

**ISOLATION AND IDENTIFICATION OF BACTERIA FROM SELECTED
WARDS WASTE WATER IN UNIVERSITY OF BENIN TEACHING
HOSPITAL (UBTH)**

BY

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CERTIFICATION

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

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APPROVAL

This project work was carried out by **Marvellous Oziohu OHIDA** in partial fulfilment of the award of a Bachelor of Science, B.Sc (Hons) degree in the Department of Microbiology, University of Benin, Benin City.

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DEDICATION

This project work is dedicated to God Almighty, for bringing me this far in life. I am truly grateful.

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I sincerely appreciate my parents, Apostle and Mrs. Patience Ohida Aliu, for their unwavering love, prayers, and support. Their encouragement has been a pillar of strength throughout my academic journey. I also extend my heartfelt gratitude to my siblings for their constant motivation and support.

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ABSTRACT

Hospital wastewater is a significant reservoir of pathogenic bacteria and antibiotic-resistant strains, posing serious public health and environmental risks. This study investigates the bacteriological analysis of wastewater samples collected from different wards at the University of Benin Teaching Hospital (UBTH), Benin City, Edo State, Nigeria. A total of four wastewater samples were obtained from different drainage outlets using sterile 500 mL glass bottles and transported under cold conditions to the Microbiology Laboratory of the University of Benin for analysis. The Total Heterotrophic Bacterial Count (THBC) varied across the sampled wards, with the highest bacterial load recorded in the laundry department ($8.00 \pm 0.05 \times 10^4 \log_{10}$ cfu/mL) and the lowest in the eye clinic ($3.12 \pm 0.23 \times 10^4 \log_{10}$ cfu/mL). Morphological, biochemical, and cultural analyses identified multiple bacterial species, including *Salmonella* sp., *Bacillus* sp., *Escherichia coli*, *Enterobacter* sp., *Pseudomonas* sp., *Streptococcus* sp., *Staphylococcus* sp., and *Klebsiella* sp. Among these, *Staphylococcus* sp. and *Pseudomonas* sp. were the most widely distributed, occurring in all sampled wards, while the eye clinic exhibited the lowest bacterial diversity. Antibiotic susceptibility testing revealed high resistance patterns among bacterial isolates, with *Pseudomonas* sp. displaying the highest resistance to multiple antibiotics such as Levofloxacin, Cefotaxime, Amoxicillin, and Azithromycin. Among Gram-

positive bacteria, *Staphylococcus* sp. exhibited the highest resistance, particularly to Rocephin and Erythromycin. The multiple antibiotic resistance (MAR) index ranged from 0.20 (*Klebsiella* sp. and *Bacillus* sp.) to 0.70 (*Pseudomonas* sp.), indicating substantial resistance to antibiotics. The findings highlight the potential health risks associated with hospital wastewater discharge and underscore the need for improved wastewater treatment practices to mitigate the spread of antibiotic-resistant bacteria. This study provides valuable data for hospital infection control and public health policies in Nigeria.

CHAPTER ONE

INTRODUCTION

1.0. Background of the study

Wastewater from healthcare facilities, particularly hospital effluents, represents a significant source of environmental contamination. This type of wastewater, often referred to as hospital wastewater (HWW), contains a unique combination of pathogenic microorganisms, chemical pollutants and pharmaceuticals that are not commonly found in other wastewater sources (Verlicchi *et al.*, 2012). Hospitals produce wastewater through a variety of activities, including patient care, laboratory analyses, radiological imaging and the use of disinfectants and antiseptics. Due to the presence of these contaminants, hospital effluent is typically more hazardous than domestic wastewater, making its management a public health priority. However, in many settings, including urban centers such as Benin City in Nigeria, hospital wastewater is often discharged directly into sewage systems or nearby water bodies without adequate treatment. This discharge leads to the widespread dissemination of potentially harmful agents into the environment and poses substantial risks to human health and ecological stability, especially in urban areas with densely populated medical facilities such as the University of Benin Teaching Hospital (UBTH) in Edo State, Nigeria.

The composition of hospital wastewater is complex, comprising both organic and inorganic materials. These contaminants arise from a range of hospital activities and processes, contributing to the high toxicity and variability of HWW. The main components typically include pharmaceuticals (antibiotics, analgesics, cytostatic drugs), heavy metals (such as

mercury from dental amalgams or x-ray developers), disinfectants, and solvents (Verlicchi *et al.*, 2010). Some of the most concerning contaminants are antibiotics, which are excreted by patients and disposed of by medical staff. Residual antibiotics in HWW contribute to the selection of antibiotic-resistant bacteria within hospital effluents. Additionally, heavy metals and solvents can have toxic effects on both microorganisms and aquatic organisms, as they bioaccumulate and disrupt biological processes at multiple levels in the ecosystem (Prado *et al.*, 2014). Studies on HWW in different settings consistently reveal that the concentration of these contaminants exceeds those found in regular domestic wastewater, which reflects the unique medical and laboratory practices within healthcare facilities.

The bacteriological profile of HWW is particularly concerning due to the high concentration of pathogenic bacteria, many of which are resistant to commonly used antibiotics. These pathogens typically originate from patients undergoing treatment for various infections, as well as from medical instruments and surfaces that are regularly disinfected. Commonly isolated bacterial genera from hospital wastewater include *Escherichia*, *Pseudomonas*, *Klebsiella*, and *Staphylococcus* (Mendoza *et al.*, 2017). These bacterial genera are frequently associated with hospital-acquired infections, such as urinary tract infections, pneumonia, and wound infections. The discharge of these pathogens into the environment increases the likelihood of resistance gene transfer among bacterial populations, leading to a higher incidence of antibiotic-resistant infections in the community. The presence of these pathogens in untreated wastewater has been shown to impact local water systems, as these bacteria can survive in sewage systems and natural water bodies, potentially contaminating drinking water sources and agricultural land.

The physicochemical properties of HWW are another crucial aspect of its impact on the environment and human health. Hospital wastewater typically exhibits high levels of biological oxygen demand (BOD) and chemical oxygen demand (COD), which indicate a significant amount of organic matter that can deplete oxygen levels in aquatic environments (Kümmerer, 2009). Furthermore, HWW often contains high levels of nitrogen and phosphorus, which can lead to nutrient pollution in receiving water bodies. Excessive nitrogen and phosphorus levels promote eutrophication, a process that results in algal blooms, oxygen depletion, and the death of aquatic organisms. Additional physicochemical characteristics, such as elevated levels of heavy metals and toxic chemicals, further compromise the quality of water bodies, affecting both microorganisms and higher organisms. The treatment and safe disposal of HWW are essential to prevent these contaminants from spreading and persisting in the environment, where they can have long-term ecological consequences.

The environmental release of untreated or inadequately treated hospital wastewater poses numerous risks to both human health and ecosystems. Pathogens present in HWW can cause infections in humans, especially in populations with compromised immune systems, such as the elderly and young children. More critically, the discharge of antibiotic-resistant bacteria (ARB) into natural water bodies exacerbates the global health crisis of antibiotic resistance. When ARB enter water systems, they interact with native microbial communities, transferring resistance genes through horizontal gene transfer. This increases the potential for resistance to spread across different bacterial species, rendering common antibiotics less effective in treating infections. Additionally, pharmaceuticals, heavy metals, and disinfectants present in HWW

disrupt the biological balance in aquatic environments, harming fish, invertebrates, and plants, and potentially entering the food chain (Emmanuel *et al.*, 2005).

Research on the microbial and chemical composition of hospital wastewater has become increasingly significant, as the public health implications of such effluents are being recognized worldwide. Numerous studies have documented the persistence of multi-drug-resistant bacteria in HWW across different regions, including within Nigeria. Agunwamba *et al.* (2018) highlight the urgent need to address the environmental impact of untreated hospital wastewater in Nigeria, noting that multi-drug-resistant pathogens are widespread in healthcare effluents. Similar studies underscore the importance of developing and implementing effective treatment protocols to mitigate the risks posed by HWW. By isolating and characterizing pathogenic bacteria, researchers aim to better understand the scale of bacterial contamination and resistance profiles, ultimately supporting the development of informed policies and practices for hospital wastewater management.

The isolation and characterization of pathogenic bacteria in hospital wastewater is a critical aspect of understanding and mitigating the risks associated with HWW. This research not only highlights the health risks posed by pathogens and contaminants in HWW but also underscores the urgent need for effective wastewater treatment solutions.

1.1. Aim

The aim of this study is to isolate and identify bacteria from hospital wastewater at the University of Benin Teaching Hospital (UBTH), Benin City, Edo State, Nigeria.

The specific objectives were to:

1. enumerate and isolate bacterial from hospital wastewater samples
2. assess the percentage occurrence of bacterial isolates
3. determine the antibiotic resistance profiles of the isolated bacterial isolates

CHAPTER TWO

LITERATURE REVIEW

2.0. Hospital Wastewater (HWW)

Hospital wastewater (HWW) is distinct from other wastewater sources due to its hazardous and infectious nature. It comprises a wide range of micro and macro pollutants released from operating rooms, patient wards, laboratories, laundries, clinics, research units, radiology departments, and microbiology laboratories (Al-Enazi, 2016). The pollutants include radioactive isotopes, pharmaceutical residues, stock cultures, heavy metals, and pathogenic microorganisms, among others (Ahsan, 2012). Pharmaceutical components, such as contraceptive drugs, are associated with endocrine disruption effects, such as sex reversal in aquatic organisms, which impairs reproduction (Obasi *et al.*, 2014). Moreover, untreated hospital wastewater is a source of emerging infectious agents like prions, viroids, and toxins, posing significant environmental and public health risks (Vieno, 2007).

Despite treatment in conventional wastewater treatment plants (WWTPs), pharmaceutical and microbial contaminants often persist due to the ineffectiveness of existing methods in removing these micro-pollutants (Verlicchi *et al.*, 2010). The discharge of untreated or inadequately treated HWW can disrupt microbial communities in WWTPs, negatively impact nutrient removal processes, and introduce heavy metals that are non-biodegradable (Emmanuel *et al.*, 2009). HWW serves as a reservoir for antibiotic-resistant bacteria and genes (ARGs), which can spread in the environment, increasing public health risks (Asfaw *et al.*, 2017). The release of untreated HWW has been linked to skin infections, gastrointestinal diseases, and increased environmental contamination with resistant bacteria, fungi, and other pathogens (Ibrahim *et al.*, 2018).

2.1. Microorganisms in Hospital Wastewater

2.1.1. Pathogenic Bacteria

Hospital wastewater is a significant source of pathogenic bacteria, including both drug-resistant and opportunistic strains. Indicator bacteria such as *Escherichia coli* and *Clostridium perfringens* have been traditionally used to assess water pollution. However, the removal of coliforms does not ensure the elimination of other pathogens, such as viruses and protozoa (El-lathy *et al.*, 2009).

Studies reveal that *Bacillus* species dominate HWW (80-90%), with *Staphylococcus* and *Streptococcus* species accounting for 5-10% (Onyeleke and Istifanus, 2009). Multidrug-resistant (MDR) strains such as *Acinetobacter*, *Enterococcus*, and *Pseudomonas* species are prevalent, with *Staphylococcus aureus* being a prominent gram-positive bacterium exhibiting significant MDR levels (Chitins, 2000). Other nosocomial bacteria frequently isolated from HWW include *E. coli*, *Klebsiella*, *Proteus*, and *Enterobacter* species. Pathogenic *E. coli* strains, such as O157, are associated with foodborne outbreaks and severe diarrhea (Wasey and Salen, 2018). Opportunistic pathogens like *Pseudomonas aeruginosa* and *Proteus mirabilis* have been linked to urinary tract infections and other health complications (Armbruster *et al.*, 2018).

Hospital effluents also harbor anaerobic bacteria like *Bifidobacteriales*, *Clostridiales*, and *Bacteroidales*, originating primarily from human gut flora (Baricz *et al.*, 2018). The presence of antibiotic-resistant bacteria (ARB) in HWW highlights the challenges of antimicrobial resistance (AMR), exacerbated by the improper disposal of hospital effluents (Galvin *et al.*, 2010).

In addition to identifying resistant strains, recent studies have explored the antimicrobial potential of bacterial extracts, such as those from *Janthinobacterium lividum*, which exhibit activity against MDR pathogens (Baricz *et al.*, 2018). These findings underscore the need for advanced treatment technologies to mitigate the spread of resistant bacteria and safeguard public health.

2.1.2. Fungi

The microbiological examination of a few HWWs showed the prevalence of contagious species alongside bacterial and coliform species. The fungi have easier nutritional requirements and have higher ability to develop at lower water movement as contrasted to bacteria (Efaq *et al.*, 2017). Moreover, the contagious populaces can without much of a stretch spread their spores to outside environment, subsequently influencing the humans directly (Efaq *et al.*, 2015).

This is the justification behind its prevalence in hospital and clinical environment because nature and environment factors such as temperature, moisture, and nutrients could provide easy and favorable conditions for the extensive growth of fungal species in storage containers holding clinical waste. The fungal diseases could go from moderate to deadly state on the contamination site just as insusceptible arrangement of the influencing individual. The predominance of intrusive fungi species has been accounted to increase since thirty years, because of expansion in number of immunocompromised patients. The moderate fungi disease may include athlete foot and ring worm diseases (cutaneous contaminations) in insusceptible patients, life-threatening contaminations incorporate mucosal and Systematic infections (Srinivasan *et al.*, 2014). Neely

and Orloff, (2001), reported increase of *Aspergillus species* in the medical waste gathered from the United States and noted it more severe than other, species.

2.1.3. Viruses

The viral contaminations are in front line as contrasted to other pathogenic illnesses brought about by bacteria, fungi, and parasites. Also, the characteristics and epidemiology of virus and the study of disease transmission of infection is completely unique in relation to bacteria in light of the fact that the bacterial markers used to address water pollution can't really address viral contamination. It has been accounted for that every one of the material present in the medical clinic waste can convey viruses, which can subsequently survive over a period of 58 days (Wiederhold. 2017).The viral hepatitis is extremely normal and leading sickness. Additionally, human immunodeficiency viral infection contaminations, Hepatitis B, and Hepatitis C are among the dangerous irresistible sicknesses, communicated by direct contact of blood from infected to another. Be that as it may, it is simple to forestall, once identified at beginning phases and mandatory safeguards are followed by patients (Akim, 2016). The samples gathered from various WWTPs have been broke down for identification and characterization, and even research has been centered on removal of viruses from wastewater (Barrella *et al.*, 2009).

2.1.4. Parasites

Parasites are for the most part transmitted through fecal oral course under poor sterile conditions, polluted water and food sources, and unhygienic wastewater removal practices. The parasites in their incapable stages like cysts, eggs, and oocysts survives under natural unfriendly conditions and through numerous wastewater treatment measures due to the presence of defensive external layer. In this manner parasites have capacity to live in wastewater for broadened time-frame in

comparison to other micro-organisms such as bacteria and viruses (Koutsoumanis *et al.*, 2018). The examples gathered from clinic sewage treatment plant situated in Zaria, Nigeria contained a few eggs, cysts, and oocytes of different parasites. Around 1648 eggs, cysts, and oocysts were available per liter of wastewater.

Ascaris lumbricoides was the normal parasite found in HWW with 307 eggs for each liter (18.67%) of wastewater followed by 287 eggs (17.42%) of *Taeniaspp.*, 253 eggs (15.35%) of *Schistosomaspp.*, 176 eggs of *Toxocaraspp.*, 135 eggs (8.19%) of *Ancylostomaspp.*, 130 eggs (7.89%) of *Cryptosporidiumparvum*, and 58 eggs (3.52%) of *Giardialambila*. Besides, 92 eggs (5.58%) of *Trichuris* and *Hymenolepispp.* were likewise found in HWW. The cyst of *Entamoeba histolytica* and *A.lumbricoides* were found in many investigations and stayed viable for longer time frame in water effluents that was additionally used to water growing vegetables, in this way, entering the food chain and afterward affecting people directly. *C. parvum* causes the illness Cryptosporiadiasis and *G. lambila* causes Giardiasis, both could be hazardous whenever found in people with low immune system.

Child diarrhea is largely brought about by the *Cryptosporidium species* (Shanan *et al.*, 2015).*Taenia species* can cause cysticerocosis in people which can attack the muscles, brain and can eventually cause onset of seizures in grown-ups, while it can likewise cause *bovin cysticerocosis* in cattles after drinking of contaminated water.

The principle side effects of parasitic disease ranges from regurgitating, malabsorption, nausea, vomiting and stomach cramps, (Kumar *et al.*, 2014). Hospital effluents analyzed have shown that they are polluted with organic and inorganic poisons released into the sewage. In their

examination work, (Hameed and Al-Enazi, 2023), found that samples gathered from various segment of the hospital examined for physical and compound attributes, the pH from various segments shows a satisfactory range for disposal into the environment, while salinity was incredibly high and varies starting with one segment then onto the next. It additionally contains unsafe substances like pharmaceuticals residue, dangerous synthetic substances, microorganisms and radio isotopes which lead to chemical, biological and physical danger to the environment and affect the general wellbeing.

2.2. Hospital Wastewater Composition

Wastewater is any water, whose quality has been compromised adversely by human action. Its origin is basically domestic, domestic, agricultural and commercial exercises (Akter *et al.*, 1999). Hospital wastewater involves both the fluid and the dissolved substance created inside the hospital environment. Much of the time, hospital wastewater is like city wastewater which contains contaminations. Antibiotics resistant bacteria and viruses, drug to some degree metabolized, radioactive components and other heavy metals and harmful substance mixtures, for example, Cu, Fe, Cd, Pb, Hg, Ni, Pt, Cyanide, Phenol and others (Emmanuel *et al.*, 2005; Ekhaise and Omavwoya, 2008; Lenz *et al.*, 2007). The hazardous substances found in hospital effluents released in municipal wastewater collection point ultimately end up in the aquatic ecosystem, where they cause contamination of the system and alter the biological balance (Xiang *et al.*, 2018).

Olalla *et al.*,(2018) in their study found the existence of some selected psychiatric pharmaceuticals from three different hospitals in Shanghai China with a view to elucidate the effects of the substances in surface water, ground water, soil and plant. High concentrations of

Lorazepam, Carbamazepine and Diazepam were found in surface water while Lorazepam was higher in ground water. The study found low risk of these drugs in aquatic organisms, but indicated that regular discharge of such affluent might be hazardous to the environment.

Hospital wastewater is accounted for to contain high measure of microbes including bacteria, viruses, protozoa and helminthes, which are effectively transmitted through water (Amouei *et al.*, 2015) announced total coliforms (TC) in wastewater of the clinics in Iran were between 1.8×10^9 and 2.4×10^3 MPN/100 ml. Different researches in Iranian clinics showed that TC numbers in wastewater in many hospitals of Tehran University of Medical Sciences were between 2.2×10^6 and 3.8×10^8 MPN/100 mL, and in Hospital of Iran University of Medical Sciences this was 1400 MPN/100 mL (Sarafraz *et al.*, 2007). Ekhaise and Omavwoya, (2008) described bacterial in wastewater from University of Benin Teaching Hospital (UBTH), Benin City, Nigeria.

They revealed total heterotrophic bacterial counts upsides of 1.9×10^7 to 78.3×10^{12} cfu/ml and total coliform counts of 1.2×10^3 to 1.6×10^3 MPN/100ml. The bacterial isolated were *Klebsiella*, *Pseudomonas*, *Escherichia*, *Serratia*, *Staphylococcus*, *Streptococcus*, *Proteus* and *Bacillus*. The bacterial genera, *Klebsiella*, *Pseudomonas* and *Serratia* were the most often circulated isolate in the hospital wastewater. Though different microorganisms in water are viewed as basic elements in adding to various waterborne outbreaks, they play numerous beneficial roles in wastewater influents. Usually, microorganisms are utilized in the auxiliary treatment of wastewater to eliminate disintegrated organic matter. The organisms are utilized in fixed film systems, suspended film systems or lagoon systems, depending upon the preference of the treatment plant. Their essence during the diverse treatment stages can enhance the degradation of solids, bringing

about less sludge creation (Ward-Paige *et al.*, 2005a). Wastewater organisms are likewise involved with nutrient recycling, like phosphate, nitrogen and heavy metals. If nutrients that are trapped in dead materials are not separated by microorganisms, they won't ever be available to assist with supporting the existence of different life forms. Microorganisms are likewise liable for the detoxification of corrosive mine seepage and different poisons in wastewater (Ward-Paige *et al.*, 2005).

2.2.1. Physicochemical Characteristics of Hospital Wastewater

Heavy metals of concern in wastewater treatment include arsenic, cadmium, chromium, cobalt, copper, iron, lead, mercury, silver, and zinc. While some metals, such as calcium, cobalt, chromium, copper, and zinc, are essential as macro and micronutrients for the growth and development of living organisms, others, such as silver, cadmium, gold, lead, and mercury, have no biological role and are considered non-essential (Hussein *et al.*, 2005).

Metals are among the most persistent pollutants in wastewater. Unlike organic pollutants, heavy metals cannot be degraded but instead accumulate in the food chain, posing significant risks to human health and ecological balance. Their presence in wastewater is primarily due to discharges from hospitals, residential homes, industrial sources, and groundwater infiltration (Hussein *et al.*, 2005; Silvia *et al.*, 2006). The risks associated with heavy and trace metal contaminants in water arise from their persistence in natural environments and their potential to bioaccumulate in successive levels of the food chain (Fuggle, 1983). Uncontrolled discharge of hospital wastewater significantly contributes to eco-toxicological hazards, posing threats to both the environment and human health.

To mitigate these risks, small-scale healthcare facilities should establish proper septic tanks and soakaway systems. Proper design, construction, operation, and monitoring of these systems are crucial to prevent odor nuisance, contamination, flooding, and other related issues. Regular maintenance and periodic monitoring of these systems are essential to ensure their effectiveness and minimize environmental impacts.

2.3. Pharmaceuticals in Hospital Wastewater

Drugs are huge constituents of medical wastewater Schuster *et al.*, (2008). Up to 90% of the medication residue can be found in the effluent leaving the treatment plant Cooper, (Siewicki and Phillips, 2008). Drugs include in excess of 4000 molecules with various physico-chemical and biological properties and distinct methods of biochemical activity (Boxall *et al.*, 2012). Some of the most representative pharmaceuticals found in receiving waters are analgesics, antibiotics, lipid controllers, anti-inflammatory, antiepileptic, and sedatives which are resistant to biological processes (Clara *et al.*, 2005; Anjaneyulu *et al.*, 2005).

2.3.1. Fate and Effects of Pharmaceuticals in Hospital Wastewater

Pharmaceutical compounds enter the environment through both direct and indirect routes. The direct route involves excretion of active pharmaceutical ingredients via urine and feces, while the indirect route occurs through improper disposal of unused or leftover drugs, such as flushing them into sewer systems (Daughton and Ruhoy, 2009).

According to Halling-Sørensen *et al.* (1998), the fate of pharmaceuticals in wastewater can be categorized into three pathways:

1. **Complete Mineralization:** Some substances, such as aspirin, undergo mineralization to carbon dioxide and water, effectively eliminating them from the environment (Richardson and Bowron, 1985).
2. **Adsorption to Sludge:** Lipophilic substances that are not readily degradable tend to bind to sludge during wastewater treatment.
3. **Transformation to Persistent Metabolites:** Certain lipophilic substances are metabolized into hydrophilic derivatives, which may evade wastewater treatment processes and enter receiving water bodies. These metabolites, if biologically active, can have adverse effects on aquatic organisms when discharged into streams via treatment plant effluents.

The presence and persistence of pharmaceuticals in aquatic environments are influenced by several processes, including aerobic and anaerobic biodegradation, as well as abiotic transformations such as UV-light degradation, hydrolysis, and sediment sorption.

Hydrolysis, a common abiotic process, is often negligible for most human pharmaceuticals due to their resistance to this reaction. In contrast, photolysis—both direct and indirect—is a significant mechanism for the degradation of pharmaceuticals in surface waters. Direct photolysis occurs when pharmaceutical compounds absorb sunlight, while indirect photolysis involves natural photosensitizers that facilitate the breakdown of the compounds (Nikolaou *et al.*, 2007).

Pharmaceuticals in hospital wastewater is a significant environmental worry as minute concentration of endocrine disruptors, some of which are drugs that have detrimental effects on

aquatic species and possibly on human wellbeing and development. These products when released on the ground or in the water bodies accumulate in the system through the food chain. Worries about their potential danger was brought up in 1999 with the issue attracting great interest after the presence of drugs in waterway water was connected to feminization of fish living downstream of wastewater treatment plant (WWTP) outfalls (Larsson *et al.*, 2007). Other adverse impacts include intersex fish (animals with both male and female sex attributes), males producing eggs, reproductive issues, sperm damage, changing of genders for example male becoming females and females becoming male (alter female to male ratio), feminizing impact on male fish, it makes adult females have larger livers, organ failure, inability to develop.

2.4. Pathogens in Hospital Wastewater

The effluents created from hospital exercises/activities is a contributory factor to the regular environmental contamination load (Eze and Onwurah, 2015). As reported by (Wang, *et al.*, 2018), drug mixtures and microorganisms are often recognized in the environment due to the generally use and improper handling. The concentrations of microbes in wastewater range from 10^7 to 10^{10} . Their review uncovered that the effluents just as the receiving waters would prompt a potential health hazard to the surrounding communities. Osulale and Okoh (2015) assessed the physicochemical and microbiological characteristics of the final effluents of two WWTPs in the Eastern Cape Province of South Africa over a time of one year. They reported that both WWTPs showed the presence of *Escherichia coli* in counts ranging somewhere in the range of 0 and 1.86×10^4 colony forming units (cfu)/100 ml and *Vibrio* counts also ranging somewhere in the range

of 0 and 9.93×10^3 cfu/100mL, demonstrating the two of them have inert health dangers to human and different organisms.

Likewise, Jacob *et al* (2015) examined the health hazard related with waterborne pathogens in three big rivers in France dependent on a long term monitoring program. *Clostridium perfringens*, *Cryptosporidium parvum* oocysts, *Giardia duodenalis* cysts were found in one of the streams where major agricultural activities were noticed, while adenovirus was found at a higher concentration in the other two streams. In spite of the high concentration of antibiotics compounds in hospital wastewater, bacteria, viruses, and parasitic worm eggs generally have solid resistance in the environment and could survive in sewage for over a long period. Accordingly it isn't unexpected that multidrug-resistant bacteria (MDRB) are found in the hospital wastewater (HWW). The major MDRB incorporate ESBL-creating *Escherichia coli*, vancomycin safe enterococci, and *Pseudomonas aeruginosa*.

Le *et al.*, (2018) researched and found the occurrence of 19 antibiotics resistance to 10 antibiotics and 15 antibiotic-resistant genes (ARGs) in raw effluent treatment stages utilizing conventional activated sludge (CAS) and MBR systems. ARGs are mostly developed by the following components: (1) target bypass: antibiotics can't get to their target enzymes because of mutational changes or loss on the enzyme compounds; (2) efflux pump: the intracellular concentration of antibiotic agents diminishes because of the change in cell membrane structure; (3) antibiotic inactivation: antibiotics particles are straightforwardly deactivated; or (4) target alteration: the action sites of antibiotics are altered. Therefore the development of ARGs might happen through various routes (Chen *et al.*, 2019). One significant concern of the development

of antibiotics resistant bacteria is the transfer of resistant gene to antibiotics sensitive microbial species. Through food chain, people could get in contact with multidrug-resistant bacteria (MDRB) and become ill.

It could even prompt the outbreak of infectious illness (Liang and Messenger 2018). There is also the danger of treatment failure when human beings or animals are infected with the bacteria which already developed antibiotics-resistant gene and other toxicity associated with DNA damage, as well as oxidative pressure (Cecconet *et al.*, 2017).

Apart from antibiotics agents, there are likewise different compounds, for example, radiation and X-beam compounds, which can instigate change of DNA and hence cause abnormal sicknesses. Pathogens are likewise a huge problem of HWW. It changes among the environment and people.

2.5. Impact of Hospital Wastewater

2.5.1. Impact of hospital wastewater on organisms

The antibiotics in hospital wastewater might be hazardous to aquatic organisms. It is accounted for that antibiotics usually utilized in medication like ciprofloxacin, ofloxacin, and metronidazole are difficult to be biodegraded in water, and their reproductive toxicity influences biological organisms in the water environment and humans. Unnecessary exposure of antibiotics into the water environment can prompt expanded resistance of bacteria to antibiotics.

Studies have shown that *Vibrio parahemolyticus*, *Vibriolal ginolyticus*, and *Aeromonas hydrophila*, which are associated with aquatic creatures diseases, have created resistance to commonly used antibiotics Pharmaceuticals in water and might go about as endocrine disruptors

(Garrido Maestu *et al.*, 2014). Aquatic organisms also accumulate heavy metals and radioactive materials in their bodies.

Bioaccumulation can happen directly from the water or through feeding. Nuclide can be moved along the marine food chain (food web), and some can be extended along the food chain. Mutation may occur due to genes of the organism being exposed to heavy metals or their nuclide, which can affect the growth and reproduction of offspring. Furthermore, persistent compounds including heavy metals and nuclides can effectively accumulate in the environment, and consequently can harm the ecosystem, and lead to the increment of infections (Loreto-Gómez *et al.*, 2018). Excessive nutrient cause biological degradation in ground water lakes and streams by using up oxygen (Eutrophication), bringing about to alga bloom and plankton in the water body. This is viewed as a serious environmental problem. Eutrophication frequently brings about the disintegration of water quality and depletion of broke down oxygen in water bodies making these water bodies become dead zone. The process of eutrophication damages the natural equilibrium in the aquatic environment, by increasing the growth of simple plants, which annihilates the equilibrium in the ecological system and significantly diminishes the biodiversity of the ecosystem by death of several desirable species.

Pathogens in hospital wastewater can contaminate host cells and may by implication cause problems on the host cell, for example, genotoxic agents, tumor proteins, pathogen replication, and inflammatory reactions, and subsequently influencing the enactment of the DDR pathway. This can lead to tremendous pressure on the stability and integrity of the cell genome (Lamothe *et al.*, 2015). Also, increase in pathogen in the environment might prompt the increase in the

conveying of pathogens by organism, in this way growing the spread of pathogenic microscopic organisms. A few organisms like fish are known to convey pathogens in their mouth, stomach, digestion tracts, and excretory cavities. In addition, oysters which are closely related to pathogens in shellfish can spread typhoid fever.

2.5.2. Impact of hospital wastewater on humans

Hospital wastewater contains countless of large quantity poisonous/toxic drugs, microorganisms, and so on, which can settle or accumulate in drinking water and oceanic food sources. There are numerous manners by which people come into contact with the pollutants of HWW: directly drinking contaminated source water; taking aquatic animals and plants grown in contaminated water; and breathing in these poisonous and unsafe substances after unpredictable toxins entering the air.

When people come in contact with harmful substances, many issues could happen. For instance, the immune system could be compromised. It was likewise discovered that drugs could induce problem of the reproduction in human beings and the effect was a lot greater in aquatic organisms like fish (Kosjek *et al.*, 2011). The presence of cyclophosphamide and of orfamide in water could prompt cancer risk to people.

In cancer and tumor treatment, huge measures of the antitumor agents are prescribed. The greater part of the unused agents and their metabolites would end up in the HWW, metropolitan wastewater treatment measures can't eliminate drugs; they would remain in the effluent and finally get into receiving water bodies, and afterward causes genotoxic and cancer-causing consequences for people. Because of the absence of explicit risk appraisal systems, cancer-

causing agents (carcinogens) in the water environment can't be precisely evaluated, however existing studies have had the option that they greaterly affect infants and youngsters than adults (Kuemmerer *et al.*, 1997). Likewise, there is a great chance of synergism between different various trace amounts of toxic and harmful substances present in water bodies, which can't be disregarded.

The effect of HWW on people, in addition to direct exposure intake, can also be affected by the bio-concentration of the food chain and the flow of substances through the biological chain, which is considered as bioaccumulation. It is another significant pathway by which pharmaceuticals are transported to human. For instance, antibiotics agents are a significant class of drugs.

Antibiotics released into the water with hospital wastewater are accumulated in drinking water and aquatic food varieties. People can accumulate antibiotics in the body by taking water which are contaminated by antibiotics or taking the aquatic products gotten from the antibiotic polluted waste. It then, at that point, can make bio amplifications to increase their concentration, and afterward lead to persistent toxicity, hypersensitive responses. It will likewise break the equilibrium of normal flora in the human body; consequently sway on different physiological functions of individuals to undermine human health.

Also, antibiotics can create drug resistance genes qualities in movement and transformation, and drug resistance genes transmits mutation in different bacteria (Rysz *et al.*, 2004). At the point when these medication resistance qualities are transferred to pathogenic bacteria they increase the danger to human wellbeing. It could likewise inherent from one generation to another.

HWW contains an assortment of chemical, physical, or organic compounds, which might make long-lasting harm to cell structure or function, forestall development, cause underdeveloped embryo or even embryo death. The danger brought about by HWW mainly influences in microcephaly, cardiovascular and facial edema, and axials.

Above all, it can cause teratogenesis, which implies that if a mother was disfigured, the likelihood of the baby's abnormality will be especially huge (Pérez-Alvarez *et al.*, 2018), likewise the outbreak of infections in neighboring communities like cholera, hepatitis A and hepatitis E, Typhoid fever and schistosomiasis.

2.6. Hospital Wastewater Guidelines Throughout the Globe

The effective survey of guidelines, regulations, and explicit standards at the global level regarding the handling and management of hospital wastewater (HWW) has revealed significant challenges. Worldwide, the primary guiding document, *Safe Management of Wastes from Health-Care Activities*, was made available by the World Health Organization (WHO) between 1999 and 2013, with updates in 2013 (USEPA, 2013). According to these guidelines, the direct release of hazardous fluids and chemical wastes—such as photochemicals, aldehydes, colorants, and pharmaceuticals—into sewers is strictly prohibited. Separate collection and pretreatment are required for wastewater from clinical laboratories.

Pretreatment methods may include sediment filtration, acid-base neutralization, or autoclaving. Non-hazardous chemicals like syrups, vitamins, or eye drops can be discharged into the sewer

without pretreatment, while radioactive wastewater must be collected and stored until its radioactivity levels decrease to safe limits.

Globally, four significant guidelines focusing on the management of HWW include:

- National Pollutant Discharge Elimination System (EPA, 2015)
- Safe Management of Waste from Healthcare Activities (WHO, 2013)
- Release of Patients After Radionuclide Treatment (IAEA, 2005)
- Release of Patients After Treatment with Unsealed Radionuclide (CRP, 2009)

2.6.1. Global Scenario of Hospital Wastewater Treatment

A working group comprising WHO personnel, clinical professionals, hospital engineers, and administrators from 19 nations concluded that managing HWW involves raising awareness, segregation, and pretreatment of waste (WHO, 2013). Most countries do not differentiate between wastewater originating from hospitals and that from urban areas.

Potentially hazardous effluents are often discharged directly into public sewage systems without prior toxicity identification (Santoro *et al.*, 2015). Implementation of these guidelines often lags behind their establishment. For instance, WHO (2013) and European Directives (EDI, 2010) identify mercury as a hazardous substance with an allowable limit of up to 5% in effluents from hospital wastewater treatment plants (HWWTPs). However, the United Kingdom and Europe contribute over half of the mercury, silver, tin, copper, and zinc from dental amalgams originating in hospitals.

In many countries, including Denmark, Greece, Italy, Iran, Taiwan, Korea, Ethiopia, Saudi Arabia, India, Nepal, and Vietnam, few studies have been conducted to address HWW treatment

(Kumari *et al.*, 2020). Countries like China and Japan have experienced outbreaks of intestinal and cancerous infections, prompting the adoption of specific on-site pretreatment for HWW before discharge into municipal sewerage systems.

In the United States, the primary legislation regulating wastewater discharge into surface water bodies is the Clean Water Act (1972) and its updated version (2002). Based on this act, specific guidelines and permits for handling and treating HWW contamination were established. The management of HWW emphasizes utilizing advanced control technologies before discharging effluents into surface waters or city wastewater treatment plants. Publicly owned treatment works must comply with local effluent discharge standards.

The United States Environmental Protection Agency (USEPA) has identified toxic and hazardous substances, including the antibiotic erythromycin and five synthetic hormones. Following the SARS outbreak, US organizations transferred membrane biofilm reactor (MBR) technology to Chinese hospitals due to its high efficiency in removing bacteria and viruses (Kumari *et al.*, 2020). In the United Kingdom, the impacts of pharmaceutical waste in HWW are less evident than in other nations, despite the significant production, use, and disposal of drugs. In 2000, the country consumed over 100 tons per year of the top 25 pharmaceutical compounds (Kumari *et al.*, 2020). Discarded pharmaceuticals are regulated under the Controlled Waste Regulations, 1992 (EDC, 1991).

Before 1990, no specific regulations existed for managing HWW in Europe, as hospital discharges were assumed to be comparable to municipal wastewater. Consequently, member states implemented their own guidelines. The European Union Water Framework Directive (EU-

WFD) of 1991 mandated preauthorization for releasing industrial waste into domestic wastewater systems to safeguard water quality. Europe has approximately 2.6 hospitals per 10,000 inhabitants, with about 530 beds per hospital (ranging from 320 in Spain to 800 in Germany). Hospital wastewater discharge in Europe averages 0.3-0.7 m³/bed/day, with an annual antibiotic consumption of approximately 10,000 tons. A significant portion of these antibiotics ends up in wastewater through urine and feces (Kumari *et al.*, 2020).

According to the United Nations Development Program (UNDP, 2013), Africa lacks comprehensive guidelines for managing HWW. Hospital wastewater is typically co-treated with urban wastewater in metropolitan sewer systems (NSPRC, 1998). This practice, common in countries such as Australia, Iran, Egypt, India, and Japan, is referred to as co-treatment. Countries like Ghana, Lesotho, and Eritrea have no formal regulations for HWW treatment, while Nigeria, Gambia, and Kenya are signatories to the Stockholm Convention (2009). In South Africa, USEPA guidelines are applied for Class A sludge treatment from hospitals, which is freely used in agriculture without additional monitoring. Sludge undergoes further treatment (air-drying, composting, or long-term storage) to meet national standards for effluent discharge (UNDP, 2013).

Reinthal *et al.* (2003) studied the antibiotic resistance of *E. coli* in three different wastewater treatment plants (WWTPs), including one treating hospital effluents. All WWTPs demonstrated a 2.3-log reduction in total *E. coli*, but the effluents still released over 10² cfu/mL into the environment. This contributes to the spread of resistant bacteria. Resistant strains to 16 of 24 tested antibiotics were observed, with the highest prevalence in the hospital wastewater treatment

plant effluent. Additionally, quinolone-resistant *E. coli* was identified exclusively in this plant, highlighting the risks posed by untreated or inadequately treated HWW.

2.7. Antimicrobial Resistance in the Environment

Antimicrobial resistance (AMR) is a significant issue posing a serious worldwide health danger, particularly affecting low- and middle-income countries, both in terms of public health and financial burden. Recent studies indicate the role of resistance networks in the transmission of AMR organisms, driven by complex interactions between clinical factors (e.g., human health, animal husbandry, and veterinary medicine) and environmental components (e.g., persistence of AMR in wastewater) (Nour *et al.*, 2020).

The World Health Organization (WHO) has recognized 12 bacterial species and their accompanying AMR profiles that pose the highest danger to human health. These primarily include Gram-negative bacteria and the most common etiologic agents associated with hospital- or community-acquired infections. These AMR bacteria have been classified into three categories: critical, high, and medium priority, based on their impact on human health and the urgency of developing new antimicrobial drugs to treat resistant infections.

The critical category includes *Acinetobacter baumannii* (carbapenem-resistant), *Pseudomonas aeruginosa* (carbapenem-resistant), and members of the *Enterobacteriaceae* family, including *Klebsiella* spp., *Escherichia coli*, *Serratia* spp., and *Proteus* spp. (carbapenem-resistant and extended-spectrum β -lactamase (ESBL)-producing), which are associated with severe and often life-threatening diseases, such as bloodstream infections and pneumonia. The high-priority

category includes *Enterococcus faecium* (vancomycin-resistant); *Staphylococcus aureus* (methicillin-resistant, vancomycin-intermediate, and resistant); *Helicobacter pylori* (clarithromycin-resistant); *Campylobacter* spp. (fluoroquinolone-resistant); *Salmonella* spp. (fluoroquinolone-resistant); and *Neisseria gonorrhoeae* (cephalosporin- and fluoroquinolone-resistant), which are causative agents of more common infections such as gastrointestinal infections and gonorrhea.

The medium-priority category includes *Streptococcus pneumoniae* (penicillin non-susceptible) and *Haemophilus influenzae* (ampicillin- and fluoroquinolone-resistant). Antibiotics are among the most effective and essential drugs used in therapeutic applications; however, their indiscriminate use has led to their presence in the environment. The misuse and overuse of antibiotics in human medicine, veterinary practices, agriculture, and aquaculture have contributed to the emergence of antibiotic-resistant genes (ARGs) and antibiotic-resistant bacteria (ARB), diminishing the efficacy of antibiotic compounds and raising significant concerns.

Studies have shown that resistant bacteria can persist even where antibiotic concentrations are low. Sub-inhibitory concentrations of antibiotics, similar to those found in some aquatic and soil environments, can promote resistance among bacteria (Kümmerer, 2009). Just as natural antibiotics have existed for billions of years, ARGs are also ancient (Von, 2016). Evidence of ARGs has been found in places with minimal human activity, such as β -lactamase genes in remote Alaskan soil, suggesting the environment serves as a reservoir for ARGs (Allen, 2009). Antibiotics have also been detected in isolated environments such as Lechuguilla Cave, New

Mexico, which has been isolated for millions of years. Strains resistant to more than 14 different antibiotics have been identified in this environment (Bhullar *et al.*, 2012). Similarly, ancient permafrost samples containing resistant components have been dated to at least 5,000 years ago (Perron *et al.*, 2015). Such findings enhance our understanding of the prevalence of resistance genes in the environment long before human use of antibiotics.

In recent years, evidence has shown the assembly of these resistance genes from environmental reservoirs into pathogenic bacteria, posing health risks to humans and animals and demonstrating a connection between environmental and clinical resistance (Martí *et al.*, 2014). Vilanova *et al.* (2005) observed significant reductions in bacterial populations during sewage treatment but noted the persistence of vancomycin and erythromycin-resistant *Enterococci* in similar proportions. This finding suggests a lack of specific removal of bacterial populations during wastewater treatment processes.

Chitnis *et al.* (2000) described a case in an Indian hospital where the wastewater treatment plant (WWTP) effluent and waste sludge were reused (after sanitation) as irrigation water and manure, respectively, to reduce costs. The study demonstrated that the hospital significantly contributed to the abundance of antibiotic-resistant strains. Chlorination was crucial to inactivate 7.5×10^3 cfu/mL of multiple antibiotic-resistant strains (MARS) in wastewater. After chlorination, no MARS were detected. However, chlorination with sodium hypochlorite was associated with the production of absorbable organic halogens (AOX), which have been linked to toxicity in *Daphnia magna* (Emmanuel *et al.*, 2004). The authors identified a positive correlation between AOX levels in hospital wastewater and toxicity.

CHAPTER THREE

MATERIALS AND METHODS

3.0. Study Area

The study was conducted at the University of Benin Teaching Hospital (UBTH), Benin City, Edo State, Nigeria.

3.1. Sample Collection

Wastewater samples were collected from the drainage outlets of the targeted wards. A total of four (4) samples, one from each ward, were collected using sterile 500 mL glass bottles. The bottles were pre-labeled with information including sample source, date, and time of collection.

Samples were transported in an icebox at 4°C to the Microbiology Laboratory of the University of Benin for immediate processing.

3.2. Preparation and Sterilization of Media

Test tubes, beakers, conical flasks, Petri-dishes, McCartney bottles, stirring glass rod and measuring cylinder. All glassware which include MacCartney bottles, Petri dishes, test tubes, conical flasks, measuring cylinders and pipettes, were sterilized at 160 °C for 1 hr in a hot-air-oven before use. The media used in this study were sterilized at 121 °C for 15 min in an autoclave. Agar media, agar slant and biochemical reagents were prepared freshly and refrigerated at 3-4 °C. Aseptic conditions were ensured during inoculation and subculturing.

3.2.1. Preparation of Nutrient Agar

Nutrient agar was used for isolation of bacteria from wastewater sample. This was prepared according to manufacturer's instruction by dissolving 28g of Nutrient Agar in 100ml of distilled water in a conical flask. The conical flask was then plugged with cotton wool, wrapped in aluminium foil and sealed properly with paper tape. The mixture obtained was then boiled to ensure proper homogenization. The media was then sterilized by autoclaving at 121°C at 15psi for 15 minutes.

3.2.2. Simmon's Citrate Agar (SCA)

This agar was prepared by dissolving 24.28g of the agar powder in 1 litre of distilled water. The medium was allowed to soak for some minutes and swirled to mix, homogenized by heating and then dispensed into bijou bottles before autoclaving at 121°C for 15 minutes. The medium was allowed to solidify in a slant position.

3.2.3. Triple Sugar iron (TSI) agar

This agar was prepared following manufacturer's direction, by dissolving 65g in 1000ml of distilled water. The medium was allowed to soak for some minutes and swirled to mix, homogenized by heating to boil and then dispensed into sterile test tubes plugged with cotton wool and aluminium foil before autoclaving at 121°C for 15 minutes. The medium was allowed to solidify in a slanted position.

3.3. Enumeration and Isolation of Bacteria

Serial dilution was performed to reduce the bacterial load in the wastewater samples to a manageable level for effective isolation and enumeration of bacteria. A sterile physiological saline solution was prepared and used as the diluent. This solution was sterilized in an autoclave at 121°C for 15 minutes to eliminate contaminants. Wastewater sample (1ml) was aseptically transferred into a test tube containing 9 mL of sterile physiological saline, creating a 10^{-1} dilution. The test tube was vortexed or gently shaken to ensure uniform mixing. From the 10^{-1} dilution, 1 mL was transferred into another test tube containing 9 mL of sterile saline, creating a 10^{-2} dilution. This process was repeated serially up to a 10^{-6} dilution, ensuring proper mixing at each step.

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 agar (cooled to about 45°C) was poured into each Petri dish and gently swirled to ensure even distribution of the inoculum. Plates containing Nutrient Agar, were incubated at 37°C for 24-48 hours for the isolation of bacterial colonies. After the incubation period, distinct colonies were counted using a

colony counter. The number of colony-forming units (CFUs) per milliliter was calculated based on the dilution factor.

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

3.3.1. Preparation of Pure Cultures

One single colony was identified and re-streaked as a primary inoculant on the surface of a nutrient agar plate medium. After achieving a pure culture, the same colony was streaked onto a nutrient agar slant. These cultures were incubated at 37°C for 24 hrs.

3.4. Identification of Bacteria

3.4.1. Gram Staining

This was carried out according to the method of Fawole and Oso (2007). It was used to differentiate bacterial isolates into Gram-negative or Gram-positive. Thin smears of the bacterial isolates were made from the pure culture on clean, grease free slide. The smears were air dried and then heat fixed by passing the slide over flame. The smears were flooded with crystal violet for 60 seconds, drained and flooded with Gram's iodine for another 60 seconds then rinsed with distilled water. The smears were then decolorized using 70% alcohol for 5 seconds and quickly rinsed with distilled water. The smears were then counter stained with safranin for 30 seconds, rinsed with distilled water and allowed to air dry. The slides were examined under the oil

immersion (X100) objective. The Gram-positive cells appear blue, while the Gram-negative cells were indicated with a pink colouration.

3.4.2. Potassium Hydroxide (KOH) test

. Two drops of 3% solution of KOH were applied on a clean glass slide and a loopful of pure bacterial growth was stirred in a circular motion in the slide. The loop was occasionally raised and observed for the presence of a string of the mixture. The solution was observed to be of a viscous and mucoid consistency indicating a Gram-negative bacterium. No reaction (absence of stringing) indicates a Gram-positive bacterium (Roberts and Sandle, 2008).

3.4.3. Biochemical Tests

The biochemical characteristics of each isolates were determined to identify them, including catalase test, citrate utilization test, oxidase test, urease test, methyl red test, indole test, coagulase test, sugar fermentation, starch hydrolysis and triple sugar iron fermentation.

3.4.3.1. Catalase Test

Catalase test was carried out by making a suspension of fresh culture of the test organisms using sterile distilled water on a clean glass microscope slide, and few drops of hydrogen peroxide (H_2O_2) were added using a dropping pipette. Formation of bubbles indicates positive result, while lack of bubbles indicates negative result.

3.4.3.2. 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 contain citrate as significant energy source 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.

3.4.3.3. Oxidase Test

The oxidase test was carried out to detect the presence of a cytochrome oxidase or indophenol oxidase that will catalyze electrons between electron donors in the bacteria and a redox dye known as tetramethyl-*p*-phenylene-diamine. The dye would be reduced to deep purple colour if it is a positive reaction.

Several reagents can be used for this study but Kovacs oxidase reagent (1% tetra-methyl-*p*-phenylenediamine dihydrochloride) in water, was used. The filter paper was saturated with a Kovacs oxidase reagent solution, and a speck of the pure culture was smeared on it with a platinum loop. It was allowed and observed for colour development within 10 - 60 seconds. The appearance of a deep purple-blue/blue colour indicated oxidase production, and the negative result was when no colour changed.

3.4.3.4. Indole Test

Spot indole test was carried out using a fresh culture of the test organism. Several drops of 1% *p*-dimethylaminocinnamaldehyde reagent were placed on a piece of filter paper. A loopful culture

of the test organism was rubbed on the reagent saturated area of the filter paper. Positive result is shown by the presence of a blue to blue-green colour change within 2-3 minutes while negative results remain colourless or appears light pink.

3.4.3.5. Triple sugar iron (TSI) agar test

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 TSI 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 H₂S, 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 H₂S production. Bubbles, cracks, or bottom-raised space in the slanted agar indicated gas production (formation of CO₂ and H₂).

3.5. Antibiotic Susceptibility Test

The identified bacterial colonies were subjected to antibiotic susceptibility testing (AST) to determine their resistance or susceptibility to commonly used antibiotics in the locality. The test was conducted using the disc diffusion method, with standard antibiotic discs manufactured by

Oxoid, UK. The specific antibiotics tested included : Pefloxacin, Gentamycin, Ampiclox, Zinnacef, Amoxicillin, Rocephin, Ciprofloxacin, Erythromycin, Levofloxacin, Azithromycin, Cefotaxim, Saprifloxacin, and Tarivid.

For the assay, bacterial cultures grown for 18–24 hours were standardized to 1.5×10^8 cells/mL according to the McFarland standard. Using a sterile loop, the bacterial inoculum was evenly streaked onto Mueller-Hinton Agar (MHA) plates. The antibiotic discs were carefully placed on the plates using sterile forceps. The plates were then incubated at 37°C for 24 hours. Following the 2017 AST guidelines established by the Clinical Laboratory Standards Institute (CLSI), the inhibition zones around each antibiotic disc were measured in millimeters using a ruler. Results were interpreted based on the (CLSI, 2020) standards, categorizing the bacterial isolates as Resistant (R), Intermediate (I), or Sensitive (S).

CHAPTER FOUR

RESULTS

The present study carried out a bacteriological analysis of wastewater samples obtained from different wards in the University of Benin Teaching Hospital, Benin City, Edo State.

The total heterotrophic bacterial count (THBC) of the wastewater samples obtained from various wards in the hospital varied considerably and is presented in Figure 4.1. The highest bacterial count was recorded in the laundry department, with a mean value of $8.00 \pm 0.05 \times 10^4 \log_{10}$ cfu/mL. Conversely, the lowest bacterial count was observed in the eye clinic, with a mean value of $3.12 \pm 0.23 \times 10^4 \log_{10}$ cfu/mL.

Table 4.1 shows the morphological, biochemical and cultural characteristics of the bacterial isolates obtained from the wastewater samples. The bacteria isolated from the wastewater samples include, *Salmonella* sp., *Bacillus* sp., *Escherichia coli*, *Enterobacter* sp., *Pseudomonas* sp., *Streptococcus* sp., *Staphylococcus* sp., and *Klebsiella* sp.

The distribution of bacterial isolates across the different hospital wards is presented in Table 4.2. Among the identified bacteria, *Staphylococcus sp.* and *Pseudomonas sp.* were the most widely distributed, as they were present in all four sampled locations. The **eye clinic** exhibited the lowest bacterial diversity, with only *Klebsiella sp.*, *Staphylococcus sp.*, and *Pseudomonas sp.* detected.

Figure 4.2 presents the percentage frequency of occurrence of the bacterial isolates from the wastewater samples, revealing variations in their distribution across the selected hospital wards. Among the identified bacterial species, ***Pseudomonas sp.*** exhibited the highest frequency of occurrence, accounting for **17.82%** of the total isolates. *Salmonella sp.* had the lowest frequency of occurrence at **7.92%**.

Table 4.3 and 4.4 present the results of the antibiotic sensitivity test on Gram-positive and Gram-negative bacterial isolates recovered from wastewater samples collected from various locations.

Among the Gram-positive bacterial isolates, *Staphylococcus sp.* exhibited the highest resistance, showing reduced susceptibility to multiple antibiotics, while *Bacillus sp.* demonstrated relatively lower resistance. *Streptococcus sp.* showed intermediate susceptibility to most antibiotics, with notable resistance to Rocephin and Erythromycin.

Among the Gram-negative bacterial isolates, *Pseudomonas sp.* displayed the highest resistance, particularly against Levofloxacin, Cefotaxime, Amoxicillin, and Azithromycin, while *Enterobacter sp.* exhibited the lowest resistance, being susceptible to Ciprofloxacin, Gentamycin,

and Pefloxacin. *Escherichia coli* and *Salmonella* sp. demonstrated moderate resistance, with *E. coli* showing notable resistance to Azithromycin and Amoxicillin.

The MAR index of the bacterial isolates recovered from the wastewater samples is presented in Figure 4.3. The MAR index is a crucial indicator of bacterial resistance, reflecting the extent to which isolates exhibit resistance to multiple antibiotics. Among the isolates, *Pseudomonas* sp. had the highest MAR index (0.70), indicating strong resistance to multiple antibiotics. This was followed by *E. coli* and *Enterobacter* sp., both with a MAR index of 0.60, signifying significant resistance. *Staphylococcus* sp. had a MAR index of 0.50, while *Streptococcus* sp. exhibited moderate resistance with a MAR index of 0.30. *Klebsiella* sp. and *Bacillus cereus* had the lowest MAR indices of 0.20.

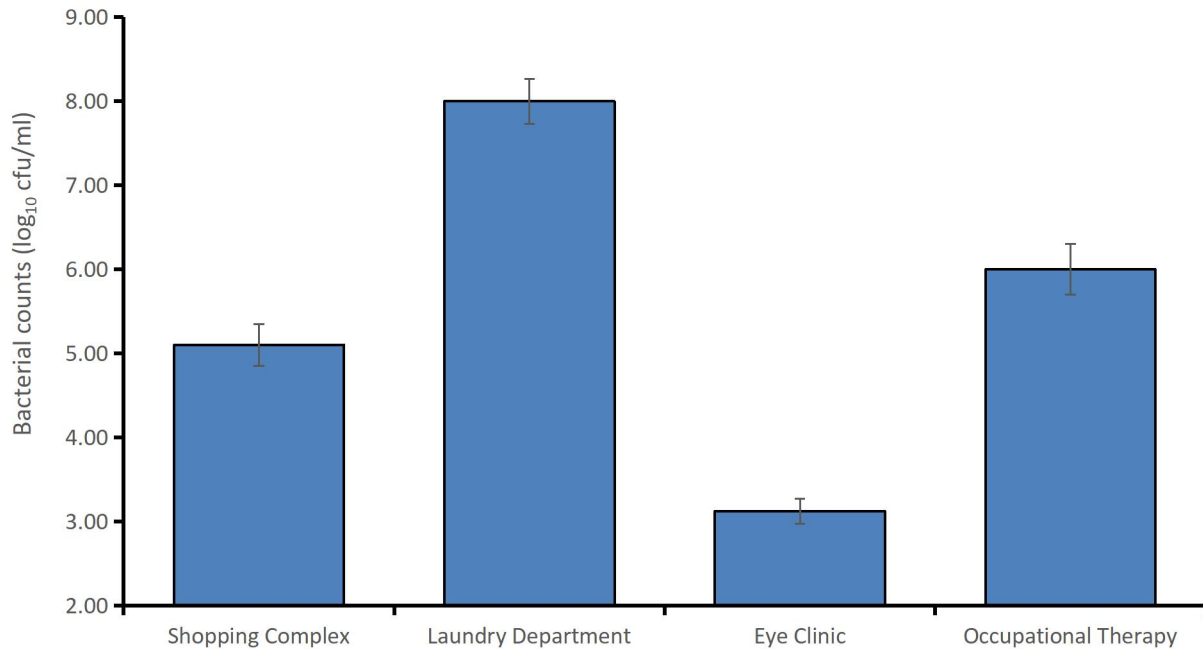


Figure 4.1: Total bacterial count (\log_{10} cfu/mL)

Table 4.1: Cultural, Morphological, and Biochemical Characteristics of Bacteria isolates

Elevation	Raised	Flat	Flat	Flat	Raised	Flat	Raised	Flat
Margin	Entire	Undulate	Undulate	Entire	Undulate	Entire	Entire	Entire
Color	Cream	Cream	Cream	Yellow	Green	White	White	Cream
Shape	Circular	Irregular	Irregular	Circular	Irregular	Circular	Circular	Circular
Size	Medium	Large	Large	Medium	Medium	Small	Small	Large
Gram Stain	-	+	-	-	-	+	+	-
Cell Type	Rod	Rod	Rod	Rod	Rod	Cocci	Cocci	Rod
Arrangement	Pair/Chains	Disperse	Disperse	Disperse	Disperse	Chains	Clusters	Pair/Chains
Color (Gram Reaction)	Pink	Purple	Pink	Pink	Pink	Purple	Purple	Pink
KOH String Test	+	-	+	+	-	-	-	-
Catalase	+	+	+	+	+	-	+	-
Indole	-	-	+	-	-	-	-	+
Citrate	-	+	-	+	+	+	+	+
Oxidase	-	-	-	-	+	-	-	-
Glucose	+	+	+	+	+	+	+	+
Sucrose	-	+	-	+	-	+	+	-
Lactose	-	+	+	+	-	+	-	+
Gas Formation	+	-	+	+	-	-	+	-
H₂S Formation	+	-	-	-	-	-	-	-
TSI (Slant/Butt) Reaction	K/AG H ₂ S	A/A	A/AG	A/A	K/AG	A/A	A/A	K/A
Identity	<i>Salmonella</i> sp.	<i>Bacillus</i> sp.	<i>E. coli</i>	<i>Enterobacter</i> sp.	<i>Pseudomonas</i> sp.	<i>Streptococcus</i> sp.	<i>Staphylococcus</i> sp.	<i>Klebsiella</i> sp.

KEY:

+: Positive to test, -: Negative to test. (A)acid; (K) alkaline; (G) gas production (bubble); (H₂S) hydrogen sulphide (black precipitate); (KOH) Potassium hydroxide test; (TSI) Triple sugar iron test;

Table 4.2: Distribution of Isolated Bacteria

Isolates	Shopping Complex	Laundry Department	Eye Clinic	Occupational Therapy
<i>E. coli</i>	+	+	-	+
<i>Enterobacter</i> sp.	-	+	-	+
<i>Klebsiella</i> sp.	+	-	+	+
<i>Streptococcus</i> sp.	+	+	-	+
<i>Staphylococcus</i> sp	+	+	+	+
<i>Bacillus cereus</i>	-	+	-	+
<i>Pseudomonas aeruginosa</i>	+	+	+	+

Key: Present (+), Absent (-)

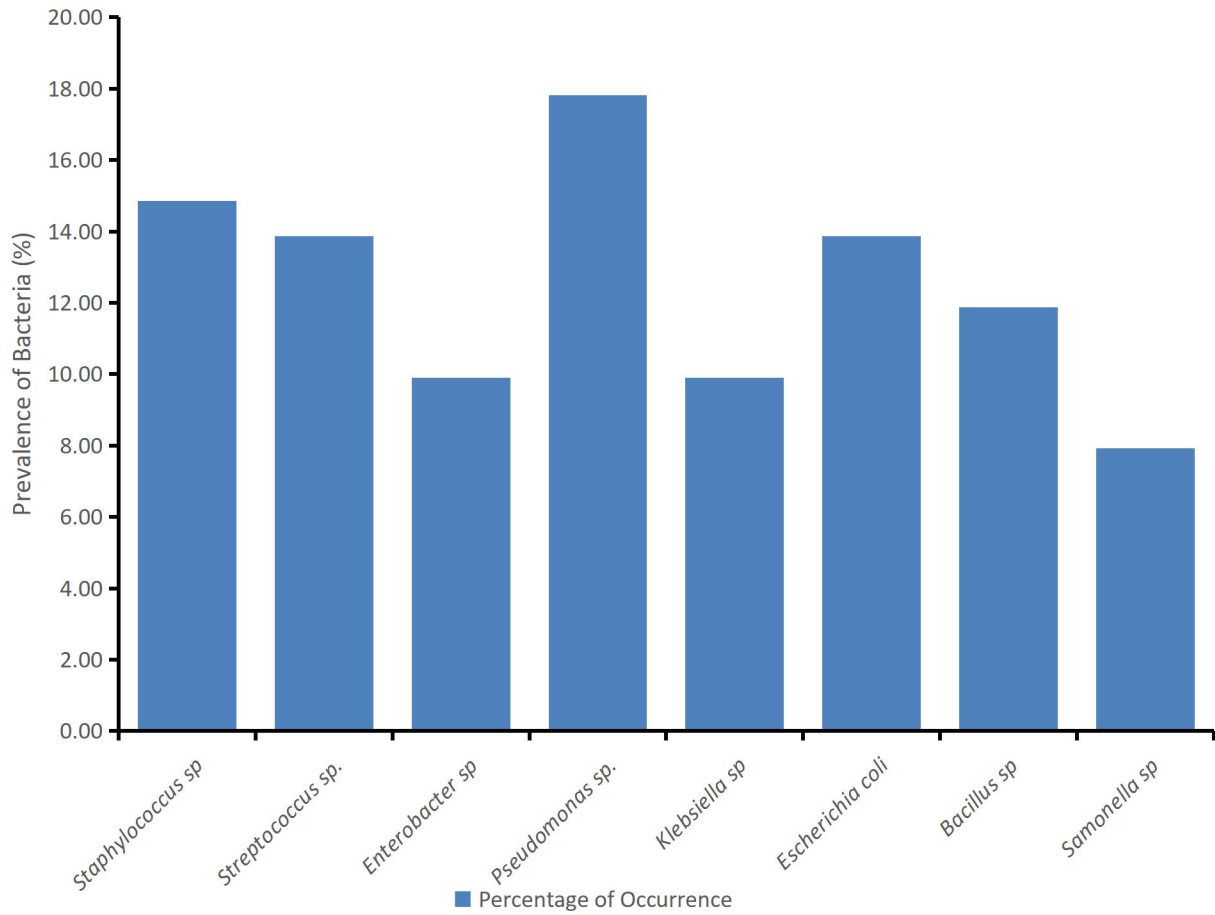


Figure 4.2. Percentage of occurrence of the bacterial isolates

Table 4.3: Antibiotic sensitivity test on Gram positive bacterial isolates

ISOLATES	PEF	CN	APX	Z	AM	R	CPX	AZ	LEV	E
<i>Staphylococcus</i>	26(S)	8(R)	9(R)	0(R)	10(R)	0(R)	24(S)	26(S)	27(S)	16(S)
Sp.										
<i>Bacillus sp</i>	16(I)	14(I)	16(I)	18(S)	14(I)	6(R)	20(S)	24(S)	24(S)	10(R)
<i>Streptococcus sp</i>	18(I)	12(I)	20(S)	10(R)	12(I)	9(R)	22(S)	18(S)	16(S)	6(R)

KEY:

Resistance (R) = 0-10mm

Intermediate (I) = 11-16mm

Sensitive (S) = 17mm and above

PEF: Pefloxacin, CN: Gentamycin, APX: Ampiclox, Z: Zinnacef, AM: Amoxicillin, R:

Rocephin, CPX: Ciprofloxacin, E: Erythromycin, LEV: Levofloxacin, AZ: Azithromycin

Table 4.4: Antibiotic sensitivity test on Gram negative bacterial isolates

ISOLATES	LEV	CF	SP	CPX	AM	AU	CN	PEF	OFX	AZ
<i>Escherichia coli</i>	9(R)	0(R)	12(R)	14(I)	0(R)	6(R)	18(S)	20(S)	20(S)	10(R)
<i>Pseudomonas sp.</i>	0(R)	0(R)	10(R)	20(S)	2(R)	10(R)	22(S)	8(R)	12(I)	0(R)
<i>Salmonella sp.</i>	0(R)	12(I)	16(I)	16(I)	14(I)	18(S)	18(S)	20(S)	20(S)	14(I)
<i>Klebsiella sp.</i>	6(R)	10(I)	18(S)	20(S)	10(I)	16(I)	20(S)	22(S)	16(S)	10(R)
<i>Enterobacter sp</i>	10(R)	0(R)	6(R)	20(S)	2(R)	10(R)	22(S)	18(S)	12(I)	0(R)

KEY:

Resistance (R) = 0-10mm

Intermediate (I) = 11-16mm

Sensitive (S) = 17mm and above

LEV: Levofloxacin, CF: Cefotaxim, SP: Saprifloxacin, CPX: Ciprofloxacin, AM: Amoxicillin,
 CN: Gentamycin, PEF: Pefloxacin, OFX: Tarivid, SP: Sparifloxacin, AZ: Azithromycin

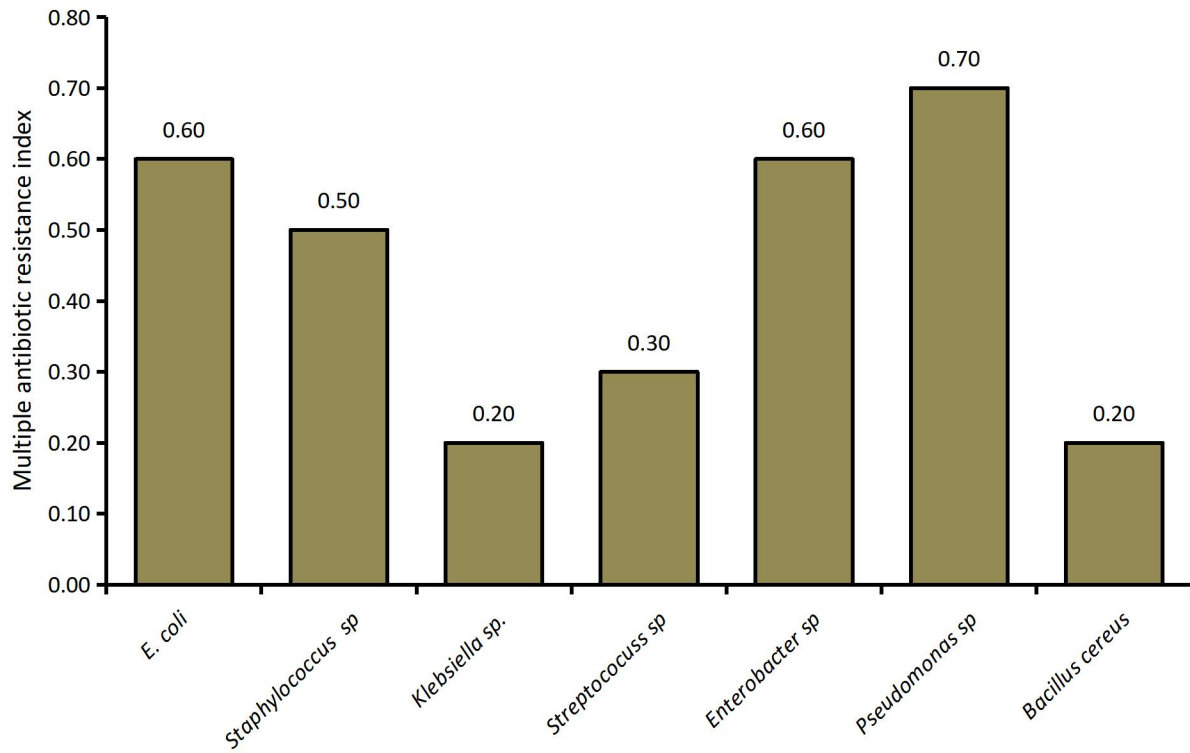


Figure 4.3. Multiple antibiotics resistance index of the bacterial isolates

DISCUSSION

The findings from this study have significant implications for understanding the microbial load and antibiotic resistance profiles within wastewater samples from different wards of the University of Benin Teaching Hospital. The bacteriological analysis conducted provides insights into the extent of microbial contamination and the potential risks to public health.

The total heterotrophic bacterial counts observed in this study ranged from 3.12 ± 0.15 to $8.0 \pm 0.27 \times (\log_{10} \text{ cfu/ml})$, with the highest bacterial load recorded in samples from the Laundry Department (B), followed by the Occupational Therapy (D) and Shopping Complex (A). The lowest count was observed in the Eye Clinic (C). The variation in bacterial counts among the wards suggests differing levels of contamination, likely influenced by specific activities and waste management practices in each area. This observation is consistent with studies that have highlighted the high microbial load in hospital wastewater, which is subject to diverse contaminants, including body fluids and clinical waste (Carraro *et al.*, 2016). Similar findings were reported by Alwabr *et al.*, (2016), who recorded mean bacterial counts exceeding 10^5 cfu/mL in hospital wastewater. The results suggest that hospital wastewater samples, especially from high-traffic areas like the laundry department, are prone to higher bacterial loads due to the continuous use of water and exposure to various biofluids.

The study identified several bacterial isolates from the hospital wastewater, including *Staphylococcus sp.*, *Escherichia coli*, and *Pseudomonas sp.*. These findings align with those of Nyadjou *et al.*, (2018), who also reported the presence of *E. coli* and *Pseudomonas sp.* in wastewater samples, suggesting significant contamination. These findings also agree with Andy and Okpo, (2018) who also identified the same bacterial isolates from effluent and waste dump

site soil of selected hospitals in Calabar metropolis, Nigeria. From the result, more Gram-negative organisms (especially members of the *enteriobacteriaceae*) were isolated than Gram-positive organisms. This is in agreement to similar study by Sintayehi, 2011, who reported some potential pathogenic bacteria such as *Salmonella* sp, *Shigella* sp, *Staphylococcus aureus* and *E. coli* in hospitals waste effluents.

The study's frequency distribution analysis revealed that *Pseudomonas sp.* was the most frequently isolated bacterium, followed by *Staphylococcus sp.* and *E. coli*. These results align with findings by Isichei-Ukah and Enabulele (2018), who examined the prevalence and antimicrobial resistance of *Pseudomonas aeruginosa* in environmental and clinical sources in Benin City, Nigeria. Similarly, Oviasogie *et al.* (2010) reported that *Pseudomonas aeruginosa* was the most prevalent Gram-negative bacterium isolated from hospital waste, accounting for 25% of all isolates. Comparable bacterial isolates in hospital waste have also been documented by other researchers (Bolaji *et al.*, 2011; Vichal *et al.*, 2011). The presence of *Pseudomonas sp.* is particularly concerning due to its known high resistance to disinfectants and antibiotics. Additionally, the identification of *Staphylococcus sp.*, often linked to nosocomial infections (Noskin *et al.*, 2005), highlights the potential hazards associated with hospital wastewater.

The occurrence of these bacteria has implications for public health, as they are associated with a range of diseases. *E. coli* and *Pseudomonas sp.*, for example, are known to cause gastrointestinal, urinary tract, and respiratory infections, which can be severe and potentially life-threatening (Alwabr *et al.*, 2016). The isolation of these bacteria from hospital wastewater samples is consistent with findings from studies such as those by Oyeleke and Istifanus (2009), where *E. coli*, *Pseudomonas sp.*, and *Staphylococcus sp.* were frequently identified in hospital waste soil

samples. The presence of these organisms in wastewater poses risks of contaminating surface and groundwater, potentially impacting public health.

Antibiotic susceptibility testing demonstrated variable resistance patterns among the bacterial isolates. The results showed that Gram-positive bacteria such as *Staphylococcus sp.*, *Bacillus sp.*, and *Streptococcus sp.* exhibited resistance to antibiotics like Ampiclox and Erythromycin, while Gram-negative bacteria, including *Pseudomonas sp.* and *Escherichia coli*, displayed multidrug resistance. Notably, *Pseudomonas sp.* had the highest multiple antibiotic resistance (MAR) index at 0.7, suggesting a significant potential for antibiotic resistance development. The MAR index is an essential parameter for evaluating potential health risks associated with antimicrobial resistance.

The high resistance levels observed, particularly in *Pseudomonas sp.*, are alarming due to the intrinsic resistance of this bacterium to many antibiotics and its potential to acquire further resistance mechanisms through mutation and horizontal gene transfer (Sader *et al.*, 2023). This finding correlates with the resistance patterns reported by Alwabr *et al.*, (2016), where infections caused by *Pseudomonas sp.* and *E. coli* were noted for their difficulty in treatment. Such multidrug resistance complicates clinical management and increases the risk of morbidity and mortality.

The MAR indices of the isolates ranged from 0.1 to 0.7, suggesting that some strains have acquired mechanisms to resist multiple antibiotics. This could lead to the emergence of multidrug-resistant (MDR) strains, posing challenges for healthcare systems and requiring more advanced and targeted therapeutic interventions (Medina and Pieper, 2016). This trend is also

supported by Kunwar *et al.*, (2019), who discussed mechanisms such as drug inactivation and reduced drug permeability as primary strategies bacteria use to combat antibiotics.

The presence of antibiotic-resistant bacteria in hospital wastewater represents a significant public health concern. The risk of these pathogens spreading through contaminated water sources, direct contact, and potential airborne transmission can contribute to the development of healthcare-associated infections and community outbreaks (WHO, 2014). The findings of this study, particularly the high prevalence of *Pseudomonas sp.* and *E. coli*, underscore the urgent need for enhanced sanitation and waste management strategies within healthcare settings. Effective antimicrobial stewardship programs, coupled with routine monitoring of hospital wastewater, are essential in mitigating the spread of resistant pathogens (Majumder *et al.*, 2020).

The presence of potentially pathogenic and antibiotic-resistant bacteria in hospital wastewater suggests that immediate action is required to minimize the risk to public health. Public health agencies should prioritize interventions such as improved wastewater treatment protocols, hygiene awareness campaigns, and stringent regulations for waste disposal in hospital environments.

CONCLUSION

The study highlighted the presence of pathogenic and antibiotic-resistant bacterial isolates in hospital wastewater, with *Pseudomonas sp.* showing the highest MAR index. These findings call for improved waste management practices, antimicrobial stewardship programs, and regular monitoring to prevent the spread of resistant pathogens. Further research should focus on long-term trends in antibiotic resistance in hospital wastewater and its broader impact on public health.

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