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Characterization of Bacterial Pathogens Isolated from Hospital Wastewater Environments

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Abstract: Hospital wastewater (HWW) contains many contaminants, including chemicals, medical wastes and infectious microbes, posing a hazard to the public health. This study aimed to isolate, identify, and assess the antimicrobial susceptibility of the bacteria from tap water and HWWsamples from different Egyptian hospitals. Isolated bacteria from water samples were identified biochemically and by Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) system and assessed for susceptibilities to antibiotics utilizing the Kirby-Bauer disk diffusion and broth microdilution assay. Thirty-five bacterial isolates have been isolated from both water samples. Most isolated bacteria were from HWW samples including *Staphylococcus aureus (*25.5%*), Klebsiella spp. (*17%*)* and *Proteus mirabilis (*17%*)*, followed by *E. coli* (3%), *Citrobacter* spp. (3%) and *Pseudomonas putida (*3%). The total resistance to antibiotics by Gram-negative bacteria obtained from HWW was 87% to amoxicillin followed by imipenem (74%), doxycycline (67%) and azithromycin (67%). On the other hand, the resistance to amikacin and meropenem were 17% and 26%, respectively. The MDR isolates within the study were from HWW, constituting 83% of the isolates. The MIC assay results indicated that all *Klebsiella spp., E. coli, P. mirabilis, Citrobacter spp. and P. putida* isolates from HWW exhibited 100% resistance to colistin. Furthermore, the resistance to meropenem was 93%, while the resistance to amikacin was 26%. Communities may be seriously at risk if these antibiotic-resistant bacteria (ARB) from HWW get released into the environment. Preventative interventions must be taken to avoid this dissemination to the environment and communities.

Keywords: Antibiotic susceptibility, Sewage, MIC, MALDI-TOF VITEK® MS, Kirby-Bauer disk.

1. INTRODUCTION

Resistance to antimicrobial agents is an important health issue globally. It represents a serious risk to the efficient management of infectious diseases and can lead to increased mortality, morbidity and healthcare costs.[1] The influence of HWW on the antimicrobial resistance problem is an important area of concern.[2] Excessive antibiotic use and improper disposal have led to the emergence of new ARB which complicate the treatment of pathogenic infections.[3] HWW may include a varied level of antibiotic resistance than other types of aquatic environments due to different antibiotics use patterns. In addition some specific antibiotics, are utilized only in hospitals, such as piperacillin, vancomycin and cefotiam.[4] Furthermore, the emergence of new ARB and ARGs is associated chlorine disinfection resistance [6] and development of horizontal gene transfer (HGT) by plasmids, transposons and integrons in water.[7] Without appropriate HWW treatment, ARBs obtained from healthcare facilities can spread and survive in various conditions, resulting in increasing MDR pathogens.[8] Clonal proliferation in HWW is eventually facilitated by selective agents in the environment such as antibiotics, high temperatures and minerals.[11] ARB monitoring in hospitals and wastewater systems is crucial for patient and population protection.[12] Culture-dependent analysis is used in traditional water quality monitoring to screen for particular indicator microbial species, including fecal coliforms

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such as *E. coli.*[13] These cultural methods are still in use today all over the world due to their specificity and availability.[14] However, non-pathogenic environmental bacteria cannot be grown in ordinary culture media, which limits their application. This challenge can be solved utilizing modern molecular approaches.[15] Various global studies assessed the presence of antibiotics, antibiotic resistance genes (ARGs) and ARB in the effluents of HWW.[16,17,18] In Egypt a study detected the presence of MDR bacteria in the effluent of HWW[19] and another one detected the presence of extended spectrum Beta-Lactamase producing bacteria in wastewater.[20] Antibiotic concentrations in HWW vary depending on drug categories, antibiotic consumption, duration, season, city and hospital type. [21] The objectives of the present study were to isolate, identify and assess the antimicrobial susceptibility profiles of bacteria obtained from inflow tap water and outflow wastewater samples collected from various Egyptian hospitals.

2. METHODS

2.1. Site Description and Sample Collection:

A total of twenty samples (ten tap water & ten wastewater samples) were collected from five different hospitals in Cairo, Egypt in summer and winter seasons from August 2022 to February 2023. From each hospital 500 mL of midstream tap water and 100 mL of HWW were collected in sterilized labeled bottles. Regarding HWW samples, the worker in charge of sanitation in the hospital was informed to collect the wastewater samples from each hospital public sink by suction using sterile 50 cm syringe.

2.2. Isolation and identification of bacteria

2.2.1. Microbiological and biochemical identification

Following collection, samples were transferred to the laboratory on an ice tank and processed within two hours. Samples of tap and wastewater were filtered by using 0.45 polyethylene sulfonate membranes filter (Sartorius Stedim Biotech, Sweden) by closed laboratory filtration unit (Sartorius Göttingen Germany), then the membrane filter was placed on blood agar and Muller Hinton agar plates (HIMEDIA, Mumbai, India) [30]and incubated at 37°C for 24 h for aerobic bacterial isolation. Following incubation, representative colonies were selected according to the morphology of the colonies and Gram stain and subcultured on several selective and differential media, such as MacConkey agar and Mannitol salt agar (Lab M, Heywood, UK). The identification of bacterial species was done using a variety of media and biochemical tests. Regarding Gram-negative bacteria, MacConkey agar, eosin methylene blue (EMB), triple sugar iron agar (TSI), (Lab M, Heywood, UK), oxidase test (Oxoid, Basingstoke , England), indole production test (Oxoid® Limited, Basingstoke, UK) , citrate utilization test, urease test and phenylalanine deaminase test (Lab M, Heywood, UK) were used according to the identification scheme of Koneman *et al.*, 1997 and Cheesbrough (2005).[22,23] Identification of Gram positive bacteria was carried out using mannitol salt agar (Lab M, Heywood, UK), catalase and DNAase tests (Lab M, Heywood, U) according to the identification scheme of Procop *et al.*, 2020. [24] All used media were sterilized by autoclaving at 121°C.

2.2.2. Matrix–Assisted Laser Desorption/Ionization–Time of Flight - Mass Spectroscopy analysis (MALDI-TOF- MS)

Biochemically unidentified isolates were detected using MALDI-TOF-VITEK® MS mass spectroscopy (bioMérieux, Marcy-l'Étoile, France) in Children's Cancer Hospital, 57357, Cairo, Egypt. Strains have been subcultured on blood agar media at 37ºC for 24 h, then bacterial colonies were scraped from plates and mixed with a particular medium, and then placed onto the mass spectrometer's MALDI targeted plate. After the solution had dried for 24 hours at 37ºC, a drop of a-cyano-4-hydroxy cinnamic acid (CHCA) matrix solution had been added to the target plate. Advanced spectrum classifier (ASC) software was used to identify the isolated bacterium by comparing the collected spectra with the typical spectra of each organism in the VITEK MS 1.1 database.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by qualitative Kirby-Bauer disk diffusion method and quantitative broth micro dilution assay for MIC determination. [25,26]

2.3.1. Kirby-Bauer disk diffusion method

The isolated Gram-negative bacteria were subjected to susceptibility testing against 13 antibiotic discs, while the isolated Gram-positive bacteria were also tested against 10 antibiotics. All antibiotic discs were supplied from Oxoid® Limited, Basingstoke, UK; (HIMEDIA Mumbai, India); and (CONDALAB, Madrid, UK).

To prepare bacterial inoculums, freshly cultured bacterial isolates were suspended in 4-5 milliliters of normal saline and the turbidity was adjusted to match a 0.5 McFarland (equivalent to 1.5 x 10^8 CFU/mL) standard solution. Subsequently, the suspension was transferred to the Mueller-Hinton agar plate surface. Antibiotic standard discs were added to each plate, which were then aerobically incubated for 18 to 24 h at 37°C. Following incubation, the recommendations of Clinical & Laboratory Standards Institute were followed in measuring and interpreting the diameter of the zone of inhibition. (CLSI, 2020) .[27]

2.3.2. Broth micro dilution assay

The MIC of the antibiotics had been determined using the broth microdilution assay. Gentamicin (GEN) and levofloxacin (LEV) were used for MIC determination of Gram-positive bacterial isolates and colistin (CT), amikacin (AK) and meropenem (MEM) were used for MIC determination of Gram-negative bacteria in accordance with the CLSI 2020 guidelines. These stock solutions were prepared at a concentration of 1024 µg/mL for colistin, amikacin, meropenem and levofloxacin (CLSI, 2020) and at a concentration of 2028 µg/mL for gentamicin.

One hundred microliters of Muller-Hinton broth (MHB) (Oxoid® Limited, Basingstoke, UK) were distributed into each well of the 96 multi-well microtiter plates. Next, 100 µL of the test antibiotic stock solution was added to the first line of the microtiter plate and stirred by pipetting the solution up and down with a micropipette, then serial dilution was performed. Five microliters of freshly prepared bacterial suspension (1.5 x 10^8 CFU/mL) were added to each well and positive as well as negative control experiments were performed.[28]All plates were incubated at 37 ˚C for 18–24 h. The least antimicrobial concentration at which there was no visible growth was known as the MIC. The wells were inspected visually and the antibiotic concentration corresponding to the well within visible growth was recorded as MIC, which was interpreted according to CLSI 2020 guidelines.[27]

3. RESULTS

3.1. Bacterial Isolates

Thirty-five bacterial isolates were isolated from 20 samples (ten tap water and ten wastewater) from five major hospitals in Cairo, Egypt. Out of 35 isolates, 24 (68.5%) were obtained from HWW, and 11 (31.5%) were from tap water. Figure 1 revels the distribution of bacterial pathogens in tap and wastewater samples from different hospitals in Egypt. A chi-square test of independence revealed a statistically significant difference in the distribution of isolates between the two sources ($\chi^2 = 4.83$, $p = 0.028$).

3.2. Identification of bacterial isolates

3.2.1. Identification of bacterial isolates from tap water and HWW samples by microbiological and biochemical tests

Twenty-seven bacterial isolates were detected using different microbiological and biochemical tests. *Klebsiella* spp. were the most frequently identified Gram-negative bacteria from wastewater (17%), followed by *E. coli* (3%), and *Citrobacter* spp. (3%). Regarding Gram-positive bacteria, *Bacillus* species were isolated from tap water (28.5%), while *S. aureus* was isolated from wastewater (25.5%), as illustrated in table 1.

3.2.2. Identification of bacterial isolates using MALDI-TOF VITEK® MS

Eight Gram-negative isolates had been identified using MALDI-TOF VITEK® MS. The identified bacterial isolates were *P. mirabilis* (17%), *P. putida* and *E. coli* (3%) as shown in table 2. Totally the most frequently isolated bacteria from both water samples were *S. aureus* (25.7%), *Klebsiella* spp. (17.1%), *P. mirabilis* (17.1%) followed by *E. coli* 2 (11.4%), *Citrobacter* spp. (2.8%) and *Pseudomonas putida* 1 (2.8%). While 28.5% *Bacillus* species were isolated from hospital tap water (Figure 2).

3.2.3. Seasonal variation of bacterial isolates from tap water and HWW samples among different hospitals

There were no clear seasonal differences in bacterial isolates in the summer and winter in tap water and HWW samples. 11.4% of *Klebsiella* spp. (4 of 35) and *Pseudomonas putida isolate* were detected in summer, while 11.4% of *Proteus mirabilis* (4 of 35%) was detected in winter (Figure 3).

3.3. Results of the Kirby Bauer disc diffusion test

3.*3.1. For Gram positive isolates from wastewater*

S. aureus isolates were fully resistant to amoxicillin (100%). The resistance to azithromycin, amikacin and clindamycin were 67%, 56% and 45%, respectively. On the other hand, 100% of *S. aureus* were sensitive to vancomycin and doxycycline and 78% were sensitive to erythromycin (Table 3 and figure 4).

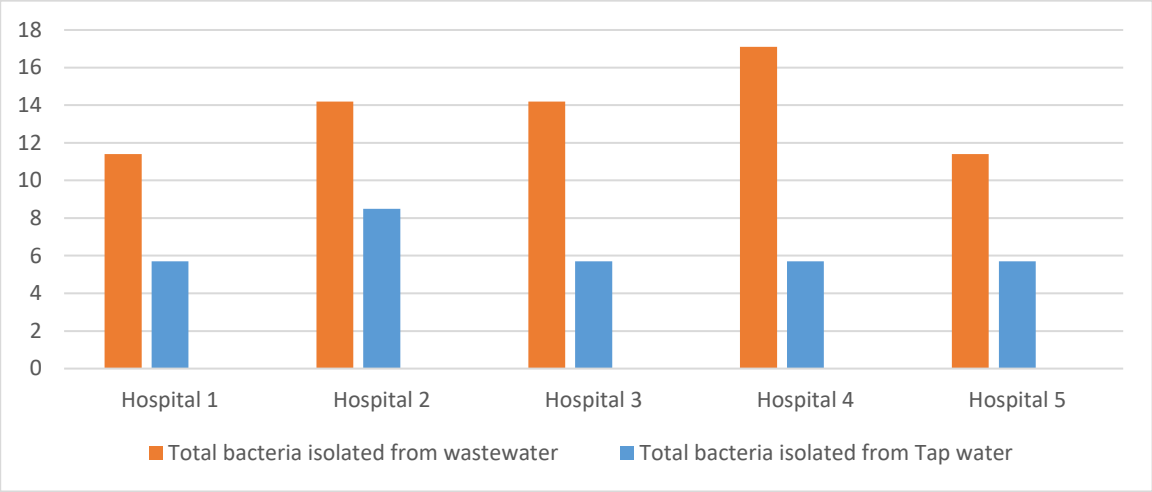

Figure 1. Distribution of bacterial isolates among tap water and HWW from different hospitals in Egypt

Table 1. Frequency of bacterial isolates from tap water and HWW samples identified by microbiological and biochemical tests

Type of bacteria	**Tap water No. (%)**	**Wastewater No. (%)**
Gram-negative bacteria		
***Klebsiella* spp.**	**-**	**6 (17%)**
E. coli	**1 (3%)**	**-**
***Citrobacter* spp.**		**1 (3%)**
Gram-positive bacteria		
S. aureus	**-**	**9 (25.5%)**
***Bacillus* spp.**	**10 (28.5%)**	**-**
Total (27out of 35)	**11 (31.4%)**	**16 (45.8%)**

Table 2. Bacterial isolates identified by MALDI-TOF

Type of bacteria	Tap water	Wastewater
Gram-negative bacteria		
P. mirabilis	**-**	**6 (17%)**
P. putida	**-**	**1 (3%)**
E. coli	**-**	**1 (3%)**
Total (8 out of 35)	**-**	**8 (22.8%)**

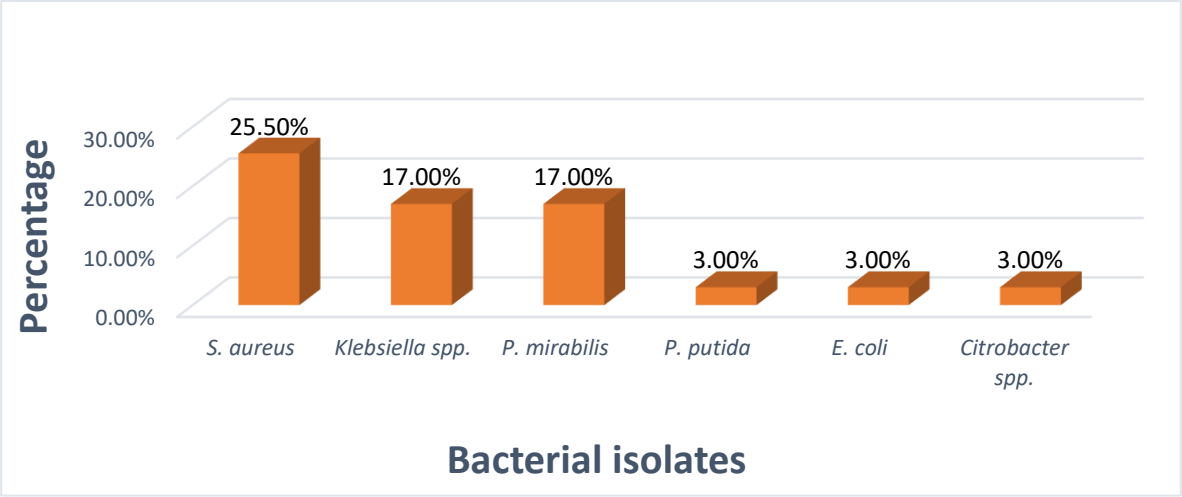

Figure 2. The frequency of bacterial isolates from HWW among the five hospitals.

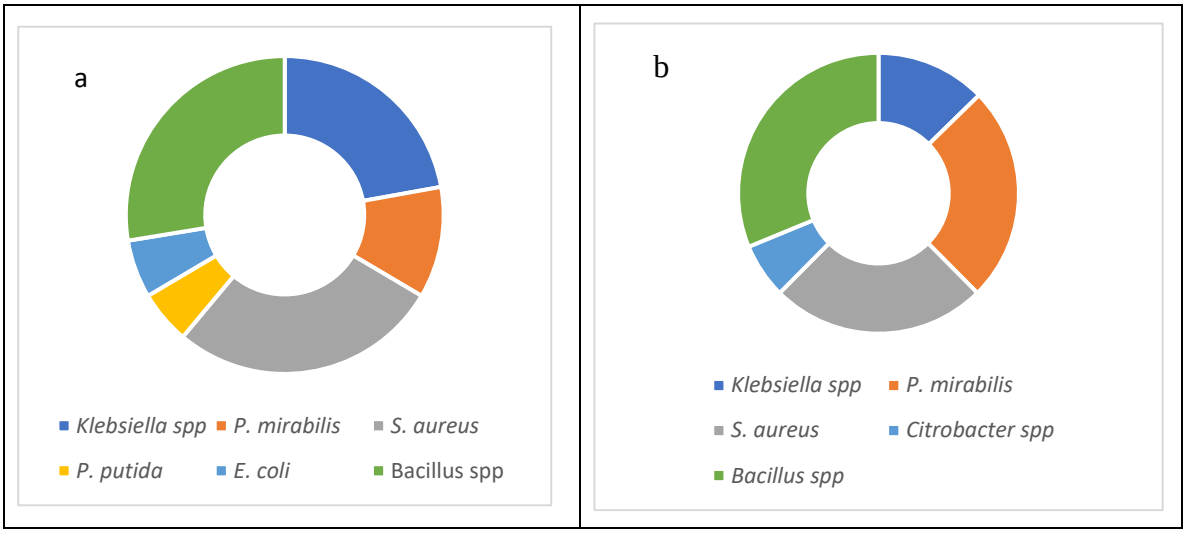

Figure 3. Bacterial diversity in HWW and tap water samples from different hospitals in different seasons. a: Summer b: Winter

Table 3. Resistance profile of *S. aureus* by Kirby Bauer disc diffusion method

Bacterial isolate	***S. aureus* (n=9)**									
	AML	**TPZ**	**VA**	**AK**	**CIP**	**AZM**	**E**	**DA**	**DO**	**C**
Antibiotic resistance N (%) Out of 9 isolates	**9 (100)**	**3 (33.3)**	**-**	**5 (55.5)**	**3 (33.3)**	**6 (66,6)**	**2 (22.2)**	**4 (44.4)**	**-**	**4 (44.4)**

AK: Amikacin, AZM: Azithromycin, AML: Amoxicillin, CIP: Ciprofloxacin, C: Chloramphenicol, DA: Clindamycin DO: Doxycycline, E: Erythromycin, TPZ: piperacillin-tazobactam, VA: Vancomycin

3.3.3 Gram-negative isolates from HWW

Gram-negative bacterial isolates from HWW samples showed multiple drug resistance profiles to standard antibiotics. Results of the Kirby-Bauer disk diffusion method revealed that 100% of *Klebsiella* spp. were resistant to amoxicillin. The resistance to doxycycline and chloramphenicol was 83% for each, followed by azithromycin, aztreonam and imipenem (67% for each). Intermediate resistance was detected against ofloxacin, ciprofloxacin, cefepime, cefoxitin and piperacillin-tazobactams, representing 50% for each. Only one isolate was resistant to meropenem (17%), while no resistance was observed for amikacin (Figure 5).

The result of Kirby-Bauer disk diffusion method revealed that 83% of *Proteus mirabilis* was resistance to amoxicillin, imipenem and cefepime. The resistance to ciprofloxacin, doxycycline, azithromycin and cefoxitin was 67% for each. Intermediate resistance was detected against ofloxacin and piperacillin-tazobactams, representing 50% for each. On the other hand, 83% of *Proteus mirabilis* were sensitive to amikacin and 67% were sensitive to chloramphenicol, meropenem and aztreonam (Figure 6).

Pseudomonas putida isolate was fully resistant to the most tested antibiotics and only susceptible to amikacin, doxycycline, and azithromycin. On the other hand, *E. coli* isolate was sensitive to most antibiotics tested and resistant to azithromycin and doxycycline only. The resistance profile of *Citrobacter* spp. isolate detected resistance to only amoxicillin, imipenem and azithromycin (Table 4).

The total resistance to antibiotics shown by Gram-negative bacteria obtained from HWW were 87% to amoxicillin followed by imipenem (73%), doxycycline and azithromycin (67%). (Table 4). *P. mirabilis* was resistant to 14 different antibiotics from 9 different classes, showing the greatest degree of MDR within Gram-negative bacterial isolates. This was followed by *Klebsiella* spp., which showed resistance to 12 antibiotics and *P. putida*, which was resistant to 11 antibiotics. Notably, 66% *P. mirabilis* and 83% *Klebsiella* spp., were resistant to more than five antibiotics from different classes. The MDR was 83% within the study. All MDR isolates were from HWW, constituting 83% (20 out of 24) of the isolates.

3.3.4. Gram-negative isolates from tap water

Only one *E. coli* isolate was recovered from tap water; it showed no resistance and was fully sensitive to the all-tested antibiotics.

3.4. Results of micro broth dilution assay

3.4.1. For Gram positive isolates from wastewater

The MIC values of gentamicin and levofloxacin were determined for *S. aureus* isolates, which revealed that 77% of *S. aureus* isolates were resistant to gentamicin MIC > 16 µg/mL, while 67% were resistant to levofloxacin MIC > 4 µg/mL. Two isolates (22.2%) of *S. aureus* were resistant to both antibiotics (Table 5).

3.4.2. Gram negative isolates from wastewater

The MIC was determined for meropenem, amikacin and colistin against Gram-negative isolates from HWW. Hundred percent colistin resistance was detected among all isolated Gram-negative bacteria. A high percentage of resistance to meropenem, 93% (14 out of 15), was observed, while the resistance was 26% (4 out of 15) to amikacin.

All of *Klebsiella* spp. (100%) were resistant to meropenem; the MIC was ≥128 µg/mL, while 50% were resistant to amikacin; the MIC was ≤ 32 µg/mL. 100% of *Klebsiella* spp. were resistant to colistin; the MIC was ≥ 8 µg/mL. Regarding *Proteus mirabilis*, 100% were resistant to colistin; MIC was ≥ 32 µg/mL. In addition, 83% of *Proteus mirabilis* were resistant to meropenem; MIC was ≥ 32 µg/mL, while no resistance to amikacin was detected; MIC was ≤ 32 µg/mL for 100% of *Proteus mirabilis.*

Pseudomonas putida isolate was resistant to both meropenem (MIC = 256 µg/mL) and colistin (MIC = 64 µg/mL), while it was intermediate resistant to amikacin (MIC = 32 µg/mL). *E. coli* isolate was resistant to meropenem (MIC = 128 µg/mL), amikacin (MIC = 64 µg/mL) and colistin (MIC = 16 µg/mL). *Citrobacter* spp. isolate was resistant to both meropenem and colistin, while it was intermediately resistant to amikacin. The MIC value for *Citrobacter* spp. isolate was 8 µg/mL for meropenem and was 16 µg/mL for colistin. (Table 6).

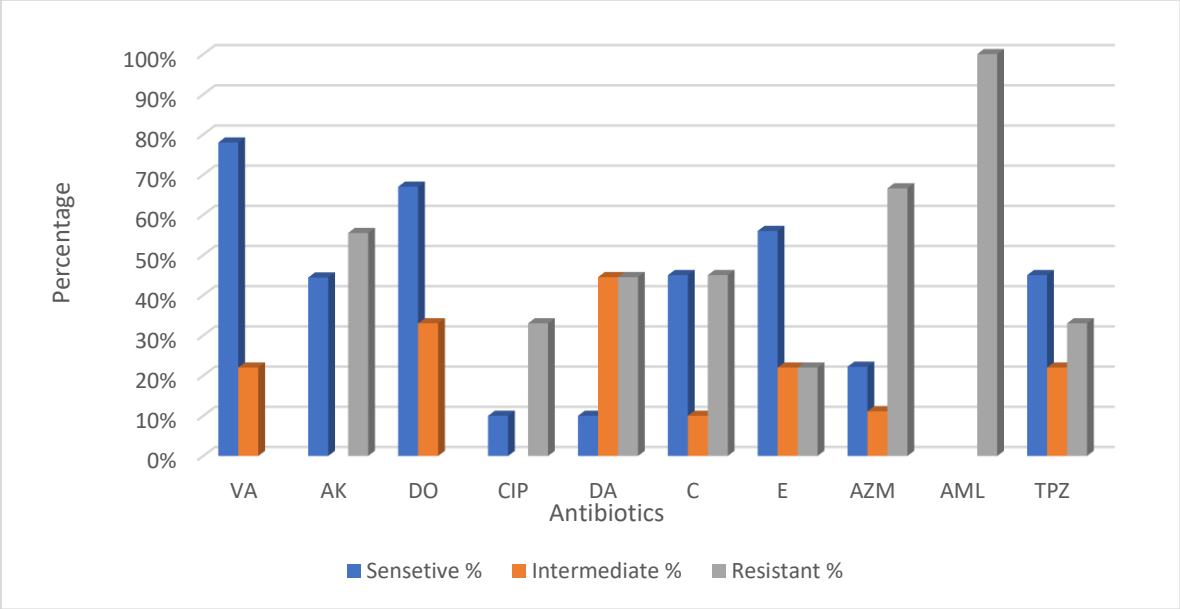

Figure 4. Resistance profile of *S. aureus* by Kirby Bauer disc diffusion method.

Table 4. Resistance rate of Gram-negative isolates from wastewater to standard antibiotics by Kirby Bauer disc diffusion**.**

***Antibiotics No. (%) of resistant isolates**	**Types of bacteria and number of tested isolates No. (%)**					
	***P. mirabilis* n=6**	***Klebsiella* spp. n=6**	***E. coli* n = 1**	***P. putida* n = 1**	**Citrobacter spp. n = 1**	**Total number of resistant isolates out of 15 (100%)**
AML	5(83)	6(100)	-	1(100)	1(100)	13(87)
TPZ	3(50)	3(50)	-	1(100)	-	7(47)
AT	2(33)	4(67)	-	1(100)	-	7(47)
FOX	4(67)	3 (50)	-	1(100)	-	8(53)
FEP	5(83)	3 (50)	-	1(100)	-	9(60)
IPM	5(83)	4(67)	-	1(100)	1(100)	11(74)
MEP	2(33)	1(17)	-	1(100)	-	4(27)
AK	1(17)	-	-	-	-	1(7)
DO	4(67)	5(83)	1(100)	-	-	10(67)
CIP	4(67)	3(50)	-	1(100)	-	8(53)
OFX	3(50)	3(50)	-	1(100)	-	7(47)
C	2(33)	5(83)	-	1(100)	-	8(53)
AZM	4(67)	4(67)	1(100)	-	1(100)	10(67)

** AML: Amoxicillin, TPZ: Piperacillin-tazobactams, FOX: Cefoxitin, CIP: Ciprofloxacin, OFX: Ofloxacin, MEM: Meropenem, FEP: Cefepime, IPM: Imipenem, AK: Amikacin, DO: Doxycycline, AZM: Azithromycin, C: Chloramphenicol, AT: Aztreonam*

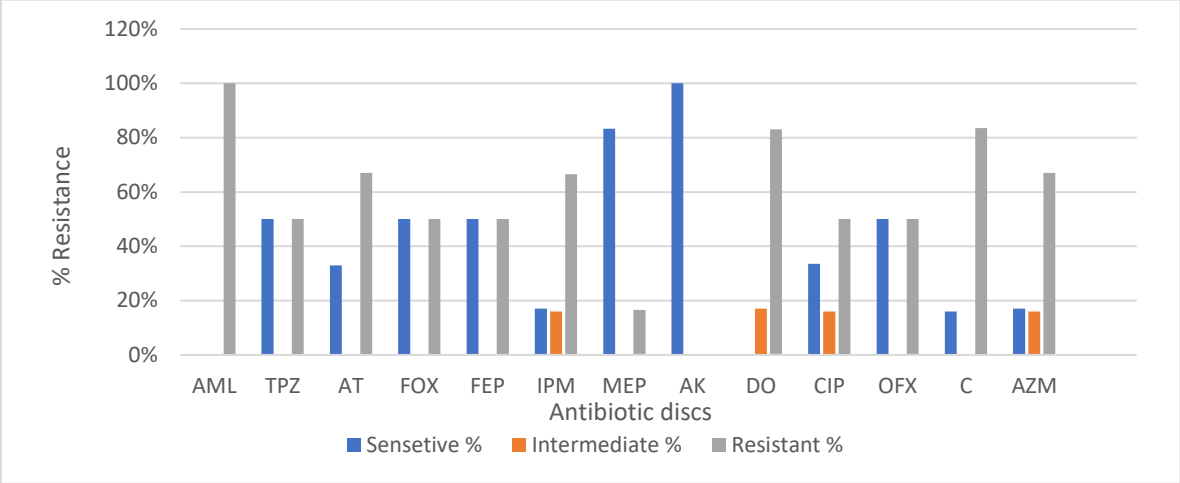

Figure 5: Resistance profile of *Klebsiella species* isolates (n=6) by Kirby Bauer disc diffusion method

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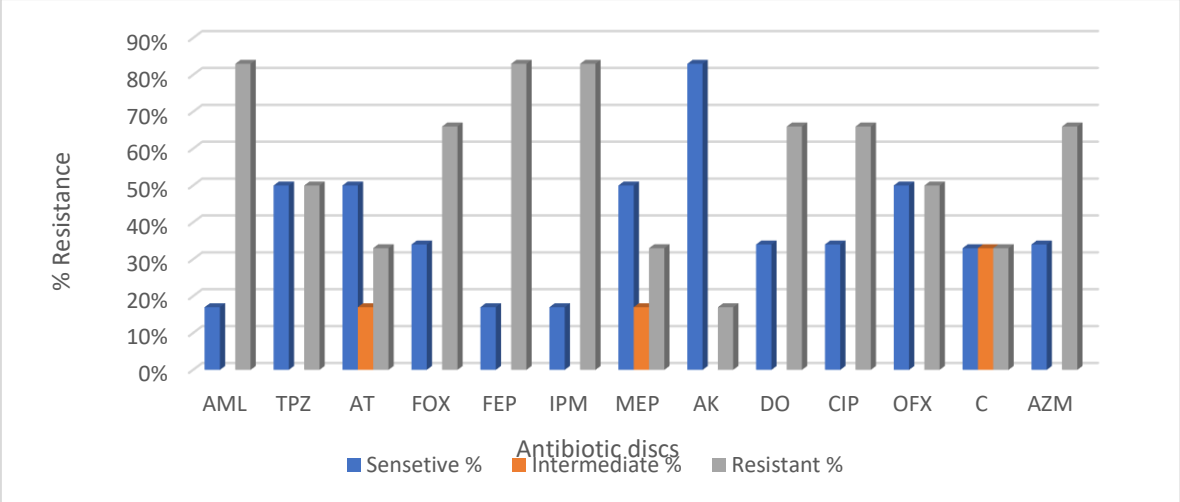

Figure 6. Resistance profile of *Proteus mirabilis* isolates (n=6) by Kirby Bauer disc diffusion method

Table 5. MIC of levofloxacin and gentamicin in *S. aureus* isolates

Antibiotic Conc. µg/ mL	Gentamicin	Levofloxacin
	S. aureus No. (%)	*S. aureus* No. (%)
2028	1 (11%)	0 (0)
1024	0 (0)	0 (0)
512	0 (0)	1 (11%)
256	1(11%)	0 (0)
128	3 (34%)	0 (0)
64	1 (11%)	1 (11%)
32	1 (11%)	2 (22%)
16	0 (0)	2 (22%)
8	0 (0)	0 (0)
4	0 (0)	0 (0)
2	2 (22%)	1 (11%)
1	0 (0)	0 (0)
0.5	0 (0)	2 (22%)
Total	9	9

Table 6. MIC of meropenem, amikacin and colistin of different Gram-negative bacteria isolated from HWW

M. O.	*P. mirabilis* (n=6)			*Klebsiella* spp. (n=6)			*P. putida* (n=1)			*E. coli* (n=1)			*Citrobacter* spp. (n=1)		
Conc µg/mL	MEM No. (%)	AK No. (%)	COL No. (%)	MEM No. (%)	AK No. (%)	COL No. (%)	MEM No. (%)	AK No. (%)	COL No. (%)	MEM No. (%)	AK No. (%)	COL No. (%)	MEM No. (%)	AK No. (%)	COL No. (%)
1024	0	0	1 (17)	0	0	0	0	0	0	0	0	0	0	0	0
512	0	0	1 (17)	2 (33)	0	0	0	0	0	0	0	0	0	0	0
256	1 (17)	0	0	2 (33)	0	0	1 (100)	0	0	0	0	0	0	0	0
128	2 (33)	0	0	2(33)	1 (17)	0	0	0	0	1 (100)	0	0	0	0	0
64	1 (17)	0	2 (33)	0	2 (33)	2 (50)	0	0	1 (100)	0	1 (100)	0	0	0	0
32	1 (17)	3 (50)	2 (33)	0	2 (33)	2 (17)	0	1 (100)	0	0	0	0	0	1 (100)	0
16	0	1 (17)	0	0	1 (17)	0	0	0	0	0	0	1 (100)	0	0	1 (100)
8	0	2 (33)	0	0	0	2 (33)	0	0	0	0	0	0	1 (100)	0	0
4	00	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	1 (17)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	6	6	6	6	6	6	1	1	1	1	1	1	1	1	1

MEM: Meropenem, AK: Amikacin, COL: Colistin

4. DISCUSSION

HWW is a critical habitat for microbial pathogens due to the high burden of ARG and residual antibiotics in their sources. [29] The current study aimed to isolate, identify and determine the antimicrobial susceptibility profile of bacteria isolated from inflow tap water and outflow HWW samples obtained from various hospitals in Egypt. About 35 bacterial isolates were isolated from both tap and HWW. Among those isolates, 68.5% were obtained from HWW (Gram-positive and Gram-negative bacteria) and 31.5% were from tap water (Gram-positive and Gram-negative bacteria).

This result was consistent with a study carried out by **Aleem *et al.*, (2021)** [30] where there was a higher prevalence of Gram-negative bacteria from hospital water sources (about 78% were Gram-negative and 22% were Gram-positive). In contrast, **Godinho *et al.*, (2024)** [31] found that the rate of Gram-positive bacteria isolated from wastewater samples was 50%.

In the present study, the HWW had a higher rate of bacterial isolation than the hospital tap water, A chi-square test of independence revealed a statistically significant difference in the distribution of isolates between the two sources ($\chi^2 = 4.83$, $p = 0.028$).

In current study, the most frequently identified bacteria from HWW were *S. aureus* (25.7%), *P. mirabilis* (17%), *Klebsiella* spp. (17%), *E. coli* (11.4%), *P. putida*. (2.8%) and *Citrobacter* spp. (2.8%). Also, about 28.5% of *Bacillus* spp. were isolated from hospital tap water. Similar results were obtained in a study conducted by **Picão *et al.*, (2013)** [32] on HWW samples, where the most isolated spp. in the hospital sewage was belong to Enterobacteriaceae family (61%) and the most detected members were *Klebsiella* spp., *E. coli*, *Enterobacter* spp. and *Citrobacter spp.* Additionally, similar to our results, **Moges *et al.*, (2020)** [33] reported that the most predominant isolated bacteria from HWW were *Klebsiella* spp. (26.6%), *Pseudomonas* spp. (16.8%), *Citrobacter* spp. (11.5%), *E. coli* (11.5%), and *S. aureus* (8.2%).

Also, our results were in accordance with Aleem *et al.*, (2021)[30] who revealed that the most commonly isolated bacteria found in hospital water surfaces were *Klebsiella* spp. (13%), *S. aureus* (13%), *Pseudomonas* spp. (10%), *E. coli* (9%), *Enterococcus* spp. (6%), *Proteus* spp. (1%), *Citrobacter* spp. (1%) and *Serratia* spp. (1%). Moreover, **Njoya et al., (2022)**[34] detected that *P. penneri*, *P. mirabilis* and *P. vulgaris* species are more abundant in HWW. The increased abundance of *Protus mirabilis and Klebsiella* spp. compared to *E. coli* in our study might be due to their ability to form biofilms in wastewater which made them highly resistance to disinfectant applied.[35]

In addition to biochemical identification, eight Gram-negative isolates (22%) were identified by MALDI-TOF VITEK® MS. The identified bacterial isolates were *Proteus mirabilis* (17%), *Pseudomonas putida* (3%) and *E. coli* (3%). A study by **Suzuki *et al.*, (2018)** [36] applied MALDI-TOF MS for the detection of coliform bacteria from municipal sewage, river water and groundwater, the dominant genera were *Klebsiella* spp., *Enterobacter* spp. and *Serratia* spp., respectively.

In this study, only *Bacillus* species (28.5%) and *E. coli* (3%) recovered from the hospital tap water. The genus Bacillus is large, comprising more than 60 species that are mostly saprophytes, widely distributed in nature, spreading from soil to water, plants, animals and shows a great diversity of strains and species **logan *et al.*, (2015)** [37]. A study carried out by **Farhana *et al.*, (2024)** [38] isolated 11 *E. coli* isolates from drinking water. High level of *E. coli* may be due to the fact that most strains were nonpathogenic and play a vital role in the human digestive system **(Kaur *et al.*, 2020)** [39]. So, future work for genotypic characterization of the isolated *E.coli* will be carried out to determine whether it was pathogenic or free living.

In our investigation, the broth microdilution assay and disk diffusion method were used to screen susceptibility to antibiotics. Kirby-Bauer disk diffusion method revealed that 83% of *P. mirabilis* were resistant to amoxicillin, imipenem and cefepime. The resistance to ciprofloxacin, doxycycline, azithromycin and cefoxitin was 67% for each. On the other hand, 83% of *Proteus mirabilis* were sensitive to amikacin and 67% were sensitive to chloramphenicol, meropenem and aztreonam (Table 4). This was in occurrence with Pierre et al., (2011) [40] who showed that all species including genus Proteus expressed MDR pattern. Also, a lower rate of resistance to aminoglycosides (25%) has been observed by a study carried out by **Paiva *et al.*, (2017)** [41]. Also, our results were comparable to

Njoya et al., (2022)[34] who isolated *Proteus* spp. expressed 95.10% resistance rate to β-lactams and 81.09% quinolones and moderate resistance to gentamicin (41.8%) and 87.5% were sensitive to amikacin. The presence of *proteus* spp. in HWW, which enriched with different stressors influences its susceptibility to antibiotics by facilitating the acquisition of resistance mechanisms. [34]

The results of the Kirby-Bauer disk diffusion method revealed that 100% of *Klebsiella* spp. were resistant to amoxicillin. The resistance to doxycycline and chloramphenicol was 83% for each, followed by azithromycin, aztreonam and imipenem (67% for each). On the other hand, only one isolate was resistant to meropenem (17%), while no resistance was observed for amikacin (Table 4). Similar results were reported by Aleem *et al., (*2021)[30] where *Klebsiella* spp. had demonstrated an increasing incidence of resistance to commonly used antibiotics, including carbapenems (52%) and ciprofloxacin (84%).

In our study *E. coli* isolated form wastewater was resistant to azithromycin and doxycycline. This was in contrast to a study conducted in northern Portugal by **Ferreira *et al.*, (2007) [42]**, who carried out a study on wastewater effluent stated that 30% of *E. coli* isolates were resistant to amoxicillin and tetracycline. On the other hand, *E. coli* isolate recovered from tap water in the current study was fully sensitive to all antibiotics tested by both assays.

In this study *Pseudomonas putida* was isolated from HWW and identified using MALDI-TOF MS. It was resistant to most tested antibiotics and was only susceptible to amikacin, doxycycline, and azithromycin (Table 4). Similarly, **Moges et al., (2020) [33]** find that Gram-negative isolates frequently exhibited multiple drug resistance to widely used antibiotics, with *Pseudomonas* species exhibiting resistance to ten different antibiotics.

The resistance profile of *Citrobacter* spp. to commonly tested antibiotics was lower compared with the higher resistance profiles of *Protus mirabilis, Klebsiella spp. and Pseudomonas putida.* It was fully resistant to azithromycin, amoxicillin and imipenem, while no resistance was detected against cefoxitin and cefepime (Table 4). In contrast, **Addae-Nuku et al., (2022) [43]** detected high resistance rates of *Citrobacter* spp. against cefuroxime, ceftazidime, and cefepime, ranging between 20% to 30%.

S. aureus isolates were 100% resistant to amoxicillin. The resistance to azithromycin, amikacin and clindamycin were 67%, 56% and 45%, respectively. On the other hand, 100% of *S. aureus* were sensitive to vancomycin and doxycycline and 78% were sensitive to erythromycin (Table 3). Similarly, a study by **Okhonlaye et al., (2018)[44]** found that *Staphylococcus* spp. isolates were MDR with 100% resistance to amoxicillin, 83% resistance to gentamycin and 78% resistance to ciprofloxacin. MDR profile was observed in *S. aureus* isolates to commonly used antibiotics. About 78% of *S. aureus* isolates had resistance to more than three antibiotics from different classes. MDR profile was observed in *S. aureus* isolates to commonly used antibiotics. Similar result was stated by **Moges et al., (2020)[33]** who detected that all of the *S. aureus* isolates were 100% resistant to ampicillin and MDR was also frequently seen in Gram-positive isolates to widely used antibiotics.

In the current study, 83% of total bacteria recovered from HWW were MDR, especially among *Protus mirabilis, Klebsiella* spp. and *S. aureus* isolates. This was similar to **Rodríguez *et al.*, (2020) [45],** where 91% of the isolates were MDR. The reason for the increase in antibiotic resistance among the isolated bacteria is due to the hospital-based therapies that result in a high rate of antibiotic consumption and antibiotic residue excretion. [42]

In the current study, the resistance rate to meropenem by broth micro dilution was 93% among all Gram-negative isolates, while the resistance to amikacin was 26%. Hundred percent resistance to colistin was detected among all isolated Gram-negative bacteria (Table 6).

Hundred percent of *Klebsiella* spp. were resistant to colistin by broth microdilution assay (MIC ≥ 8 µg/mL) (Table 6). This was in contrast to **Aleem *et al., (*2021) [30]** where the *Klebsiella* spp. isolates showed lower resistance to colistin (30%). Fifty percent of *Klebsiella* spp. were resistant to amikacin; the MIC was ≥ *32* µg/mL. This was comparable to **Paiva *et al.*, (2017) [41],** where a lower rate of resistance to aminoglycosides (25%) has been observed. The resistance to meropenem reaching 100% (MIC ≥128 µg/mL). The emergence of carbapenem resistance in *Klebsiella* species has become a substantial clinical problem, most

commonly due to overexpression of efflux pumps that expel carbapenems, mostly meropenem. [46]

Hundred percent of *Proteus mirabilis* were resistant to colistin; MIC was ≥ 32 µg/mL. In addition, 83% of *Proteus mirabilis* were resistant to meropenem; MIC was ≥ 32 µg/mL (Table 6). *Proteus mirabilis* is naturally resistant to several antibiotics including colistin. The higher levels of resistance to carbapenem commonly occur due to the loss of porins, reduced expression of penicillin binding proteins (PBPs), or acquisition of several antibiotic resistance genes, including carbapenemase genes**.** [47]

Seventy-seven percent of *S. aureus* isolates were resistant to gentamicin MIC > 16 µg/mL, while 67% were resistant to levofloxacin MIC > 4 µg/mL (Table 5). A study by **Alarjani *et al.*, (2022)** [48] detected higher resistance to gentamicin and ciprofloxacin in *S. aureus* isolates from HWW samples. Increased gentamicin resistance was mainly due to the acquisition of aminoglycoside-modifying enzymes [49], while higher resistance to levofloxacin may be due to the increased usage of fluoroquinolones in hospitals to eradicate MRSA colonization in patients.[50]

Stringent rules and infection control measures in hospitals, should be implemented alongside HWW management to guarantee that antibiotic resistance does not spread in the environment. Furthermore, when wastewater is reused for agricultural purposes, further steps must be performed to eradicate these bacteria before releasing the water back into the environment. To effectively combat bacterial resistance in wastewater systems, it is recommended to employ bacteriophages for targeted bacterial lysis, conduct biofilm assays on isolated strains to understand biofilm formation capabilities, genetically characterize resistant bacterial strains to identify resistance mechanisms, develop detection methods based on genetic insights, implement tailored treatment strategies and establish regular monitoring and surveillance protocols to track resistance trends. By integrating these approaches, a comprehensive strategy can be formulated to address bacterial resistance in wastewater, ultimately enhancing water quality and public health outcomes.

5. CONCLUSION:

Our study revealed high MDR rate of bacteria isolated from HWW. Water contamination by antimicrobials resulted in higher resistance due to selection pressure exerted. The prevalence of Gram-negative bacteria from HWW was higher than Gram-positive bacteria in our study. The most resistant pathogens found in HWW were *S. aureus, P. mirabilis* and *Klebsiella* spp. and higher antibiotic resistance was found against amoxicillin, colistin, imipenem, meropenem, doxycycline and azithromycin. The study reflects a good treatment process for income water where all isolates from tap water were sensitive to tested antibiotics.

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List of Abbreviations:

HWW: Hospital wastewater
AMR: Antimicrobial Resistance
ARB: Antimicrobial-resistant bacteria
HGT: Horizontal gene transfer
MDR: Multiple drug resistance
MIC: Minimum inhibitory concentration (MIC)
MALDI-TOF: Matrix Assisted Laser Desorption Ionization Time-of-Flight

REFERENCES

1. Aijaz M, Ahmad M, Ansari MA, Ahmad, S. Antimicrobial Resistance in a Globalized World: Current Challenges and Future Perspectives. International Journal of Pharmaceutical Drug Design.2023; 1(1):7-22.

2. Fatimazahra S, Latifa M, Laila S, Monsif K. Review of hospital effluents: special emphasis on characterization, impact, and treatment of pollutants and antibiotic resistance. Environmental Monitoring and Assessment. 2023;195(3): 393.

3. Hendriksen RS, Munk P, Njage P, van Bunnik B, McNally L, Lukjancenko O. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nature communications. 2019; 10:1124.

4. Kümmerer K, Henninger A. Promoting resistance by the emission of antibiotics from hospitals and households into effluent. Clinical microbiology and infection. 2003; 9 (12): 1203–1214.

5. Zagui GS, Moreira NC, Santos DV, Darini ALC, Domingo JL, Segura-Muñoz SI Andrade LN. High occurrence of heavy metal tolerance genes in bacteria isolated from wastewater: a new concern. Environmental Research. 2021; 196: 110352.

6. Beattie RE, Skwor T, Hristova KR. Survivor microbial populations in post-chlorinated wastewater are strongly associated with untreated hospital sewage and include ceftazidime and meropenem resistant populations. Science of the Total Environment. 2020; 740:140186

7. Jiang N, Zhang X, Zhou Y, Zhang Z, Zheng X. Whole-genome sequencing of an NDM-1- and OXA-58-producing Acinetobacter towneri isolate from hospital sewage in Sichuan Province, China. Journal of global antimicrobial resistance. 2019;16: 4–5.

8. Wang Q, Wang, P, Yang, Q. Occurrence and diversity of antibiotic resistance in untreated HWW. Science of the Total Environment. 2018; 621: 990-999.

9. Fajardo A, Martínez-Martín N, Mercadillo M, Gal´an JC, Ghysels B, Matthijs S. The neglected intrinsic resistome of bacterial pathogens. PLoS One. 2008; 3(2): e1619

10. Jutkina, J., Marathe, N. P., Flach, C. F., & Larsson, D. G. J. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. Science of the total Environment. 2018; 616: 172-178.

11. Klümper U, Recker M, Zhang L, Yin X, Zhang T, Buckling A. Selection for antimicrobial resistance is reduced when embedded in a natural microbial community. The ISME journal. 2019; 13(12): 2927-2937.

12. Zhang X, Lü X, Zong Z. Enterobacteriaceae producing the KPC-2 carbapenemase from hospital sewage. Diagnostic microbiology and infectious disease. 2012; 73(2): 204-206.

13. McLain JE, Cytryn E, Durso LM, Young, S. Culture-based methods for detection of antibiotic resistance in agroecosystems: Advantages, challenges, and gaps in knowledge. Journal of environmental quality. 2016; 45(2): 432-440.

14. Lee HW, Yoon SR, Kim SJ, Lee HM, Lee JY, Lee JH. Identification of microbial communities, with a focus on foodborne pathogens, during kimchi manufacturing process using culture-independent and-dependent analyses. LWT-Food Science and Technology. 2017; 81: 153-159.

15. Yamin D, Uskoković V, Wakil AM, Goni MD, Shamsuddin SH, Mustafa FH. Current and future technologies for the detection of antibiotic-resistant bacteria. Diagnostics. 2023; 13(20):3246.

16. Timraz K, Xiong Y, Al Qarni H, Hong PY. Removal of bacterial cells, antibiotic resistance genes and integrase genes by on-site HWW treatment plants: surveillance of treated hospital effluent quality. Environmental Science: Water Research & Technology. 2017; *3*(2): 293-303.

17. Le T.-H, Ng C, Chen H, Yi XZ, Koh TH, Barkham TMS. Occurrences and characterization of antibiotic-resistant bacteria and genetic determinants of HWW in a tropical country. Antimicrobial Agents Chemotherapy 2016: 60 (12): 7449–7456.

18. Lutterbeck CA, Colares GS, Dell'Osbel N, Silva FP, Kist L T, Machado ÊL. Hospital laundry wastewaters: a review on treatment alternatives, life cycle assessment and prognosis scenarios. Journal of Cleaner Production. 2020; 273:12285.

19. Mehanni MM, Gadow SI, Alshammari FA, Modafer Y, Ghanem KZ, El-Tahtawi N F. Antibiotic-resistant bacteria in hospital wastewater treatment plant effluent and the possible consequences of its reuse in agricultural

irrigation. Frontiers in Microbiology. 2023; 14: 1141383.

20. Amine AEK. Extended Spectrum Beta-Lactamase Producing Bacteria in Wastewater Alexandria, Egypt. International Journal of Bioscience, Biochemistry, Bioinformatics. 2013; 3: 605-608.

21. Coutu S, Rossi L, Barry DA, Rudaz S, Vernaz N. Temporal variability of antibiotics fluxes in wastewater and contribution from hospitals. PLoS One. 2013; *8*(1): e53592.

22. Koneman EW, Allen SD, Janda W, Schreckenberger P, Winn W. Diagnostic microbiology. The nonfermentative gram-negative bacilli. Philedelphia: Lippincott-Raven Publishers. 1997;253-320.

23. Cheesbrough, M. District laboratory practice in tropical countries, part 2: Cambridge university press. 2005.

24. Procop GW Church, DL. Hall GS. Janda WM. Koneman's color atlas and textbook of diagnostic microbiology: Jones & Bartlett Publishers. 2020.

25. Aw B. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Patholology. 1966; 45: 493-496.

26. Jorgensen JH, Turnidge JD. Susceptibility test methods: dilution and disk diffusion methods. Manual of clinical microbiology. 2015; 1253-1273.

27. Wayne, P. "Clinical and laboratory Standards Institute (CLSI)(2020) Performance standards for antimicrobial susceptibility testing: Background, Organization, Functions, and Processes." J Clinical Microbiology.58.3 (2010): 01864-0186419.

28. Lalitha M. Manual on antimicrobial susceptibility testing. Performance standards for antimicrobial testing: Twelfth Informational Supplement.2004; 56238: 454-456.

29. Singh R, Singh AP, Kumar S, Giri BS, Kim KH: Antibiotic resistance in major rivers in the world: A systematic review on occurrence, emergence, and management strategies. Journal of Cleaner Production. 2019, 234:1484-505.

30. Aleem M, Azeem AR, Rahmatullah S, Vohra S, Nasir S, Andleeb S. Prevalence of bacteria and antimicrobial resistance genes in hospital water and surfaces. Cureus. 2021;13(10).

31. Godinho O, Lage OM, Quinteira S. Antibiotic-Resistant Bacteria across a Wastewater Treatment Plant. Applied Microbiology. 2024; *4*(1): 364-375.

32. Picão R C, Cardoso JP, Campana EH, Nicoletti A G, Petrolini FV, Assis DM. The route of antimicrobial resistance from the hospital effluent to the environment: focus on the occurrence of KPC-producing *Aeromonas* spp. and Enterobacteriaceae in sewage. Diagnostic microbiology and infectious disease. 2013; *76*(1): 80-85.

33. Moges F, Endris M, Belyhun Y, Worku W. Isolation and characterization of multiple drug resistance bacterial pathogens from waste water in hospital and non-hospital environments, Northwest Ethiopia. BMC research notes. 2014; *7*:1-6.

34. Njoya AM, Eheth JS, Poutoum Y, Metsopkeng CS, Moldovan CV, Belengfe SC. Proteus bacteria species from hospital sewage and Mfoundi River in Yaounde (Cameroon, Central Africa): Comparison of the diversity, abundance and susceptibility against some β-lactams, Quinolones and Aminoglycosides antibiotics. Journal of Advances in Microbiology Research. 2022.

35. Shadkam S, Goli HR, Mirzaei B, Gholami M, Ahanjan M. Correlation between antimicrobial resistance and biofilm formation capability among *Klebsiella pneumoniae* strains isolated from hospitalized patients in Iran. Annals of Clinical Microbiology and Antimicrobials. 2021; 20: 1-7.

36. Suzuki Y, Niina K, Matsuwaki T, Nukazawa K, Iguchi A. Bacterial flora analysis of coliforms in sewage, river water, and ground water using MALDI-TOF mass spectrometry. Journal of environmental Science and Health, Part A. 2018; *53*(2): 160-173.

37. Logan NA, Vos PD. Bacillus. Bergey's manual of systematics of archaea and bacteria. 2015; 1-163.

38. Farhana N, Akhtar K, Hossain A, Khanam, F. Molecular characterization of antimicrobial

resistance organisms from drinking water and wastewater in a metropolitan city. medRxiv. 2024; 2024-04.

39. Kaur R, Yadav B, Tyagi, RD. Microbiology of hospital wastewater. In Current developments in Biotechnology and bioengineering, Elsevier. 2020; 103-148.

40. Pierre P. Multiresistant bacteria in the environment: research in the effluents of the city of Toulouse. France: University of Limoges. 2011.

41. Paiva MC, Reis MP, Costa PS, Dias MF, Bleicher L, Scholte LL. Identification of new bacteria harboring qnrS and aac (6′)-Ib/cr and mutations possibly involved in fluoroquinolone resistance in raw sewage and activated sludge samples from a full-scale WWTP. Water research. 2017; 110: 27-37.

42. Ferreira da Silva M, Vaz-Moreira I, Gonzalez-Pajuelo M, Nunes OC, Manaia CM. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. FEMS microbiology ecology. 2007; *60*(1): 166-176.

43. Addae-Nuku DS. Multidrug-Resistant Bacteria In Hospital Wastewater Of The Korle Bu Teaching Hospital (Doctoral dissertation, University Of Ghana).2022.

44. Okhonlaye O, Oluwatosin O. Occurrence of multidrug resistant Staphylococcus aureus and Klebsiella pneumoniae isolated from clinical and environmental samples in Ondo state, Nigeria. Journal of Advances in Microbiology. 2018; 10(2): 1-10.

45. Rodríguez EA, Garzón LM, Gómez ID, Jiménez JN. Multidrug resistance and diversity of resistance profiles in carbapenem-resistant Gram-negative bacilli throughout a wastewater treatment plant in Colombia. Journal of global antimicrobial resistance. 2020; 22: 358-366.

46. Orias F, Perrodin Y. Characterisation of the ecotoxicity of hospital effluents: a review. Science of the Total Environment. 2013; 454: 250-276.

47. Girlich D, Bonnin RA, Dortet L, Naas T. Genetics of acquired antibiotic resistance genes in Proteus spp. Frontiers in microbiology. 2020; 11: 256.

48. Alarjani, K. M., and Skalicky, M. Antimicrobial resistance profile of Staphylococcus aureus and its in-vitro potential inhibition efficiency. Journal of Infection and Public Health. 2021; 14(12): 1796-1801.

49. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. Drug resistance updates. 2010; 13(6): 151-171.

50. Limoncu MH, Ermertcan S, Çetin ÇB, Cosar G, Dinç G. Emergence of phenotypic resistance to ciprofloxacin and levofloxacin in methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* strains. International Journal of Antimicrobial Agents. 2003; 21(5): 420-424.