5 McFarland standard, ensuring uniform inoculum density. Using a sterile cotton swab, the standardized inoculum was evenly spread on the surface of Mueller-Hinton Agar (MHA) plates to achieve a confluent lawn of growth. Commercially prepared antibiotic discs (Oxoid, UK) were aseptically placed on the inoculated agar plates using sterile forceps, ensuring adequate spacing between the discs to prevent overlapping of inhibition zones. The antibiotic discs and their respective concentrations used in this study were as follows: Pefloxacin (10 µg), Gentamicin (10 µg), Ampicillin (30 µg), Azithromycin (30 µg), Amoxicillin (30 µg), Rifampicin (20 µg), Ciprofloxacin (10 µg), Streptomycin (10 µg), Cotrimoxazole (30 µg), and Erythromycin (15 µg). The inoculated plates were incubated inverted at 37 °C for 24 hours, after which the zones of inhibition around each antibiotic disc were carefully measured using a transparent metric ruler. The results were interpreted according to the CLSI (2020) standard interpretive chart and categorized as Sensitive (S), Intermediate (I), or Resistant (R) based on the diameter of the inhibition zones (in millimeters).

3.8.1 Multiple Antibiotic Resistance (MAR) Index

This index is obviously a good tool which identifies the region where the isolates were obtained. Whether they are from places of high or low risks or from arears where antibiotics are abused. This tool becomes necessary for health risk assessment. According to Davis and Brown (2016), an index of ≥ 0.2 and above is indicative of a 'high-risk' contamination source. In this study the MAR index was determined by employing the methods delineated by Chitanand *et al.* (2010). The formula below was used to decipher MAR index of bacterial isolates.

$$MAR\ index = \frac{y}{nx}$$

$$where\ y = number\ of\ resistance\ scored,$$

$$n = number\ of\ isolates\ and$$

$$x = total\ number\ of\ antibioitics$$

It is a general established rule that MAR index greater than 0.2 is indicative of the fact that the bacterium originates from areas where antibiotics have been abused (or regularly used) or worse still from areas of high-risk source of contamination.

3.9. Statistical analysis

Data obtained in this study were collected and analysed using Microsoft excel and by statistical package for social scientist (SPSS) version 22.0 (SPSS Inc., Chicago, IL, USA). Normal distributed data was expressed as mean ± standard deviation and means were compared by analysis of variance (Ogbeibu, 2014).

CHAPTER FOUR

RESULTS

Table 4.1 and Figure 4.1 present the mean total bacterial counts of wastewater samples from selected hospitals (Mount Gilead Hospital, Ebony Health Center, Echos Hospital, and Purity Clinic) over a three-week period. The lowest bacterial load was recorded at Purity Clinic in week 3 ($1.6 \pm 0.20 \times 10^3$ CFU/mL), while the highest was observed at Mount Gilead Hospital in week 3 ($5.6 \pm 0.38 \times 10^3$ CFU/mL). Ebony Health Center generally exhibited higher bacterial counts across the sampling weeks, with the highest value occurring in week 2 ($5.2 \pm 0.34 \times 10^3$ CFU/mL). Echo Hospital recorded moderate bacterial loads, with the peak value in week 2 ($4.7 \pm 0.31 \times 10^3$ CFU/mL).

Table 4.2 presents the cultural, morphological, and biochemical characteristics of the bacterial isolates recovered from wastewater samples. The isolates were identified as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Enterococcus* sp. and *Pseudomonas aeruginosa*.

The distribution of bacterial isolates across the selected hospitals is shown in Table 4.3. *Escherichia coli* was the most widely distributed, occurring in wastewater samples from all four hospitals. *Klebsiella* sp. and *Proteus* sp. were detected in three hospitals each, while *Staphylococcus aureus* was found in three hospitals as well. *Pseudomonas aeruginosa* was present in two hospitals, whereas *Enterococcus* sp. was only isolated from two hospitals.

The frequency of occurrence of the bacterial isolates is presented in Table 4.4. *E. coli* had the highest frequency of occurrence (27.3%), followed by *Klebsiella* sp. and *Proteus mirabilis*

(18.2% each). *Staphylococcus aureus* and *Pseudomonas aeruginosa* were isolated at equal frequencies of 13.6% each, while *Enterococcus* sp. had the lowest frequency (9.1%).

The antibiotic susceptibility profiles of the bacterial isolates are shown in Tables 4.5a and 4.5b. Among Gram-positive isolates, *Staphylococcus aureus* was resistant to ampicillin, amoxicillin, and cotrimoxazole, but sensitive to ciprofloxacin, pefloxacin, and gentamicin, while *Enterococcus* sp. showed multidrug resistance with sensitivity only to ciprofloxacin. For Gram-negative isolates, *E. coli* and *Klebsiella* sp. were resistant to ampicillin, amoxicillin, and cotrimoxazole, but sensitive to ciprofloxacin and gentamicin. *Pseudomonas aeruginosa* and *Proteus* sp. also exhibited multidrug resistance, though both remained sensitive to ciprofloxacin. Overall, fluoroquinolones (ciprofloxacin and pefloxacin) and gentamicin were the most effective antibiotics, whereas beta-lactams and cotrimoxazole were largely ineffective.

The Multiple Antibiotic Resistance (MAR) indices of the bacterial isolates are presented in Table 4.6. The MAR index values ranged from 0.3 to 0.5, with *Enterococcus* sp. and *Pseudomonas aeruginosa* recording the highest indices (0.5), indicating resistance to half of the antibiotics tested. *Proteus* sp. also showed a high MAR index of 0.4. In contrast, *E. coli*, *Klebsiella* sp. and *Staphylococcus aureus* had relatively lower MAR indices of 0.3. These findings imply that hospital wastewater in Benin City harbors bacteria with significant resistance to multiple antibiotics, suggesting possible selective pressure from indiscriminate antibiotic use.

Table 4.1: Mean Total Bacterial Count ($\times 10^3$ cfu/mL) of Wastewater Samples from Selected Hospitals Over Three Weeks

Weeks	Mount Gilead Hospital	Purity Clinic	Ebony Health Center	Echos Hospital
Week 1	$4.2 \pm 0.40 \times 10^3$	$2.1 \pm 0.21 \times 10^3$	$4.6 \pm 0.32 \times 10^3$	$3.1 \pm 0.29 \times 10^3$
Week 2	$3.9 \pm 0.42 \times 10^3$	$1.8 \pm 0.23 \times 10^3$	$5.2 \pm 0.34 \times 10^3$	$4.7 \pm 0.31 \times 10^3$
Week 3	$5.6 \pm 0.38 \times 10^3$	$1.6 \pm 0.20 \times 10^3$	$4.9 \pm 0.30 \times 10^3$	$3.4 \pm 0.28 \times 10^3$

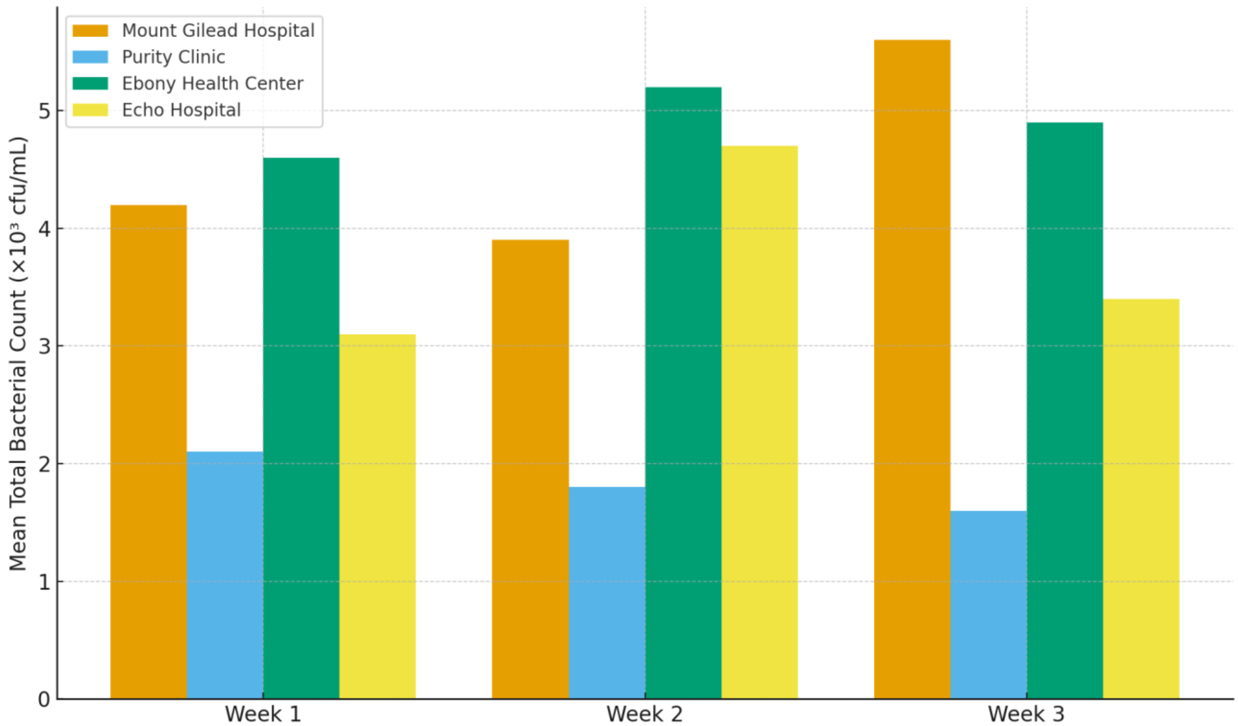

Figure 4.1. Mean Total Bacterial Count (×10³ cfu/mL) of Wastewater Samples from Selected Hospitals Over Three Weeks

Table 4.2: Cultural, Morphological and Biochemical Characteristics of Bacterial Isolates

Characteristics	1	2	3	4	5	6
Elevation	Raised	Flat	Flat	Flat	Raised	Flat
Margin	Entire	Undulate	Entire	Irregular	Entire	Irregular
Colony Colour	Golden yellow	Cream	Cream	Colourless	Cream/White	Greenish (pigmented)
Colony Shape	Circular	Irregular	Circular	Irregular	Circular	Irregular
Colony Size	Medium	Large	Large	Large	Small	Medium
Gram Stain	+	–	–	–	+	–
Cell Type	Cocci	Rod	Rod	Rod	Cocci	Rod
Arrangement	Clusters	Disperse	Disperse	Disperse	Chains	Disperse
Gram Reaction Color	Purple	Pink	Pink	Pink	Purple	Pink
KOH String Test	–	+	+	+	–	+
Catalase	+	+	+	+	–	+
Indole	–	+	–	+	–	–
Citrate	+	–	+	+	–	+
Oxidase	–	–	–	–	–	+
Glucose	+	+	+	+	+	+
Sucrose	–	–	+	+	–	–
Lactose	–	+	+	–	–	–
Gas Formation	–	+	+	+	–	+
H_2S Formation	–	–	–	+	–	–
TSI (Slant/Butt)	K/A	A/AG	A/AG	K/A (H_2S + G)	K/K	K/A
Identity	*Staphylococcus aureus*	*Escherichia coli*	*Klebsiella* sp.	*Proteus* sp.	*Enterococcus* sp.	*Pseudomonas aeruginosa*

Keys:

(+) Positive test; (–) Negative test
(A) Acid; (K) Alkaline; (G) Gas; (H_2S) Hydrogen sulphide
KOH = Potassium hydroxide test
TSI = Triple sugar iron test

Table 4.3: Distribution of Bacterial Isolates

Bacterial Isolates	Mount Gilead	Purity Clinic	Ebony Health Center	Echos Hospital
Escherichia coli	+	+	+	+
Klebsiella sp.	+	–	+	-
Staphylococcus aureus	+	+	–	+
Pseudomonas aeruginosa	+	–	+	–
Proteus sp.	+	–	+	+
Enterococcus sp.	–	+	–	+

Table 4.4: Frequency of Occurrence of Bacterial Isolates

Bacterial Isolate	**Frequency**	**Percentage (%)**
Escherichia coli	6	27.3
Klebsiella sp.	4	18.2
Staphylococcus aureus	3	13.6
Proteus sp.	4	18.2
Enterococcus sp.	2	9.1
Pseudomonas aeruginosa	3	13.6

Table 4.5a: Antibiotics Sensitivity Test for Gram-Positive Bacteria

Bacteria Isolates	PEF	CN	APX	Z	AM	R	CPX	S	SXT	E
Staphylococcus aureus	20 (S)	18 (S)	10 (R)	14 (I)	9 (R)	11 (I)	22 (S)	15 (I)	10 (R)	13 (I)
Enterococcus sp.	10 (R)	16 (I)	8 (R)	12 (I)	7 (R)	10 (R)	18 (S)	14 (I)	9 (R)	12 (I)

Table 4.5b: Antibiotics Sensitivity Test for Gram-Negative Bacteria

Bacteria Isolates	PEF	CN	APX	Z	AM	R	CPX	S	SXT	E
Escherichia coli	14 (I)	19 (S)	9 (R)	12 (I)	8 (R)	11 (I)	21 (S)	18 (S)	10 (R)	15 (I)
Pseudomonas aeruginosa	10 (R)	17 (S)	8 (R)	13 (I)	9 (R)	10 (R)	19 (S)	15 (I)	9 (R)	14 (I)
Klebsiella sp.	13 (I)	18 (S)	10 (R)	14 (I)	8 (R)	12 (I)	20 (S)	22 (S)	10 (R)	13 (I)
Proteus sp.	18 (S)	16 (I)	9 (R)	11 (I)	7 (R)	10 (R)	18 (S)	14 (I)	9 (R)	12 (I)

Keys:

0–10 mm = Resistant (**R**)
11–16 mm = Intermediate (**I**)
≥17 mm = Sensitive (**S**)
PEF: Pefloxacin (10 µg)
CN: Gentamicin (10 µg)
APX: Ampicillin (30 µg)
Z: Azithromycin (30 µg)
AM: Amoxicillin (30 µg)
R: Rifampicin (20 µg)
CPX: Ciprofloxacin (10 µg)
S: Streptomycin (10 µg)
SXT: Cotrimoxazole (30 µg)
E: Erythromycin (15 µg)

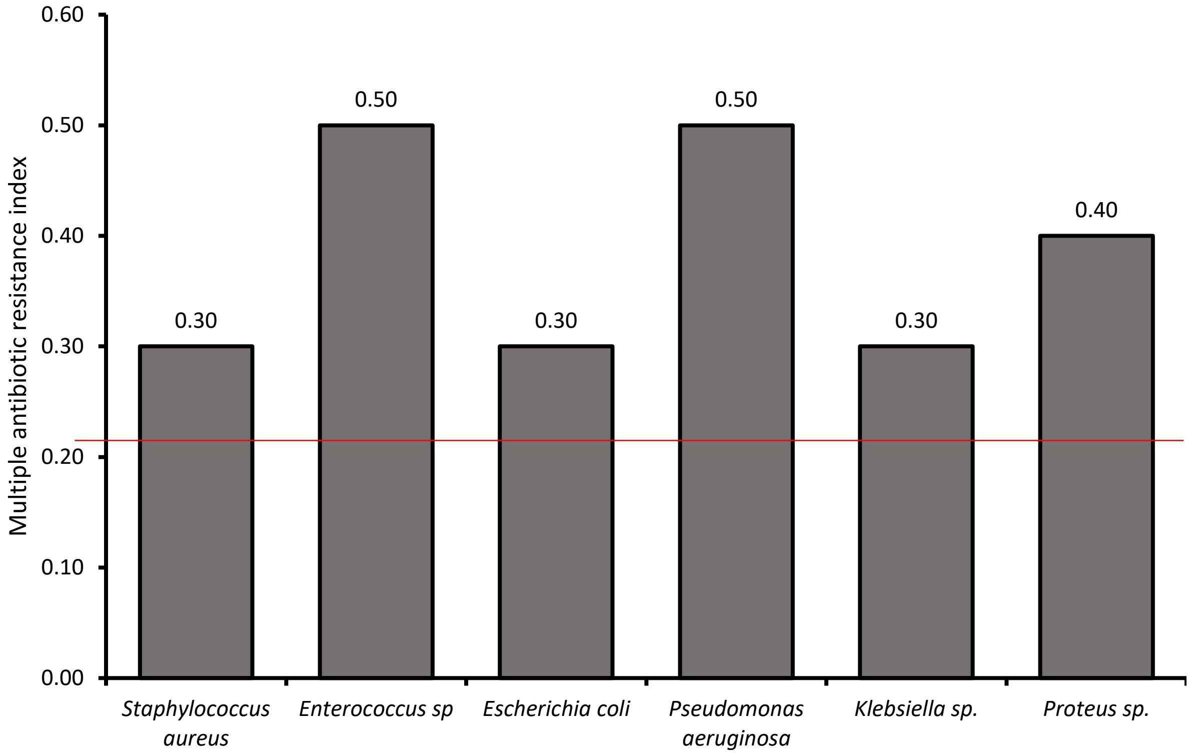

Figure 4.2: Multiple Antibiotic Resistance (MAR) Index of Bacterial Isolates

CHAPTER FIVE

DISCUSSION

Hospital wastewater is a complex mixture of water, chemicals, and pathogenic microorganisms that originate from hospital activities, including patient care, laboratory work, and medical procedures. Such effluents are recognized as significant reservoirs of antibiotic-resistant bacteria (ARB) and other microbial contaminants, posing a major threat to public and environmental health (Kümmerer, 2009; Rizzo *et al*., 2013; Fatimazahran *et al.*, 2023). The presence of these microorganisms in hospital effluents is often associated with the indiscriminate use of antibiotics and inadequate wastewater treatment processes (Haque *et al*., 2018). In Benin City, like in many urban areas in Nigeria, hospital wastewater is commonly discharged into municipal sewage systems or directly into nearby water bodies without sufficient treatment, creating a potential pathway for the dissemination of multidrug-resistant pathogens into the environment (Męcik *et al*., 2024; Tilahun *et al.*, 2025). This study seeks to evaluate the bacteriological quality, distribution, and antibiotic resistance patterns of bacteria isolated from wastewater collected from selected private hospitals in Benin.

The mean total bacterial counts of wastewater samples varied across the hospitals and sampling weeks, with Mount Gilead Hospital recording the highest bacterial load ($5.6 \pm 0.38 \times 10^3$ CFU/mL in Week 3) and Purity Clinic the lowest ($1.6 \pm 0.20 \times 10^3$ CFU/mL in Week 3) (Table 4.1, Figure 4.1). Ebony Health Center consistently exhibited high bacterial counts, peaking at $5.2 \pm 0.34 \times 10^3$ CFU/mL in Week 2. These variations may reflect differences in patient turnover, hospital size, sanitation practices, and the types of medical procedures performed (Michael *et al*., 2018). These findings align with studies by Deguenon *et al*. (2022) and Eze *et al*. (2016), who reported high microbial loads in hospital wastewater in Benin and Enugu, Nigeria, respectively.

The bacterial counts observed in this study also corroborate with previous studies indicating that hospital effluents typically harbor high microbial loads, which include both pathogenic and opportunistic bacteria (Kümmerer, 2009; Rizzo *et al*., 2013). High microbial loads in wastewater are particularly concerning as they may serve as hotspots for horizontal gene transfer of antibiotic resistance genes (ARGs) among bacteria (Haque *et al*., 2018).

Six bacterial species were identified in the wastewater samples: *Escherichia coli*, *Klebsiella sp.*, *Proteus* sp., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus* sp. (Table 4.2). Among these, *E. coli* was the most widely distributed, occurring in all ho*sp*itals, with a frequency of 27.3% (Table 4.4). *Klebsiella sp.* and *Proteus sp.* were detected in three hospitals each, while *S. aureus* was present in three hospitals. *P. aeruginosa* and *Enterococcus sp.* were less common, found in two hospitals each.

The dominance of *E. coli* suggests significant fecal contamination, as this species is a recognized indicator of fecal pollution in wastewater (Okoh *et al*., 2010). *Klebsiella sp.* and *Proteus sp.* are opportunistic pathogens capable of causing urinary tract infections, wound infections, and sepsis (Patel *et al*., 2015). The detection of *S. aureus* and *P. aeruginosa* reflects the presence of clinically relevant pathogens that can survive and proliferate in wastewater environments, highlighting potential public health risks (Rizzo *et al*., 2013).

The antibiotic susceptibility tests revealed multidrug resistance among both Gram-positive and Gram-negative isolates (Tables 4.5a and 4.5b). Among Gram-positive bacteria, *S. aureus* exhibited resistance to ampicillin, amoxicillin, and cotrimoxazole but remained sensitive to ciprofloxacin, pefloxacin, and gentamicin. *Enterococcus* sp. showed multidrug resistance, being sensitive only to ciprofloxacin.

Gram-negative bacteria also demonstrated concerning resistance patterns. *E. coli* and *Klebsiella* sp. were resistant to beta-lactams and cotrimoxazole but sensitive to fluoroquinolones and gentamicin. Similarly, *P. aeruginosa* and *Proteus* sp. exhibited multidrug resistance, although both were susceptible to ciprofloxacin. Overall, fluoroquinolones (ciprofloxacin and pefloxacin) and gentamicin were the most effective antibiotics, whereas beta-lactams and cotrimoxazole were largely ineffective. These patterns are consistent with findings from studies in Nigeria and other West African countries, which have reported high levels of antibiotic resistance in hospital wastewater (Hotor *et al*., 2025). The widespread resistance to commonly used antibiotics such as ampicillin and amoxicillin suggests selective pressure from frequent antibiotic use in hospital settings, which promotes the survival of resistant strains (Haque *et al*., 2018; Rizzo *et al*., 2013).

The MAR indices of the isolates ranged from 0.3 to 0.5, with *Enterococcus* sp. and *P. aeruginosa* recording the highest indices (0.5), indicating resistance to half of the antibiotics tested (Figure 4.2). *Proteus sp.* also exhibited a high MAR index of 0.4, whereas *E. coli*, *Klebsiella sp.*, and *S. aureus* had lower indices (0.3).

MAR indices greater than 0.2 are indicative of environments with high antibiotic exposure and selective pressure (Krumperman, 1983). The high MAR values observed in this study suggest that hospital wastewater in Benin City constitutes a reservoir for multidrug-resistant bacteria, emphasizing the need for proper wastewater management and treatment to mitigate the spread of resistant pathogens into the environment. Similar MAR index values have been reported in studies by Deguenon *et al*. (2022) and Eze *et al*. (2016), highlighting the widespread nature of antibiotic resistance in hospital wastewater in Nigeria. The presence of multidrug-resistant bacteria in hospital wastewater has serious implications for public health. Discharge of untreated effluents into municipal sewage or water bodies can facilitate the dissemination of antibiotic resistance genes to environmental bacteria, posing risks to communities through contaminated water, food, or recreational activities (Rizzo *et al*., 2013). This is particularly concerning in low- and middle-income countries like Nigeria, where wastewater treatment infrastructure is often inadequate. Studies by Fatima *et al*. (2022) and Deguenon *et al*. (2022) emphasize the need for effective wastewater treatment and stringent infection control measures to mitigate the spread of antibiotic resistance.

Furthermore, the detection of clinically significant pathogens such as *S. aureus*, *E. coli*, and *P. aeruginosa* indicates potential occupational hazards for hospital staff and wastewater workers, as well as risks for patients and surrounding communities. This underscores the urgent need for regulatory enforcement of hospital effluent treatment and monitoring programs.

5.1 Conclusion

This study has demonstrated that hospital wastewater in Benin City, Nigeria, harbors a diverse community of bacteria. The microbial loads varied across hospitals and sampling weeks, with certain facilities exhibiting consistently higher bacterial counts, indicating differences in wastewater management and hygiene practices. The antibiotic susceptibility testing revealed that both Gram-positive and Gram-negative isolates exhibited multidrug resistance, particularly to beta-lactams and cotrimoxazole, while remaining largely susceptible to fluoroquinolones and gentamicin. The Multiple Antibiotic Resistance (MAR) indices ranged from 0.3 to 0.5, with *Enterococcus sp.* and *Pseudomonas aeruginosa* showing the highest resistance, highlighting the potential for hospital wastewater to act as a reservoir of multidrug-resistant bacteria. These findings emphasize the public health risks associated with the discharge of untreated or inadequately treated hospital wastewater into the environment. The study underscores the urgent need for effective wastewater treatment, regular microbial monitoring, and stringent antibiotic stewardship programs to curb the dissemination of antibiotic-resistant bacteria. Hospital wastewater in Benin City represents a significant source of environmental contamination and a potential contributor to the spread of antimicrobial resistance. Addressing these challenges through improved management practices, policy enforcement, and public awareness is essential to protect both human health and the surrounding environment.

5.2 Recommendations

Based on the findings of this study, the following recommendations are made:

1. **Implementation of Effective Wastewater Treatment:** Hospitals should adopt effective wastewater treatment systems, including disinfection and microbial load reduction, before effluent discharge.
2. **Regular Monitoring of Hospital Effluents:** Routine microbiological and antibiotic resistance surveillance should be conducted to detect emerging multidrug-resistant bacteria.
3. **Rational Use of Antibiotics:** Hospitals should enforce antimicrobial stewardship programs to minimize indiscriminate antibiotic use and reduce selective pressure for resistant strains.
4. **Public Awareness and Policy Enforcement:** Government and health authorities should enforce regulations on hospital effluent management and educate stakeholders about the environmental and health risks associated with untreated wastewater.

5. **Further Research:** Future studies should investigate the presence of antibiotic resistance genes in hospital wastewater and assess the effectiveness of treatment technologies in mitigating ARB dissemination.

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