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THESIS

Presented by

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OPTION: Microbiology and Quality Control

THEME

The impact of hospital wastewater from Tlemcen University Hospital Center on multi-drug resistant bacteria

Presented on **13 June 2024**

Before the jury composed of:

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Dedication

This thesis is dedicated to my beloved mom for her endless love and support.

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Abstract

Multi-drug resistance is a major global threat, especially in healthcare settings. Hospitals generate wastewater that acts as a reservoir for multidrug-resistant bacteria. Hospital wastewater, if untreated or poorly treated, can disseminate resistant pathogens into the environment, posing significant risks to public health and ecological systems. This study aims to qualitatively assess the presence of bacteria and multidrug-resistant bacteria in the hospital wastewater of Tlemcen University Hospital.

Hospital wastewater samples were collected from 3 different units of the hospital which are the Pneumo-phthisiology, Hematology, and Neurosurgery units. Bacterial isolation was performed using selective and differential agar media to selectively promote the growth of target ESKAPE bacteria. Antibiotic susceptibility testing was carried out using the Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute guidelines to determine resistance profiles.

The research revealed the presence of bacteria in the hospital wastewater, including the occurrence of multidrug-resistant bacteria. Predominantly, MDR bacteria from the *Enterobacteriaceae* family were found in the Hematology unit, while MDR *Pseudomonas spp.* were prevalent in the Pneumo-phthisiology unit. The study also identified Staphylococcus spp., which demonstrated susceptibility to the tested antibiotics. However, *Pseudomonas spp*. exhibited high resistance to Ciprofloxacin and Tobramycin across multiple units.

The findings highlight the crucial need for efficient hospital wastewater treatment to prevent the spread of antibiotic resistance. MDR bacteria in wastewater pose major threats to public health.

Keywords: antibiotic resistance, hospital wastewater, multidrug-resistant bacteria, ESKAPE bacteria, public health

Résumé

La multirésistance aux médicaments constitue une menace mondiale majeure, en particulier dans les établissements de soins de santé. Les hôpitaux génèrent des eaux usées qui servent de réservoir à des bactéries multirésistantes. Si les eaux usées des hôpitaux ne sont pas ou mal traitées, peuvent disséminer des agents pathogènes résistants dans l'environnement, posant ainsi des risques importants pour la santé publique et les systèmes écologiques. Cette étude vise à évaluer qualitativement la présence de bactéries et de bactéries multirésistantes dans les eaux usées hospitalières du CHU de Tlemcen.

Des échantillons d'eaux usées hospitalières ont été collectés dans 3 différentes unités de l'hôpital, à savoir les unités de pneumo-phtisiologie, d'hématologie et de neurochirurgie. L'isolement bactérien a été réalisé à l'aide de milieux gélosés sélectifs et différentiels pour favoriser sélectivement la croissance des bactéries ESKAPE cibles. Des tests de sensibilité aux antibiotiques ont été effectués à l'aide de la méthode de diffusion sur disque de Kirby-Bauer conformément aux directives du Clinical and Laboratory Standards Institute afin de déterminer les profils de résistance.

La recherche a révélé la présence de bactéries dans les eaux usées de l'hôpital, notamment l'apparition de bactéries multirésistantes. Les bactéries MRA de la famille des Enterobacteriaceae ont été principalement trouvées dans l'unité d'hématologie, tandis que les bactéries MRA Pseudomonas spp. étaient fréquents dans l'unité de Pneumo-phtisiologie. L'étude a également identifié Staphylococcus spp., qui a démontré une sensibilité aux antibiotiques testés. Cependant, Pseudomonas spp. a présenté une résistance élevée à la ciprofloxacine et à la tobramycine dans plusieurs unités.

Les résultats mettent en évidence la nécessité cruciale d'un traitement efficace des eaux usées hospitalières pour prévenir la propagation de la résistance aux antibiotiques. Les bactéries MDR présentes dans les eaux usées constituent une menace majeure pour la santé publique.

Mots clés : résistance aux antibiotiques, eaux usées hospitalières, bactéries multirésistantes, bactérie ESKAPE, santé publique

ملخص

تمثل مقاومة الأدوية المتعددة تهديدًا عالميًا كبيرًا، خاصة في أماكن الرعاية الصحية. تولد المستشفيات مياه الصرف الصحي التي تعمل كمستودع للبكتيريا المقاومة للأدوية المتعددة. يمكن لمياه الصرف الصحي في المستشفيات، إذا لم تتم معالجتها أو تمت معالجتها بشكل سيئ، أن تنشر مسببات الأمراض المقاومة في البيئة، مما يشكل مخاطر كبيرة على الصحة العامة والنظم البيئية. تهدف هذه الدراسة إلى التقييم النوعي لوجود البكتيريا والبكتيريا المقاومة للأدوية المتعددة في مياه الصرف الصحي التابعة للمستشفى الجامعي تلمسان.

تم جمع عينات مياه الصرف الصحي في المستشفى من ثلاث وحدات مختلفة في المستشفى وهي وحدات أمراض الرئة وأمراض الدم وجراحة الأعصاب. تم إجراء العزلة البكتيرية باستخدام وسائط أجار انتقائية وتفاضلية لتعزيز نمو بكتيريا ESKAPEالمستهدفة بشكل انتقائي. تم إجراء اختبار الحساسية للمضادات الحيوية باستخدام طريقة نشر قرص-Kirby Bauerوفقًا لإرشادات معهد المعايير السريرية والمخبرية لتحديد ملفات تعريف المقاومة.

وكشف البحث عن وجود بكتيريا في مياه الصرف الصحي بالمستشفى، بما في ذلك وجود بكتيريا مقاومة للأدوية المتعددة. في الغالب، تم العثور على البكتيريا المقاومة للأدوية المتعددة من عائلة Enterobacteriaceae في وحدة أمراض الدم، في حين تم العثور على البكتيريا المقاومة للأدوية المتعددة .Pseudomonas spp كانت سائدة في وحدة أمراض الرئة. كما حددت الدراسة أيضًا المكورات العنقوديةStaphylococcus spp ، والتي أظهرت قابليتها للمضادات الحيوية التي تم اختبارها. ومع ذلك، الزائفة النيابة. أظهرت مقاومة عالية للسيبروفلوكساسين والتوبراميسين عبر وحدات متعددة.

تسلط النتائج الضوء على الحاجة الماسة لمعالجة مياه الصرف الصحي في المستشفيات بكفاءة لمنع انتشار مقاومة المضادات الحيوية. تشكل البكتيريا المقاومة للأدوية المتعددة الموجودة في مياه الصرف الصحي تهديدات كبيرة للصحة العامة .

الكلمات المفتاحية: البكتيريا المقاومة للأدوية المتعددة، الصحة العامة، بكتيرياESKAPE ، مقاومة المضادات الحيوية، مياه الصرف الصحي في المستشفيات.

List of Abbreviations

AMR: Antimicrobial resistance

ARB: Antibiotic-resistant bacteria

ARGs: Antibiotic-resistant genes

Amp: ampicillin

BOD: Biochemical Oxygen Demand

BOD5: Biochemical Oxygen Demand 5 days of incubation

BMW: Biomedical waste

COD: Chemical oxygen Demand

CIP: Ciprofloxacin

DA: Clindamycin

DNA: Deoxyribonucleic acid

E.coli : Escherichia coli

Ecs : Emerging contaminants

EHS : Environmental, Health, and Safety

ESBLs: extended-spectrum β-lactamases

ESKAPE: Enterococcus faeciumi, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.

HWW: Hospital WasteWater

IMP: imipenemase

K. pneumoniae: Klebsiella pneumonia

MRSA: Methicillin-Resistant Staphylococcus Aureus

MDR: Multidrug resistance

MWW: Municipal Wastewater

NA: Nalidixic acid

OFX: Ofloxacin

OXA: Oxacillinase

P. aeruginosa : Pseudomonas aeruginosa

PMQR: plasmid-mediated quinolone resistance

SARS: Severe acute respiratory syndrome

SARS-CoV2: Severe acute respiratory syndrome coronavirus 2

S. aureus : Staphylococcus aureus

STX: Trimethoprim-sulfamethoxazole

TOB: Tobramycin

TSS: Total Suspended solid

UV: Ultraviolet

WHO: World Health Organization

WWTP: Wastewater treatment plants

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Introduction

Introduction

Wastewater is defined as any water with a high concentration of contaminants, either soluble or not. All types of waste and contaminants are produced by human activity, whether domestic, agricultural, or industrial, and these materials are transported in liquid form **(Benets, 2000; Emmanuel, 2001)**.

Hospitals are essential to both the advancement of medical research and knowledge as well as the welfare of people. They support healthcare by offering continuing services to address complicated medical issues. Nonetheless, a lot of waste water is produced as a result of these activities. Hospital wastewaters (HWW) are extremely complex effluents that contain bacteria, excrement from patients, disinfectants, metabolized drugs **(Emmanuel *et al*., 2005),** antibiotic compounds, and possibly multidrug-resistant (MDR) genes **(Chang *et al*., 2010; Galvin *et al*.,2010; Chagas *et al*.,2011).** The type and amount of HWW and biomedical waste (BMW) released, the services and amenities offered, and the hospital's size all significantly impacts the waste management techniques employed **(Patel *et al*., 2019).**

Hospital wastewater (HWW) is dangerous and contagious and differs greatly from wastewater released from other sources. It is made up of a variety of micro- and macro pollutants that are released from radiology, wards, laboratories, operation (surgery) rooms, research units, and microbiology laboratories **(Kaur *et al.,* 2020)**. HWW are therefore thought to be hotspots for antibiotic resistance, creating a setting conducive to the spread of antibiotic-resistance genes. They are becoming increasingly contaminated with gram-negative bacteria that carry multiple bla genes (e.g. blaNDM, blaKPC, blaCTX-M, and blaSHV) **(Chagas *et al*.,2011; Zhang *et al*.2012)**.

Due to the careless use of antibiotics, the spread of antibiotic resistance in the environment and clinical settings has become a global problem. The World Health Organization (WHO) in **2019** has identified Antimicrobial Resistance (AMR) as one of the major 10 public health threats that the world is currently facing **(EClinicalMedicine, 2021)**. AMR is one of the biggest threats to both global development and public health, according to estimates, bacterial AMR caused 4.95 million deaths worldwide in 2019 and it directly caused 1.27 million deaths **(Antimicrobial Resistance Collaborators, 2022).** Hospital effluents may be the most hazardous due to the nature and importance of the xenobiotics and microorganisms they carry, as aquatic environments are the primary disposal sites for human waste **(Anssour *et al*.,2013).**

Introduction

Over the past two decades, the presence of antibiotics in water bodies and the ensuing emergence of microbial resistance have drawn the attention of scientists and the general public as a potentially concerning issue **(Kümmerer., 2009; Rodríguez-Gil *et al*., 2010).**

In the past decade, much research on HWW has focused on a limited number of pharmaceutical substances (mainly antibiotics and anti-inflammatory medications), their fate in the water management cycle, and their effects on the environment. There is limited research or inadequate knowledge of hospital effluents as sources of antibiotic resistance genes (ARG) **(Benamara, 2022).** In reality, several countries release hospital wastewater directly into the municipal sewer system without any prior treatment or quality standards because they view it as domestic waste and pre-release treatment for HWWs has been legalized in other nations such as Italy and France **(Verlicchi et al., 2010).** Hospital effluents are regarded as industrial in very few countries and undergo pre-treatment before being released into the municipal sewer network **(Carraro *et al*., 2016).**

To address the identified knowledge gaps, this study aimed to evaluate the existence of multidrug-resistant bacteria in wastewater at Tlemcen's University Hospital and to better understand the role of hospital effluents as a source of a wide range of antibiotic-resistance genes (ARG).

The objectives of this study are to qualitatively assess the presence of bacteria and multidrug-resistant bacteria in the hospital wastewater of Tlemcen University Hospital, describe the qualitative aspects of hospital effluent, determine its possible impact on multi-drug resistant bacteria, and provide recommendations to mitigate the spread of antibiotic resistance.

Literature review

CHAPTER 1: ANTIBIOTIC RESISTANCE

1. **Multi-drug Resistant Bacteria**

Antibiotic-resistant bacteria were initially identified in the late 1950s, when the majority of *S. aureus* isolates became resistant to penicillin, which was previously the antibiotic of choice for treating them **(Stapleton & Taylor, 2002)**. Since the 1960s, when new medication classes like methicillin and vancomycin were created to combat antibiotic resistance, the issue of antibiotic resistance has not been an issue of significant concern globally **(McGuinness *et al*.,2017).** Subsequently, following the development of these new antibiotics, antibiotic resistance has progressed during the next decades as a result of bacteria developing a wide range of antibiotic resistance mechanisms that have shielded them from the effects of these medications **(Aslam *et al*., 2018).**

The World Health Organisation (WHO), in **2017**, produced a list of "priority pathogens" that are resistant to antibiotics. The twelve groups of bacteria on the list are the most dangerous to human health due to their resistance to available antibiotics **(Mulani *et al*., 2019).** According to how urgently new antibiotics are needed, the list is split into three categories that are ***critical, high,*** and ***medium priority*** **(De Oliveira *et al*., 2020).**

The most 'critical category' of all comprises multidrug-resistant bacteria that pose a particular threat in hospitals, carers, and among patients, particularly the ESKAPE, which incorporates six extremely virulent and drug resistant bacterial pathogens that are, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp*. **(Mulani *et al*., 2019)**. Their resistance to a broad spectrum of antibiotics poses a global health risk since it frequently facilitates the spread of bacterial infections with high rates of morbidity and mortality **(Benamara, 2022).**

Table N° 1: World Health Organization (WHO) list of antibiotic-resistant 'priority pathogens' **(WHO,2017)** https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

PRIORITY PATHOGEN	RESISTANCE
First Priority: Critical	
Acinetobacter baumannii	carbapenem-resistant
Pseudomonas aeruginosa	carbapenem-resistant
Enterobacteriaceae	carbapenem-resistant, ESBL-producing
Second Priority: High	
Enterococcus faecium	vancomycin-resistant
Staphylococcus aureus	methicillin-resistant, vancomycin-intermediate and resistant
Helicobacter pylori	clarithromycin-resistant
Campylobacter spp.	fluoroquinolone-resistant
Salmonellae	fluoroquinolone-resistant
Neisseria gonorrhoeae	cephalosporin-resistant, fluoroquinolone-resistant
Third Priority: Medium	
Streptococcus pneumoniae	penicillin-non-susceptible
Haemophilus influenzae	ampicillin-resistant
Shigella spp.,	fluoroquinolone-resistant

2. Main multi-drug resistant bacteria

2.1. Enterobacteriaceae

Enterobacteriaceae is a family of Gram-negative, non-spore-forming bacteria that includes *Shigella, Proteus, Citrobacter, Salmonella, Escherichia coli (E. coli),* and *Enterobacter*. These bacteria are motile due to peritrichous cilia, with the exception *of Klebsiella, Shigella,* and *Yersinia.* They are the animals' and humans' natural hosts of the digestive tract. The most significant members of this family are *Escherichia coli, Klebsiella pneumonia,* and *Proteus mirabilis,* as they account for 80% of human infections **(Nnadozie & Odum, 2019).**

Antibiotic resistance in Enterobacteriaceae is increasing globally, including resistance to extended-spectrum cephalosporins, penicillins, and monobactams. Plasmid-mediated enzyme production hydrolyzes the β-lactam ring of antibiotics, leading to resistance. ESBLs, or extended-spectrum β-lactamases, are formed by point mutations in existing wide-spectrum β-lactamases **(Miyagi and Hirai, 2019).**

Moreover, a large group of b-lactamases known as carbapenemases, which are divided into classes ABC and D, are in charge of rendering carbapenem antibiotics inactive. Imipenem and meropenem are two examples of carbapenem antibiotics that are used as last resorts for treating severe infections brought on by ESBL producers. Treating infections caused by pathogens resistant to carbapenem presents significant challenges and is associated with exceptionally high mortality rates due to the lack of antibiotic alternatives **(Khalili & Izadpanah, 2015)**.

Anssour *et al*. **(2013**) conducted a study in Algeria that emphasized hospital effluents' significant potential function as suppliers of resistance genes to natural habitats for the first time in Algeria. The PMQR determinants found were qnrB1, qnrB2, qnrB9, qnrB19, qnrS2, and aac(6')-Ib-cr. qnrB2, qnrB9, qnrB19, and blaCMY-4 and qnrB19 for the first time in non-clinical settings.

In this study by Anssour *et al***. (2013),** extended-spectrum beta-lactamases, plasmidic AmpC (pAmpC), and associated plasmid-mediated quinolone resistance (PMQR) determinants in cefotaxime-resistant coliforms were isolated from hospital effluent in Algiers, and isolates showed the highest quantity of blaCTX-M genes, followed by blaTEM-1, and a small quantity of pAmpC genes (blaCIT). CTX-M+ isolates had ISEcp1B association with blaCTX-M, and most of them had class 1 integrons. blaCTX-M-15, blaCTX-M-3, and blaCMY-4 genes were

revealed by sequencing. Inc L/M conjugative plasmids included blaCTX-M-3 and blaCTX-M-15 **(Anssour *et al*., 2013**).

2.2. Acinetobacter spp.

Acinetobacter is a ubiquitous bacterium that is Gram-negative and a member of the Moraxellaceae family. Acinetobacter strains were naturally resistant to drugs like ampicillin, cephalotin, tetracyclines, and chloramphenicol in the early 1970s. However, with the advent of carboxypenicillins, second- and third-generation cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones, it has become more challenging to identify active compounds on these bacteria because *Acinetobacter* species are well-known for their remarkable capacity to develop resistance mechanisms to most new antibiotics. **(Kyriakidis et al., 2021)**

Acinetobacter baumanni is the most common pathogen found in the most severe hospital environments. Acinetobacter infections are a type of ESKAPE pathogen that pose a threat to public health due to their high fatality rates and ability to cause severe and invasive infections, primarily nosocomial infections. The development of multidrug resistance (MDR) in this microorganism in recent years is primarily the result of widespread antibiotic misuse **(Kyriakidis *et al*., 2021).**

In **2017**, the World Health Organization (WHO) declared carbapenem-resistant *A. baumannii* (CRAB) to be one among the critical focus of antibiotic research and development. Since carbapenem resistance is frequently associated with a broad range of antibiotic co-resistance, it was selected as a marker.

Three primary mechanisms by which antibiotic resistance can be acquired in *A. baumannii* are by decreasing membrane permeability (e.g., reduced porin permeability or increased efflux) to prevent antibiotics from reaching the target, changing antibiotic targets, that is the antibiotic target is modified by either genetic mutation or post-translational change and the inactivation of antibiotics by enzymes, where antibiotics can lose their effectiveness through hydrolysis or modification (**Kyrikidis et al., 2021).**

2.3. Methicillin- Resistant staphylococcus aureus

Staphylococci are Gram-positive bacteria that are members of the Staphylococcaceae family. Hospitals are the usual settings for the isolation of coagulation-negative staphylococci, which are frequently found in *Staphylococcus epidermidis* and *Staphylococcus hemolyticus*. Coagulase-positive staphylococci make up the second category of staphylococci. Of these, Staphylococcus aureus is the most prevalent and dangerous species, causing nosocomial and community infections.

Seventy to ninety percent of cases of *Staphylococcus aureus* secrete penicillinase, which leads to resistance to penicillin G, penicillin A (ampicillin, amoxicillin, etc.), ureidopenicillin (piperacillin), and carboxypenicillins (ticarcillin) **(Bekhti & Belhadi, 2019a).**

The mecA gene encodes a novel penicillin-binding protein PLP2a, which renders Staphylococci resistant to all beta-lactam antibiotics and methicillin (oxacillin) **(Belhadi & Bekhti, 2019b)**.

Beta-lactam antibiotics similar to penicillin do not work on MRSA. Still effective against MRSA are glycopeptides (like vancomycin and teicoplanin), linezolid, tigecycline, daptomycin, and even some new beta-lactams like ceftaroline and ceftobiprole. Conversely, MRSA has proven to be remarkably versatile, emerging and proliferating in a range of epidemiological settings over time including hospitals, the community, and, more recently, animals **(Belhadi & Bekhti, 2019c)**

The development of a vancomycin-resistant strain is a result of horizontal transfer between environmental enterococci strains that already possess innate vancomycin resistance and Staphylococcus acquiring a resistance plasmid **(Belhadi & Bekhti, 2019d**)

2.4. Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram-negative bacillus. It is distinguished by its high nutritional flexibility, which allows it to adapt to harsh environments **(Botelho *et al*., 2019a).**

It is a widespread environmental pathogen that can cause a wide range of acute and chronic nosocomial infections, including severe respiratory infections in individuals with weakened host defenses **(Jurado-Martín *et al*., 2021; Moradali *et al*., 2017).**

These strains are characterized by their high level of innate resistance to a broad range of antibiotics, such as penicillins G, A, and M, first and second generation cephalosporins and some third generation cephalosporins, cotrimoxazole, kanamycin, macrolides, cyclins, chloramphenicol, first generation quinolones, and rifampicin (**Botelho et al., 2019b**).

The overexpression of efflux pumps, a decrease in the permeability of the outer membrane, and the acquisition or mutation of resistance genes that encode for proteins that regulate the passive diffusion of antibiotics over the outer membrane are the three primary mechanisms of resistance in this bacterium (**Henrichfreise et al., 2007; Langendonk et al., 2021**). In addition, *P. aeruginosa* can gain resistance by overexpressing AmpC β-lactamases due to gene mutations (**Berrazeg et al., 2015).**

To treat MDR P. aeruginosa, colistin is used in conjunction with anti-pseudomonas agents such as imipenem, piperacillin, aztreonam, ceftazidime, or ciprofloxacin (**Pungcharoenkijkul et al., 2020).** Fosfomycin, combined with aminoglycosides, cephalosporins, and penicillins, has effectively cured drug resistance in P. aeruginosa **(Ontong *et al.*, 2021; Pachori *et al.*, 2019).**

2.5. Enterococci

Enterococci are Gram-positive, facultative anaerobes that are common gastrointestinal commensals that may endure in a variety of harsh and stressful conditions **(García-Solache & Rice, 2019).**

E. faecalis and *E. faucium* are the causes of human enterococcal infections. Despite *E. faecium's* greater resistance to several antimicrobial drugs and its potential to cause significant morbidity and death in immunocompromised hosts, *E. faecalis* is the most harmful species **(Shiadeh *et al.*, 2019).**

E. faecium is considered multi-drug resistant bacterium because it is resistant to aminoglycosides such as tobramycin, kanamycin, and gentamicin and can produce AMEs such as aminoglycoside nucleotidyltransferases (ANTs), aminoglycoside acetyltransferases (AACs), and aminoglycoside phosphotransferases (APHs**) (Kim *et al.*, 2021).**

In addition, mutations in the *rpsL* gene, which encodes the ribosomal protein S12, can cause significant resistance to streptomycin **(Jubeh *et al.*, 2020).** Also, resistance to fluoroquinolones in *E. faecium* is often caused by mutations in the gyrA and parC genes,

which encode subunits A of DNA gyrase and topoisomerase IV, or the efflux transporter NorA, which removes these medicines **(Jubeh *et al*., 2020**).

3. Antibiotic resistant bacteria present in Hospital Wastewater in Algeria

In the guide for discovery, research and development of new antibiotics for drug resistant bacterial infections by World Health Organisation (**WHO: 2017**), the 10-year trend of prevalence of resistance for each multi drug resistant bacterium in Algeria is shown as in **Table N° 2**

The most prevalent resistant bacteria to different antibiotics in Algeria include Acinetobacter baumannii, Campylobacter spp, Enterobacter spp, Enterococcus faecium, E.Coli, Klebsiella spp, Staphylococcus aureus and Streptococcus pneumoniae **(WHO, 2017).**

Table N° 2: Antibiotic resistant bacteria and their prevalence of resistance in Algeria

Antibiotic-resistant bacterium	Prevalance of resistance in Algeria (10-year trend)	Reference
Acinetobacter baumannii, carbapenem-resistant (CR)	>50 %	WHO, 2017
Campylobacter spp., fluoroquinolone-resistant (FQR)	31-50%	
Enterobacter spp., third generation cephalosporin-resistant (3GCR)	31-50%	
Enterobacter spp., carbapenem-resistant (CR)	<5%	
Enterococcus faecium, vancomycin-resistant (VR)	5-15%	

Escherichia coli, third generation cephalosporin-resistant (3GCR)	16-30%	WHO, 2017
Escherichia coli, carbapenem-resistant (CR)	<5%	
Haemophilus influenzae, ampicillin-resistant (AmpR)	5-15%	
Helicobacter pylori, clarithromycin-resistant (ClaR)	<5%	
Klebsiella spp., third generation cephalosporin-resistant (3GCR)	16-30 %	
Klebsiella spp., carbapenem-resistant (CR)	<5%	
Salmonella Typhi, fluoroquinolone-resistant (FQR)	5-15%	
Staphylococcus aureus, methicillin-resistant (MR)	31-50%	
Streptococcus pneumoniae, penicillin-non-susceptible (PNS)	31-50%	

4. Antimicrobial resistance

4.1. Main Antimicrobial Resistance Mechanisms

There is not a single mechanism responsible for the quick spread of AMR among bacteria, there are a number of mechanisms responsible for the spread of resistance. AMR frequently comes from intricate procedures. For that reason, antibiotics are divided into different groups according to their mechanism of action.

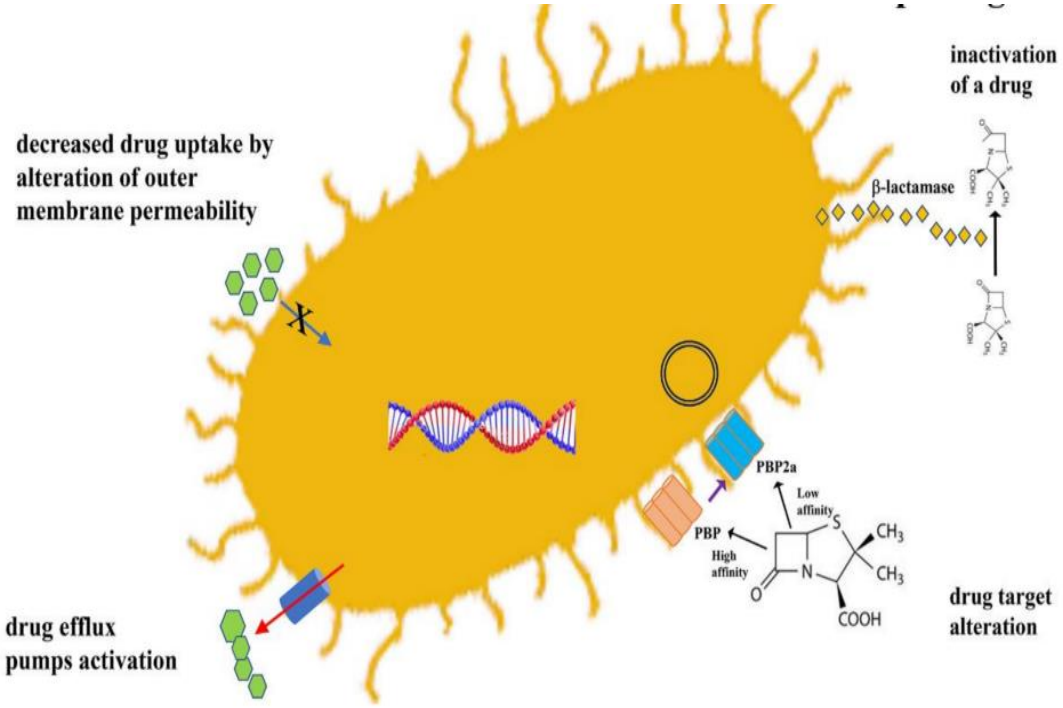
Table N° 3 focuses on antibiotics that are closely linked to antibiotic resistance although there are many classes of antibiotics. It summarises the primary antibiotic groups' modes of action and resistance.

Drug inactivation, drug target modification, reduced drug uptake, and drug efflux pump activation are the primary mechanisms of resistance (**Benkő *et al*., 2020; Reygaert, 2018**). The **Figure N° 1** shows these primary mechanisms of resistance.

Table N° 3: Antimicrobial groups, their mode of action and resistance mechanisms

Antimicrobial Groups	Mechanism of Action	Resistance Mechanism	Reference
β-Lactams **Penicillins** Cephalosporins Carbapenems	Inhibits cell wall production	Beta-lactamase production Penicillinase Cephalosporinase Carbapenemase	**(Mancuso *et al.*, 2021)**
β-Lactamase inhibitors	Block beta-lactamase enzyme activity.	Extended-spectrum beta-lactamase (ESBL)	
Aminoglycosides, Chloramphenicol Macrolides, Tetracyclines	Binding to bacterial 30S or 50S proteins can inhibit ribosome assembly and protein production.	Multiple factors, including enzymatic modification, target site modification, and efflux pumps.	
Fluoroquinolone	Inhibit DNA replication	Multiple factors, including target-site gene alterations, efflux pumps, and modifying enzymes.	
Sulfonamides and trimethoprim	Inhibit folic acid metabolism	Resistance genes transmit horizontally by transposons and plasmids, resulting in drug-insensitive versions of target enzymes.	

Figure N° 1: Primary Antimicrobial Resistance mechanisms in ESKAPE bacteria **(Mancuso *et al.*, 2021)**



4.2. Spread of Antimicrobial resistance

Since it has long been shown that AMR develops spontaneously over time through several pathways, the description of AMR as a process brought on by antibiotic misuse is inadequate **(Cepas & Soto, 2020; Mir Saleem et al., 2019)**. To put it another way, overuse of antibiotics in both people and animals speeds up this natural process, which encourages the spread of AMR **(Iramiot *et al.*, 2020; Malik & Bhattacharyya, 2019).**

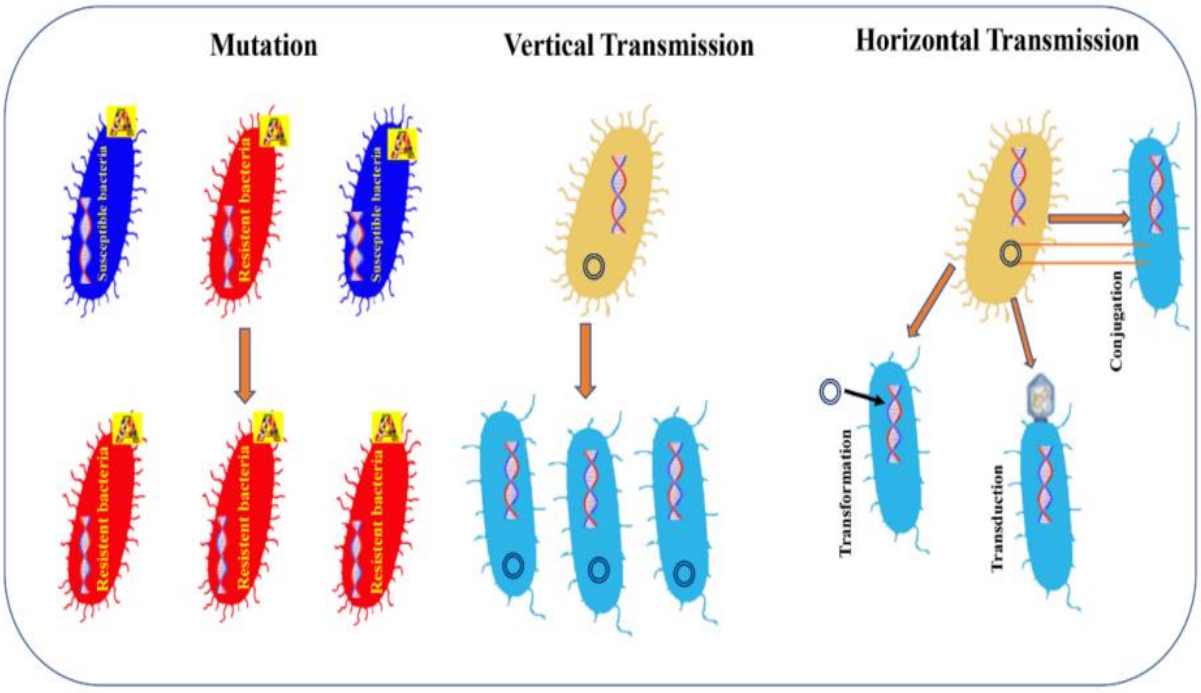
Resistance may be divided into two categories: acquired resistance and natural resistance. Natural resistance is further divided into intrinsic and induced resistance **(Reygaert, 2018).** Examples of intrinsic resistance include ampicillin and vancomycin resistance in *Escherichia coli*, as well as resistance to first- and second-generation cephalosporins in *Pseudomonas aeruginosa*. Intrinsic resistance occurs when bacterial species are naturally resistant to specific classes of antibiotics and is clearly independent of prior antibiotic exposure **(Sandner-Miranda *et al.*, 2018).**

Natural resistance in bacteria can be caused by activation of genes due to exposure to antibiotics **(Ben *et al*., 2019**).

Two different pathways can result in acquired resistance,the first is by a mutation that happens in the cell's DNA during replication or DNA transfer, wherein mutant strains can pass the mutation on to their children by a vertical transfer (**Friedrich, 2019; Reygaert, 2018).** The second mechanism via which bacteria Resistance is achieved is by conjugation, transposition, and transformation, that is horizontal gene transfer (**Sun *et al*., 2019).**

The **Figure N° 2** shows how the spread of antibiotic resistance is achieved . It can be either natural or acquired resistance by horizontal or vertical gene transfer.

Figure N° 2: Antibiotic-resistance spread



CHAPTER 2: HOSPITAL WASTEWATER

1. Definition

Wastewater is any kind of water whose quality has deteriorated due to human activity, such as that from domestic sources, agriculture, human excretion, pharmaceuticals, and healthcare facilities **(Buelow *et al*., 2018).**

Hospital effluents are an example of human-caused pollution in action. Hospital wastewater (HWW) is a complex mix of chemicals and biological constituents that are regularly released. This mixture includes active principles from pharmaceutical drugs, chemicals, disinfectants, detergents, radioactive markers, iodinated contrast media, nutrients, and bacteria with antimicrobial resistance genes excreted during laboratory diagnostics and research **(Verlicchi *et al*. 2010).**

2. Characteristics of Hospital Wastewater

2.1. Microbiological composition

Hospital wastewater, rich in organic and inorganic debris, creates an ideal environment for the growth and spread of microorganisms like viruses, fungi, and bacteria.

Infectious viruses, including SARS and MERS, spread through water and can cause severe illness. These viruses can cause epidemics or pandemics, posing a risk to the entire community. The SARS-CoV-2 pandemic is the most recent example of virus severity **(Revilla Pacheco et al., 2021).**

According to HWW microbiology research by **Eyekele *et al* (2009**), the research found a high concentration of pathogens, with Bacillus bacteria accounting for 80%-90% and Staphylococcus and Streptococcus ranging from 5% to 10%.

Numerous studies have found high levels of coliform and other bacteria in HWW in various countries, including Escherichia coliform (*E. coli*), total coliform, thermotolerant coliform, Streptococcus, Mycobacterium, and *Pseudomonas aeruginosa* **(Khan *et al*., 2020; Majumder *et al*., 2021a).**

HWW contains a diverse range of bacteria, including anaerobic bacteria like Bifidobacteriales, Clostridales, and Bacteroidales, which are believed to originate from the human gut.

Even though hospital effluents have high concentrations of bacteria like *E. Coli* and *total coliform*, these microorganisms should not be viewed as innocuous markers of fecal contaminations, but rather as pathogens that spread antibiotic resistance because of their exposure to high drug and antibiotic concentrations.

Acinetobacter, Enterococcus, and Pseudomonas species are among the bacteria with drug-resistance properties found in wastewater from hospitals and laboratories. The most prevalent pathogenic Gram-positive bacterium with a high level of multidrug resistance (MDR with drug resistance) is *Staphylococcus aureus*.

2.2. Emerging contaminants

High concentrations of emerging contaminants (ECs) in various water matrices have been caused by excessive medication use, endangering both humans and aquatic life.

Due to their extensive use in medical facilities, pharmaceutically active compounds (PhACs) have been detected in the majority of water matrices among the different ECs **(Majumder *et al.*, 2021a).** More than 300 PhACs have been found in various HWWs, along with their metabolites and trans products (**Khan *et al.*, 2021).**

Hospital effluents contain a range of chemical contaminants, including disinfectants and surfactants, that can be harmful to biotic components in addition to PhACs and contrast media. Most of the ECs identified in hospital wastewater are present at quantities beyond anticipated criteria for no discernible effects.

It has been noted that antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs)—like the antiepileptic medication carbamazepine—are more frequently found in HWW.

Additionally, studies have found X-ray contrast media, like iopromide and iomeprol, in hospital effluents from different countries **(Parada et al. 2021)**.

2.3. Physico-chemical characteristics

Measurements of other specific macropollutants, such as total and free chlorine, detergents, disinfectants, oil and grease, total nitrogen, heavy metals, and microbiological indicators (total coliform, faecal coliform, or Escherichia coli), as well as toxicity, are necessary when a wastewater sample is considered to be a particular effluent, such as that from a hospital (**Carraro *et al*., 2016a**).

The Environmental, Health, and Safety (EHS) Guidelines state an overview of the fundamental physio-chemical indicators that are typically required by law in order to determine the quality of a generic wastewater sample: pH and temperature (usually about 40 C), Total Suspended Solid (TSS), Chemical Oxygen Demand (COD), BOD, also known as Biochemical Oxygen Demand (BOD5 **(Carraro et al., 2016b).**

BOD is classified by the US Clean Water Act as a conventional pollutant. The usual upper limits are 300 mg/l for disposal into sewer systems and 10 mg/l for direct environmental disposal **(Biochemical Oxygen Demand (BOD) in Wastewater Treatment | AOSTS, 2018).**

In order to determine whether there is a correlation between the wastewater quality parameters, many studies compared the municipal wastewater (MWW) of various nations with the raw hospital wastewater (HWW). It was found that the parameters, such as BOD, COD, and TSS, in the hospital effluents were 2-3 times higher than MWW (**Verlicchi *et al*., 2010b).**

2.4. Heavy metals

HWW contains a variety of heavy metals as well. They among them have a variety of harmful consequences that vary depending on the metal. They could cause chronic illnesses like lead poisoning, mercury toxicity, or they could be immediate deadly poisons like asernic and cr6+ chromium IV **(Hassan Omer, 2020).**

Mercury (Hg) is commonly used in diagnostics, diuretics, and disinfectants, resulting in continuous detection. Platinum has been found in hospital effluents from oncology patients treated with cis- and carbo-platinum. **Verlicchi *et al.* (2010b)** and **Khan *et al*. (2020**) found that approximately 98% of unmetabolized metals were discharged in HWW within 24 hours of usage.

3. Hospital Wastewater contaminants into the aquatic environment

Unmetabolized fractions of ECs and other pollutants have an impact on the total organic components and solids content of HWW. Hospital effluents are typically dumped untreated into freshwater streams in low- and middle-income countries, leading to elevated levels of organic debris, pathogens, and ECs in aquatic environments **(Parida *et al*., 2022)**.

When HWW flows into home sewers and is treated alongside MWW at WWTPs, co-treatment is employed in a number of nations, including Algeria, Iran, Japan, Egypt, Australia, South Africa, India, and Thailand **(Parida *et al.,* 2022)**. According to **Patel et al. (2019)**, most municipal wastewater treatment facilities are not built to handle such complex organic pollutants. Municipal WWTPs can only remove a portion of the ECs as a result. As a result, the primary source of these ECs' release into different water matrices is usually municipal WWTPs (**Patel *et al*., 2019**).

Moreover, these municipal WWTPs' treated sewage is widely applied as fertilizer in farming. Consequently, some ECs might seep into the groundwater from the soil **(Khan et al., 2021)**. However, the majority of high-income countries have WWTPs on their premises where HWW is pre-treated before being dumped into municipal sewers **(Verlicchi *et al*, 2018).**

This treatment targets HWW specific pollutants, so it may remove organics, pathogens, and ECs at much higher levels. However, the operation and maintenance of on-site HWW treatment is expensive and energy-intensive **(Parida *et al*., 2022**).

4. Hazards associated with HWW discharge into the aquatic environment

The most common contaminants found in HWWs include antibiotics, analgesics and anti-inflammatories, psychiatric medications, b-blockers, anesthetics, disinfectants, chemicals from laboratory operations, and X-ray contrast media (**WHO, 2013).**

These compounds are excreted primarily as unmetabolized substances, metabolites, or conjugated with inactivating substances in the urine and less so in the feces. These substances behave differently in the WWTP due to differences in their solubility, volatility, molecular weight, adsorbility and biodegradability. If they are not neutralized in the wastewater treatment process, they are released in surface waters that have received treatment (**Verlicchi *et al*., 2010).**

5. Health and environmental risks

The highly infectious illnesses that the HWW spreads quickly across society, hospitals, healthcare facilities, and the surrounding environment represent a major threat to human health **(Akın, 2016).**

For many pathogenic microorganisms, such as bacteria, viruses, fungi, and parasites, the HWW can serve as an excellent growth medium. Hospital wastewaters contain a variety of resistant bacteria as well as antibiotic residues, which may prevent susceptible bacteria from growing and thereby increase the amount of resistant bacteria in the receiving water. When resistant bacteria are released into the environment, they can either serve as reservoirs for antibiotic-resistant genes (ARGs) that could endanger public health or as vectors carrying transmissible genes **(Asfaw et al., 2017).**

A variety of harmful microorganisms, such as bacteria, fungi, yeasts, algae, viruses, protozoa, parasites, and bacteriophages, make up HWW. Hospital effluent is often treated and released with household wastewaters without any prior treatment (**Chonova *et al.*, 2016**). If left untreated, the pathogens in the receiving water can persist in soil or water for a considerable amount of time before entering the food chain and posing a danger to human health and infectious disease (**Gurel *et al.*, 2007**).

Heavy metal ions are also present in hospital wastewater, and the majority of these contaminants have the ability to effectively block biological activity in treatment systems. In reality, heavy metals represent a concern to the environment and human health since they are not biologically degradable pollutants and are transportable pollution sources (**Emmanuel *et al.*, 2009; Ortega *et al.*, 2008**).

Materials and Methods

Materials and Methods

1. Presentation of the site: Centre Hospitalo-Universitaire (CHU) de Tlemcen

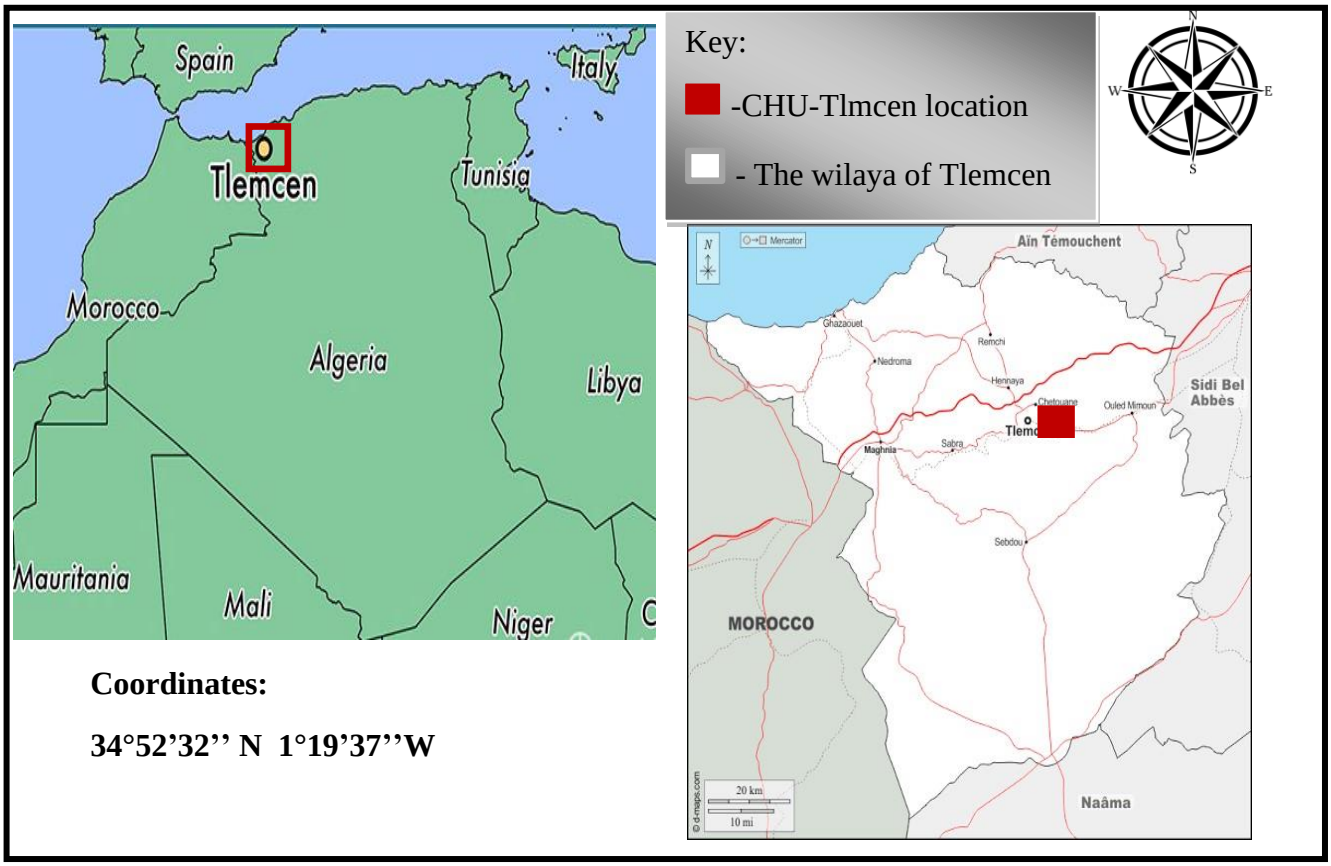
The Dr. Tidjani Damerdji University Hospital is a suburban structure situated in the city of Tlemcen which is in the northwest of Algeria, close to the Moroccan border (**Figure N° 3**). The facility takes up thirteen hectares of land. It serves a population of 1.5 million people and has 646 beds with 44 specialized departments and laboratories (**Centre Hospitalo-Universitaire Dr Tidjani Damerdji De Tlemcen, n.d.).**

This hospital was chosen because of its importance as a significant healthcare centre in the region, as well as its contribution to hospital wastewater generation.

2. Hospital Wastewater sample collection

Hospital wastewater samples were taken from the University Hospital Center-Tlemcen on 27 March 2024. Sampling was done at the Neurosurgery, Pneumo-phthisiology, and Haematology units of the hospital prior to discharge into the wastewater collection pipe. Chloride concentrations in the samples were determined using a titration technique with silver nitrate solution (AgNO3), which revealed that all samples had a chloride value of 0 g/mL.

For each sampling location, samples were collected in a sterile 0.2 Litre glass reagent bottle **(Figure N° 4)**, and they were then transported on ice in a cooler box to the laboratory for microbiological analysis.



Coordinates:

34°52’32’’ N 1°19’37’’W

Figure N° 3: Geographic location of the Dr. Tidjani Damerdji University Center Hospital-Tlemcen.

Figure N° 4: The 3 reagent bottles with samples from 3 different units of the hospital.

3. Isolation and identification of bacteria

3.1. Culture methods: Isolation of ESKAPE bacteria

Bacterial isolation was performed using selective and differential culture media to encourage the growth of target bacteria while inhibiting the growth of other microorganisms. The targeted bacteria were of the ESKAPE group of bacteria. For each sample, the following agar plates were inoculated:

- MacConkey Agar plates
- Cetrimide Agar plates.
- Nutritive Agar plates
- Mannitol Salt Agar

Approximately 100 microliters of each sample were inoculated onto the surface of each agar plate using a sterile spreader. Then the inoculated plates were incubated aerobically at 37°C for 24 hours except mannitol salt agar plates which were incubated for about 48 hours to allow for bacterial growth. After incubation, each of the plates was examined for growth, and subcultures were done and incubated at 37°C for 24 hours (including for 3 of the plates that had mixed bacterial growth) to obtain pure cultures.

3.1.1. Revival of Stored Bacterial Subcultures

Due to an extended storage period, the bacterial subcultures required revival before proceeding with further experiments. The stored subcultures were carefully inoculated into 10 ml of nutrient broth. The inoculated broth was incubated at 37°C for 24 hours after vortexing at 150 rpm to ensure optimal aeration and revival of the bacterial cells. Following the revival, 100 microliters of the broth culture were spread onto the initial different selective and differential agar plates (MacConkey Agar, Cetrimide Agar, Mannitol Salt Agar) to confirm the identity and purity of the bacterial isolates, then the plates were incubated at 37°C for 24 hours (Mannitol Salt Agar plates were incubated for about 48 hours). After incubation, isolated colonies were picked and used for antimicrobial susceptibility testing and other subsequent analyses.

3.2. Identification of bacteria

Pure bacterial colonies were examined visually for morphological properties such as form, size, colour,texture and any other distinctive features. These insights helped to identify the bacterial isolates. The isolates were to be confirmed as ESKAPE bacteria. Based on these visual observations, bacterial isolates were tentatively identified to the genus level.

4. Antimicrobial susceptibility testing

4.1 The method

The antimicrobial susceptibility of isolated bacteria was determined using the Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines **(M100 ED34 | Performance Standards for Antimicrobial Susceptibility Testing, 2021.)**. Used the disk diffusion assay on Mueller-Hinton agar plates. The bacterial culture was diluted in a tube of sterile saline to obtain turbidity equivalent to 0.5 McFarland standard, ensuring the bacterial concentration was standardized. A sterile cotton swab was dipped into the suspension and swabbed evenly across the entire surface of a Mueller Hinton agar plate in 3 different dimensions. This was done to obtain a semi-confluent growth following incubation. Antibiotic-impregnated paper disks were placed on the agar surfaces.The antimicrobial disks used were CIP (5µg), STX (25 µg), TOB (10 µg), CTX (30 µg), DA (2 µg), OFX (5 µg), and NA (30 µg) **(Table 4**). The plates were incubated at 37°C for 18 hours after which the zones of inhibition around the antimicrobial disks were measured and interpreted according to the breakpoints of the CLSI.

Table 4: Antibiotics used

Antibiotic	Class	Charge of the disk
Ciprofloxacin (CIP)	Fluroquinolone	5 µg
Trimethoprim-sulfamethoxazole (STX)	Dihydrofolate reductase inhibitor + sulfonamide	25 µg
Tobramycin (TOB)	Aminogycoside	10 µg
Clindamycin (DA)	Lincosamide	2 µg
Ofloxacin (OFX)	Fluroquinolones	5 µg
Nalidixic acid (NA)	Quinolone	30 µg

4.2 The interpretation

The CLSI guidelines were used for the interpretation of the bacterial isolates being resistant, susceptible, or intermediate **(M100 ED34 | Performance Standards for Antimicrobial Susceptibility Testing, 2021)**. A bacterial isolate was regarded as multidrug-resistant when it showed resistance to 2 or more classes of antibiotics.

Results and Discussions

1. Isolated bacteria in the hospital wastewater

1.1. Bacterial Revival and Growth in nutrient broth

The stored bacterial subcultures were successfully revived in nutrient broth. After 24 hours of incubation at 37°C, the bacteria demonstrated robust growth, indicating successful revival **(Figure 5).**

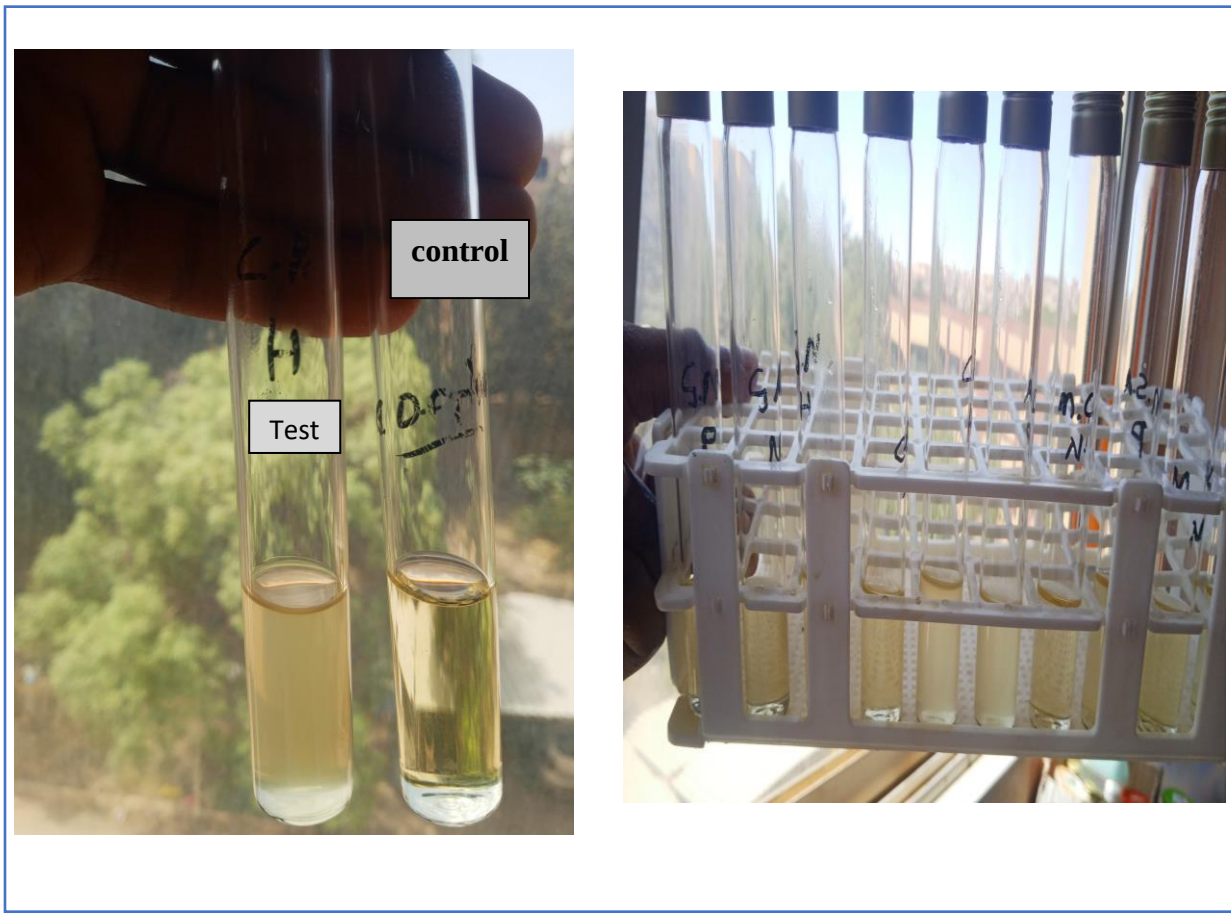


Figure 5: Growth of revived bacteria in nutrient broth.

1.2. Macroscopic characterization of Isolated bacteria

After sub-culturing the isolates to create pure cultures, the pure bacterial colonies were visually evaluated for morphological traits in order to provisionally identify the bacterial isolates at the genus level.

The isolates revealed various appearances after incubation. The identification method depended primarily on visual observations of colony morphology, including size, color, texture, and other distinguishing characteristics.

1.2.1. *Enterobacteriaceae* Isolates - MacConkey Agar plates

The isolates showed some appearances after about 24 hours of incubation at 37°C on Mac Conkey Agar plates. The different macroscopic aspects are summarized in **Table 5** and presented in **figure 6.**

Table 5 : Description of the macroscopic aspects of the isolates selected on Mac Conkey agar from 3 units of the hospital.

The isolate	Macroscopic aspects on Mac Conkey Agar
Mac-P	The colonies are large , round, and smooth-edged. They have a notably mucoid texture, shiny and glistening. This mucoid appearance is so visible. The colonies are opaque and abundant. No distinctive odor was noticed.
Mac- H	Small , round, mucoid and pink colonies. Transparent and no distinct odor.
Mac- N	Few colonies were observed. 2 distinct different morphology of colonies were observed. All the colonies look small. The other, very round with smooth edges, pinkish, mucoid. The other looks pale or a bit colorless, and translucent

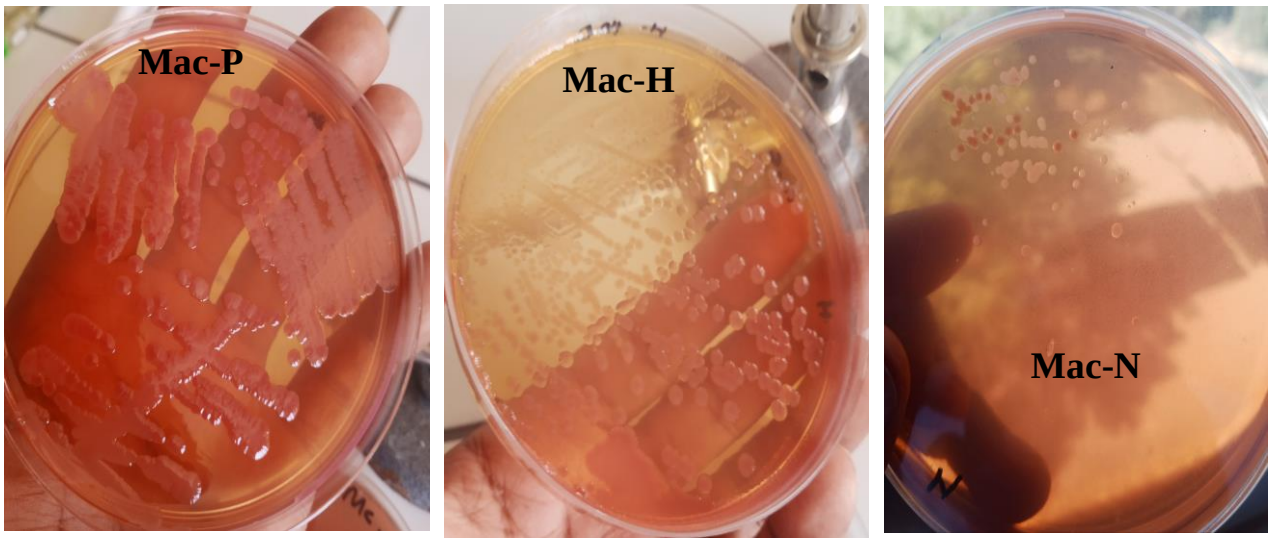


Figure 6: Photos of the macroscopic aspect of the Enterobacteriaceae isolates after 24 hours of incubation.

Mac-P: *Enterobacteriaceae* isolates from the Pneumo-phthisiology unit

Mac-H*: Enterobacteriaceae* isolates from the Haematology unit

Mac-N: *Enterobacteriaceae* isolates from the Neurosurgery unit

1.2.2. *Pseudomonas spp.* Isolates- Cetrimide Agar plates

On Cetrimide Agar plates (CA), After 24 hours of incubation at 37°C, some appearances were observed on 2 plates from 2 different hospital units.

The macroscopic aspects of the Isolates are summarized in **Table 6** and presented in **Figure 7.**

Figure 7: Photos of the macroscopic aspect of the Pseudomonas spp. isolates after 24 hours of incubation.

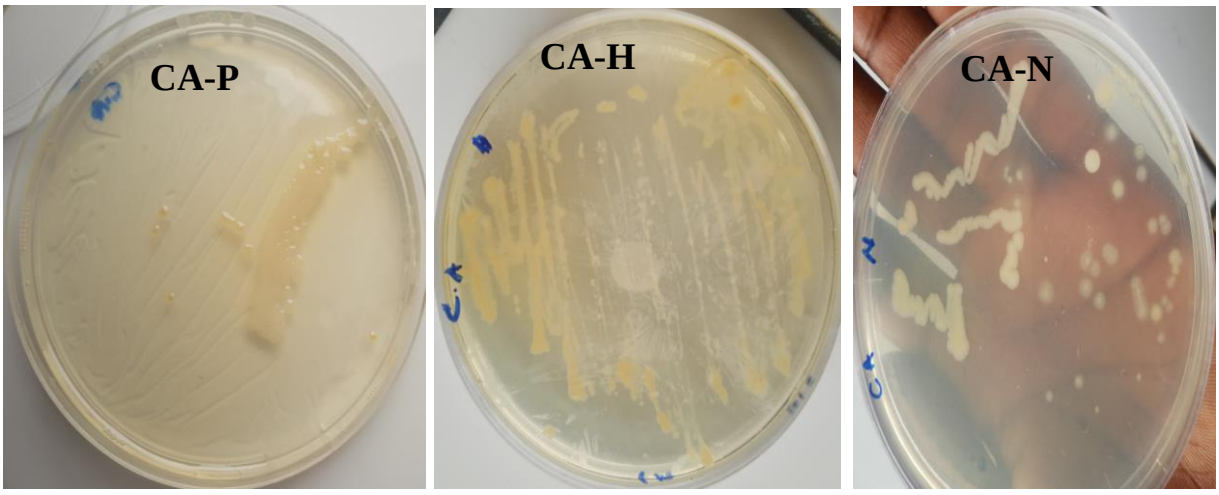


Table 6: Description of the macroscopic aspects of the isolates selected on Cetrimide Agar from 3 units of the hospital

The isolate	The macroscopic aspects on Cetrimide agar plates
CA-P	The colonies were cream, smooth, round, and have a moist appearance. No significant coloration on the plate
CA-H	The colonies were yellowish to cream, appeared to be dry, rough, and wringed
CA-N	Colonies were white to off-white, smooth, and had a moist to slightly mucoid appearance. No pigment coloration

CA-P: *Pseudomonas spp*. isolates from the Pneumo-phthisiology unit

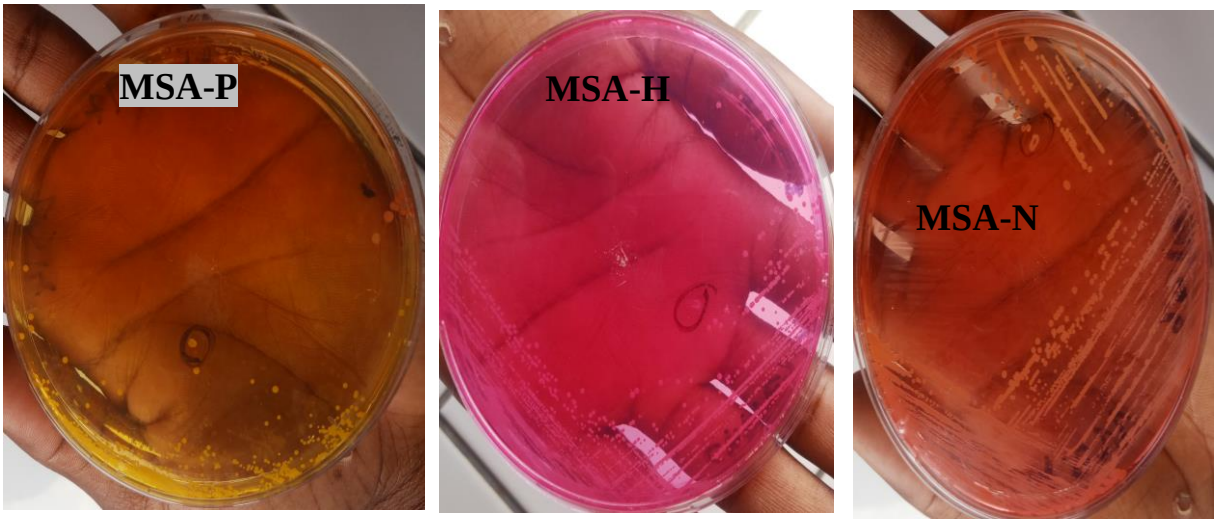
CA-H*: Pseudomonas spp.* isolates from the Haematology unit

CA-N: *Pseudomonas spp*. isolates from the Neurosurgery unit

1.2.3. *Staphyloccocus spp.* Isolates – Chapman Agar plates

The isolates showed some appearances after about 24-48 hours of incubation at 37°C on Mannitol Salt Agar plates. The different macroscopic aspects are summarized in **Table 7** and presented in **Figure 8.**

Figure 8: Photos of the macroscopic aspect of the *staphylococcus spp.* isolates after 24 hours of incubation.



MSA-P: *Staphyloccocus spp.* isolates from the Pneumo-phthisiology unit

MSA-H*: Staphyloccocus spp.* isolates from the Haematology unit

MSA-N: *Staphyloccocus spp.* isolates from the Neurosurgery unit

Table 7: Description of the macroscopic aspects of the isolates selected on Mannitol Salt Agar from 3 units of the hospital

THE ISOLATE	THE MACROSCOPIC ASPECTS ON MANNITOL SALT AGAR PLATES
MSA-P	The colonies are pale in color. They are small and medium in size, round, smooth, and have a convex shape. They are slightly glistening or shiny in appearance. The agar around the colonies remained pink, indicating that mannitol fermentation did not occur.
MSA-H	The colonies were yellow, shiny, and had a convex shape. The Mannitol salt Agar changed colour from pink to yellow (to indicate acid production due to mannitol fermentation(pointing to *Staphylococcus aureus)*
MSA-N	The colonies were small to medium size and pale in color. They were smooth and convex in shape and glistening. The agar slightly changed the color

1.2.4. Growth of Bacteria on Nutritive Agar plates

Nutritive Agar was utilized to provide a non-selective medium that supports the growth of a broad spectrum of bacteria from hospital wastewater samples. This acted as a control and allowed for the assessment of the overall bacterial load and diversity present in the samples.

After incubating the Nutritive Agar plates for 24 hours at 37°C, a diverse array of bacterial colonies was observed in all the plates from three different units of the hospital.

The colonies varied significantly in their appearance, which was indicative of the heterogeneity of the bacterial population. Key observations included the colonies' size, shape, texture, and color differences in all the plates (**Figure 9)**. Other colonies were large, round, smooth-edged with a mucoid texture, and others were small, round with different colors, some whitish, and pale, others translucent.

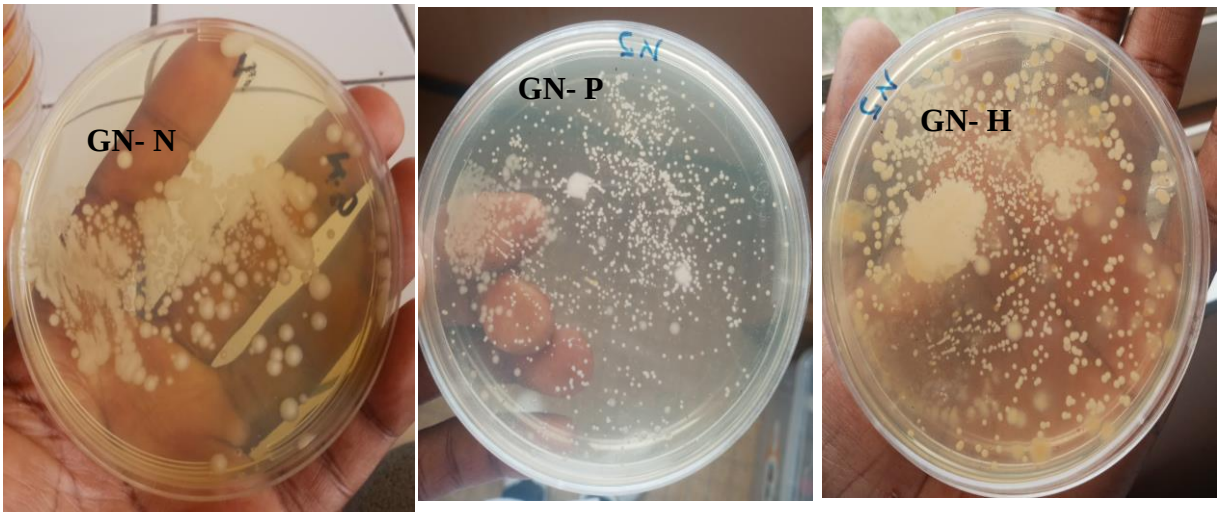


Figure 9: Photos of the macroscopic aspect of different bacteria on Nutritive Agar after 24 hours of incubation.

1.3. Discussion

This study was carried out with one of the aims of searching for the presence of different bacteria species present in the Hospital wastewater of Tlemcen Hospital. The bacterial organisms present in the hospital wastewater sampled from Neurosurgery, Pneumo-phthisiology, and Haematology units comprised of Gram-negative enteric bacteria, *staphylococcus spp*., *Pseudomonas spp*., and many other different bacterial species isolated on nutrient agar from all the units.

The variety of bacteria retrieved from the wastewater emerging from the three units may reflect the bacterial contamination managed at the units of the hospital during the study period, and it aligns well with many previous hospital wastewater evaluation studies. For instance , **Anssour *et al*.(2016),** who conducted their study in an Algerian hospital recovered different bacteria of the *Enterobacteriaceae* family such as *E. coli,* and *K. pneumoniae*, same family as retrieved in this study.

Similarly**, Daoud *et al*. (2018),** conducted their study as well in a Lebanese hospital where they recovered a wide range of bacteria which they found to be *E. coli, Enterobacter cloacae,* and *K. pneumoniae,* which all fall under the Enterobacteriaceae family of bacteria

Le *et al*.(2016) also identified *Acinetobacter junii, Comamonas testosteroni, Enterobacter spp*., and *Pseudomonas spp*. as bacteria present in hospital wastewater in a hospital Singapore. The identification of *Pseudomonas spp*. in the study of **Le et al.(2016)** aligns well with the findings of *Pseudomonas spp.* and *Enterobacteriaceae* bacteria in this study although our study did not recover other bacterial species found in the study of **Le et al (2016)**.

Similarly, **Moges *et al*.(2014)** identified 13 different bacterial species—*E. coli, Enterobacter spp., Pseudomonas spp., Citrobacter spp., K. pneumoniae, Klebsiella oxytoca, Klebsiella ozaenae, Shigella spp., S. aureus, Providencia spp., Edwardsiella spp., Serratia spp.,* and *Morganella spp*.—from the wastewater of Gondar University Hospital in Ethiopia. The families of all these bacteria overlapped with those isolated in our study.

The presence of several bacterial species in hospital wastewater as evidenced in this study has important consequences for public health and environmental management. Gram-negative enteric bacteria, such as *E. coli* and *K. pneumoniae,* are well-known pathogens that cause healthcare-associated infections (HAIs), putting human lives at risk **(Blot et al., 2022 ; Szabó**

et al., 2022). The presence of these bacteria in hospital wastewater highlights the significance of good infection control measures in healthcare institutions to prevent pathogen spread.

The presence of *Staphylococcus spp.* in the hospital wastewater of this study emphasizes the potential for environmental contamination and the necessity for stringent hygiene standards to reduce the risk of HAIs. *Staphylococcus spp.*, especially *S. aureus*, are opportunistic infections that frequently appear on human skin and mucous membranes **(Rossi et al., 2024)**. While some strains are harmless, others can cause serious infections such as surgical site infections and bloodstream infections **(Munro et al., 2024).**

Pseudomonas spp. can cause a wide range of infections, particularly in immunocompromised patients and those with indwelling medical devices **(Hernández-Jiménez *et al.*, 2022)**. The presence of *Pseudomonas spp*. in the hospital wastewater in this study is a public health concern.

The study advances the understanding of the microbial ecology of hospital wastewater by highlighting the presence of a variety of bacterial species, including Gram-negative enteric bacteria, Staphylococcus spp., and Pseudomonas spp. These findings highlight the need of strong infection control methods and environmental management strategies in reducing the risk of HAIs and contamination.

2. Antimicrobial susceptibility test of isolated bacteria

2.1. Study of the sensitivity of isolates to antibiotics

The sensitivity of the isolates was tested on Muller Hinton Agar using the Kirby-Bauer disk diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines. The following antibiotics used for the test: Ciprofloxacin (CIP, 5 µg), Cephalothin (KF, 30 µg), Trimethoprim-sulfamethoxazole (STX, 25 µg), Tobramycin (TOB, 10 µg), Cefotaxime (CTX, 30 µg), Clindamycin (DA, 2 µg), Fusidic acid (FA, 10 µg), Ofloxacin (OFX, 5 µg), Ceftriaxone (CRO, 30 µg), Nalidixic acid (NA, 30 µg).

2.1.1. The sensitivity of *Enterobacteriaceae* isolates to antibiotics

The antibiotic discs used on *Enterobacteriaceae* isolates were Ciprofloxacin (CIP, 5 µg), Trimethoprim-sulfamethoxazole (STX, 25 µg), Tobramycin (TOB, 10 µg), Ofloxacin (OFX, 5 µg), and Nalidixic acid (NA, 30 µg). This was according to the CLS1 standard guidelines (2021).

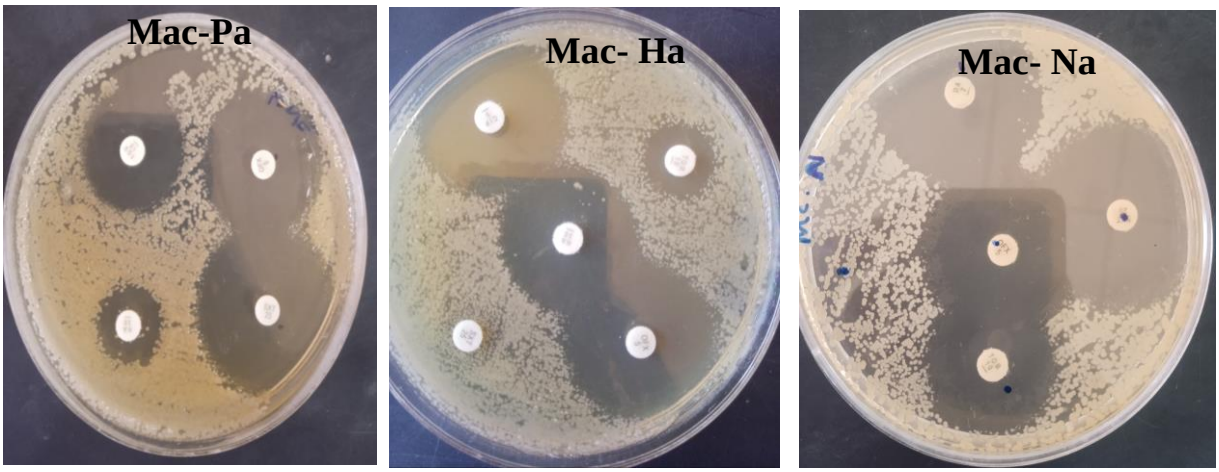
The results were obtained by measuring and comparing the diameters of the inhibition zones obtained to the standardized interpretation charts by the Clinical and Laboratory Standards Institute (2021). **Table 8** shows the description of the results and **figure 10** shows the antibiogram of the isolates.

By comparing the diameters of the inhibition zones obtained against the critical CLSI values, it turned out that the *Enterobacteriaceae* isolate from the Pneumo-phthisiology unit was susceptible to Trimethoprim-sulfamethoxazole, Tobramycin, and Ofloxacin, but resistant to Nalidixic acid.

In the Hematology unit, the *Enterobacteriaceae* isolate showed sensitivity to Ciprofloxacin, Ofloxacin, and Nalidixic acid. However, it was resistant to Trimethoprim-sulfamethoxazole and Tobramycin, classifying it **as multidrug-resistant (MDR)** since it is resistant to at least two antibiotics. This indicates the presence of MDR bacteria in this unit.

The isolate from the Neurosurgery unit displayed sensitivity to all tested antibiotics: Trimethoprim-sulfamethoxazole, Tobramycin, Ofloxacin, and Nalidixic acid.

Figure 10: Antibiogram of the *Enterobacteriaceae* isolates



Mac-Pa: *Enterobacteriaceae* isolate antibiogram from the Pneumo-phthisiology unit.

Mac-Ha: *Enterobacteriaceae* isolate antibiogram from the Haematology unit

Mac-Na: *Enterobacteriaceae* isolate antibiogram from the Neurosurgery unit

Table 8: The results of the antimicrobial disc susceptibility tests of the *Enterobacteriaceae* isolates.

Isolate	**Antibiotic Disc**	**Zone diameter(mm)**	**Comparison to CLSI values**	**Interpretive category**
Mac-Pa	CIP	-	-	-
	STX	33	≥ 16	Susceptible
	NA	11	≤ 13	Resistant
	TOB	22	≥15	Susceptible
	OFX	27	≥16	susceptible
Mac-Ha	CIP	30	≥ 26	Susceptible
	STX	0	≤ 13	Resistant
	NA	27	≥19	Susceptible
	TOB	12	≤ 12	Resistant
	OFX	31	≥16	Susceptible
Mac-Na	CIP	-	-	-
	STX	32	≥ 16	Susceptible
	NA	30	≥19	Susceptible
	TOB	21	≥15	Susceptible
	OFX	29	≥16	Susceptible

Mac-Pa: *Enterobacteriaceae* isolate antibiogram from the Pneumo-phthisiology unit.

Mac-Ha: *Enterobacteriaceae* isolate antibiogram from the Haematology unit

Mac-Na: *Enterobacteriaceae* isolate antibiogram from the Neurosurgery unit

2.1.2. The sensitivity of *Pseudomonas spp.* isolates to antibiotics

The antibiotics disc used for *pseudomonas spp* isolates were Ciprofloxacin (CIP, 5 µg), Tobramycin (TOB, 10 µg), and Ofloxacin (OFX, 5 µg), according to the CLS1 standard guidelines (2021).

The diameter measurements were measured and compared to the standard interpretation charts by the Clinical and Laboratory Standards Institute (2021) **(Table 9)** and the antibiogram isolates presented in **Figure 11.**

From the Pneumo-phthisiology unit, the *Pseudomona*s isolate exhibited susceptibility to Ofloxacin but exhibited strong resistance to two different antibiotics: Ciprofloxacin and Tobramycin. Notably, these two antibiotics belong to different classes, thereby classifying it as **MDR bacteria**.

From the Hematology unit, the *Pseudomonas* isolate showed susceptibility to Tobramycin and Ofloxacin, with an intermediate resistance to Ofloxacin. From the Neurosurgery unit, the *Pseudomonas* isolate exhibited sensitivity to Ofloxacin, intermediate resistance to Tobramycin, and a very strong resistance to Ciprofloxacin.

Figure 11: Antibiogram of the *Pseudomonas spp.* isolates

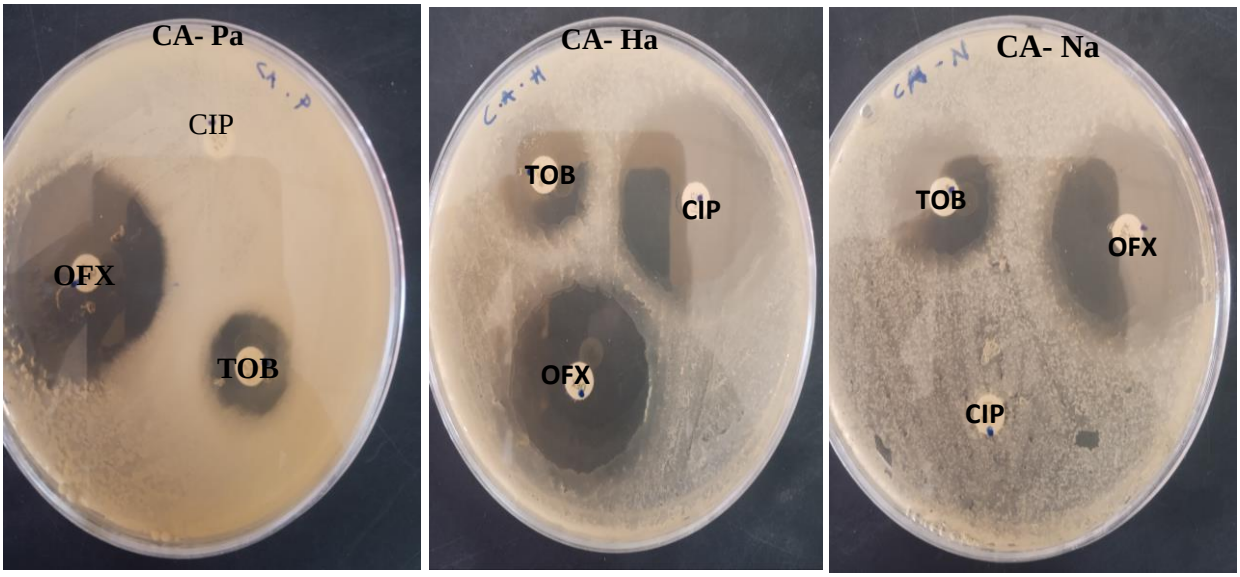


Table 9: The results of the antimicrobial disc susceptibility tests of *Pseudomonas spp.* Isolates

Isolate	Antibiotic Disc	Zone diameter(mm)	Comparison to CLSI values	Interpretive category
CA-Pa	CIP	0	≤18	Resistant
	TOB	12	≤ 12	Resistant
	OFX	31	≥16	Susceptible
CA-Ha	CIP	33	≥25	Susceptible
	TOB	13	13-14^	Intermediate
	OFX	21	≥ 16	Susceptible
CA-Na	CIP	0	≤18	Resistant
	TOB	13	13-14^	Intermediate
	OFX	27	≥ 16	Susceptible

CA-Pa: *Pseudomonas spp.* isolate antibiogram from the Pneumo-phthisiology unit.

CA-Ha: *Pseudomonas spp.* isolate antibiogram from the Haematology unit

CA-Na: *Pseudomonas spp.* isolate antibiogram from the Neurosurgery unit

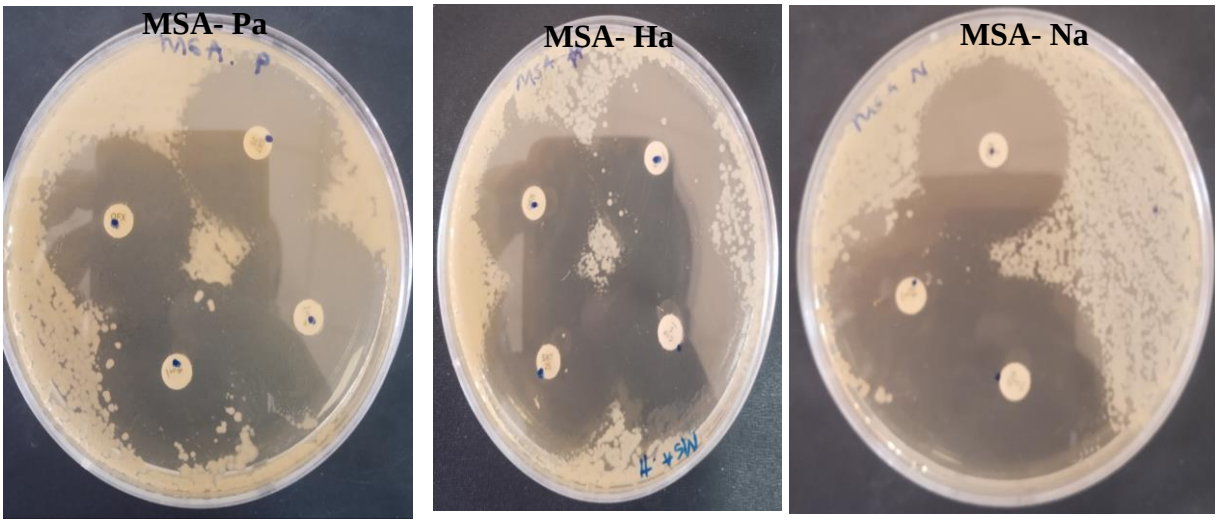
2.1.3. The sensitivity of *staphylococcus spp.* isolates to antibiotics

The antibiotics discs used for the antimicrobial susceptibility test were Ciprofloxacin (CIP, 5 µg), Trimethoprim-sulfamethoxazole (STX, 25 µg), Ofloxacin (OFX, 5 µg), and Clindamycin (DA, 2 µg) according to the CLS1 standard guidelines (2021). **Figure 12** shows the isolates of the *staphylococcus spp*. isolates and **Table 10** clearly shows the comparison of the measured diameter to the standard interpretation charts by the Clinical and Laboratory Standards Institute **(2021).**

Results and Discussions

By comparing the diameters of the inhibition zones obtained against the values from CLSI (2021), all the *staphylococcus spp*. isolates from all the 3 sampling units exhibited sensitivity to the antibiotics tested. They were all sensitive to Ciprofloxacin, Trimethoprim-sulfamethoxazole, Ofloxacin, and Clindamycin.

Figure 12: Antibiogram of the *staphylococcus spp*. isolates



MSA-Pa: *Staphloccocus spp.* isolate antibiogram from the Pneumo-phthisiology unit.

MSA-Ha: *staphyloccocus spp.* isolate antibiogram from the Haematology unit

MSA-Na: *Staphyloccocus spp.* isolate antibiogram from the Neurosurgery unit

Table 10: Shows the results of the antimicrobial disc susceptibility tests of *staphylococcus spp.* Isolates

Isolate	Antibiotic Disc	Zone diameter(mm)	Comparison to CLSI values	Interpretive category
MSA-Pa	CIP	27	≥21	Susceptible
	STX	28	≥ 16	Susceptible
	DA	22	≥21	Susceptible
	OFX	28	≥18	susceptible
MSA-Ha	CIP	33	≥21	Susceptible
	STX	31	≥ 16	Susceptible
	DA	30	≥21	Susceptible
	OFX	28	≥ 18	Susceptible
MSA-Na	CIP	37	≥21	Susceptible
	STX	-	-	-
	DA	22	≥ 21	Susceptible
	OFX	31	≥ 18	Susceptible

MSA-Pa: *Staphloccocus spp.* isolate antibiogram from the Pneumo-phthisiology unit.

MSA-Ha: *staphyloccocus spp.* isolate antibiogram from the Haematology unit

MSA-Na: *Staphyloccocus spp.* isolate antibiogram from the Neurosurgery unit

2.2. Discussion

This study additionally assessed the occurrence of multidrug resistance among hospital wastewater isolates collected. Our findings revealed the presence of MDR bacteria in two out of the three sampled units at Tlemcen Hospital. Notably, in the Hematology unit, isolates of *Enterobacteriaceae* demonstrated resistance to antibiotics from two distinct classes. Similarly, within the Pneumo-phthisiology unit, *Pseudomonas spp*. exhibited strong resistance to multiple antibiotics.

The significant antibiotic resistance rates, particularly multidrug resistance, align with the high rates reported in many recent studies among clinical isolates. Several studies indicated the prevalence of multi-drug resistance bacteria in clinical settings **(Donkor *et al.*, 2018; Anafo *et al.*,2021)**. For instance, **Merradi *et al.* (2023),** who conducted a study at the University Hospital Center of Batna in Algeria reported high resistance of the hospital-isolated bacteria with the highest resistance noted in Tobramycin and penicillin antibiotics. Similarly, the occurrence of enterobacterial-resistant strands is consistent with the study carried out at Tlemcen Hospital by **Tani et al. (2013)**, where they identified resistance traits in the isolated *Enterobacteriaceae* from the surgery unit of the hospital with a high level of resistance to ciprofloxacin.

In this study, we observed a strong resistance rate of *Pseudomonas spp*. to ciprofloxacin, actually from the Pneumo-phthisiology and Neurosurgery units. This is consistent with various previous studies that reported the resistance *of Pseudomonas spp*. to this antibiotic from hospital wastewater isolates. **Roulová *et al.* (2022)** isolated *Pseudomonas aeruginosa* from hospital wastewater in the Czech Republic and reported that the highest resistance rates were observed for ciprofloxacin, followed by gentamicin and meropenem. Of the 3 antibiotics reported to be with the highest resistance rate by the study of **Roulová *et al.* (2022)** , this study only tested ciprofloxacin antibiotic. Similarly, **Magalhães *et al.* (2016)** found substantial resistance to ciprofloxacin in *P. aeruginosa* isolates from raw and treated hospital wastewater.

Ciprofloxacin is very effective for treating *P. aeruginosa* infections, and resistance to it can greatly complicate treatment strategies. Ciprofloxacin is the most efficient fluoroquinolone against this bacterium and is thus widely used **(Rehman et al. 2019).** High fluoroquinolone concentrations in hospitals might cause ciprofloxacin resistance by overexpressing efflux

pumps or acquiring resistance genes through horizontal gene transfer **(Xu *et al*. 2021).** Ciprofloxacin resistance in *P. aeruginosa* is highly associated with hospital wastewater and this claim is supported by studies by **Govender *et al*. (2021**) and **Luczkiewicz *et al.* (2015),** who reported low levels of resistance to ciprofloxacin in *P. aeruginosa* strains obtained from municipal wastewater.

Another key finding of this study is the susceptibility of all the *staphylococcus spp*. to the tested antibiotics with zero rate of resistance. These findings were contrary to various study reports, for instance, a study by **Merradi *et al*. (2023b)** at the University Hospital Center of Batna in Algeria, who reported a high resistance of *staphylococcus aureus* to different antibiotics including tobramycin, kanamycin, and gentamicin. Their study found MRSA isolates to be highly resistant to penicillin and cefoxitin (100%), oxacillin (95.24), tetracyclin (66.67%), gentamycin (66.67%), tobramycin (71.43%), kanamycin (76.2%) and ofloxacin (71.43%). There were no tested antibiotics in our study that overlapped with the tested antibiotics in the study of **Merradi *et al*. (2023b).** Also, a study by **Hailu *et al*.(2016**) opposed the findings of this study, their study reported resistance rates of 7.9% to clindamycin, 34.6% to oxacillin, 42.6% to tetracycline, 23.1% to trimethoprim + sulfamethoxazole (SXT), 6.4% to chloramphenicol, 0% to ciprofloxacin, 14.1% to erythromycin and 65.4% to penicillin, and in our study, *staphylococcus spp*. were susceptible to STX. The sensibility of all the *staphylococcus spp.* to tested antibiotics could be due to efficient treatment and cleaning ways, or the limited choice antibiotics disks.

The detection of MDR bacteria in hospital wastewater has profound implications for both public health and environmental safety since hospital wastewater is a potential reservoir for antibiotic-resistant bacteria, which can disseminate into the broader environment, contributing to the global spread of antimicrobial resistance. The study underscores the necessity for stringent infection control measures within hospital settings. Moreover, the presence of resistant *Pseudomonas spp*. highlights the need for effective wastewater treatment processes capable of reducing bacterial load and antibiotic residues.

General Conclusion

General conclusion

The study of antimicrobial resistance in hospital wastewater is a critical area of research given the rising global threat posed by resistant bacteria. The essential objectives of this study were to qualitatively assess the presence of bacteria and multidrug-resistant bacteria in the hospital wastewater of Tlemcen University Hospital and provide recommendations to mitigate the spread of antibiotic resistance which were perfectly addressed.

The findings of the study revealed a significant presence of antibiotic-resistant bacteria in the hospital's wastewater and this underscores the role of healthcare facilities as hotspots for the emergence and spread of AMR. The results showed that several bacterial isolates exhibited resistance to multiple classes of antibiotics, highlighting the complexity and severity of the issue.

In light of these findings, numerous recommendations can be made to mitigate the impact of AMR in hospital wastewater. The Tlemcen University Hospital should adopt advanced treatment technologies capable of effectively removing or neutralizing resistant bacteria and their genetic material from wastewater. Establishing routine surveillance programs for AMR in hospital effluents can help in early detection and prompt response to emerging resistance patterns. It is also important to promote the rational use of antibiotics within healthcare settings, as this can reduce the selective pressure that drives the emergence of resistant strains. The responsible authorities can also strengthen policies and regulations regarding the disposal of hospital wastewater to ensure compliance with environmental standards and safeguard public health.

The study had several limitations, including its focus on a single hospital and the sampling conducted on a single day from only 3 units. This restricted scope may limit the generalizability of the findings to broader healthcare settings and temporal variations. Future research efforts would benefit from expanding the geographic scope to include multiple hospitals and implementing more comprehensive sampling strategies such as sampling across different seasons and hospital departments. Furthermore, challenges related to obtaining samples, such as bureaucratic hurdles, constrained the study's ability to collect samples and

data consistently and extensively. Overcoming these challenges in future studies will be essential for obtaining more robust and representative findings.

Future research should focus on exploring innovative treatment methods of hospital wastewater such as nanomaterial-based filtration systems, assessing the effectiveness of existing interventions, and also a deep understanding of the long-term impacts of AMR on environmental ecosystems. Collaborative efforts at local, national, and international levels will be crucial in combating this global health threat.

This study contributes to the growing body of evidence that hospital wastewater is a critical point of intervention in the fight against multi-drug resistance.

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ملخص

تمثل مقاومة الأدوية المتعددة تهديدًا عالميًا كبيرً ا، خاصة في أماكن الرعاية الصحية. تولد المستشفيات مياه الصرف الصحي التي تعمل كمستودع للبكتيريا المقاومة للأدوية المتعددة. يمكن لمياه الصرف الصحي في المستشفيات، إذا لم تتم معالجتها أو تمت معالجتها بشكل سيئ، أن تنشر مسببات الأ مراض المقاومة في البيئة، مما يشكل مخاطر كبيرة على الصحة العامة والنظم البيئية. تهدف هذه الدراسة إلى التقييم النوعي لوجود البكتيريا والبكتيريا المقاومة للأدوية المتعددة في مياه الصرف الصحي التابعة للمستشفى الجامعي تلمسان.

تم جمع عينات مياه الصرف الصحي في المستشفى من ثلاث وحدات مختلفة في المستشفى وهي وحدات أمراض الرئة

وأمراض الدم وجراحة الأع صاب. تم إجراء العزلة البكتيرية باستخدام وسائط أجار انتقائية وتفاضلية لتعزيز نمو بكتيريا ESKAPEالمستهدفة بشكل انتقائي. تم إجراء اختبار الحساسية للمضادات الحيوية باستخدام ط ريقة نشر قرص-Kirby

Bauerوفقًا لإرشادات معهد المعايير السريرية والمخبرية لتحديد ملفات تعريف المقاومة.

وكشف البحث عن وجود بكتيريا في مياه الصرف الصحي بالمستشفى، بما في ذلك وجود بكتيريا مقاومة للأدوية المتعددة. في الغالب، تم العثور على البكتيريا المقاومة للأدوية المتعددة من عائلة Enterobacteriaceae في وحدة أمراض الدم، في حين تم العثور على البكتيريا المقاومة للأدوية المتعددةPseudomonas spp. كانت سائدة في وحدة أمراض الرئة. كما حددت الدراسة أيضًا المكورات العنقوديةStaphylococcus spp ، والتي أظهرت قابليتها للمضادات الحيوية التي تم اختبارها. ومع ذلك، الزائفة النيابة. أظهرت مقاومة عالية للسيبروفلوكساسين والتوبراميسين عبر وحدات متعددة.

تسلط النتائج الضوء على الحاجة الماسة لمعالجة مياه الصرف الصحي في المستشفيات بكفاءة لمنع انتشار مقاومة المضادات الحيوية. تشكل البكتيريا المقاومة للأدوية المتعددة الموجودة في مياه الصرف الصحي تهديدات كبيرة للصحة العامة

الكلمات المفتاحية: البكتيريا المقاومة للأدوية المتعددة، الصحة العامة، بكتيرياESKAPE ، مقاومة المضادات الحيوية، مياه الصرف الصحي في المستشفيات.

Abstract

Multi-drug resistance is a major global threat, especially in healthcare settings. Hospitals generate wastewater that acts as a reservoir for multidrug-resistant bacteria. Hospital wastewater, if untreated or poorly treated, can disseminate resistant pathogens into the environment, posing significant risks to public health and ecological systems. This study aims to qualitatively assess the presence of bacteria and multidrug-resistant bacteria in the hospital wastewater of Tlemcen University Hospital.

Hospital wastewater samples were collected from 3 different units of the hospital which are the Pneumo-phthisiology, Hematology, and Neurosurgery units. Bacterial isolation was performed using selective and differential agar media to selectively promote the growth of target ESKAPE bacteria. Antibiotic susceptibility testing was carried out using the Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute guidelines to determine resistance profiles.

The research revealed the presence of bacteria in the hospital wastewater, including the occurrence of multidrug-resistant bacteria. Predominantly, MDR bacteria from the Enterobacteriaceae family were found in the Hematology unit, while MDR Pseudomonas spp. were prevalent in the Pneumo-phthisiology unit. The study also identified Staphylococcus spp., which demonstrated susceptibility to the tested antibiotics. However, Pseudomonas spp. exhibited high resistance to Ciprofloxacin and Tobramycin across multiple units.

The findings highlight the crucial need for efficient hospital wastewater treatment to prevent the spread of antibiotic resistance. MDR bacteria in wastewater pose major threats to public health.

Keywords: antibiotic resistance, hospital wastewater, multidrug-resistant bacteria, ESKAPE bacteria, public health

Résumé

La multirésistance aux médicaments constitue une menace mondiale majeure, en particulier dans les établissements de soins de santé. Les hôpitaux génèrent des eaux usées qui servent de réservoir à des bactéries multirésistantes. Si les eaux usées des hôpitaux ne sont pas ou mal traitées, peuvent disséminer des agents pathogènes résistants dans l'environnement, posant ainsi des risques importants pour la santé publique et les systèmes écologiques. Cette étude vise à évaluer qualitativement la présence de bactéries et de bactéries multirésistantes dans les eaux usées hospitalières du CHU de Tlemcen.

Des échantillons d'eaux usées hospitalières ont été collectés dans 3 différentes unités de l'hôpital, à savoir les unités de pneumo-phtisiologie, d'hématologie et de neurochirurgie. L'isolement bactérien a été réalisé à l'aide de milieux gélosés sélectifs et différentiels pour favoriser sélectivement la croissance des bactéries ESKAPE cibles. Des tests de sensibilité aux antibiotiques ont été effectués à l'aide de la méthode de diffusion sur disque de Kirby-Bauer conformément aux directives du Clinical and Laboratory Standards Institute afin de déterminer les profils de résistance.

La recherche a révélé la présence de bactéries dans les eaux usées de l'hôpital, notamment l'apparition de bactéries multirésistantes. Les bactéries MRA de la famille des Enterobacteriaceae ont été principalement trouvées dans l'unité d'hématologie, tandis que les bactéries MRA Pseudomonas spp. étaient fréquents dans l'unité de Pneumo-phtisiologie. L'étude a également identifié Staphylococcus spp., qui a démontré une sensibilité aux antibiotiques testés. Cependant, Pseudomonas spp. a présenté une résistance élevée à la ciprofloxacine et à la tobramycine dans plusieurs unités.

Les résultats mettent en évidence la nécessité cruciale d'un traitement efficace des eaux usées hospitalières pour prévenir la propagation de la résistance aux antibiotiques. Les bactéries MDR présentes dans les eaux usées constituent une menace majeure pour la santé publique.

Mots clés : résistance aux antibiotiques, eaux usées hospitalières, bactéries multirésistantes, bactérie ESKAPE, santé publique