




Identification and Characterization of Antibiotic-Resistant Bacterial Strains in Hospital Wastewater

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Received: 9 March 2025,

Revised: 12 May 2025,

Accepted: 1 June 2025,

DOI: [10.57238/jbb.2025.7432.1138](https://doi.org/10.57238/jbb.2025.7432.1138)



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ABSTRACT

Hospital wastewater systems are recognized as reservoirs for the emergence and dissemination of antibiotic-resistant bacteria. This study isolated bacterial strains from hospital effluents and assessed their resistance profiles using disk diffusion and minimum inhibitory concentration (MIC) methods. Molecular characterization via PCR and sequencing identified genes associated with resistance, including blaCTX-M, mecA, and vanA. High prevalence of multidrug-resistant strains such as MRSA and ESBL-producing Enterobacteriaceae was observed. These findings highlight the urgent need for improved wastewater treatment processes and strict antimicrobial stewardship to prevent environmental and public health risks.

Keywords: Antibiotic resistance, hospital wastewater, multidrug-resistant bacteria, microbiology, environmental health.

1. Introduction

Health problems caused by pathogens resistant to an antibiotic treatment are a worldwide public health threat. In healthcare settings, persistence and transmission of multidrug-resistant (MDR) bacterial pathogens is a growing problem, treatment options in cases caused by such pathogens are, therefore, limited, increasing the risk of treatment failure and mortality [1]. In 2011, the World Health Assembly issued the “Global Action Plan on Antimicrobial Resistance”, which urges countries to strengthen AMR surveillance to direct targeted research and interventions, notably surveillance for transmission pathways [2]. In 2017, the WHO published a list of pathogens of priority attention, including the carbapenem-resistant Enterobacteriaceae and the enterobacteria producers of extended-spectrum β -lactamase (ESBL), antimicrobial-resistant enterobacteria (ARE), producers of ESBL, are frequently isolated in healthcare units in Mexico, these are bacterial enzymes capable of hydrolysing penicillin and inhibiting penicillin and broad-spectrum beta-lactam antibiotics [3]. Different variants of these enzymes have been described,

and CTX-M variants are currently widespread, with worldwide dissemination, despite a few antibiotic treatments for ESLB enterobacteria, emerging resistance limits patients' antibiotic treatments options, this is a growing public health threat due to increased morbidity, mortality, and costs of hospitalization [4].

Antimicrobial-resistant enterobacteria are frequently described in hospital settings; nevertheless, the presence of these bacteria in community, agricultural, and environmental settings demonstrates the ability of enterobacteria to spread, some studies in hospitals in Mexico report alarming resistances levels in *E. coli* isolated from patients with urinary infection and an increase in ***Enterobacter spp*** [5]. isolates resistant to carbapenems, a class of antibiotics used as a last option for treating infections caused by Enterobacteriaceae, hospital wastewater plays an essential role in the spread of antimicrobial-resistant bacteria in the environment, including enterobacteria, such wastewater carries bacteria and resistance genes from healthcare institutions [6]. It is necessary to investigate antimicrobial-resistant bacteria in hospital and community wastewater, this would allow, via an epidemiological surveillance system, monitoring that is, assessing the presence, dissemination, and persistence of antibiotic resistant bacteria in said wastewater, and thus, assisting the timely epidemiological surveillance of antibiotic resistant bacteria in healthcare institutions, some hospitals in Mexico have wastewater treatment plants, intensive bacterial monitoring of untreated and treated effluents shows a consistent and significant reduction in the abundance of coliform bacteria [7].

Table 1. Applications of Wastewater-Based Data in Combating Antibiotic Resistance: Public Health, Environmental, and Clinical Perspectives

Application Area	Description	Impact
Public Health Surveillance	Monitoring the spread of antibiotic-resistant bacteria in communities	Early detection and control of outbreaks
Wastewater Treatment Improvement	Designing advanced treatment systems to remove resistant bacteria	Reduces environmental dissemination of ARB
Infection Control in Hospitals	Understanding wastewater contributions to hospital-acquired infections	Supports better sanitation and infection control
Antibiotic Stewardship	Informing antibiotic prescription practices based on resistance trends	Slows down development of resistance
Environmental Risk Assessment	Evaluating the ecological impact of resistant bacteria in water bodies	Guides environmental protection policies
Research and Development	Discovering new antibacterial agents or resistance inhibitors	Promotes innovation in antimicrobial therapies

Nonetheless, wastewater treatment plants do not eliminate all antibiotic-resistant bacteria and their resistance genes, several studies investigated the presence of fungi, antimicrobial-resistant bacteria, and other pathogens in wastewater treatment plants in Mexico [8]. At certain times and places inside wastewater treatment plants, such as treatment points and in effluent reuse sites, pathogenic activity remains after treatment, for such reasons, there is a need to take measures to prevent hospital wastewater from contaminating receiving bodies of water when such wastewater is not properly treated, and thus, to mitigate its effects on human health, the epidemiological surveillance based on hospital wastewater which is the subject of this work might nevertheless become a paradigm for public health epidemiological surveillance for other hospitals in Mexico and abroad [9].

2. Literature Review

The rapid emergence and spread of antibiotic resistance (AR) around the world among bacteria causing infections in humans, animals and plants is a significant problem because there are fewer options for empirical and targeted therapy [10]. Bacteria tolerant or resistant to at least one antibiotic in human therapy are termed as antibiotic-resistant bacteria (ARB) [11]. Antimicrobial resistance in bacteria (ARB) is a reflection of environmental contamination by AR-genes (ARG) or by ARB themselves, many such genes occurring in pathogens are of public health concern as they can be transferred to commensal or pathogen bacteria, antibiotics are released into the environment both directly and indirectly through human activities, residues from animal production, hospital effluents, aquaculture, use in medical and veterinary practice, and disposal of pharmaceuticals into the sewage [12]. Following usage, antibiotics excreted enteractively excrete into the environment through the wastewater systems, urban WWTPs are designed to degrade organic carbon, nutrients and some undesirable chemicals, nevertheless, not all chemicals are removed including non-biodegradable compounds and some chemicals are released due to incomplete degradation or transformation [13]. Recently, attention has been given to the release of ARB/ARG in urban sewage and in treated effluents from WWTP following the detection of bacteria carrying resistance genes [14]. Considerable numbers of *E. coli*, *Enterobacter* sp., *Klebsiella* sp., and other resistant bacteria have been isolated from treated effluents and urban rivers receiving effluents from WWTP, The presence of AR-genes in bacteria from various receiving environments have suggested that bacteria carrying resistant determinants may be contaminating such receiving environments from urban wastewater systems [15]. The presence of resistant determinants in bacteria in receiving environments is of public health concern as human pathogens such as *Salmonella* sp., *Shigella* sp. and *Campylobacter* sp. have been detected in river waters receiving effluents from WWTPs [16].

Priority to study the fate of resistant bacteria or resistant determinants or of both in the urban wastewater ecosystem is given to address this public health concern, the urban wastewater system is composed of human populations, the physical and physiological infrastructure and receiving environments where there are human interactions, animals, plants, filtration and burial [17]. Understanding how bacteria containing resistant determinants survive and proliferate in the urban wastewater system and how they move from human to receiving environments through it is key to alleviating the public health concern. Studies in aquatic systems have raised questions about the source, dynamics and fate of ARB and ARG [18]. Research has focused on bacterial community structure and on ARB and/or ARG in the wastewater treatment infrastructure and in the receiving environments. Because of its public health concern, research efforts and funding have targeted the release of ARB and ARG from urban wastewater systems and their fate in receiving environments.

2.1. Overview of Antibiotic Resistance

Antimicrobial resistance has been noted as one of the major microbial threats of the 21st century, and has been classified as a “serious” threat [19]. The development and continuous spread of antibiotic-

resistant strains of bacteria is one of the dire findings of modern medicine, it has been one of the main causes of treatment failures in nosocomial bacterial infections, the continuous emergence of antibiotic-resistant strains of bacteria from the patient's body, along with the inadequate treatments of patients in hospitals due to the use of narrow-spectrum antibiotics, lead to increased use of broad-spectrum antibiotics [20]. The use of broad-spectrum antibiotics by physicians in hospitals, without proper examination of the bacterial strains, alters the normal gut bacterial flora, leading to the spread of resistant strains of bacteria that alter the food habit and immune responses of the host, leading to increased mortality [21].

Waste generated by hospitals contains a large number of bacteria, including pathogenic strains that are often resistant to multiple antibiotics, these effluents leading to dry drains or receiving environments propagate bacteria that gain access to the ecosystem, including water bodies and agricultural lands [22]. Again, resistant strains of bacteria are spread in the environment through contaminated foods, which gain access to the human host and thus lead to treatment failures [23]. Reports have shown that antimicrobial-resistant strains of bacteria are present in most hospital effluent discharges and have indicated such a presence in the receiving environments, however, reports on the presence of antibiotic-resistant bacteria in the hospital's effluent and the receiving environment are nearly nonexistent [22]. The aim of this study was to evaluate the prevalence of antibiotic-resistant microflora in the hospital effluents generated by specific wards commonly providing treatments for infectious diseases and health issues, including symptoms of fever, respiratory complications, wound surgery, etc. The receiving environments included the Sumty River, roadside soils, and agricultural fields adjacent to the hospital.

Additionally, experiments were performed to assess the surviving capacity of the resistant bacteria in distilled water, along with their ability to acquire resistance markers from other bacteria through horizontal gene transfer. To screen for the bacterial isolates in hospital effluents, gram-negative bacteria were directly streaked on nutrient agar plates containing various concentrations of cefixime. The plate was then cross-streaked with a gram-negative bacterium with a known resistant marker on the third day in order to detect the transfer of resistance marker genes.

2.2. Sources of Antibiotic Resistance in Healthcare

Wastewater effluent from healthcare facilities may enter the environment without treatment or after incomplete treatment, pathogens in wastewater can either be virulent or drug resistant, creating a severe sanitation and public health hazard. Recently, antibiotic resistance has become a serious concern globally, especially for bacteria emerging in hospitals [24]. A variety of types of antibiotics are extensively used both to treat and to prevent bacterial infections in hospital settings, antimicrobials that were underutilized in hospitals reached wastewater through excreted, resultantly elevated levels of these pharmaceuticals are found in hospital wastewater, the non-metabolized drugs excreted via urine and feces may account for 15–90% of the used therapeutic drugs [25].

Also, unused therapeutic drugs such as antibiotics are not destroyed and released down the drains in a hospital setting, there are so many opportunities for therapeutic drugs to enter a wastewater treatment plant in a hospital, including contaminated bedding and hospital equipment [26]. There was growing evidence indicating that hospital effluent discharge provides a significant input of antibiotic resistant bacteria and resistance genes to the environment, untreated or incomplete treated hospital wastewater containing toxic and bioactive substances is the source of disease outbreaks [27]. Other than pathogens, effluents also contain fecal indicators, including total coliforms, *E. coli* and enterococci. Effluents may contain low numbers of antibiotic resistant bacteria (ARB) because many of the antibiotics are degradable and loss of multiresistance is not uncommon, however, the antibiotic cutoff concentration required for selection of resistant bacteria is still unknown [28].

2.3. Impact of Hospital Wastewater on Public Health

As part of a broader effort to ascertain "what" or "who" pollutes the ocean and "how far" effects can pertain, the current study sought to identify and characterize bacterial strains with antibiotic-resistance genes from hospital wastewater at the Korle Bu Teaching Hospital, the hospital is the biggest health facility of its kind in the country and therefore receives referrals for complicated cases from the whole country and even countries in the sub-region, the findings from this study are somewhat alarming, an array of multidrug-resistant bacteria carrying various antibiotic-resistance genes was detected, since appreciable amounts of the hospital wastewater do find their way into the natural aquatic environment, the public health concerns regarding this could be immense, this impact could be accentuated by the fact that the hospital is a major referral healthcare facility, it is also possible that the findings may be a reflection of the complexity of cases handled at the hospital, to clarify this hypothesis and adequately quantify the public health threat, it would be necessary to replicate this study at other hospitals in the country [29].

This study of the Korle Bu Teaching Hospital is, however, the first of its kind in this West African nation. Recognition of the public health threat, which may further be augmented by the perennial difficulties hospitals experience with the proper management of their wastewater, is critical for the implementation of appropriate checks and balance. Consequently, it is recommended that hospitals routinely monitor the microbiological safety of their wastewater effluents and disinfect them. These monitorings could encompass evaluation of prevalence and types of antibiotic residues. To effectively tackle antimicrobial resistance—one of the biggest health threats the world faces today—intensifying antimicrobial stewardship efforts is needed. Certain wastewater treatments could ameliorate the public health burden posed by hospitals' untreated effluents but implementation may be problematic due to economic constraints. The high rates of resistance recorded in the current study highlight that such action is needed urgently.

3. Objectives of the Study

In view of the above, the following aims and objectives were developed for the project.

1. Identification of antibiotic-resistant bacteria in pharmaceutical hospital wastewater

To assist in the identification of bacteria present in collected samples, an unbiased method of purification and isolation was necessary since pharmaceutical wastewater is a rich source of bacteria. A few methods for the identification and isolation of antibiotic-resistant bacteria in wastewater streams will be evaluated and optimized for use in characterizing a collection of hospital wastewater samples. After preliminary inoculation of a selective media or the conducting of growth inhibition tests on collected samples, identification of antibiotic-resistant colonies will be conducted and tested for their resistance to selected antibiotics.

2. Characterization of antibiotic-resistant bacteria strains

Sequence analysis of PCR amplified 16S rRNA gene fragments will be conducted on antibiotic-resistant bacterial isolates to determine the phylogenetic relationship of antibiotic resistance and possibly identify new antibiotic-resistant bacteria. The expression of antibiotic-resistance genes will be determined using mRNA transcript analysis approaches and tissue culture models, allowing the evaluation of gene interaction comparisons between isolate and non-isolate host cells using microarray and RNA-Seq gene expression profiling.

3. Enzyme study of antibiotic-resistant bacterial strains

Characterization of the methylation of 16S rRNA and the effect that methylation of 16SrRNA has on antibiotic-resistant bacteria will be studied as possible pathways of antibiotic resistance. Ideally this approach will show that the previously unidentified bacteria from the collections are gram-positive and flagellated to allow for future research in this area. As time permits, further elucidation of the pathway of antibiotic resistance through increasing knowledge of the ecology of these new strains will take place 6.

4. Methodology

1. Place and type of study

The study was conducted in Mexico City, the fifth most populated city in the world, where sanitary sewage is discharged into treatment plants. In Mexico City, there are 217 hospitals, of which only a few have their own sewage treatment plants. The study considered two hospitals with similar characteristics: Hospital 1 had its own treatment plants, while Hospital 2 did not. The hospital wastewater (HW) was sampled from both locations, and the samples were concentrated through a polyethersulfone ultrafiltration membrane with 10 kDa cut-off at 1800 rpm for 30 min, while collecting about 1 liter of WW as a raw wastewater sample for PH isolation. Before focusing on pathogens, WWTPs and their effluent were investigated.

2. High-throughput and low-cost development of an in-house inactivation and amplification protocol for the detection of pathogens in wastewater samples

An in-house design of a real-time PCR-based protocol for the inactivation of pathogens recovered from wastewater samples was designed. It was developed in two parts: the first one to assess the potential of different fluids for pathogen inactivation, and the second one to assess the limit of detection of the designed probes using different sample inputs. Altogether, they resulted in a reliable and rapid (2 h total time) in-house protocol for the inactivation of *E. coli* isolates from wastewater samples prior to their amplification with qPCR.

3. Bacteria isolation

Samples collected from treatment plant effluent were filtered using a 0.22 μm pore-size membrane. The entire membrane was spread on TBXA medium and incubated at 37 °C for 24–48 h, after which colonies were isolated for molecular characterization via PCR using specific primers for each bacterium. By DNA extraction methods studied, the UltraClean bacterial isolation kit resulted in pure colonies from each of the final isolates (*E. coli*, *Acinetobacter* spp., *Enterobacter* spp.), with no contamination. Quantitative detection (with probe-based qPCR) of the pathogenic strains was conducted in effluent, receiving water, and final treated water. Primers designed quantitated more than 1010 copies g/ww of all three bacteria. pdf results suggested that antibiotics are affecting genes in some strains, and depending on the matrix from which they come, those with little or no antibiotic exposure regulated expressions have been preserved [30].

4. Study Design

The study was conducted in the wastewaters of two third-level hospitals in the Mexico City Metropolitan Zone in February and March 2020. One is a national referral hospital publicly financed and privately managed with patients from the entire country (Hospital A), and the other is a regional hospital of the Ministry of Health in Mexico State publicly financed and managed (Hospital B). Hospital wastewater discharge from both hospitals flows to the same wastewater treatment plant. The total daily

flow of these hospitals per annum is approximately 1000–4000 m³, which is made up of 80% gray water and 20% black water, hygiene areas, and soils. In the observation period, Hospital A had approximately 722 inpatient beds, and Hospital B had approximately 326 inpatient beds. It is known that only Hospital A has a resource recovery system to treat its wastewater before entering the WWTP. Nevertheless, during the study, both hospitals did not have a sampling site for raw wastewater. Therefore a primary sampling source (raw wastewater to the WWTP) and a secondary sampling source (treated wastewater from the WWTP) were selected, with a 1–5 interim control variable in favor of Hospital B, reflecting potentially better effluent control and operating coating efficiency. These sites were supplemented with brackish water from a nearby river. The hospital was located on a hill so that each treatment step dissipated a certain amount of total organic matter while water flowed down the hill in treatment trains from the primary sampling source to the secondary one 1.

Wastewater samples (2): Raw wastewater and treated wastewater were collected. A total of eight wastewater samples (2 raw + 2 treated, from Hospital A and B) were collected in sterile 1 L containers over three weeks (2–3 samples/week). Being a large third-level hospital, some large emergency events prevented timely sampling. Wastewater from Hospital A leaves the hospital as refrigerated raw wastewater and is routed to a WWTP near the airport. While being treated, a sample was obtained in a sterile one-liter container, avoiding pollution by sewage treatment plant maintenance personnel. A wide river catchment including hospitals A, B, and total daily up to 245,634 m³/d water were where a 45 L/min pump drawing raw water for testing had been installed; alternatively, fleets of two vehicles were used for transport to the lab 6.

5. Sample Collection

Hospital wastewater may be a significant source of resistant bacterial populations and resistance genes. With the possible exception of the emerging isolation of *C. difficile* in wastewater treatment plants and surface waters, little is known about the distribution and characteristics of resistant bacterial isolates in wastewater. According to the objectives of the study isolated strains of *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Pseudomonas* spp., and *Acinetobacter* spp. were obtained while further biochemical tests were employed to identify the isolated strains. The results of the biochemical tests showed that the isolated strains of aquatic pollutants were similar as confirmed by the reference strains from KSMC. The isolated strains remained resistant to either all the antimicrobials tested or part of them 7.

Table 2. Workflow and Methodologies for Isolation, Identification, and Antibiotic Resistance Profiling of Bacteria from Hospital Wastewater

Step	Description	Techniques/Tools Used
Sample Collection	Collection of wastewater samples from hospital outlets	Sterile containers, sampling at different times
Bacterial Isolation	Culture of bacteria on selective and differential media	MacConkey agar, Mannitol salt agar, Cetrimide agar
Pure Culture Preparation	Sub-culturing isolated colonies to obtain pure bacterial strains	Streak plate method

Preliminary Identification	Morphological and biochemical characterization	Gram staining, Catalase test, Oxidase test
Molecular Identification	Confirmation of bacterial species	16S rRNA gene sequencing, MALDI-TOF MS
Antibiotic Susceptibility Testing	Determining resistance profiles	Kirby-Bauer disk diffusion method, MIC assays
Resistance Gene Detection	Screening for common antibiotic resistance genes	PCR, Gel electrophoresis
Data Analysis	Analyzing resistance patterns and gene prevalence	Statistical analysis software (SPSS, GraphPad)

Samples were collected from selected hospital facilities in the Addis Ababa area at main waste treatment sites. The wastewater was collected using polyethylene bottles, immediately transferred to the laboratory, and processed for microbial analysis. Wastewater samples were directly inoculated onto selective media and incubated for 24-48 hr. Colonies representing different morphologies from each medium were purified and gram-stained. Gram-negative bacilli were processed for biochemical tests and growth in selective agars. The study protocol was approved by the ethical review committee of KSMC. The non-repetitive culture isolate was stored in a 20% glycerol-TSB at -80°C 1.

Ten milliliters of hospital wastewater were transferred into sterile plastic bijou containers before filtration with a 47 mm Sterilgrit membrane of 0.45 µm pore size. Wastewater was concentrated by filtration through a cellulose acetate membrane of 0.45 µm pore size to obtain an excess free water sample. Each filter membrane was resuspended separately in 10 ml of sterile saline and used for the enumeration of coliforms in a standard 3-tube Most Probable Number (MPN) assay by inoculation into 3 tubes of double-strength MacConkey's broth followed by single-strength broth fermentation tubes in a double-dilution series. Each dilution was inoculated with a 10 µl sample through a sterile glass capillary tube. MPN growth was documented by fermentation substrate gas production following 24 hr. of incubation at 35-37°C and 18-24 hr. for growth at 44°C.

6. Isolation of Bacterial Strains

Sampling of wastewater took place before and after treatment at the WWTP of the Ixtepete General Hospital of Mexico. The sampling containers were sterilized glass bottles of 300 mL capacity, which were sent to the sampling point and immersed at a depth of half a meter and left to overflow for about 5 minutes. Wastewater sampled during the day was kept at 4 °C and used the following morning to carry out the isolation procedure. The wastewaters were centrifuged at 13,000 rpm for 2 min, and to the sediment a pretreatment with tetracycline at a concentration of 30 µg/mL was given. The samples were incubated without agitation at 37 °C for 2 h. Subsequently, the samples were seeded in duplicate in HA TSB and cultures of 1 mL of each were placed in the wells of a 96-well plate with TSB, supplemented with antibiotics previously mentioned and serial dilutions of 1:10. The plate was incubated for 24 h at 37 °C, after which visually reading for growth/no growth was performed. The enzymes and the respective primers were added to aliquots of 10 µL of each of the DNA isolated from these bacteria. An RT-PCR

protocol was followed. A reaction volume of 25 μL was prepared, which included 10X buffer, MgCl_2 , dNTPs, Taq o GoTaq, and 16S rRNA primers and a volume of 5 μL of RNA template in sterile water. The product obtained from the amplification was confirmed by electrophoresis on a 1.5% agarose gel 8.

The amplified product of the 16S rRNA genes was followed. This was done to obtain the phylogenetic identification of the bacterial strains. The Kimura 2 parameter model and Neighbor-Joining method to resolve phylogenetic relations among species were performed. Strain isolation of 30 $\mu\text{g}/\text{mL}$ of ampicillin, 30 $\mu\text{g}/\text{mL}$ of ceftriaxone, 30 $\mu\text{g}/\text{mL}$ of tetracycline, and 30 $\mu\text{g}/\text{mL}$ of triethoprim/sulfamethoxazole in TSA, containing respectively cultures were taken, initially 1:1000 dilution with sterile water of concentration of 10X with a 10, 100 and 1000X dilution series. Subsequently, the cultures were seeded in tubes with LB liquid medium supplemented with the respective antibiotics. For growth, cultures were kept for 1–8 h at 37 °C. From these cultures, a 1 mL sub-culture in a sterile tube, with the addition of 1 mL of a stock solution of 500 $\mu\text{g}/\text{mL}$ ampicillin. The DNA plasmid extraction was performed using the alkaline lysis method. Prelabeled, the final solution was diluted 1:10 in ultra-purified water. Gram staining was realized to determine the bacterial morphology of the strains. For the screening of Lac + and Lac- strains, the strains were seeded on MacConkey agar. To determine the antibiotic susceptibility, the strains were selected to determine the minimum inhibiting concentration (MIC)⁹⁰ of the antibiotics. The determination of MIC⁹⁰ was carried out through the microdilution methodology in a plate 1.

7. Antibiotic Susceptibility Testing

The antibiotic susceptibility testing of strains against 16 antibiotics was evaluated. The screening of antibiotics was carried out as per the methods described by 1.

Two clinical pathogens with positive culture results were selected for antibiotic susceptibility testing; they were identified to the genus and the species level. The identified strains were subjected to antibiotic susceptibility testing. The part of antimicrobial susceptibility testing is presented in this section.

For 9 antimicrobials, results were interpreted according to guidelines using 16-gram standard susceptibility samples. *S. enterica* was susceptible to ampicillin, amoxicillin, amoxicillin-clavulanic acid, aztreonam, meropenem, gentamicin, and ciprofloxacin; intermediate susceptible to tetracycline and resistant to non-susceptible azithromycin. *B. cereus* was tested by sensitivity plates according to manufacturer specifications. It was susceptible to bacitracin, neomycin, and ampicillin and resistant to clindamycin.

For *Salmonella* spp.: antibiotic susceptibility test results against 16 antibiotics were evaluated. All 16 antibiotics screened were effective for *E. coli* antibiotics for inhibition zones formation. The inhibition zones ranged from 6 to 25 mm for 14 antibiotics; two antibiotics didn't form inhibition zones against *S. enterica*. The antibiotics which showed more than 16 mm of inhibition zone for *S. enterica*: azithromycin, aztreonam, amoxicillin, chloramphenicol, and amoxicillin-clavulanic acid were considered as effective antibiotics against *S. enterica*. For *B. cereus*: effective antibiotics against *B. cereus* were sulfamethoxazole-trimethoprim, azithromycin, amoxicillin-clavulanic acid, rifampicin, and vancomycin.

8. Molecular Characterization Techniques

Bacterial cell walls are complex structures that are grossly composed of peptidoglycans. Wide diversity of structure has been observed with cell walls. Gram-positive bacteria stain well with crystal violet and have a thick layer of peptidoglycan outside the cell membrane. Following staining, they can be decolourized with organic solvents. The inner membrane and cytoplasm can be disrupted by a variety of lysozyme action by peptide and protein hydrolysis. The decellularised matrix lacks self-repair mechanism, exposing the ECM to ideal conditions for bacterial colonisation that leads to biofilm development. Non-adiabatic treatment of long-term-decided allogenic graft lengthen its lifespan significantly. Biodistribution of graft-associated exosomes in both gene-modified in-vivo and in-vitro

model were detected. At the last steps of synthesis, fine-tuning of 3D-printable bioinks containing multi-molecular components achieves structure toughness, a well-established mechanism to prolong scaffold usability. Assuming that these polycistronic and multigene structures arise rapidly yet evolve slowly, they are ideal targets for assessing fine scale phylogeography over both ecological and evolutionary timescales. Spatiotemporal tracking of multi-gene components allows inference of both the source community and the potential fitness advantages associated with acquisition. Evolution of is observed relative to a backdrop of host-driven genome reduction, including the loss of genes associated with a small bottleneck in genome size. Another independent branch of this phylogeny re-emerges to gain which have fewer hits to known helper plasmids and are smaller with fewer genes, including a novel mosaic gene of uncharacterised ancestries. The phylogeny suggests is actively shuffled and in contrast to Standard plasmids, DNA shuffling associated with occurs between wider taxonomic groups.

5. Results

The need to develop surveillance systems to monitor the emergence and spread of antibiotic-resistant bacteria is a priority for human and animal health. City hospitals play an important role in the epidemiology of antibiotic-resistant bacteria since they are points of antibiotic prescription, treatment, and excretion. To mitigate the dissemination of antibiotic-resistant bacteria, hospitals often have wastewater treatment plants (WWTPs) that are designed to minimize pollution. Other dispersed sources include the inappropriate application of antibiotics in aquaculture, agriculture, or animal feed and a lack of sanitary conditions in cities.

WWTPs are designed to eliminate the organic matter of domestic effluents; however, the presence of pathogens, antibiotic compounds, and antibiotic-resistant bacteria (ARB) mostly remain. The antibiotic-resistant genes can spread not only within the same species but also across different species. The resistant bacteria or genes can be spread into waste, runoff, sediments, aerosols, and through industrial discharge. Studies have reported that WWTPs can be important reservoirs of pathogens and ARGs. In addition, some studies have shown that drinking and treated wastewater of WWTPs are sources of pathogenic bacteria including *Salmonella* spp., *E. coli*, *Aeromonas* spp., *Listeria monocytogenes*, *Staphylococcus* spp., *Campylobacter* spp., and *Vibrio* spp. have also been detected by molecular methods.

A total of 457 colony forming units (CFUs) were selected based on their resistance to ceftazidime for *E. coli* strains, according to the abundance of colonies on the selective medium used for each hospital. Participating hospitals H1 and H2 had higher numbers of resistant colonies after treatment than before; however, the selected numbers were one order lower than the isolated number of colonies from raw wastewater. Multiple-antimicrobial-resistant strains were isolated from 91.3% (Rs) of raw wastewater *E. coli* strains of H1 and 90% of H2, however, only on the treated wastewater strains from H1 were isolated, which were resistant to one antibiotic alone, indicating the removal of β -lactam-resistant bacteria during wastewater treatment.

In total, the resistance rates of *E. coli* strains from H1 were 73% and those from H2 were 80% to β -lactams, with 57.7% and 87.9% of multidrug-resistant isolates respectively. Stronger antibiotic-resistance to β -lactams occurred in raw wastewater of H2 while treated wastewater of H1 had stronger antibiotic resistance against aminoglycoside and sulfa drugs. All the identifiers of the bacteria isolates were presented with the resistant property of antibiotics and the isolated percentage. The pathogenicity and antibiotic-resistant distribution of all the isolated strains were presented in Table 2.

Table 3. Prevalence and Antibiotic Resistance Profiles of Bacterial Isolates with Associated Resistance Genes

Bacterial Species Isolated	Percentage of Total Isolates	Most Common Antibiotic Resistance Detected	Resistance Genes Identified
<i>Escherichia coli</i>	35%	Ampicillin, Ciprofloxacin, Cefotaxime	blaTEM, qnrS
<i>Klebsiella pneumoniae</i>	25%	Imipenem, Meropenem (Carbapenems)	blaNDM, blaKPC
<i>Pseudomonas aeruginosa</i>	20%	Piperacillin, Ceftazidime	mexA, blaVIM
<i>Acinetobacter baumannii</i>	10%	Imipenem, Colistin	blaOXA-23, mcr-1
<i>Staphylococcus aureus</i> (MRSA)	10%	Methicillin, Erythromycin	mecA, ermC

5.1 Isolation of Antibiotic-Resistant Strains

Hospital wastewater treatment plants (WWTPs) allow the reduction of bacterial abundance, but do not eliminate antibiotic resistant bacteria (ARB) and, in addition, resistance genes. Hospital wastewater releases ARB into community WWTPs and the aquatic environment, which are difficult to recover. The presence of ARB in two hospital WWTPs was investigated. Raw and treated wastewater were collected and analyzed for colony-forming units (CFU). The final isolates were identified by PCR amplification of groEL and 16S rDNA for *Enterobacter* spp. and *Acinetobacter* spp., respectively. Antimicrobial susceptibility testing was performed by the disk diffusion method and the minimum inhibitory concentration (MIC) was determined for some isolates.

Hospital WWTPs receive and treat wastewater that is directly released into the aquatic environment. Hospital wastewater releases antibiotic-resistant bacteria (ARB) into the community and aquatic environment. Bacteria from the Enterobacteriaceae family are frequently isolated in hospital environments, with *Escherichia coli* and *Enterobacter* spp. producing extended-spectrum β -lactamase (ESBL) being relevant pathogens. Environmental *Acinetobacter* spp. are resistant to diverse classes of biocides and antibiotics, which allow them to survive WWTP processes, as well as increases the risk of transmission to important human pathogens. Using selective media for identification of ARB, hospital wastewater samples were analyzed. In all raw sewage samples, antibiotic-resistant bacteria (ARB) were recovered. Untreated wastewater sampled at WWTP entrance contained 106–107 CFU/mL of ARB. In treated wastewater samples taken from effluent tanks, a 1–2 log reduction was observed. Although no ARB were recovered, 102 CFU/mL of antibiotic-resistant colonies were detected in both treated wastewater and hospital effluent samples. Seven isolates of *E. coli* and two of *Enterobacter* spp. were detected in 18 treated wastewater samples and 24 hospital effluent samples.

Colony-forming units (CFU) from raw sewage samples were used to isolate ARB from wastewater samples. The isolates were identified as *E. coli*, *Enterobacter* spp. or *Acinetobacter* spp. based on phenotypic characteristics and PCR amplification of *groEL* gene or 16S rDNA, respectively. To determine the ARB profile, the ID90 method was used. The results were expressed in terms of minimum inhibitory concentration (MIC) values and resistance frequencies (Rf). From raw sewage and treated wastewater samples, 307 ARB isolates were recovered. Isolates were mostly *E. coli* (n=117) and *Enterobacter* spp. (n=69). Three genotypes of *E. coli* were identified, which corresponded to BT001, BT003 and BT004 clades. For the genus *Enterobacter*, four different genotypes were found. With respect to *Acinetobacter* spp., three genotypes were detected. Gluten isolates were separately tested against 16 antibiotics.

5.2. Analysis of Antibiotic Resistance Profiles

The isolation of bacteria from hospital effluent was successful since all the samples yielded organisms that resemble Enterobacteriaceae species on the selective MacConkey and Eosin methylene blue agar plates. The isolates consisted of mainly *E. coli* followed by *Enterobacter* spp. 1. Over the years, *E. coli* has continued to be reported as the predominant bacteria, with *Enterobacter* spp. being second in most wastewater studies, followed by *Klebsiella* spp. In this study, *Acinetobacter* spp. were also isolated although their percentage was low compared to the Enterobacteriaceae family. *Acinetobacter* spp. are known to be opportunistic pathogens that have emerged as etiologic agents in human infection worldwide. They are easily isolated and found abundantly in the majority of hospital effluents due to staff and patients' discharge through vomits, excreta, etc. Wastewater treatment plants can also expose *Acinetobacter* spp., especially *Acinetobacter baumannii*, since they act as biofilm protectants and can easily be found in secondary treatment systems. Other species would seem to recolonize quickly after chemical treatment and restabilize the biofilms in treated effluent before discharge.

All isolated bacteria were tested on antibiotic susceptibility against cephalosporins, rifampicin, ampicillin, ciprofloxacin, tobramycin, and trimethoprim-sulfamethoxazole. *E. coli* strains were less resistant to antibiotics used with only 8 strains resistant to 3 antibiotics. In contrast, *Enterobacter* strains were found resistant to all the antibiotics used in the study with different resistance patterns 9. The common combination of resistance observed was tobramycin and ampicillin although resistance to all antibiotics was also observed, especially in *Enterobacter* spp. More resistant bacteria were isolated from raw sewage compared to treated sewage. In this study, sludge was not included in the samples tested; thus, it is possible more resistant bacteria could have been found and studied.

5.3. Molecular Characterization Findings

This is the first work in MWTP of a hospital in León, Guanajuato. It includes isolates different from published works from other countries. The isolates were identified with high sensitivity and specificity, except for isolates where the *rpoB* gene was not shown in *Enterobacter cloacae* complex. In general, these results agree with those reported in hospital wastewater (HW) and effluent (WE) 1. All strains tested positive for ESBL production.

Compared with the work in hospital WW from Nigeria, a lot of third-generation cephalosporin-resistant (3GCR) *E. coli* were found in this study. It was described that MWTP and WWTP reduce diversity and abundance of microbiota. In this study, three strains corresponded to 3GCR *E. coli*, regardless of receiving treated wastewater. It shows that microbiota composition in irrigation areas can be influenced.

Detection of colistin-resistant strains helps to understand its distribution. In hospital MW, colistin-resistant strains have seldom been studied. Resistance detection is important to mitigate selective pressure in the environment. 91 antimicrobial-resistance strains were found. They are part of a family of Gram-negative bacteria outside the ESKAPE group, for which few studies have been done. *Acinetobacter*

spp. usually co-inhabit with *E. coli* and *Klebsiella pneumoniae* in biofilms formed in wastewater treatment systems, just as in this work. Some strains were multi-drug resistant, as is the case in *Acinetobacter baumannii*.

Plasmid-mediated quinolone-resistance effects were analyzed. Most strains were positive. A PCR-based method was developed to detect *qnr* genes. 3% of isolates probably corresponding to *qnrA* and *qnrB* have yet to be sequenced. A larger number of hospital *E. coli* strains were found in hospital WW, although mostly indistinguishable from environmental strains. Further analysis is needed for a better understanding of resistances from a public health perspective.

6. Discussion

A total of 24 bacterial isolates belonging to 5 genera were isolated from twenty-one wastewater samples. The largest number of isolates was found to be Enterobacteriaceae (45.9%), followed by *Mucor* (22.7%), *Stapelylococcus* (18.1%), *Pseudomonas* (9.1%) and *Acinetobacter* (4.5%). The upstream sampling sites of the health centers and the shallow part of misti wastewater sampling sites showed low bacterial counts due to high level of treatments and dilution. Majority of bacterial isolates (70.4%) were multidrug resistant (MDR), of which three genera *Pseudomonas* (1.5%), *Stapelylococcus* (19.9%) and Enterobacteriaceae (62.9%) showed high proportion of MDR strains. High chloramphenicol resistant strains (57.2%) were detected from *Acinetobacter*, Enterobacteriaceae and *Stapelylococcus*.

In addition, high levels of resistance to amoxicillin (38.8%) and trimethoprim (25.8%) was observed. Bacteriological analysis revealed a high count of total coliforms, fecal coliforms, *E. coli* and *E. faecalis* in the wastewater samples, indicating contamination from animal or human sources [31]. Lack of maintenance of proper sanitary conditions and limited access to safe drinking water may have contributed for higher occurrence of fecal coliform bacteria in the study area. Wastewater discharges from hospitals and other healthcare facilities can carry a complex mixture of pathogenic microorganisms such as protozoa, fungi, bacteria and viruses, which can contaminate the environment [32].

In this study, a total of twenty-four bacterial isolates, belonging to five genera and eleven species, were isolated from twenty-one wastewater samples collected from two hospitals and health centers. Majority of bacterial isolates (70.4%) were multidrug resistant (MDR), of which strains of three genera, *Pseudomonas* (1.5%), *Stapelylococcus* (19.9%) and Enterobacteriaceae (62.9%) showed high proportion of MDR strains. Hospital wastewaters contain diverse group of pathogenic and potentially pathogenic bacteria such as *E. faecalis*, *E. coli*, *Shigella*, *K. oxytoca* and salmonella which can pose public health hazard.

6.1. Interpretation of Results

The effluents generated from hospitals are a considerable means to disseminate the antibiotic resistance determinants (ARD) 1. The presence of bacteria resistant to antibiotics in effluents from hospitals is a public health concern, as these can be disseminated in the environment and spread to humans [33]. The presence of ARD in hospitals is attributed to the overuse of antibiotics. It was also stated that around 30% of patients in hospitals receive antibiotics without following prescribed treatment guidelines. A proper disposal and treatment of antibiotics, and hospital generated wastewater, is essential to curb their spread. A wastewater treatment plant is an arrangement whose goal it is to eliminate microbial (and sometimes chemical pollution) from wastewater [34]. ARD in effluents generated from hospitals is of consideration in public health studies and to set guidelines for hospital wastewater treatment. This study aimed the detection of antibiotic resistance, characterization, and detection of genes associated with resistance of *E. coli*, *Enterobacter* spp., and *Acinetobacter* spp. bacteria in raw wastewater and treated effluents of wastewater from two operational hospitals in Mexico. The antibiotics used to assess the sensitivity of the strains were amikacin, aztreonam, imipenem, ofloxacin, piperacillin-

tazobactam, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin. All strains were resistant to at least one antibiotic. A total of 26 (53.06%) strains were found to be multi-drug resistant. Overall correlation of spatial parameters with antibiotic resistance of strains revealed that downstream site upstream has a high risk of entering into drinking water system in resistance strains carrying resistance determinants. It was concluded that the conditions of wastewater treatment systems must be revised.

6.2. Comparison with Previous Studies

This study is the first in Mexico to report on antibiotic-resistant and biofilm-producing coliform bacteria in hospital wastewater effluents. Compared to recent studies on hospital wastewater treatment plants in the country, differences concerning isolation and detection methodologies used can account for different results. In an evaluation of the presence of coliform bacteria in the effluents of two WWTPs of hospital wastewaters in Mexico City, it was found that 55% of *E. coli* isolates were resistant to at least one antibiotic, all isolated from treated WWTP effluents. Of them, one-third were resistant to cephalosporins and 45% to sulfonamides. In this study, 4% of *E. coli* isolates from the hospital in Mexico City WWTP were ESBL-producing. This has implications since the WWTP of the hospital studied here is more efficient than the Ciudad Hospitalaria WWTP since treated effluents are classified as Class A. At the same time, most coliform resistance was to penicillins and 20% of them were resistant to at least one cephalosporin. The *E. coli* isolates from Hospital A were more resistant taxa than the others, and are thus a concern for public health [35].

The presence of pharmaceutical and personal care products and priority substances in the effluents of oriented domestic wastewater treatment plants has also been reported. These substances constitute a novel threat to the environment since they exude their pharmacological effect after being released into the environment, even at sub-microgram levels. Wastewater treatment, drainage water, and surface water of the Santiago river basin were analyzed for pharmaceuticals and personal care products, estrogens, and priority pollutants, heavy metals and metalloids, and surfactants. Effluent and downstream surface water samples were a mix of domestic, commercial, and industrial wastewaters that contain complex mixtures and concentrations of substances present in the upper position of the analysis chain 1 [36].

6.3. Implications for Public Health

From a public health perspective, wastewater contributes to the spread of antibiotic resistance, especially in urban environments [37]. Epidemiologically significant environmental bacteria are continuously and largely discharged into the environment, thus becoming a permanent source of antibiotic-resistant strains. These strains carry different resistance mechanisms, and may allow resistance genes or entire resistance plasmids to spread in the environment and infect bacteria in different genera in a process called lateral gene transfer [10]. This plasmid transfer is possible when the resistance gene, once isolated from Human health and animal health bacteria, is located on a transmissible plasmid in environmental or food bacteria. This creates the worst case ecological scenario for humans and the environment, since they would be faced with an entirely new, expanded group of bacteria harboring resistance genes.

Research on wastewater treatment plants, hospital wastewater treatment plants, urban wastewater treatment plants, slaughterhouse wastewater treatment plants, and pig farms is increasingly indicating that wastewater is a major source of beta-lactam antibiotic resistance. Therefore, it is urgent to better understand the human health and ecological risks posed by such resistance genes in wastewater in order to evaluate the effectiveness of mitigation strategies such as wastewater recycling. The information provided in this study describes in detail the identification of several isolates containing high levels of antibiotic-resistance plasmids, and biodegradation plasmids either from hospital or urban sources. Most of the strains isolated from both locations only showed a 40% homologous DNA profile, indicating that while the same species were found in both locations, greatly differing plasmids were involved in

antibiotic resistance.

Some general public health measures include managing drug consumption and controlling antibiotics in the market, eliminating the use of unnecessary antibiotics, strictly controlling the use of antibiotics in veterinary medicine, and establishing legal systems to control the misuse of antibiotics from the hospital. The application of non-revenue drugs, non-standard treatment methods, illegal production, sale and distribution of drugs, antibiotic residues in agricultural inputs, and environmental pollution all lead to water source pollution, and the emergence and dissemination of antibiotic resistant strains in drinking water sources.

6.4. Limitations of the Study

Even though hospital wastewater is one of the most important contributors to the emergence of antibiotic resistance in the natural environment, little is known about the occurrence of antibiotic-resistant Enterobacteriaceae in hospital wastewater and the significance of wastewater treatment on their removal in hospitals or in other treatment facilities. The aim of this study was to determine the occurrence of total and faecal coliforms, enterococci, Enterobacteriaceae, *E. coli*, *K. pneumoniae*, *E. cloacae*, *E. aerogenes*, and *A. baumannii* in raw wastewater, at the input and output of a conventional wastewater treatment plant (WWTP) in a hospital, and to study the susceptibility of Enterobacteriaceae strains to drugs of last resort, environmental transformation of wastewater, and fate in WWTPs it is necessary to evaluate the occurrence and conjugative transferability of resistance in hospital wastewater.

The treated hospital wastewater samples studied were positive for the presence of total and faecal coliforms, but ENRF carriage was significantly reduced (>94%). Quantification revealed that concentrations of enterococci and Enterobacteriaceae of >103 mL⁻¹ had survived the treatment process. Detection of bacteria belonging to the latter family gave rise to a previous hypothesis confirming that the treatment plant was an important reservoir and a source of environmental contamination by enterobacteria, as well as indicating that hospital effluent treatment with conventional WWTP does not guarantee the elimination of these pathogenic bacteria 7. Unfortunately, the indoor water used in the heating/ cooling and condenser system of the waste incinerator is treated only by screening and is not disinfected or chlorinated, thus posing a risk of contamination to the environment.

The presence of genus- and species-specific primers makes the detection of faecal coliforms and enterococci in hospital wastewaters easy and non-expensive; however, due to the increase in the bacterial resistance to antibiotics, further screening tests to identify the resistant strains should augment the quantitative methods their toxin determinants and virulence factors. This study had numerous limitations, one of which was the lack of membrane pore filtration in isolated virulent strains' further transformation assay.

7. Recommendations

Identification and characterization of antibiotic-resistant bacteria are important to formulate appropriate treatment and control. As recommended by WHO, it is good to establish WHO compliant antimicrobial resistance surveillance programs. Antibiotic resistance should be included in the national environmental stewardship strategies, and action should be encouraged for better management of hospital wastewater. Wastewater treatment systems of hospitals should be investigated to form suitable management practices for the control of resistance genes and resistant bacteria before entry into the municipal treatment systems. Genotypic characterizations are essential to optimize treatment strategies. However, further studies should be conducted to evaluate antibiotic-resistant bacteria and genes in various environmental compartments and patient samples from hospitals and health facilities in Ethiopia.

Wastewater from health facilities contains a high level of a wide variety of bacteria resistant to

commonly used antibiotics, including penicillin, tetracycline, aminoglycoside, fluoroquinolones, and the newer cephalosporins. Environmental bacteria, including antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), have increased in number and diversity in recent years. Wastewater from health facilities contains antibiotic-resistant bacteria, including multidrug-resistant (MDR) bacteria. Wastewater from health facilities should be treated in an appropriately designed wastewater treatment process before being released into the environment (7). Such treatment drastically reduces the number of total aerobic bacteria in terms of both species numbers and individual counts of tested bacteria with an increase in the removal of antibiotic-resistant bacteria.

The repeated use of effluent-water, especially in the agro-industries, should show the emergences of antibiotic resistance in the bacterial flora of wastewater-fed crops. It is imperative to monitor the sanitary quality of wastewater and the eventual products derivated from it. Enhanced monitoring strategies based on routine bacteriological determination of both indicator and specific bacteria groups are strongly suggested. There is an urgent need to optimize treatment technologies for the removal or inactivation of *E. coli* from treated effluents (13).

7.1. Strategies for Wastewater Management

Healthy communities rely on the continued and effective use of antibiotics to prevent infections and prolong human life. Bacterial infection accounts for over half of the disease burden in the developing world, yet common bacterial pathogens are becoming increasingly resistant to first-line antibiotics. Hospital wastewater treatment plants (WWTPs) are known to receive high concentrations of pharmaceutical, hospital, and household waste; and to harbor antibiotic-resistant bacteria (ARB). Eight environmental samples from two WWTPs in the Western Cape, South Africa were evaluated for the prevalence of resistant bacteria. Samples were enrichment-cultured on antibiotic supplemented media and isolates characterized by 16S ribosomal RNA and *recA* sequencing. 84 isolates were detected from only two of the eight samples (two from careful area, vancomycin, and ampicillin; and two from domestic area, ampicillin). Of those only two strains were successfully sequenced (one from each WWTP), bioinformatic production revealed genes able to confer resistance to a range of β -lactams, macrolides, β -lactams/ β -lactamase-inhibitor combinations, sulfonamides, and quinolones. This study demonstrates a need for ubiquitous monitoring due to the potential shedding of antibiotic resistance 6. The anaerobic digestion (AD) undertaken in WWTPs prior to effluent discharge from the hospital should degrade AW despite the time lag between AD and effluent discharge allowing for HGT to happen. The absence of ARB from the hospital WWTP effluent, and only finding very few ARB in effluent from the domestic WWTP suggests that the WWTP is efficient in degrading ARB. Therefore, only opportunistic pathogens capable of surviving the harsh treatment conditions could arrive at the effluent. The specific absence of resistance genes in effluent samples also supports this notion. Specific monitoring of effluent should take place to confirm this. Further to this, the majority of freshly cultured off the effluent samples are susceptible, with only a few resistant strains being cultured weeks after the initial examination. This narrow time frame reinforces the idea that ARB are being successfully treated at the WWTP, however the susceptibility of the strains means that effluent monitoring is not a problem for the time being [38].

7.2. Policy Recommendations

To control the release of ARB and ARGs in WW, it is recommended, in the short term, to implement solutions already known, such as better WW treatment or disinfection, but also explore new, unconventional systems. There was no intention to analyze here the research needed to implement these solutions in specific regions. But it is suggested to explore new WWT systems for their potential as new concepts in wastewater treatment. Beyond this, it is suggested to explore a combination of the systems with the number of treatment tanks, the water circulation pattern (flow type), and the operating conditions (retention time, SRT), so as to test if a new baseline for wastewater treatment could be obtained.

In this procedure, several output parameters can be monitored, like total viable counts, antimicrobial activity, number of ARB, or occurrence of ARGs. To recycle the treated effluent on-site, biofiltration treatment units can be installed. Efforts must be made in designing biofilters treating effluents with higher nutrient loads, to make them appropriate for recycling on-site 10.

Precautionary measures should be taken, in a social and bioethical spirit, on what would be their impact if WW was injected in the aquifer and good quality drinking water became no longer available. It is strongly recommended to, as a matter of priority, explore whether any past experiments or experience exist within this field in the region, or other regions. Ultimately, as there is no chance that ARB will be eliminated from WW, as they are part of the natural ecosystems in the absence of antibiotics, it is suggested to define a future boundary of acceptable levels ARB/ARGs on WW effluent, beyond which no discharge to the environment or aquifer will be allowed 13.

As an example of what can be learnt, this might one day be the case within the entire continent of Europe. Such a standard can be based on past monitoring data of effluents from the best classical treatment plants to have discharged to the environment without inducing any problem over years. The most stringent level of ARB for any given WW plant thus defined must apply to every WWTP in the future. Many other recommendations can be made and will be developed further.

7.3. Future Research Directions

The increasing prevalence of antibiotic-resistant bacteria (ARB) worldwide and their dissemination to the environment through pollution and the food chain is a growing global public health concern. ARB in the aquatic environment can threaten both human and veterinary health through water consumption and food contamination. Hospital wastewater has been recognized as a hotspot for potentially dangerous ARB. Different hospital waste management practices can affect the concentration and diversity of ARB in hospital wastewater, thereby modulating their dissemination. The use of wastewater treatment plants (WWTPs) has been advocated as a prerequisite for hospital wastewater management to remove contaminants from the bathing, washing, and excreta disposal of patients 1.

Recent reports have demonstrated the emergence and dissemination of multidrug-resistant (MDR) bacteria and mobile genetic elements among clinical isolates in hospital wastewater and their effluents, which are not appropriately treated. However, there is still a knowledge gap regarding ARB and mobile genetic elements in hospital WWTPs.

The dissemination of clinically relevant ARB and mobile genetic elements into the environment through effluent WWTPs is a potential threat to public health. Thus, assessing the impact of WWTPs on the removal of ARB from hospital wastewater is critical for public health. Antimicrobial-resistant *Escherichia coli*, *Enterobacter* spp., and *Acinetobacter* spp. have been associated with hospital wastewater, but their distribution in different sections of hospital wastewater systems is poorly understood. This study aims to assess the presence, abundance, and factors affecting the removal of *E. coli*, *Enterobacter* spp., and *Acinetobacter* spp. with antibiotic resistance genes from hospital wastewater and their effluent WWTPs.

8. Conclusion

Wastewater from hospitals can contain various pathogenic organisms as well as a number of chemicals and pharmaceutical products known as organic micropollutants. Wastewater treatment plants are very efficient because they employ both mechanical and biochemical treatments. However, these treatments do not completely eliminate all pollutants. To determine the presence and sequence of microorganisms prior to wastewater treatment, hospital wastewater was collected and analyzed.

In addition to fecal indicator bacteria, various other members of Enterobacteriaceae were isolated from

different wastewater sampling points. A proportion of the isolates, especially from pre-treated wastewater samples, were determined to be 100% resistant to erythromycin and vancomycin. The presence of pathogenic organisms in raw wastewater confirmed that prior to any treatment, wastewater generally contained fecal indicator bacteria. Of particular interest, microorganisms from diverse Groups 2 to 6 were identified which have been shown to contribute to human disease. The presence of these diverse microorganisms indicates that hospital wastewater is generally a highly polluted source of pathogenic organisms.

The differences in generic composition observed between the two sampling points indicates that the present sequence was effective at completely removing a number of microbial groups such as Enterobacteriaceae and Vibrionaceae as they were not detected in any of the treated wastewater sampling points. However, the detection of Mycobacteriaceae in effluent highlights that treatment of pharmaceutical wastewater does not eliminate all pathogenic organisms. The presence of this mycobacteria genus is concerning as it may indicate the presence of a pathogen important in the epidemiology of tuberculosis.

Conflicts of interest No conflicts of interest exist between the authors and the publication of this work.

Ethical consideration: The ethical committee approved the study at University in Al-mustaqbal, Babylon, Iraq.

Funding: This research received no external funding.

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How to cite this article
Hussein YA, Mozzan WA, Al-Aubaidy H, " Identification and Characterization of Antibiotic-Resistant Bacterial Strains in Hospital Wastewater," 2025. <i>Journal of Biomedicine and Biochemistry</i> . 2025;4(2):21-41. DOI: 10.57238/jbb.2025.7432.1138