Journal **Concepts in Structural Biology & Bioinformatics**

ANTIBIOTICS RESISTANCE ARTICLES

Research article:* *Master's research based.

Detection of multidrug-resistant bacteria in the effluents of the specialized HAMDANE Bakhta hospital in Saida

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Published: 15 January 2024

Abstract

The main objective of this study was to investigate the presence of multidrug-resistant bacteria in untreated hospital effluents from HAMDANE Bakhta Hospital in Saida and evaluate their resistance levels to certain antibiotics. From these hospital effluents, we conducted a count of the resistant flora on MacConkey medium using ceftazidime, revealing a concentration of approximately 2.6×10^6 CFU/mL. Similarly, the colistin-resistant flora was counted at a concentration of 1.4×10^5 CFU/mL, compared to an estimated total flora of around 1×10^7 CFU/mL. Furthermore, the vancomycin-resistant flora on Slanetz and Bertelay medium was present at a concentration of 2.4×10^2 CFU/mL, compared to a total flora concentration of approximately 10^4 CFU/mL. During our study, we successfully isolated 30 bacterial strains, including 12 strains of *Aeromonas hydrophila*, 3 strains of *Vibrio vulgaris*, 1 strain of *Citrobacter koseri*, 1 strain of *Proteus fluviatis*, 1 strain of *Providencia rettgri*, and 12 strains of *Enterococcus* spp.

Antibiotic susceptibility was evaluated using the antibiogram and MIC measurement. The results revealed that most strains were multidrug-resistant to various tested antibiotic families, particularly to hospital-used antibiotics such as ceftazidime, colistin, and vancomycin. These strains exhibited a high level of resistance, with MIC values exceeding 512 µg/mL. Additionally, we detected the production of extended-spectrum beta-lactamases (ESBLs) in three strains of *Aeromonas hydrophila* (Ce10, Ce15, and Ce16). Furthermore, we isolated bacteriophages effective against several bacterial strains, including *Staphylococcus*, *E. coli*, *Micrococcus*, *Salmonella*, *Enterococcus*, and others. In this study, we utilized these bacteriophages in combination with ceftazidime. However, no synergy between the two was observed against the *Enterococcus* strains isolated from hospital effluents.

Multidrug-resistant bacteria have been found in hospital effluents, posing a significant challenge for treating nosocomial infections. Further research is required to develop effective strategies, including exploring alternative combinations of antibiotics, bacteriophages, and other innovative approaches.

Keywords: Hospital effluents, antibiotic resistance, ceftazidime, colistin, vancomycin, ESBL, bacteriophages.

I. Introduction

Multiresistant bacteria (MRB) constitute a growing public health problem worldwide, and their presence in hospital effluents is a major concern. These bacteria, often referred to as "superbugs," have developed the ability to resist multiple classes of antibiotics, significantly limiting available treatment options and increasing the risk of complications and death in infected patients (Kooli, et al., 2014).

Hospital effluents, such as wastewater and medical waste, are potential sources of MRB dissemination in the environment. These effluents contain a diversity of microorganisms, including pathogenic bacteria, which may be antibiotic-resistant due to continuous exposure to antimicrobial agents in healthcare facilities (Al Aukidy, Al Chalabi, & Verlicchi, 2018).

MRB isolated from hospital effluents pose a threat to public health in several ways. Firstly, they can be directly transmitted to patients upon admission to the hospital, leading to severe and challenging-to-treat nosocomial infections (Li, Ondon, Ho, Jiang, & Li, 2022). Secondly, these bacteria can be dispersed in the environment, including surface waters, soils, and aquatic ecosystems, contributing to the spread of antibiotic resistance (Guessennd, et al., 2013).

Antibiotic resistance is often associated with the acquisition of resistance genes through horizontal transfer, enabling bacteria to rapidly and efficiently share these resistance mechanisms. Hospital effluents provide a conducive environment for this genetic transfer due to the coexistence of different bacterial species and the presence of mobile genetic elements such as plasmids and integrons (Nadella, et al., 2022).

In this study, our main objective is to characterize multiresistant bacteria isolated from the effluents of HAMDANE Bakhta Hospital. Specifically, we focus on identifying bacteria resistant to last-resort and hospital-use antibiotics, such as ceftazidime, colistin, and vancomycin, in the Saida Wilaya.

To achieve this goal, we are evaluating the antibiotic resistance profile of these bacteria, identifying the antibiotics to which they exhibit resistance. We are particularly interested in antibiotics used in the hospital setting, as resistance to these drugs can have a significant impact on the management of nosocomial infections.

Furthermore, we are exploring solutions to combat the isolated bacteria using a combination approach of phages and antibiotics. Hence, we are investigating the potential of using phages in conjunction with antibiotics to overcome resistance and improve treatment efficacy.

II. Materials and Methods

II.1. Provenance of Samples

On March 1st, 2023, at 08:43, samples were taken from the main sewage collector of all clinical services, laboratories, and the kitchen. The samples were collected in 250 ml bottles. Once the samples were taken, they were immediately transported to the microbiology laboratory at the University of Saida Dr. Moulay Tahar, where the necessary analyses were carried out.

II.2. Enumeration of total flora and resistant flora

Decimal dilutions ranging from 10-1 to 10-6 were made from the wastewater samples using a sterile solution of physiological water or TSE. Then, 100 µL of each dilution was carefully spread on the surface of appropriate selective media. For the enumeration of the total flora, the Slanetz and Burtely medium was used for *Enterococcaceae,* while the Mac Conkey medium was used for *Enterobacteriaceae* and non-enterobacteria such as *Aeromonadaceae* and *Vibrionaceae*. For the detection of vancomycin-resistant *enterococci*, the Slanetz and Burtely medium contained 8µg/mL of vancomycin. Additionally, the Mac Conkey medium contained either 2µg/mL of ceftazidim or 4µg/mL of colistin for the detection of resistant flora. The plates were incubated at a temperature of 37°C for a period of 24 to 48 hours, which was the necessary time for the development and appearance of bacterial colonies.

II.3. Purification and Identification of Bacterial Isolates

After incubation, four to five isolated colonies of the resistant flora were randomly selected from each Petri dish. Each selected colony was then streaked onto a nutrient broth. After incubation at the desired temperature, the appearance of cultures in the liquid medium was observed. At this stage, bacterial isolates were purified using the streak method. The purification process was repeated twice, each time selecting a well-isolated colony from the agar. This resulted in a culture whose purity was estimated through macroscopic and microscopic observations. After purification, replicas of these isolates were preserved. The strains were then pre-identified based on biochemical criteria (Vira, Bhat, & Chavan, 2016).

II.4. Determination of the MIC

The MIC (Minimum Inhibitory Concentration) is defined as the lowest concentration of an antibiotic that inhibits the in vitro growth of 99% of the tested bacterial population. In practice, the MIC is determined by measuring the lowest concentration of a range of antibiotic dilutions, tested in half-logarithmic increments, that results in the complete inhibition of visible bacterial growth (Wenzler, *et al.*, 2023).

In the current scenario, the MIC has been measured for five antibiotics: ampicillin, colistin, ceftazidime, gentamicin, and vancomycin.

II.4.1. Preparation of the inoculum for MIC measurement

II.4.1.1. Turbidity standard for the preparation of the inoculum

To standardize the inoculum density for a susceptibility test, a 0.5 McFarland turbidity standard was used. This original McFarland standard is obtained by mixing specified amounts of barium chloride and sulfuric acid, which causes the formation of a precipitate of barium sulfate and makes the solution turbid.

To prepare a 0.5 McFarland standard, 0.05 mL of 1.175% w/v barium chloride dihydrate ($BaCl_2$,$2H_2O$) is mixed with 9.95 mL of 1% v/v sulfuric acid (H_2SO_4). This mixture yields a suspension containing approximately 1-2 x 10^8 colony-forming units (CFU)/mL for *Escherichia coli* ATCC 25922.

Visual evaluation was used to adjust the inoculum density. An appropriate light source was used to visually compare the inoculum tube with the 0.5 McFarland standard (Cockerill, 2012).

II.4.1.2. Preparation of antibiotic stock solutions

To prepare a series of logarithmic dilutions of antibiotics, the method involves preparing an initial stock solution of 5120 µg/mL. This concentration is then subjected to successive 1/2 dilutions using sterile distilled water, to obtain a series of concentrations ranging from 1.25 µg/mL to 5120 µg/mL. The antibiotic solutions thus prepared are then stored at 4°C, protected from light, and must be used within 7 days to avoid any loss of antibiotic activity (Cockerill, 2012).

II.4.2. Microdilution method

The inoculum was prepared by suspending isolated colonies selected from a Petri dish culture for 16 to 24 hours in a physiological saline solution. The suspension was adjusted to match the 0.5 McFarland turbidity standard using physiological water and vigorously shaking. For the microdilution method, a dilution of 1/200 is desirable to obtain a final concentration of approximately 5 × 10^4 CFU/well. For this, 5 µL of the suspension were diluted in 0.1 mL of MH medium supplemented with antibiotic. Bacterial growth was observed with the naked eye after 24 hours of exposure to the antibiotic at 37°C. The MIC is the lowest concentration of antibiotic where there is no visible bacterial growth (CLSI, 2012; Rennie, Turnbull, Brosnikoff, & Cloke, 2012).

II.4.3. Agar dilution method

The agar dilution method requires a 1:10 dilution of the 0.5 McFarland suspension in physiological water to obtain a concentration of 10^7 CFU/mL. The final inoculum on MH agar supplemented with antibiotic will

be approximately 10^4 CFU per 3 mm diameter spot, using 2 µL of inoculum. The spots are allowed to dry at room temperature for 15 to 30 minutes, and then the plates are incubated at 37°C for 24 hours. The MIC is defined as the concentration in the first plate of the series that shows no microbial growth (CLSI, 2012).

II.4.4. Antibiotic Susceptibility Testing Method by Agar Diffusion

A sterile swab was used to inoculate the plates after calibrating the inoculum to 0.5 McFarland and streaking it across the MH agar surface. This process was repeated twice at 60° intervals to ensure even distribution of the inoculum. Following this step, the plates were left slightly open for 3 to 5 minutes to absorb excess moisture before applying the antibiotic discs.

Antibiotic discs (Table 1) were evenly placed on the seeded plate, ensuring a minimum distance of 24 mm between each disc. Subsequently, the plates were placed in an incubator set at 37°C within 15 minutes of disc application.

After incubating for 16 to 18 hours, the zones of inhibition were accurately measured using a ruler for subsequent interpretation based on established breakpoints outlined in CLSI M100-S (Clinical and Laboratory Standards Institute) (CLSI, 2012).

Table 1: Table of Antibiotics Utilized for Antibiogram Testing

Symbol	Antibiotic	Family	Disk Loading in mg
AUG	Amoxicillin + Clavulanic acid	Beta-lactams	30
CAZ	Ceftazidim	Beta-lactams	30
IMP	Imipenem	Beta-lactams	10
C	Chloramphenicol	Phenicoles	30
CN	Gentamicin	Aminoglycosides	10
SXT	Sulfamethoxazole/Trimethoprim	Sulfamides	25
TE	Tetracycline	Cycline	30

II.5. Detection of Extended-Spectrum Beta-Lactamases (ESBLs)

The production of extended-spectrum β-lactamase (ESBL) was studied using synergy tests on MH agar. Third-generation cephalosporin discs (C3G) (ceftazidime) were placed 2 or 3 cm away from a disk of amoxicillin + clavulanic acid (Jarlier, Nicolas, Fournier, & Philippon, 1988). The presence of an ESBL was characterized by the appearance of a "champagne cork" synergy image due to the inhibitory effect of clavulanic acid (Siu, Lu, Chen, Lin, & Chang, 2003).

II.6. Bacteriophage Research

Bacteriophages are capable of infecting various species of bacteria and demonstrating their natural antibacterial effect, which is comparable to that of an antibiotic. Bacteriophages are isolated from wastewater samples, incubated with the target bacteria, and then selected based on their ability to amplify.

II.6.1. Bacterial culture

The targeted bacteria in this study are reference strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella oxytoca*, *Listeria innocua*, and *Acinetobacter baumannii*. All bacterial strains are cultured at 37°C in Luria-Bertani (LB) growth medium.

II.6.2. Isolation and Purification of Bacteriophages

Bacteriophages are isolated from wastewater samples. Firstly, 100 µL of the sample is mixed with the target bacteria (500 µL) in 5 mL of LB medium at 37°C overnight. Then, the amplified sample is centrifuged for 30 minutes at 10000 g. The supernatant is filtered using a 0.45 µm porosity filter. A series of half dilutions up to a dilution of 10^7 of the isolated bacteriophages is prepared in $CaCl_2$ at a concentration of 11 g/L. 100 µL of each dilution is taken and spread over a sector of a bacterial lawn composed of the target bacteria. The Petri dishes containing the dilutions are then incubated at 37°C overnight (Van Twest & Kropinski, 2009).

II.7. Phage/Antibiotic Synergy

Synergy between bacteriophages and antibiotics refers to the beneficial effect obtained through the combined use of these two approaches to treat bacterial infections. This combination leverages the distinct advantages of bacteriophages, which specifically target bacteria, and antibiotics, which act directly on bacteria, to improve treatment efficacy. In our study, we evaluated the synergy between ceftazidim, an antibiotic of the cephalosporin class, and a mixture of isolated bacteriophages, with the aim of determining the joint effects of these two approaches on bacterial inhibition (Gu Liu, et al., 2020).

The protocol involved spreading the strain to be tested on LB medium. Two discs were placed on the surface of the Petri dish. The first disc contained only ceftazidime, while the second disc was loaded with 10 µL of a diluted 1/2 mixture of bacteriophages in $CaCl_2$. As a control, 10 µL of $CaCl_2$ alone was added to the LB agar. After 15 minutes, the plates were incubated at 37°C for 24 hours.

III. Results and Discussion

III.1. Enumeration of total flora and resistant flora

The main objective of our study was to isolate and characterize the resistant bacterial species present in wastewater. The results revealed an average concentration of 1×10^7 CFU/mL for the total flora on the MacConkey medium. However, we counted 2.6×10^6 CFU/mL for the ceftazidim-resistant flora and 1.4×10^5 CFU/mL for the colistin-resistant flora. On the Slanetz and Bartley medium, the total flora was enumerated at 10^4 CFU/mL, while the vancomycin-resistant flora was present at a concentration of 2.4×10^2 CFU/mL.

These results clearly indicate that the wastewater is heavily contaminated with bacteria. Our observations align with those of Ekhaise and Omavwoya, who found an average of 10^7 to 10^8 CFU/mL of Gram-negative bacteria, mainly represented by *Enterobacteria*, in hospital wastewater (Ekhaise & Omavwoya, 2008). They also noted that the number of microorganisms could increase by 1000 times in river waters after the discharge of urban wastewater (Baumont, 2004). Regarding *Enterococci*, which correspond to black colonies on the Slanetz medium and are presumed to be *Enterococci*, the enumeration revealed 10^4 CFU/mL for the total flora and 2.4×10^2 CFU/mL for the resistant flora, which is in line with the findings of Varela et al. (Varela, et al., 2013), who estimated a total number ranging from 10^2 to 10^4 CFU/mL and 10^1 to 10^3 CFU/mL for vancomycin-resistant flora.

The observed resistance in this study can be attributed to the presence of unmetabolized antibiotic residues in hospital effluents, which significantly contribute to the selection of multidrug-resistant bacteria in wastewater. This hypothesis is supported by the work of Islam (Islam, Uddin, Hakim, Das, & Hasan, 2008).

These findings highlight the importance of monitoring and properly treating hospital effluents to prevent the spread of resistant bacteria into the environment.

III.2. Isolation and identification

On MacConkey medium (Fig.1. A, B), the colonies exhibit a variety of shapes (round), sizes (small, medium, and large), and colors (light pink, dark pink, white). In contrast, on Slanetz and Bartley medium (Fig.2), the colonies are small and have a black color**.**

From these colonies, we proceeded to isolate and identify bacterial isolates resistant to colistin, ceftazidime, and vancomycin. To do this, we randomly selected four to five colonies isolated from the resistant flora from each Petri dish containing the samples. Each selected colony was then inoculated into a nutrient broth and then onto GN agar.

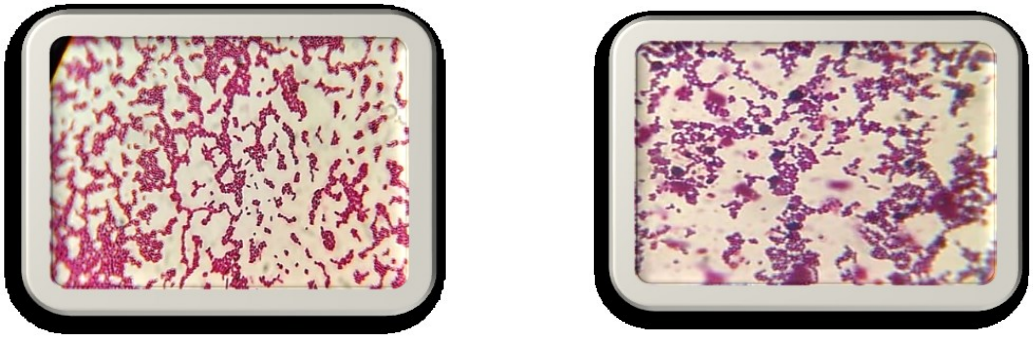
The purity of the cultures was checked by performing successive streaking on GN agar, followed by incubation at 37 °C for 24 hours. This step was repeated until colonies with the same size, shape, and color were obtained, indicating the purity of the isolated bacterial strains.

Fig.1. Appearance of colonies on MacConkey medium: (A) with Ceftazidim and (B) with Colistin.

Fig.2: Appearance of colonies on Slanetz and Bartley medium in the presence of Vancomycin.

III.3. Microscopic examination of the isolates

The bacterial isolates obtained from the MacConkey medium were examined under an optical microscope after Gram staining with a magnification of X 1000. The observed bacteria were pink in color, indicating that they do not retain the Gram stain. They were sometimes isolated and sometimes grouped and had coccobacillus or bacillus shapes. In contrast, the bacteria isolated from the Slanetz and Bartley medium were violet in color and retained the Gram stain. They had cocci or ovoid shapes. An example of the microscopic appearance of the Co3 and V1 strains is shown in Fig.3.



Co3 V1

Fig.3. The microscopic appearance of strain Co3 (coccobacillus) and V1 (cocci) at a magnification of X 1000.

III.4. Biochemical criteria

III.4.1. Catalase activity test

The results of the catalase test, conducted as part of the study of the respiratory type of Gram-negative bacteria, revealed that most strains in this group exhibited gas production (Fig.4), indicating the presence of the catalase enzyme. It should be noted that certain families of Gram-negative bacteria, such as *Enterobacteriaceae*, have been reported to be catalase positive (Taylor & Achanzar, 1972), meaning that they produce the catalase enzyme. In contrast, all Gram-positive bacterial strains were identified as catalase negative, producing no gas production, suggesting the absence of the catalase enzyme. It has been reported that certain Gram-positive families, such as *Enterococcaceae*, are catalase negative (Facklam & Elliott, 1995).

Catalase plays a crucial role in neutralizing the bactericidal effects of hydrogen peroxide, and its presence in bacteria is often associated with their pathogenic potential (Reiner, 2010).

Fig.4. The result of the catalase test for strain Co3.

III.4.2. Biochemical identification

The identification of Gram-negative bacterial strains isolated from hospital effluents was performed using the API 20E gallery. This analysis allowed the selection of 18 different strains (Table 2), including 12 strains of *Aeromonas hydrophila*. The distinction between these strains was based on their utilization and fermentation of sugars, and three strains of *Vibrio fluviatis* were also identified. In addition, one strain each of *Citrobacter koseri*, *Proteus rettgeri*, and Proteus vulgaris was also identified as shown in Fig. 5 which presents an example of results obtained from the API 20E galleries, identified as *A. hydrophila*.

A total of 12 Gram-positive strains were isolated from the Slanetz and Bartley medium. These strains were numbered from Vo1 to Vo12 to facilitate their later reference.

Table.2. Results of strain identification using the API 20E gallery

Strains	Co2	Co3	Co4	Co5	Co7	Co8	Co9	Co12	Co19	Co20	Ce9	Ce10	Ce11	Ce12	Ce13	Ce14	Ce15	Ce16
Bacterial shape	Coccobacillus	Coccobacillus	Coccobacillus	Coccobacillus	Coccobacillus	Bacillus	Coccobacillus	Coccobacillus	Coccobacillus	Coccobacillus	Bacillus	Coccobacillus	Bacillus	Bacillus	Bacillus	Bacillus	Coccobacillus	Coccobacillus
Identification%	86.6	99.1	86.6	86.6	99.9	89.8	99.8	91.9	91.8	81.2	72.5	65.1	88.7	88.7	100	100	91.3	98.8
Identification Genus/Species	*Aeromoans hydropila Gr1*	*Aeromoans hydropila Gr2*	*Aeromoans hydropila Gr1*	*Aeromoans hydropila Gr1*	*Aeromoans hydropila Gr1*	*Citrobacter koseri*	*Aeromoans hydropila Gr2*	*Aeromoans hydropila Gr2*	*Aeromoans hydropila Gr2*	*Aeromoans hydropila Gr2*	*Vibrio flavutis*	*Aeromoans hydropila Gr2*	*Vibrio flavutis*	*Vibrio flavutis*	*Providencia rettgri*	*Proteus vulgaris*	*Aeromoans hydropila Gr1*	*Aeromoans hydropila Gr1*

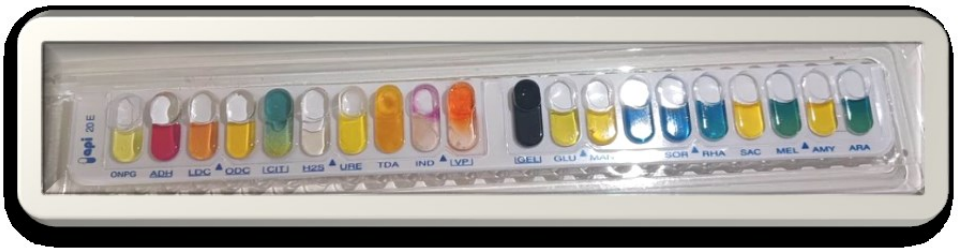


Fig.5. The API 20E gallery identification result for strain Co3.

III.5. Measurement of the MIC

The MIC is defined as the lowest concentration of an antibiotic that is capable of inhibiting bacterial growth. In our study, the determination of MIC was performed using two different techniques. The first technique is based on the use of a solid medium, while the second technique uses the microbroth dilution method in a liquid medium.

III.6. The MIC in solid medium

The determination of MIC using a solid medium has several advantages. Firstly, it allows for the evaluation of the sensitivity of multiple bacterial strains simultaneously, which is particularly useful in comparative studies. Additionally, this method offers high precision, which can even allow for the visible detection of slight bacterial growth on the solid medium, thus facilitating the interpretation of results. An example of the results obtained is presented in Fig.6.

GN medium + 8 µg/mL colistin GN medium + 16 µg/mL colistin

Fig.6. Determination of the MIC of strains against colistin: in Fig.6.A, the results show the growth of most strains at a colistin concentration of 8 µg/mL. However, upon increasing the concentration to 16 µg/mL, in Fig6.B, the growth of all strains was inhibited, except for strain Co7, which proved to be more resistant, indicating that the MIC for all tested strains is 16 µg/mL.

III.7. The microdilution method

The microbroth dilution method involves using a single 96-well plate to measure the MIC of eight strains simultaneously. This method uses a small amount of culture medium (a few tens of microliters per well). An example of the results obtained is presented in Fig. 6, shown above, where the MIC of strain Ce9 against colistin is around 8 µg/mL.

The results of the MIC obtained are presented in Tables 3 and 4. The resistance or sensitivity of bacteria is defined based on the MIC values established by the (CLSI, 2012).

Table.3. The MIC values of Gram-negative strains against different antibiotics. (S) - Susceptible, (R) - Resistant, (I) - Intermediate.

Strains		Colistin	Ceftazidime	Ampicillin	Gentamycin	Vancomycin
A. hydrophila	Co2	16(R)	>12(R)	>512(R)	256(R)	512(R)
	Co3	16(R)	8(I)	>512(R)	4(S)	4(S)
	Co4	16(R)	325(R)	>512(R)	128(R)	256(R)
	Co5	16(R)	4(S)	>512(R)	64(R)	512(R)
	Co7	>16(R)	4(S)	>512(R)	64(R)	512(R)
	Co9	16(R)	>128(R)	>512(R)	64(R)	512(R)
	Co12	>16(R)	>128(R)	>512(R)	>512(R)	>512(R)
	Co19	16(R)	64(R)	>512(R)	>512(R)	>512(R)
	Co20	16(R)	32(R)	>512(R)	64(R)	>512(R)
	Ce10	16(R)	>128(R)	>512(R)	64(R)	512(R)
	Ce15	16(R)	8(I)	>512(R)	128(R)	512(R)
	Ce16	>16(R)	>128(R)	>512(R)	64(R)	512(R)
C. koseri	Co8	16(R)	64(R)	>512(R)	256(R)	512(R)
V. fluviatis	Ce9	8(R)	>128(R)	>512(R)	256(R)	512(R)
	Ce11	8(R)	16(R)	>512(R)	4(S)	512(R)
	Ce12	8(R)	16(R)	>512(R)	16(R)	512(R)
P. rettgri	Ce13	8(R)	4(S)	>512(R)	16(R)	512(R)
P. vulgaris	Ce14	16(R)	16(R)	512(R)	256(R)	256(R)

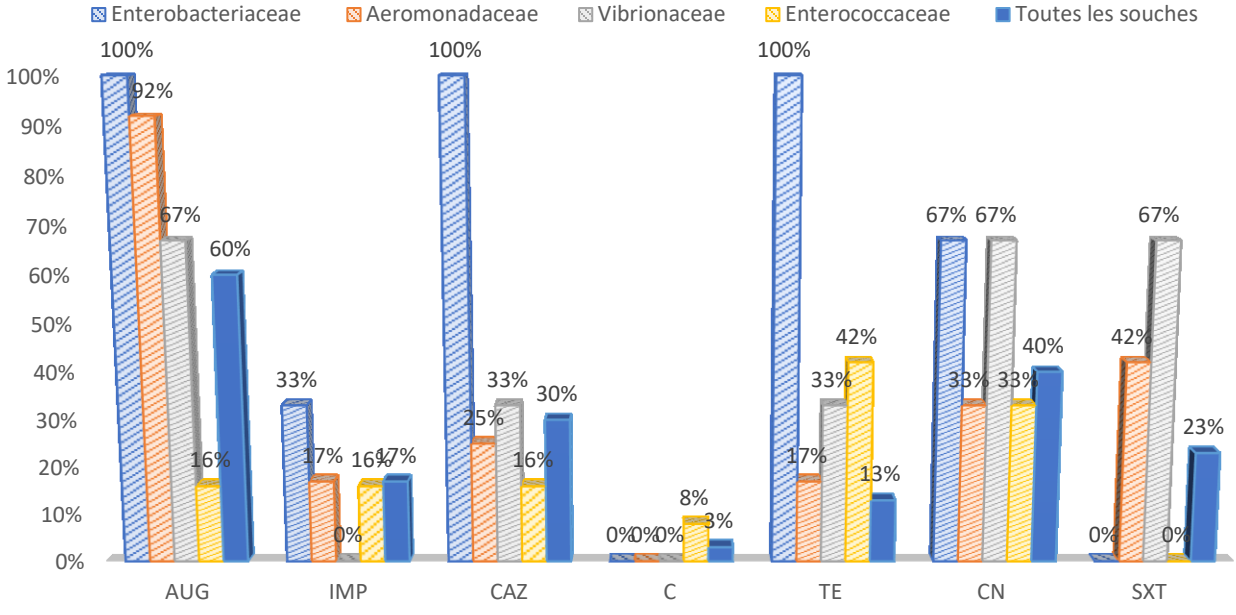
Table.4. The MIC values of Gram-positive strains of Enterococcus spp. against different antibiotics. (S) - Susceptible, (R) - Resistant, (I) - Intermediate.

Strains		Vancomycin	Ampicillin	Gentamycin
Enterococcus sp	Vo1	>1024(R)	0.5(S)	128(R)
	Vo2	>1024(R)	0.5(S)	512(R)
	Vo3	>1024(R)	0.5(S)	128(R)
	Vo4	>1024(R)	1(S)	128(R)
	Vo5	>1024(R)	64(R)	>512(R)
	Vo6	>1024(R)	1(S)	128(R)
	Vo7	>1024(R)	0.5(S)	128(R)
	Vo8	>1024(R)	64(R)	>512(R)
	Vo9	>1024(R)	32(R)	256(R)
	Vo10	>1024(R)	1(S)	128(R)
	Vo11	>1024(R)	1(S)	128(R)
	Vo12	>1024(R)	1(S)	128(R)

III.8. Determination of antibiotic resistance profiles (Antibiogram)

After 24 hours of incubation, the diameter of the zone of bacterial growth inhibition is measured for each antibiotic. This allows for the determination of the susceptibility of the bacteria to the antibiotics. The obtained results are then compared to critical values to classify the strains into three categories: Susceptible (S), Resistant (R), and Intermediate (I). This classification is based on the size of the inhibition zone and indicates whether the bacteria are susceptible, resistant, or have intermediate susceptibility to the tested antibiotic.

Based on the results of the antibiogram, we have calculated the resistance rates for different antibiotics (Fig.7).



	AUG	IMP	CAZ	C	TE	CN	SXT
Enterobacteriaceae	100%	33%	100%	0%	100%	67%	0%
Aeromonadaceae	92%	17%	25%	0%	17%	33%	42%
Vibrionaceae	67%	0%	33%	0%	33%	67%	67%
Enterococcaceae	16%	16%	16%	8%	42%	33%	0%
Toutes les souches	60%	17%	30%	3%	13%	40%	23%

Fig.7. Resistance rates of strains to different antibiotics.

III.9. Detection of broad-spectrum beta-lactamases (ESBLs)

The agar diffusion method using antibiotic discs containing enzyme inhibitors has been used to detect the production of extended-spectrum beta-lactamases (ESBLs). This method allows for the detection of the inhibition of ESBL activity by enzyme inhibitors, which is indicated by the formation of a champagne cork-like appearance on the agar.

The provided figure, Fig. 8, confirms the formation of ESBLs in three strains of *A. hydrophila* (Ce10, Ce15, and Ce16), represented by a champagne cork-like appearance. These ESBLs belong to class A and class B beta-lactamases, according to Ambler's classification. Class A ESBLs confer resistance to all penicillins and cephalosporins but not to cephamycins or carbapenems. They are inhibited by beta-lactamase inhibitors (Bradford, 2001). Class B ESBLs are active against carbapenems and cephalosporins. They are not sensitive to clavulanic acid, and their mediation can be chromosomal or plasmid-mediated (Parveau, 2011). It is also mentioned that the production of ESBLs in Aeromonas has been increasingly reported in recent years (Rodríguez, *et al.*, 2005).

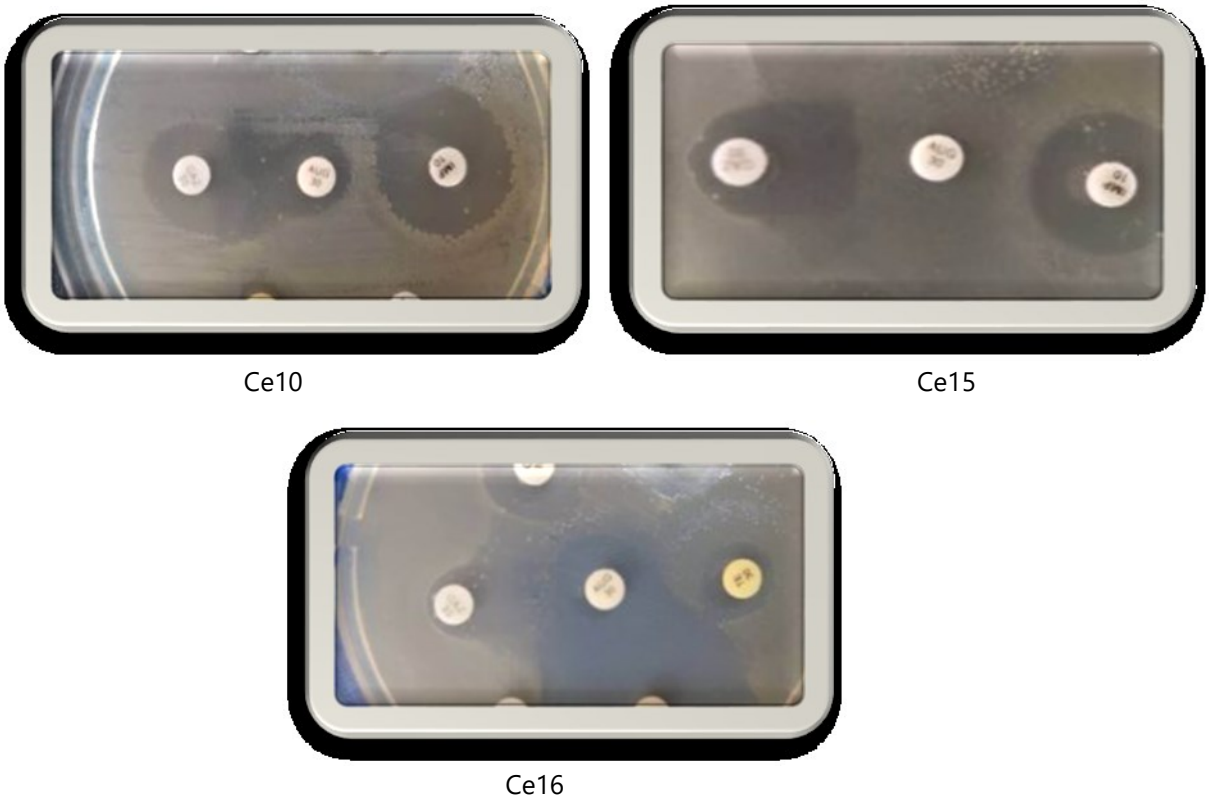


Fig.8. The production of ESBLs by various strains of *A. hydrophila* (Ce10, Ce15, and Ce16)

III.9.1. Bacteriophage detection

The bacteriophages were isolated from wastewater samples and then used to target different reference bacterial strains, including *Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella oxytoca, Listeria innocua, Acinetobacter baumannii*, as well as *Micrococcus*.

The figure presented below, Fig.9, illustrates the results of the bacteriophage lysis tests specific to *Micrococcus, Listeria,* and *Enterococcus*. In this figure, it is observed that as the dilution in CaCl2 increases, the zone of bacterial growth inhibition becomes narrower, and this was observed for the remaining strains. These results indicate that the bacteriophages have the ability to effectively lyse bacterial cells. The decrease in the inhibition zone observed with increasing dilution in CaCl2 can be explained by the decrease in the concentration of active bacteriophages.

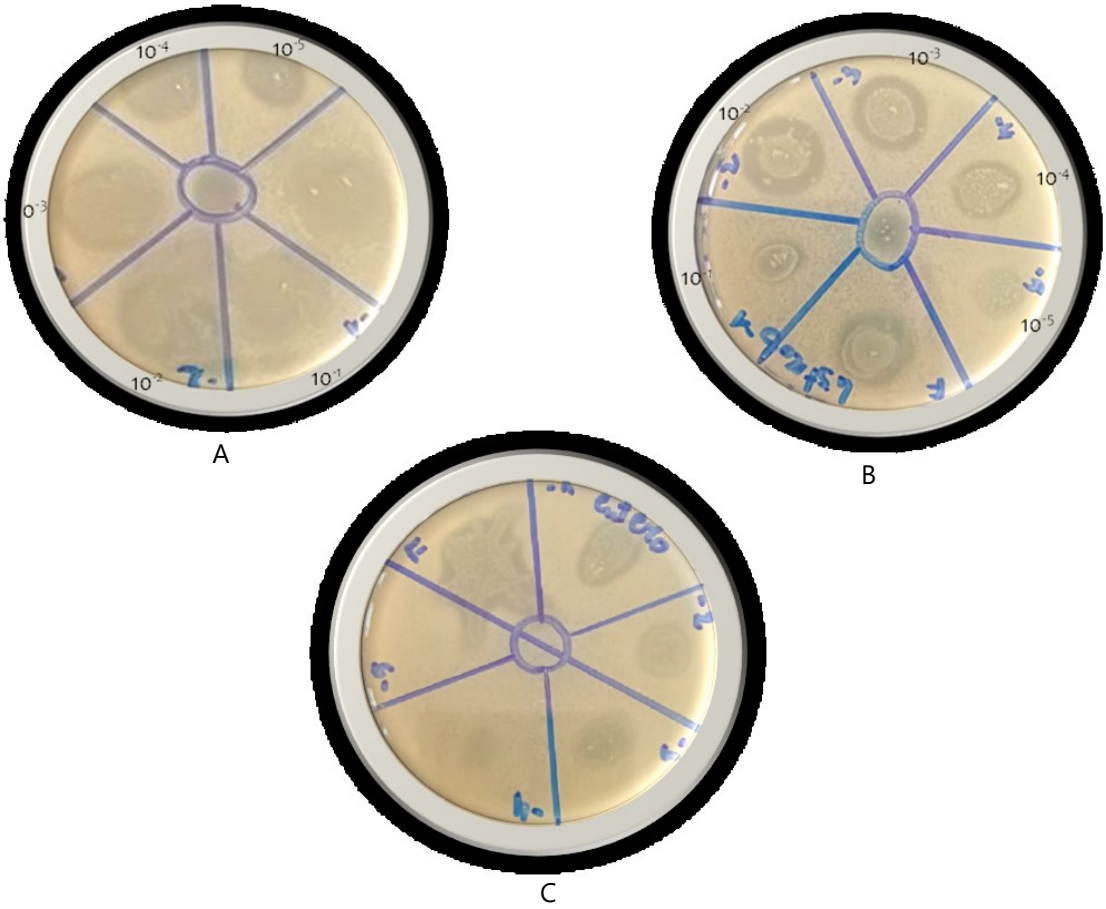


Fig.9. Results of bacteriophage-specific lysis for: *Micrococcus* (A), *Listeria* (B), and *Enterococcus* (C).

III.9.2. Result of phage/antibiotic synergy

In this part of the study, the objective was to test the synergy between bacteriophages and Ceftazidim on resistant strains of *Enterococcus*. We applied the previously isolated bacteriophage mixture to the bacterial strains and evaluated the combined effect with the addition of Ceftazidim. However, our results did not show any synergy between the bacteriophages and Ceftazidim.

IV. Conclusion

Hospital wastewater represents a major source of contamination with antibiotics and multi-drug resistant bacteria, raising significant concerns regarding public health. These wastewater streams from hospitals, rich in organic matter, create an aquatic environment conducive to bacterial proliferation. Among these microorganisms, Gram-negative bacilli such as *Enterobacteriaceae, Vibrionaceae,* and *Aeromonadaceae,* as well as Gram-positive cocci like *Enterococcaceae,* play a substantial role in human infectious diseases due to their antibiotic resistance.

In our study, we focused on the search for multi-drug resistant bacteria in the effluents of HAMDANE Bakhta Hospital, located in the Saida province. Among the different bacterial species identified, we observed a high frequency of *A. hydrophila* presence in these effluents, suggesting a probable origin of these microorganisms within the hospital environment. We also detected other species such as *P. rettgeri, P. vulgaris, C. koseri, V. fluvialis*, and *Enterococcus spp*.

The results obtained revealed high resistance to several antibiotics, particularly amoxicillin plus clavulanic acid (AUG) and hospital-use antibiotics such as ceftazidime, colistin, and vancomycin, with extremely high minimum inhibitory concentrations (MICs). On the other hand, imipenem appears to exhibit some effectiveness against these bacterial strains. For example, resistance to beta-lactams may be attributed to the production of enzymes such as extended-spectrum beta-lactamases (ESBLs) produced by *A. hydrophila* strains.

The susceptibility to other antibiotics varies depending on each bacterial family. It is important to note that these results are based on available data, and a more in-depth analysis would require a better understanding of bacterial strains, their resistance mechanisms, and the history of antibiotic use in the hospital environment.

In the face of this concerning situation, it is essential to implement effective preventive and control measures. One promising approach that we have explored is the combined use of phages and antibiotics to

combat multi-drug resistant bacteria. Phages are bacteria-specific viruses capable of selectively targeting and eliminating these microorganisms. By combining phages with antibiotics, we could enhance treatment efficacy and reduce the spread of resistant bacteria.

The preliminary results obtained in this study open up several avenues for further research. It would be relevant to expand our study to a larger geographical area over an extended period, perform strain identification using molecular and serological techniques, characterize the presence of extended-spectrum beta-lactamases (ESBLs) using PCR, and study the nationwide impact of these effluents on the environment.

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