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DISSERTATION

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Theme:

Prevalence of Extended Spectrum β-Lactamases & Carbapenemases-producing Gram-negative bacteria from wastewater in Bordj Bou Arreridj, Algeria

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ABDERAOUF

Abstracts

Abstract:

This study assesses the prevalence of Extended Spectrum β-Lactamase (ESBL)- and carbapenemase (CRB)-producing Gram-negative bacteria (GNB) from 14 wastewater samples collected in BBA. Overall, 12 samples (86%) harbored CTX^R strains, and 30 CTX^R-GNB strains were recovered, among which 11 (37%) were found to be ESBL-E, and 4 strains harbored the bla_{CTX-M} encoding gene. ESBL-E were identified as: *K. pneumoniae* (6 strains), *E. coli* (4 strains), and *Citrobacter freundii* (1 strain). Six multi-drug resistant (MDR) strains were detected among ESBL-E. In addition, 10 samples (71%) contained IMP^R strains, and 14 IMP^R-GNB strains were collected, among which 1 (7%) was CRB-GNB and MDR.

Keywords: ESBL-producing Gram-negative bacteria, Carbapenemase-producing Gram-negative bacteria, *Enterobacteriaceae*, bla_{CTX-M}, wastewater, BBA.

Résumé:

Cette étude évalue la prévalence des bactéries à Gram-négatif (BGN) productrices de β-lactamases à spectre étendu (BLSE) et de carbapénémases (CRB) à partir de 14 échantillons d'eaux usées collectés dans la ville de BBA. Au total, 12 échantillons (86 %) hébergeaient des souches CTX^R et 30 souches CTX^R-BGN ont été collectées, parmi lesquelles 11 (37 %) se sont révélées BLSE-E et 4 souches hébergeaient le gène codant pour *bla*$_{CTX-M}$. Les BLSE-E ont été identifiées comme suit : *K. pneumoniae* (6 souches), *E. coli* (4 souches) et *Citrobacter freundii* (1 souche). Six souches multirésistantes (MR) ont été détectées parmi les BLSE-E. De plus, 10 échantillons (71 %) contenaient des souches IMP^R et 14 souches IMP^R-GNB ont été collectées, parmi lesquelles 1 (7 %) était CRB-GNB et MR.

Mots-clés: Bactéries à Gram-négatif productrices de BLSEs, bactéries à Gram-negatif productrices de carbapenémases, *Enterobacteriaceae*, *bla*$_{CTX-M}$, eau usée, BBA.

ملخّص:

تقيّم هذه الدّراسة مدى انتشار البكتيريا سالبة الجرام المنتجة لإنزيم البيتالاكتاماز واسع الطيف وأيضا إنزيم الكاربابينيماز من 14 عيّنة من مياه الصرف الصحي تم جمعها في مدينة برج بوعريريج. أوضحت النتائج أنّ 12 عينة (86٪) تحتوي على سلالات مقاومة للسيفوتاكسيم، وتم جمع 30 سلالة منها، من بينها 11 (37٪) صنّفت أنها منتجة لإنزيم البيتالاكتاماز واسع الطيف، كان من بينها 4 سلالات تحتوي على المورّثة $bla_{\text{CTX-M}}$ ، وقد تم تحديد هذه السلالات على النحو التالي: *K. pneumoniae* (6 سلالات)، *E. coli* (4 سلالات)، و*Citrobacter freundii* (سلالة واحدة). كما تم الكشف عن ست سلالات متعددة المقاومة للمضادات الحيوية من بين هذه السلالات المنتجة للبيتالاكتاماز واسع الطيف. بالإضافة إلى ذلك، احتوت 10 عينات من مياه الصرف (71%) على سلالات مقاومة للاميبينام، وتم جمع 14 سلالة منها، من بينها 1 (7%) كانت منتجة لإنزيم الكاربابينيماز و متعددة المقاومة للمضادات الحيوية.

الكلمات المفتاحية: البكتيريا سالبة الغرام المنتجة للبيتالاكتاماز واسع الطيف، البكتيريا سالبة الغرام المنتجة للكاربابينيماز، البكتيريا المعوية، مياه الصرف الصحي، المورّثة $bla_{\text{CTX-M}}$ ، برج بوعريريج.

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List of Abbreviations

3GC : Third-Generation Cephalosporin.

ADH : Arginine Dihydrolase.

AK : Amikacin.

AMC : Amoxicillin-Clavulanate.

AmpC : AmpC β-Lactamase.

AMR : Antimicrobial Resistance.

AMY : Amygdalin.

API 20 E : Analytical Profile Index 20 Enterobacteriaceae.

ARA : Arabinose.

ATCC : American Type Culture Collection.

ATM : Aztreonam.

BBA : Bordj Bou Arreridj.

CAZ : Ceftazidime.

CDC : Centers for Disease Control and Prevention.

CHL: Chloramphenicol.

CIP : Ciprofloxacin.

CIT : Citrate.

CLSI : Clinical and Laboratory Standards Institute.

CRB : Carbapenemase.

CRB-E : Carbapenemases-producing *Enterobacteriaceae*.

CRB-GNB : Carbapenemases-producing Gram-Negative Bacteria.

CTX : Cefotaxime.

CTX-M : Cefotaxime-Munich β-Lactamase.

CTX^R : Cefotaxime-Resistant strains.

CTXR-GNB : Cefotaxime-Resistant Gram-Negative Bacteria.

DDST : Double-Disk Synergy Test.

DDT : Double Disc Test.

DNA : Deoxyribonucleic Acid.

DWW : Domestic Wastewater.

EDTA : Ethylenediaminetetraacetic Acid.

ESBL : Extended-Spectrum β-Lactamase.

ESBL-E : Extended-Spectrum β-Lactamase-Producing Enterobacteriaceae.

ESBL-GNB : Extended-Spectrum β-Lactamase-Producing Gram-Negative Bacteria.

ETP : Ertapenem.

FEP : Cefepime.

GEL : Gelatinase.

GEN : Gentamicin.

GLU : Glucose.

GN : Gram-Negative.

GNB : Gram-Negative Bacteria.

GPP : global priority pathogens.

H2S : Hydrogen Sulfide.

HWW : Hospital Wastewater.

IMP : Imipenem.

IMP^R : Imipenem-Resistant strains.

IMPR-GNB : Imipenem-Resistant Gram-Negative Bacteria.

IND : Indole.

INO : Inositol.

K : Kanamycin.

KPC : *Klebsiella pneumoniae* Carbapenemase.

LDC : Lysine Decarboxylase.

MAN : Mannitol.

MBL : Metallo-β-Lactamase.

mCIM : Modified Carbapenem Inactivation Method.

MDR : Multidrug-Resistant.

MEL : Melibiose.

MGE : Mobile Genetic Element.

MH : Mueller-Hinton (Agar).

MHT : Modified Hodge Test.

MRP : Meropenem.

NDM : New Delhi Metallo-β-Lactamase.

ODC : Ornithine Decarboxylase.

ONPG : - o-Nitrophenyl-beta-D-Galactopyranoside.

OXA : Oxacillinase.

PCR : Polymerase Chain Reaction.

PIA : Pasteur Institute of Algeria.

PWW : Poultry Wastewater.

RHA : Rhamnose.

S : Streptomycin.

SAC : Sucrose.

SHV : Sulfhydryl Variable β-Lactamase.

SOR : Sorbitol.

SP : Spiramycin.

SXT : Sulfamethoxazole-Trimethoprim.

TDA : Tryptophan Deaminase.

TEM : Temoneira β-Lactamase.

TOB : Tobramycin.

TTC : Ticarcillin-Clavulanate.

URE : Urease.

UV : Ultraviolet.

VIM : Verona Integron-encoded Metallo-β-Lactamase.

VP : Voges-Proskauer Test.

WHO : World Health Organization.

WWTP : Wastewater Treatment Plant.

Introduction

Introduction

The 1928 discovery of penicillin was a groundbreaking event in biology and medicine **(Bennett & Chung, 2001)**. However, by the 1940s, the first signs of penicillin resistance began to emerge among bacteria **(Knowles, 1985)**. Today, antimicrobial resistance (AMR) poses a major health challenge **(Marston *et al*., 2016)**. According to the Centers for Disease Control and Prevention (CDC), 23000 deaths each year are a direct result of infections by resistant agents; the number is even higher in Europe with 25000 deaths per year **(Brinkac *et al*., 2017)**.

In 2017, the World Health Organization (WHO) published a list of global priority pathogens (GPP), including 12 species divided in 3 tiers: critical, high and medium antibiotic resistance levels. *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and the group of *Enterobacteriaceae*, all categorized as critical pathogens, represent an absolute priority for antibiotic development strategies with the rise of resistance to carbapenems and β-lactams **(Asokan *et al*., 2019)**. Worldwide, the most used antibiotics are those belonging to the β-lactams family **(Rodriguez Villalobos & Struelens, 2006)**, however, we counted 6971 β-lactamases enzymes conferring resistance to bacteria on December, 2020, especially among Gram-negative (GN) bacteria **(Oumeima *et al*., 2022)**.

Particular attention is paid to Extended-Spectrum β-Lactamases (ESBLs), as one of the most relevant resistance mechanisms in this group **(Chenouf *et al*., 2021)**. These enzymes are responsible for resistance by hydrolyzing penicillins, cephalosporins and aztreonam **(Zenati *et al*., 2019)**, and are encoded by genes located in plasmids **(Bariz *et al*., 2019)**. The most detected types of ESBLs were TEM and SHV types until 1989 **(Labid *et al*., 2014)**. In recent years, the CTX-M type became the most common **(Gharout-Sait *et al*., 2012)**. CTX-M β-Lactamases encoded by $bla_{\text{CTX-M}}$ genes are classified into 6 groups: CTX-M-1, CTX-M 2, CTXM-8, CTX-M-9, CTX-M-25 and CTX-M-45; they guarantee a high resistance to cefotaxime **(Bariz *et al*., 2019)**. CTX-M 15 β-Lactamases belonging to the CTX-M-1 group first known in 2001 are the most frequently detected worldwide **(Nouria *et al*., 2016)**.They have been reported for the first time in Algeria in 2005 **(Touati *et al*., 2006)**.Thereafter, it was reported in distinct environments.

Even tough carbapenems are very effective antibiotics against most ESBL-producing bacteria, resistance is rising not only by reduced cell membrane permeability combined with the overproduction of AmpC β-lactamases or ESBLs, but also by the production of carbapenemases (CRB), another variety of β-Lactamases **(Schaffarczyk *et al*., 2024)**. CRB capable to hydrolyze carbapenems are classified in 3 classes according to Ambler classification:

Ambler class A enzymes, such as *Klebsiella pneumoniae (K. pneumoniae)* carbapenemase (KPC) , Ambler class B or metallo-β-lactamases (MBLs), including Verona integron-borne metallo-β-lactamase (VIM), New Delhi metallo-β-lactamase (NDM), imipenemase-type metallo-β-lactamase (IMP), and Ambler class D enzymes, such as OXA-48-like enzymes in *Klebsiella pneumoniae* **(Lade *et al*., 2023)**. At the genetic level, genes encoding CRB spread rapidly due to the variety of mobile genetic elements (MGEs) carrying them such as plasmids, integrons and transposons **(Schultsz & Geerlings, 2012)**. ESBL- and/or CRB-producing GNB are known to be multi-drug resistant (MDR), thus their presence represents a serious global threat as it limits the therapeutic options for treatment of infections.

On the other hand, wastewater from different environmental sources is considered a hotspot niche of MDR and/or pathogenic bacteria **(Kumar & Pal, 2018** ;**Korzeniewska *et al*., 2013)**. Therefore, the aim of this study is to assess the prevalence and the antimicrobial resistance patterns of ESBL- and CRB-GNB in wastewater samples collected from distinct wastewater discharge sources located in Bordj Bou Arreridj city (BBA), and to characterize the molecular features of selected ESBL-GNB.

Materials & Methods

1. Sampling

1.1. Sample sources

As illustrated in table 1 and figures 1 and 2, fourteen wastewater samples were drawn from four wastewater discharge sources located in BBA city: domestic wastewater (DWW), hospital wastewater (HWW), poultry house wastewater (PWW) and Wastewater Treatment Plants (WWTPs), during the period from February 11 to May 2024. The proportion of each sample type is given in figure 3.

Table 1: Distribution of wastewater samples (in number)

Sample source	Description /location	Sampling date	Number of samples
DWW	Residential houses in BBA (n=2)	February 11, 2024	3
HWW	General effluent of Bouzidi Lakhdar hospital in BBA (n=1)	February 26, 2024	2
PWW	Poultry house in Ch'fa, El-Khelil, BBA (n=1)	March 7, 2024	3
WWTP	Collection basin of WWTP of BBA	April 14, 2024	3
	Collection basin of WWTP of Ain Zada	May 5, 2024	3

Figure 1: DWW sampling

Figure 2: WWTP Sampling

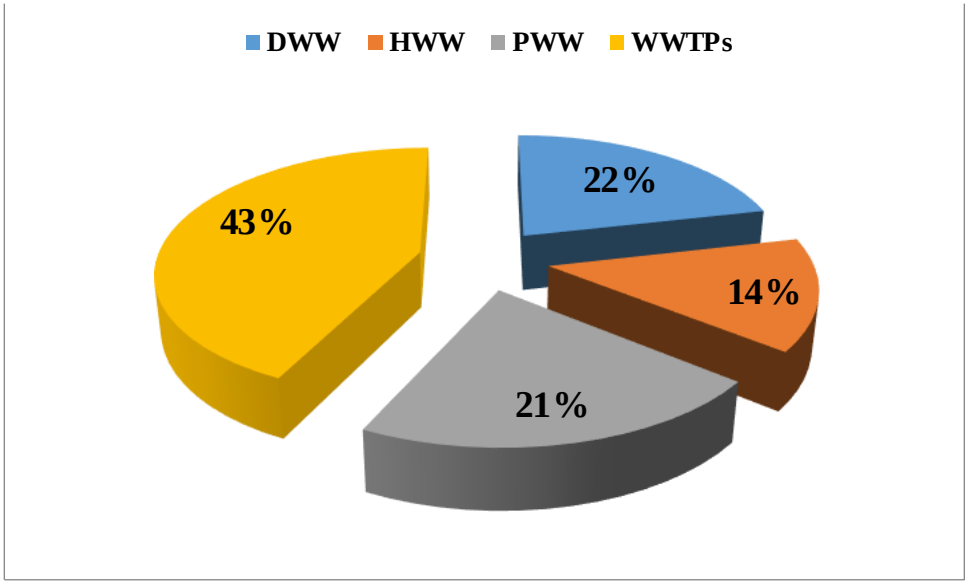

Figure 3: Distribution of wastewater samples (in percentage)

1.2. Sampling methods

1.2.1. Equipment used

Small sterile 50ml-containers were used and a special sampling 4m-rod was designed for sampling, as shown in figure 4. Wastewater samples were carefully collected and then immediately transported to the laboratory of the Department in a 4°C cooler.

Figure 4: Sampling equipment

1.3.2. Ethical and safety considerations

1.3.2.1. Permissions

Necessary permissions were obtained from competent authorities.

1.3.2.2. Safety measures

Appropriate personal protective equipment was worn during sampling.

2. Enrichment

In order to promote the growth of bacteria in wastewater samples (figures 5 and 6), enrichment was performed by transferring 1ml of each wastewater sample using a micropipette in test tubes containing 5ml of Nutrient Broth (NB). The tubes were then incubated overnight at 37°C.

Figure 5: Wastewater samples

Figure 6: Enrichment steps

3. Isolation

In order to reduce the bacterial charge and promote the growth of ESBL- and CRB-GNB, the enriched cultures were streaked onto selective media obtained by adding Cefotaxime with a concentration of 2µg/ml **(Hassen *et al*., 2020)** and Imipenem with a concentration of 2µg/ml **(Moran-Gilad *et al*., 2014)** to Mac Conkey/Hektoen media (figure 7). The T-streaking method was used in isolation.

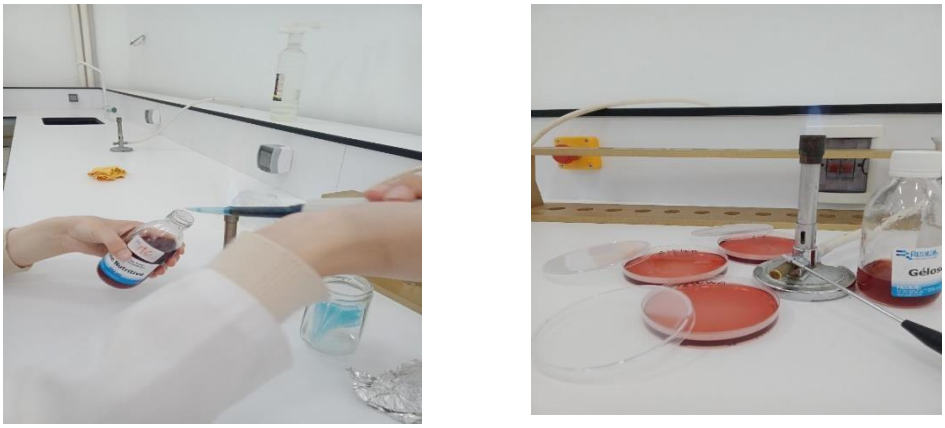

Figure 7: Preparation of selective media supplemented with antibiotics

4. Purification

After isolation, bacterial cultures were obtained with different morphological aspects of colonies. The purpose of purification is to obtain pure cultures of bacterial isolates. For that, selected colonies were streaked onto the same media using the T-streaking method. A re-streaking was needed until obtaining single-type and well-isolated colonies. The purity of the bacterial cultures is confirmed by observing one morphological aspect of colonies (figure 8).

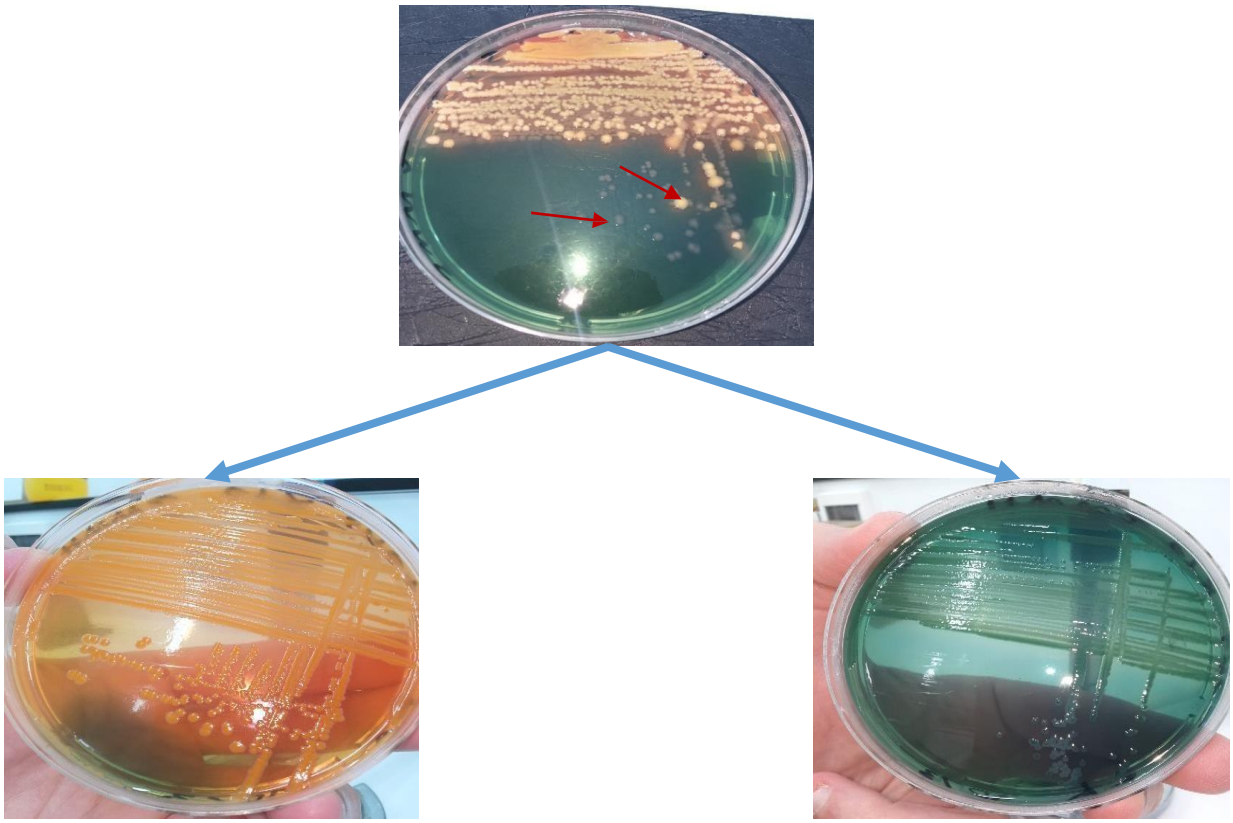

Figure 8: Purification of a bacterial culture

5. Identification

5.1. Macroscopic identification

The aim is to provide initial information about bacterial isolates grown on the selective media based on their morphological characteristics. For this purpose, the color, shape, elevation, margin, texture and all additional observable characteristics of the colonies were noted.

5.2. Microscopic identification (Gram-staining)

Using a sterile platinum loop, a small number of pure colonies is transferred on a clean glass slide and mixed with a drop of sterile water in order to prepare bacterial smears. As shown in the figure below, several stains were used **(Coico, 2006)**. Subsequently, Gram reaction, color, shape and arrangement of bacteria were noted.

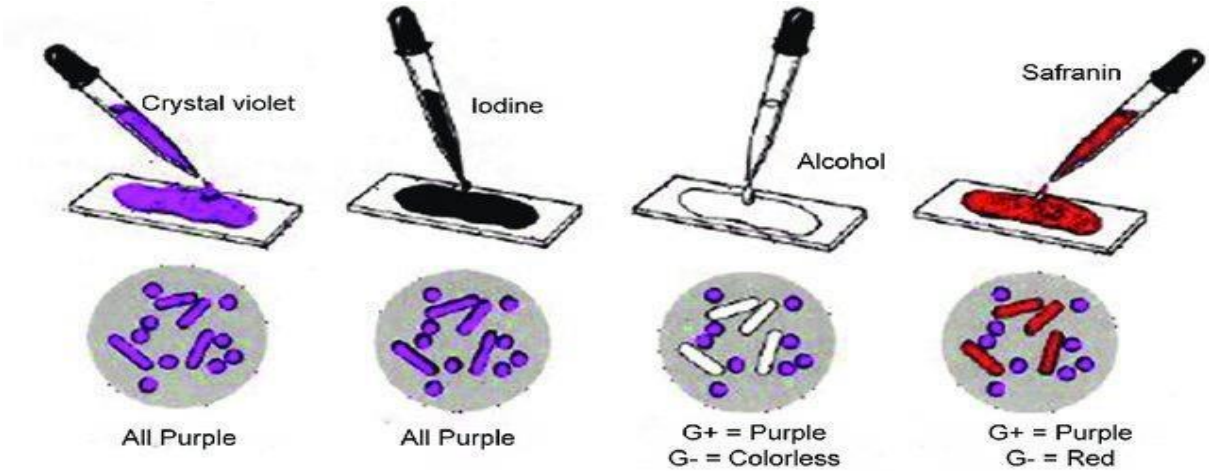

Figure 9: Gram-staining procedure **(Mubarak et al., 2017)**

5.3. Biochemical identification

5.3.1. Oxidase test

The oxidase test is based on the determination of the presence or not of cytochrome c oxidase enzyme in bacterial isolates.

Using a sterile platinum loop, a small amount of pure colonies is transferred on an oxidase test disc (figure 10). Subsequently, the color change is observed within 10-30 seconds, and two results are possible: a blue color indicates a positive result, while no color change or light color indicates a negative result **(Chavan *et al*., 2022)**.

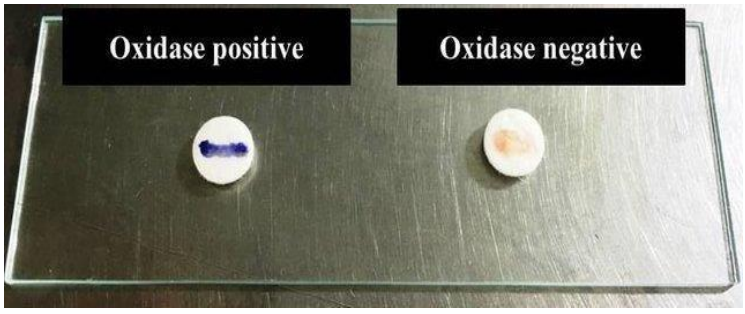

Figure 10: Oxidase test **(Sarangan et al., 2016)**

5.3.2. API 20E System

API 20 E is an identification system for *Enterobacteriaceae* and other GNB based on 21 biochemical tests that allow the determination of the bacterial strain. The principle of this system is based on the inoculation of the prepared bacterial suspension into 20 microtubes

containing 20 dehydrated substrates: ONPG, ADH, LDC, ODC, CIT, H2S, URE, TDA, IND, VP, GEL, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA (figure 11).

The preparation of the 0.5 Mac Farland Units suspension is performed by taking a few colonies from a 24h pure culture in 5 ml of sterile 0.9% sodium chloride solution. Afterwards, it was introduced into the tubes using a sterile syringe. For the CIT, VIP, and GEL tests, both parts (tube and cupule) are filled. For the remaining tests, only the tube is filled. As for the ADH, LDC, ODC, H2S, and URE tests paraffin oil was added in the cupule to create anaerobiosis.

After incubation at 36°C ± 2°C for 18-24 hours, the reagents KOVACS, TDA, VP1, and VP2 are added. The results are then read by referring to the reading table. Identification is obtained from the numerical profile using the Apiweb database.

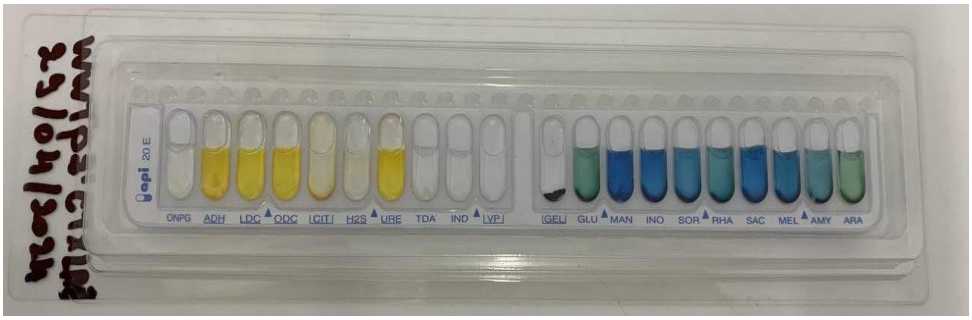

Figure 11: Inoculated API 20 E strip

6. Antibiogramme

6.1. Antimicrobial susceptibility testing

Antimicrobial susceptibility was assessed using the disc diffusion method on Mueller-Hinton agar plates **(Bauer *et al.*, 1966)**. The antimicrobial agents used and their concentration in µg/disc are the following: ticarcillin-clavulanic acid (TTC) (75-10 µg), amoxicillin- clavulanic acid (AMC) (20-10 µg), cefotaxime (CTX) (30 µg), ceftazidime (CAZ) (30 µg), cefepime (FEP) (30 µg), aztreonam (ATM) (30 µg), ertapenem (ETP) (10 µg), meropenem (MRP) (10 µg), imipenem (IMP) (10µg), gentamicin (GEN) (10 µg), tobramycin (TOB) (10 µg), amikacin (AK) (30 µg), kanamycin (K) (30 µg), streptomycin (S) (10 µg), spiramycin (SP) (30 µg), ciprofloxacin (CIP) (5 µg), chloramphenicol (CHL) (30 µg), trimethoprime- sulfamethoxazole (SXT) (1.25-23.75 µg). The results obtained were interpreted according to CLSI guidelines.

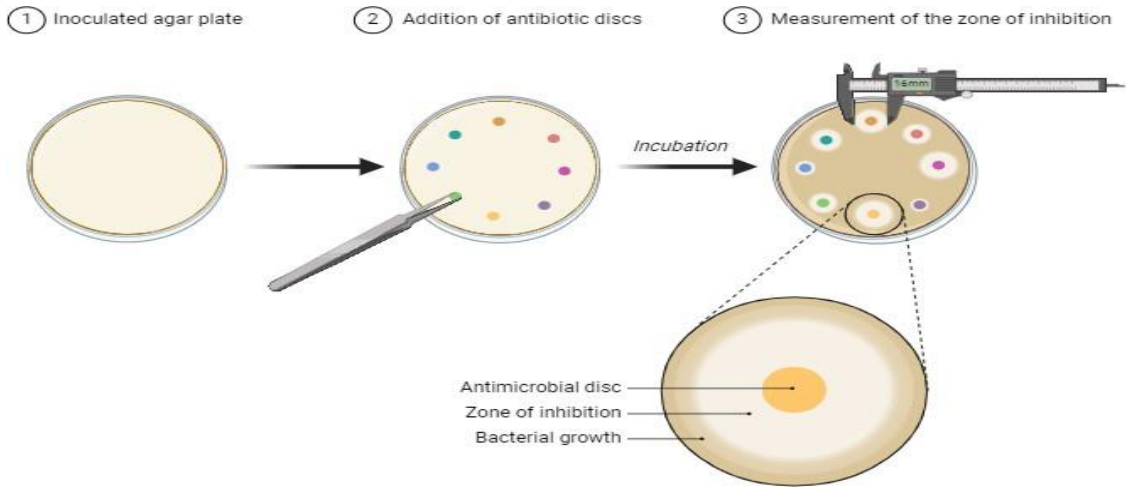

Figure 12: Disc diffusion method**(created by biorender.com based on the CDC)**

6.2. Complementary tests

6.2.1. ESBL phenotypic detection

6.2.1.1 Double-disc synergy test (DDST)

The test was conducted according to **Jarlier *et al*. (1988)**, where third-generation cephalosporin (3GC) discs Cefotaxime (CTX) (30 µg), Ceftazidime (CAZ) (30 µg) and Aztreonam (ATM) (30 µg) were placed 20 mm from a central Amoxicillin-clavulanic acid disc (AMC) (20-10 µg). The formation of a typical champagne-cork-like image between the central disc and one of the third-generation cephalosporin discs after incubation at 37°C for 24h was considered evidence of ESBL production by the bacterial strain (figure 13).

As for Pseudomonas strains, the detection of ESBL production was performed according to the protocol of the **Pasteur Institute of Algeria (PIA, 2020)**, in which the central AMC disc was replaced by a Ticarcillin-clavulanic acid disc (TTC) (75-10 µg).

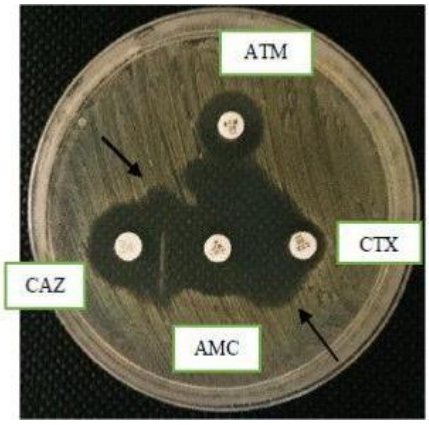

Figure 13: A positive DDST of an ESBL-producing strain **(Effendi & Witaningrum, 2021)**

6.2.1.2. Double-disc test (DDT)

An AMC disc is placed at a distance of 30 mm from a 3GC disc (CTX or CAZ). After inoculation with the strain to be tested, the discs are left to diffuse on MH (figure 14). After 1 hour, the AMC disc is replaced by a 3GC disc, and the plate is incubated at 35°C for 16-18 hours. An inhibition diameter of ≥5 mm between the second 3GC disc and the original 3GC disc is considered a positive result **(PIA, 2020)**.

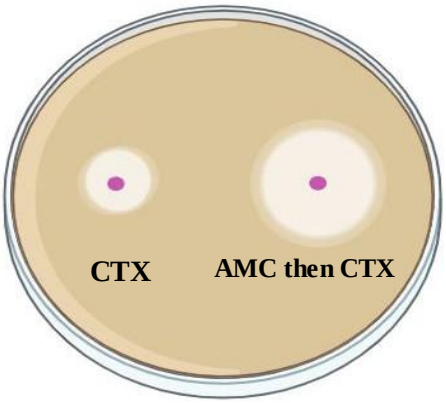

Figure 14: A double disc test for ESBL confirmation **(created by biorender.com)**

6.2.2. Carbapenemases phenotypic detection

6.2.2.1. Modified Hodge test (MHT)

MHT is also known as the clover leaf method used for the detection of carbapenemase activity **(Lee *et al.*, 2001; Caliskan-Aydogan & Alocilja, 2023)**. It is based on the inhibition of carbapenem activity against a sensitive indicator strain when in contact with a CRB- strain **(PIA, 2020)**. Consequently, negative and positive controls are needed **(Rao *et al.*, 2019)**.

The test is performed by placing a carbapenem antibiotic disc (Meropenem/Ertapenem) at the center of a plate containing Mueller-Hinton agar, previously inoculated with the reference strain "*Escherichia coli* ATCC 25922" diluted 1/10 from an inoculum with a turbidity of 0.5 McFarland Units. Then, the positive control, negative control (non-diluted standardized inoculum of *Escherichia coli* ATCC 25922), and the tested strain were inoculated in radial streaks from the disc to the edge (figure 15). The plates are incubated at 35°C for 16-20 hours **(PIA, 2020)**.

The presence of a cloverleaf-shaped zone of inhibition near the test organism was interpreted as an indication of carbapenemase production **(Balan, 2013).**

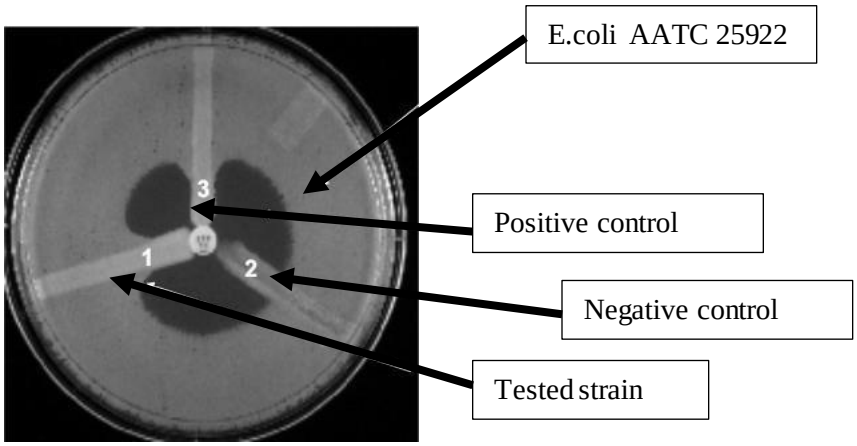

Figure 15: The Modified Hodge Test **(CLSI, 2018)**

6.2.2.2. Modified carbapenem inactivation method (mCIM)

mCIM was introduced by CLSI in 2016 **(Caliskan-Aydogan & Alocilja, 2023)**. The test allows for the evaluation of carbapenemase activity in the strain of interest by measuring the inhibition diameter of the *E. coli* ATCC 25922 strain around a carbapenem disc inactivated by the strain of interest **(Cui *et al.*, 2019)**. The test involves preparing a suspension of the strain to be tested in 2 ml of sterile sodium chloride solution, followed by adding a meropenem disc in this suspension, and incubation was performed at 35°C for 4 hours. Afterwards, the disc is retrieved and placed on the surface of a MH agar plate previously inoculated with *E. coli* ATCC 25922, with the density adjusted to 0.5 McFarland Units. The results were interpreted after incubation at 35°C for 18-24 hours **(PIA, 2020).**

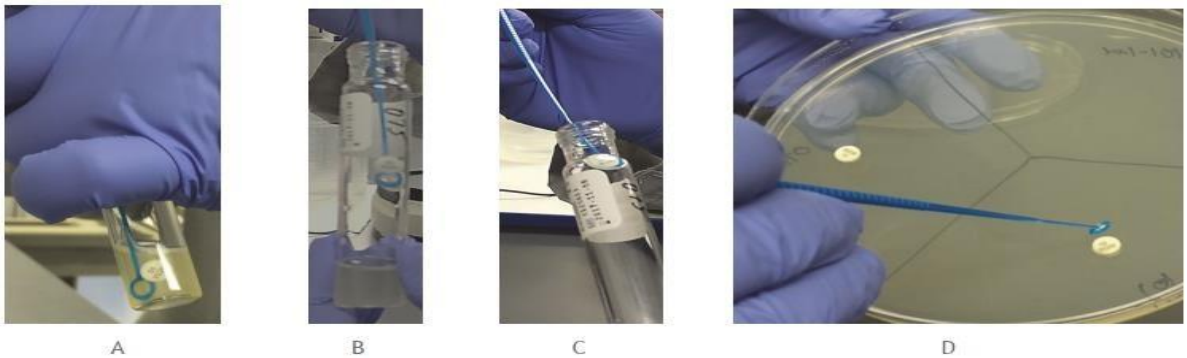

Figure 16: Protocol for applying meropenem discs for the mCIM **(CLSI, 2021)**

For test validation, it is preferable to use the meropenem discs immerged in suspensions of CRB-strains (positive control) and include them in the plate **(PIA, 2020).** Four CRB-GNB strains were included in the test as positive controls: OXA-48-*E.coli*, *E.coli*-VIM-1, KPC-3- *Klebsiella pneumoniae* and IMP-1-*Pseudomonas aeruginosa*. These strains have been provided by the Laboratory of Microbial Ecology of the university of Bejaia (figure 17).

Figure 17: Carbapenemases-producing strains used (positive control)

7. Molecular characterization

Molecular characterization of 4 selected ESBL-E was performed by Polymerase Chain Reaction (PCR) at the Gene Life Sciences Laboratory (GLSL) of Sidi Bel Abbes on May 20, 2024. The PCR technique was first introduced by Kary Mullis in 1983 **(Mullis, 1990)** allowing the *in vitro* amplification of a specific DNA sequence, defined by a pair of primers (F: Forward and R: Reverse), to produce a large number of identical copies **(Garibyan & Avashia, 2013)**.

According to the laboratory's description, the procedure initially involved the extraction of bacterial DNA by using standard method of DNA extraction. Then, PCR essays were conducted using the primer $bla_{\text{CTX-M}}$ encoding gene (F-CGATGTGCAGTACCAGTAA; R- TTAGTGACCAGAATCAGCGG; amplicon size 585bp). MyCycler (BIO-RAD, USA) thermocycler was used.

The PCR process occurred as follows: Initial denaturation at 95°C for 15 minutes, denaturation at 95°C for 50 seconds, annealing at 52°C for 40 seconds, and extension of 72°C for 1 minute. The amplification was repeated in 40 cycles followed by a final extension at 72°C for 7 minutes (figure 18).

After the PCR reaction, PCR products were electrophoresed on a 1.5% agarose gel in TAE buffer (40mM Tris, 20 mM Acetic Acid et 1mM EDTA) at pH 8), and the gel was visualized under UV light after staining with ROTI® red gel stain and observed with a UV trans- illuminator.

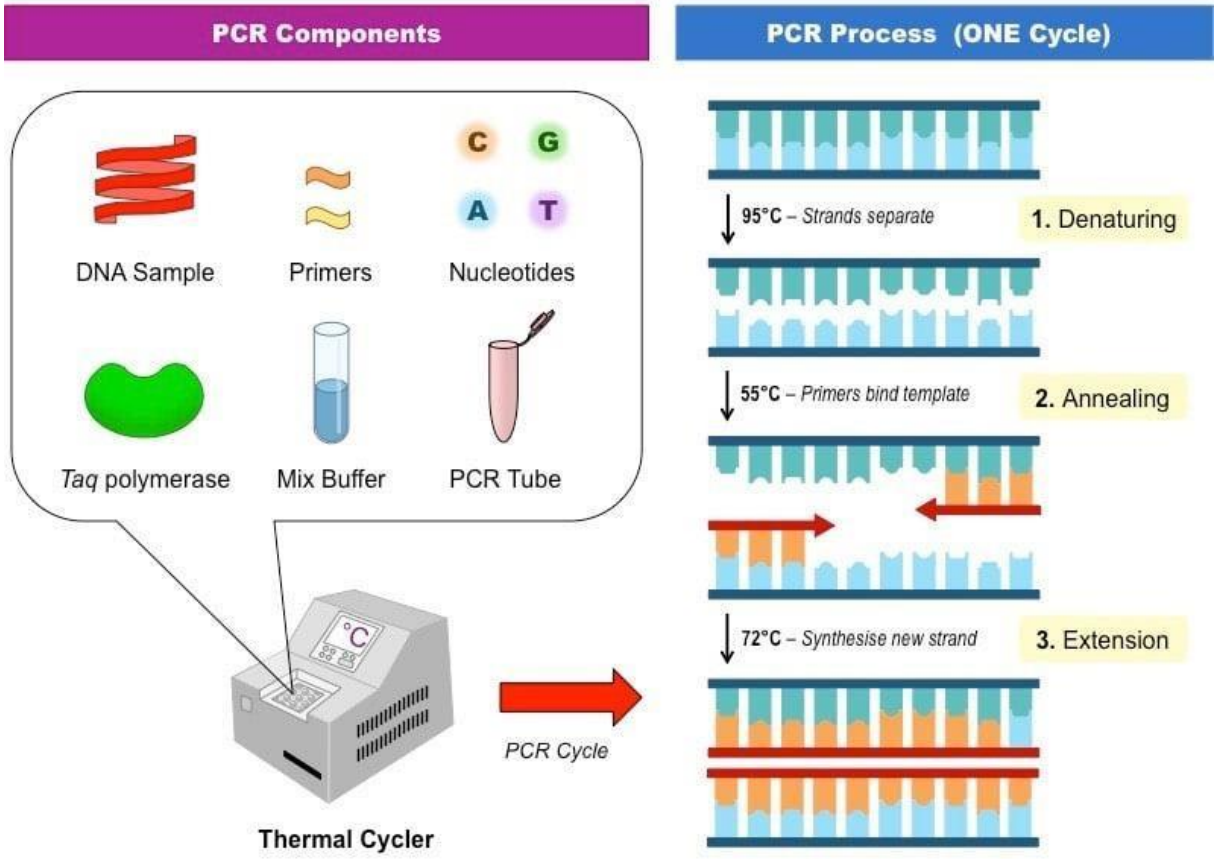

Figure 18: The PCR process **(From biochemistrybasics.com)**

Results
&
Discussion

I- Results

1. Isolation frequency of cefotaxime-resistant GNB (CTX^R-GNB)

As shown in the figure below, out of 14 wastewater samples inoculated on selective media supplemented with CTX, 12 (86%) harbored CTX^R-GNB isolates. The remaining 2 negative samples (14%) originated from DWW and PWW sources.

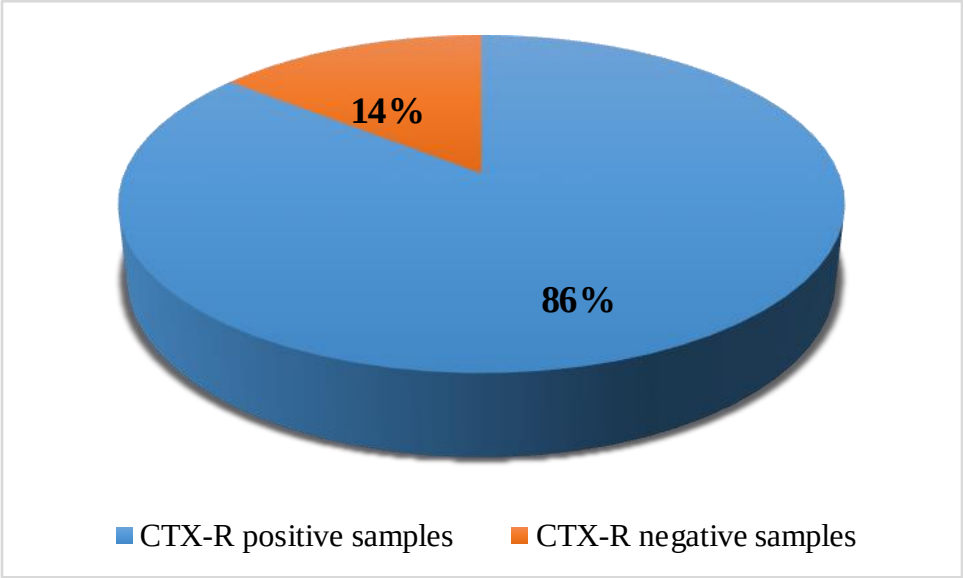

Figure 19: Isolation frequency of CTX^R-GNB

2. CTX^R-GNB Recovery

Overall, 30 CTX^R-GNB strains were recovered from 12 positive samples. The distribution and number of CTX^R-GNB isolates per sample type are given in table 2.

Table 2: Distribution of CTX^R-GNB strains per wastewater sample

Sample type	DWW	HWW	PWW	WWTP 1	WWTP 2	Total
Positive samples	**2/3**	**2/2**	**2/3**	**3/3**	**3/3**	**12**
Strains number	4	4	3	11	8	30
Rate	13%	13%	10%	37%	27%	100%

DWW: Domestic Wastewater, **HWW:** Hospital Wastewater, **PWW:** Poultry Wastewater, **WWTP:** Wastewater treatment plants

3. Prevalence of ESBL-GNB

Among the 30 CTX^R-GNB, 11 were confirmed to be ESBL producers, which indicates a prevalence of 37% of total isolates (figure 20). As illustrated in figure 21, they ESBL-GNB isolates were assigned to 3 different species belonging to the *Enterobacteriaceae* family (ESBL-E): *K. pneumoniae* (6 strains), *E. coli* (4 strains), and *Citrobacter freundii* (1 strain). In contrast, all *Pseudomonas aeruginosa* strains were negative to the synergy test.

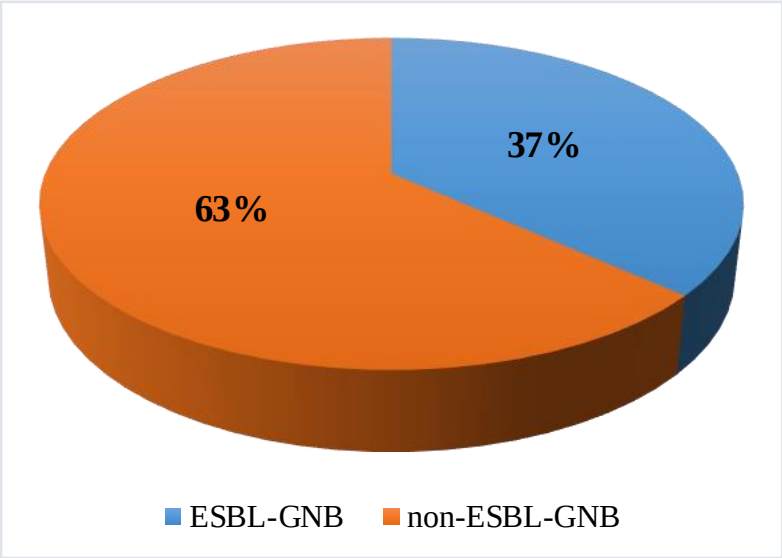

Figure 20: Prevalence of ESBL-GNB

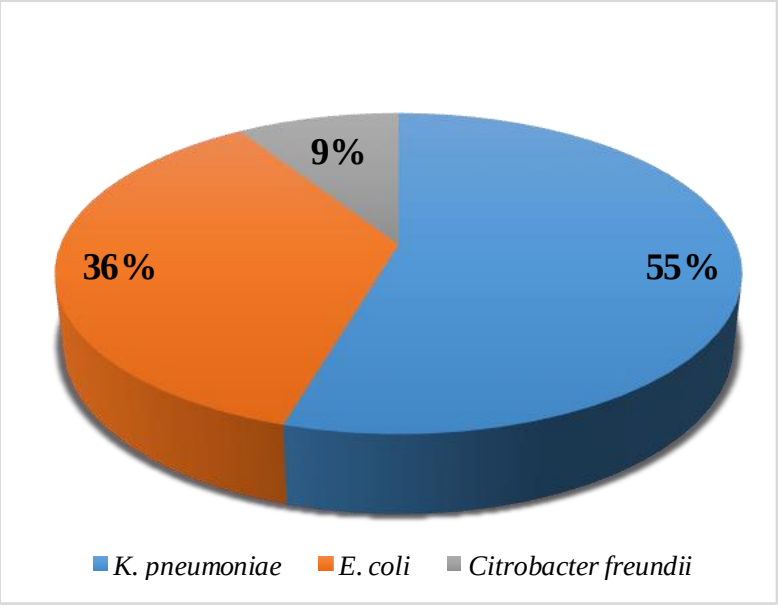

Figure 21: Distribution of ESBL-E strains

The synergy test results of the different ESBL-E recovered are shown in the figure below:

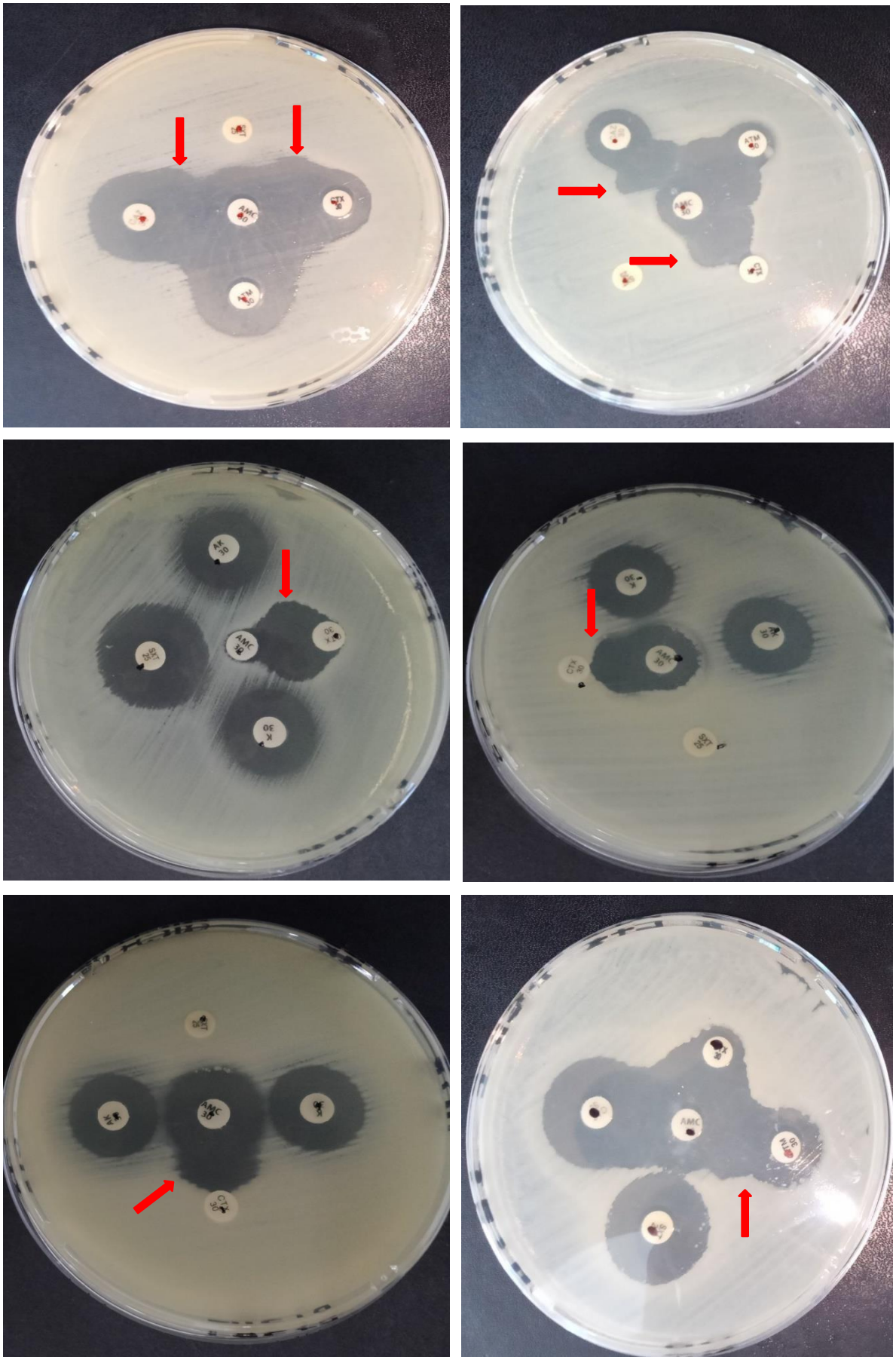

Figure 22: Positive synergy tests detected in ESBL-E

The different results of the macroscopic and microscopic identification are shown in the figures below:

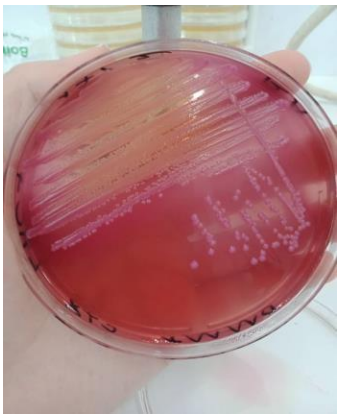

Figure 23: Lactose-positive colonies on MacConkey Agar

Figure 24: Lactose-negative colonies on MacConkey Agar

Figure 25: Lactose-positive GNB on Hektoen Agar

Figure 26: Lactose-negative colonies on Hektoen Agar

Figure 27: E. coli colonies on MacConkey Agar

Figure 28: K. pneumoniae colonies on MacConkey Agar

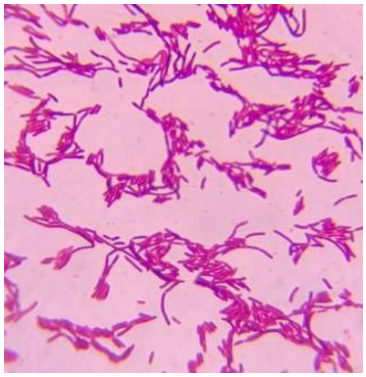

Figure 29: Gram-negative bacilli under light microscope (x1500)

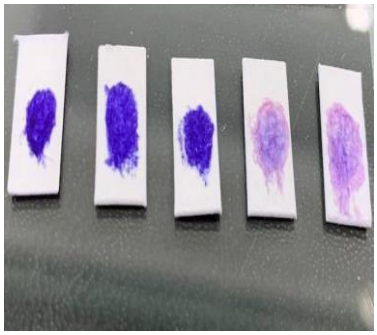

Figure 30: Oxydase test results

The different results of the API 20 E identification of ESBL-GNB are summarized in the figure below:

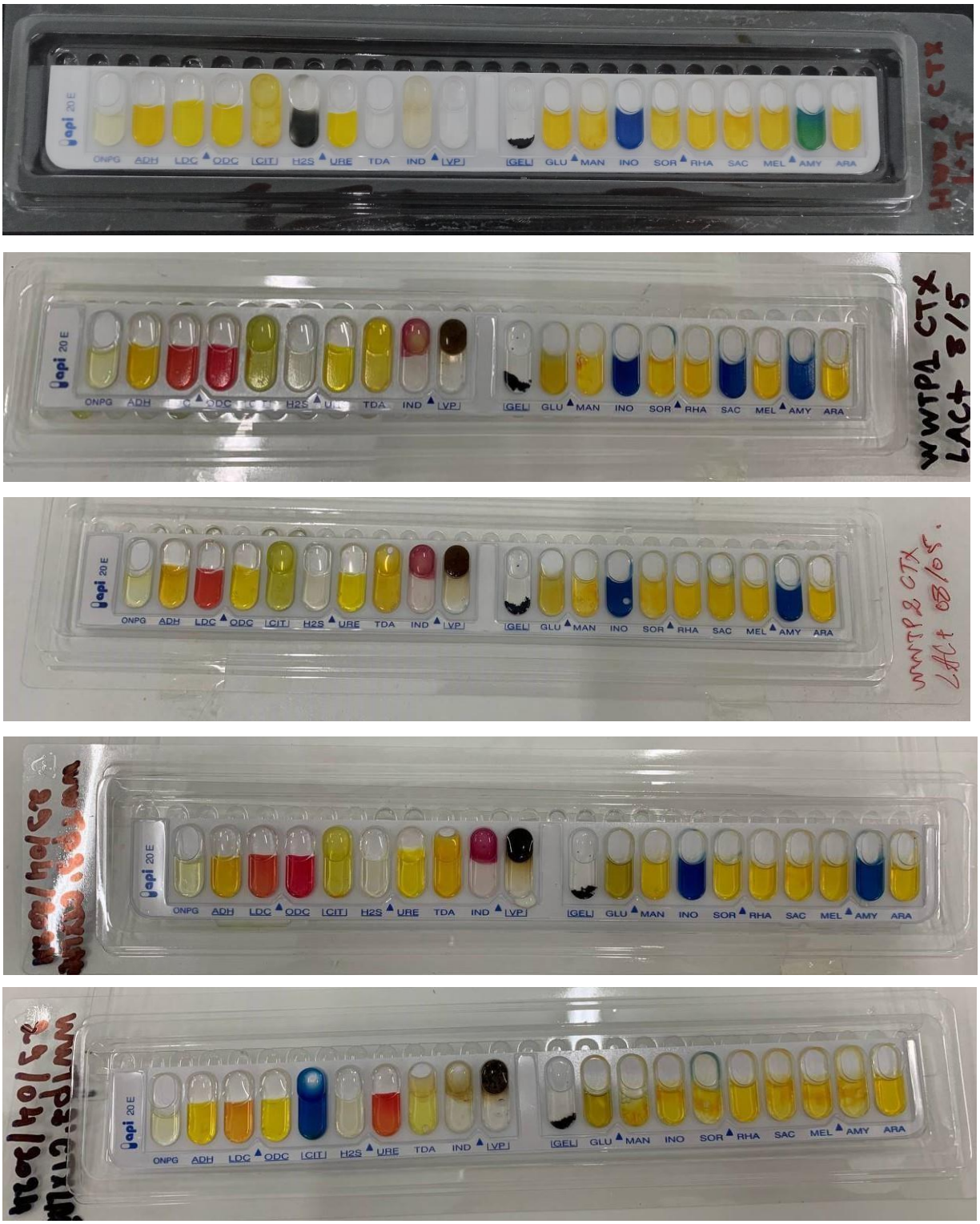

Figure 31: Biochemical identification of ESBL-E using the API 20E system

4. Antimicrobial resistance patterns of ESBL-E

The antimicrobial susceptibility testing performed using the disk diffusion method according to the guidelines of CLSI (2020) showed high resistance levels to cefotaxime (100%), trimethoprim-sulfamethoxazole (73%), aztreonam (64%). Moderate resistance was observed towards ceftazidime and ciprofloxacin (45%), while low resistance rates were noted to amoxicillin-clavulanic acid (27%), amikacin and kanamycin (18%), and tobramycin (9%). In contrast, all ESBL-GNB were susceptible to gentamicin, imipenem, ertapenem, meropenem, spiramycin, streptomycin, cotrimoxazole and chloramphenicol. The antimicrobial resistance patterns of the 11 ESBL-E strains are outlined in table 3.

On the other hand, 5 ESBL-E displayed a multi-drug resistant (MDR) phenotype, defined as resistance to ≥3 drug classes (45%).

Table 3: Antimicrobial resistance patterns of ESBL-E

Strain code	Species	Antimicrobial resistance pattern
HWW1/CTX/LAC+	*K. pneumoniae*	CTX-CAZ-ATM-SXT-AK-K (*)
HWW2/CTX/LAC+J	*C. freundii*	AMC-CTX-CAZ-ATM-SXT-AK-K (*)
WWTP1/CTX/ LAC+	*E. coli*	CTX-ATM-CIP
WWTP2/CTX/LAC+	*E. coli*	CTX-SXT
WWTP3'/CTX/LAC+	*E. coli*	AMC-CTX-CAZ-ATM-SXT-CIP-TOB (*)
WWTP3/CTX/LAC+	*K. pneumoniae*	CTX-CAZ-ATM-SXT-CIP (*)
WWTP5/CTX/LAC+	*E. coli*	CTX-AMC
WWTP1'/CTX/LAC+	*K. pneumoniae*	CTX-ATM-CIP
WWTP2'/CTX/LAC+	*K.pneumoniae*	CTX-CAZ-ATM-SXT
WWTP5/CTX/LAC+J	*K. pneumoniae*	CTX-SXT
WWTP6/CTX/LAC+	*K. pneumoniae*	CTX-SXT-CIP (*)

HWW: Hospital Wastewater, **WWTP:** Wastewater treatment plant, **AMC:** Amoxicillin-clavulanic acid, **CTX:** cefotaxime, **CAZ:** ceftazidime, **SXT:** Trimethoprime-sulfamethoxazole, **ATM:** Aztreonam, **COT:** Cotrimoxazole , **C:** Chloramphenicol, **CIP**: Ciprofloxacin, **TOB**: Tobramycin, **K:** Kanamycin**, AK**: Amikacin, **LAC+**: lactose-positive GNB, **LAC-**: lactose-negative GNB, **(*):** multi-drug resistant (MDR) phenotype

5. Antimicrobial resistance patterns of CTX^R non-ESBL-GNB

Regarding the 19 remaining CTX^R non-ESBL-GNB, the highest resistance rates were noticed against cefotaxime and trimethoprim-sulfamethoxazole (58%), amoxicillin-clavulanic acid (42%), ceftazidime, ciprofloxacin, aztreonam and chloramphenicol (21%). Interestingly, 2 strains exhibited resistance towards imipenem and kanamycin while only one strain was

resistant to amikacin and streptomycin. Conversely, 7 strains were susceptible to all antibiotics tested. Importantly, 7 strains were MDR (37%) (table 4).

Table 4: Antimicrobial resistance patterns of CTX^R non-ESBL-GNB

Number of strains	Strain Code	Antimicrobial resistance pattern
1	HWW2/CTX/LAC+S	AMC-CTX-CAZ-ATM-SXT-K (*)
1	HWW1/CTX/LAC-	AMC-CTX-ATM-SXT-AK-K-S (*)
1	PWW1/CTX/LAC-g	AMC-SXT-IMI-C-CIP (*)
1	PWW1/CTX/LAC-	AMC-CTX-CAZ-SXT-IMI-C-CIP (*)
1	WWTP4/CTX/LAC+	CTX-SXT
1	WWTP3/CTX/LAC-	CTX-ATM-SXT-C (*)
1	WWTP3'/CTX/LAC-	AMC-CTX-ATM-SXT-C (*)
1	WWTP2''/CTX/LAC+	AMC-CTX-CAZ-CIP
1	WWTP2/CTX/LAC-	CTX-SXT
1	WWTP1''/CTX/LAC+	AMC-CTX-CAZ-SXT-CIP (*)
1	WWTP4/CTX/LAC+	AMC-CTX-SXT
1	WWTP6/CTX/LAC+	CTX-SXT
7	/	Susceptible to all antibiotics

HWW: Hospital Wastewater, **PWW**: Poultry Wastewater, **WWTP**: Wastewater treatment plant, **AMC**: Amoxicillin-clavulanic acid, **CTX**: cefotaxime, **CAZ**: ceftazidime, **SXT**: Trimethoprime-sulfamethoxazole, **ATM**: Aztreonam,, **C:** Chloramphenicol, **CIP**: Ciprofloxacin, **K:** Kanamycin, **AK**: Amikacin **S:** Streptomycin, **IMI:** Imipenem, **LAC+**: lactose-positive GNB, **LAC-**: lactose-negative GNB, **(*):** multi-drug resistant (MDR) phenotype

6. Molecular characterization of ESBL-E strains

Results of the PCR/electrophoresis essays showed the detection of the *bla_{CTX-M}* encoding gene in the 4 selected ESBL-E (table 5 and figure 31).

Table 5: The selected ESBL-E strains for bla_{CTX-M} investigation

PCR's code	Original code	ESBL-E
RA1	HWW1/CTX/LAC+	*K. pneumoniae*
RA2	HWW2/CTX/LAC+J	*C. freundii*
RA3	WWTP1/CTX/LAC+	*E. coli*
RA4	WWTP3'/CTX/LAC+	*E. coli*

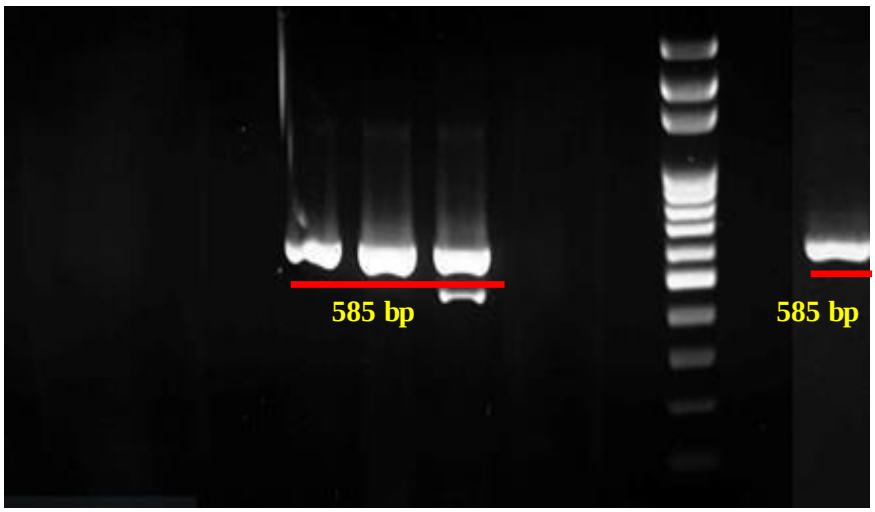

Figure 32: The bla_{CTX-M} PCR electrophoretic profile
Samples: RA3, RA1, RA4, RA2, **M:** 100pb DNA Ladder.

7. Isolation frequency of imipenem-resistant GNB (IMP^R-GNB)

As reported in figure 32, 10 of the 14 wastewater samples inoculated on selective media supplemented with IMP contained IMP^R-GNB isolates (71 %), and 14 IMP^R-GNB isolates were recovered. The distribution and number of IMP^R-GNB isolates per sample type are illustrated in table 6.

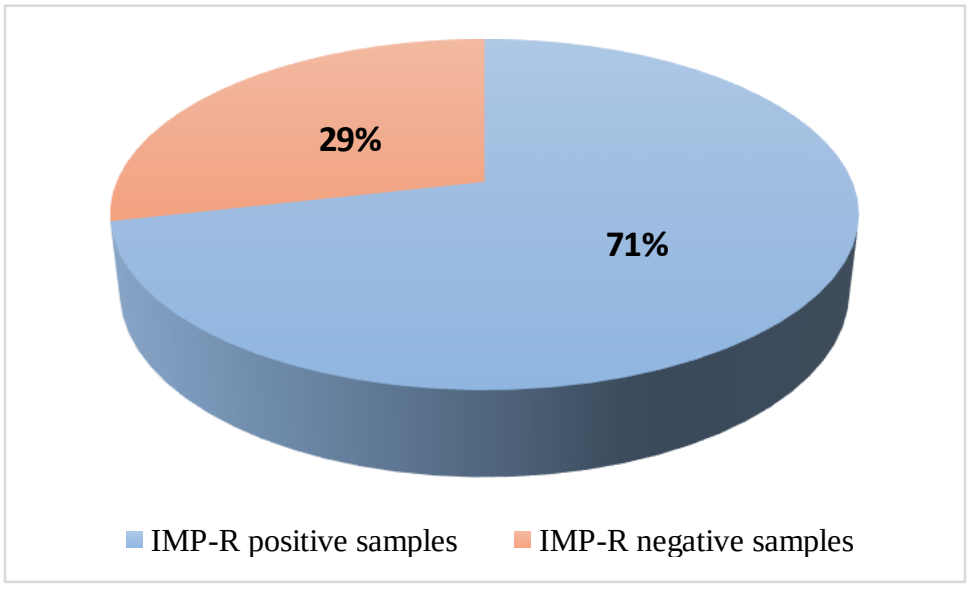

Figure 33: Isolation frequency of CTX^R-GNB

Table 6: Distribution of IMPR-GNB strains per wastewater sample

Sample type	DWW	HWW	PWW	WWTP 1	WWTP 2	Total
Positive samples	**3/3**	**2/2**	**0/3**	**3/3**	**2/3**	**10**
Strains number	5	4	0	3	2	14
Rate	36%	29%	0	21%	14%	100%

DWW: Domestic Wastewater, **HWW:** Hospital Wastewater, **PWW:** Poultry Wastewater, **WWTP:** Wastewater treatment plants.

8. Prevalence of CRB-GNB

Out of the 14 IMP^{R}-GNB strains, one was found to be CRB-GNB using the modified carbapenem inactivation method (7%), as mentioned in figures 33 and 34.

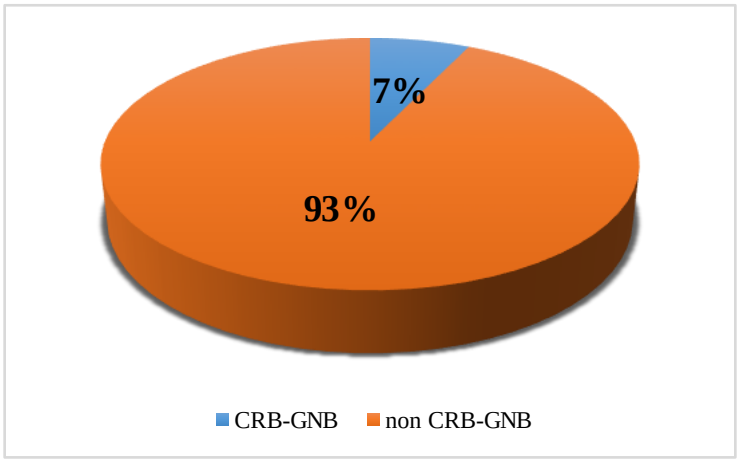

Figure 35: Prevalence of CRB-GNB

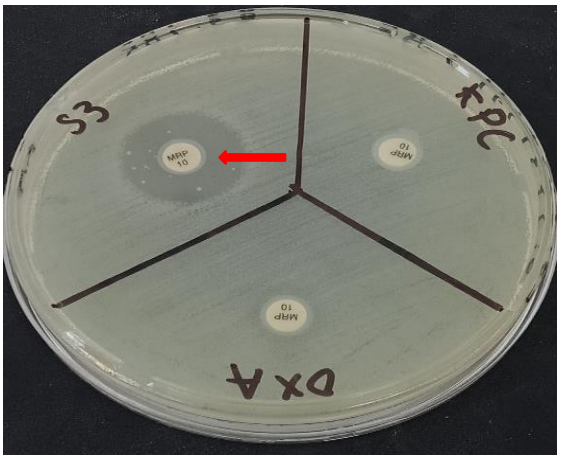

Figure 34: Positive mCIM test (meropenem diameter = 18 mm and the presence of colonies inside the inhibition zone)

It is worth mentioning that the mCIM test conducted on IMP^{R}-GNB was considered undetermined as meropenem diameter was 18 mm with no colonies inside of the inhibition zone (figure 35). As for the Hodge test, all IMP^{R}-GNB tested revealed a negative result (figure 36).

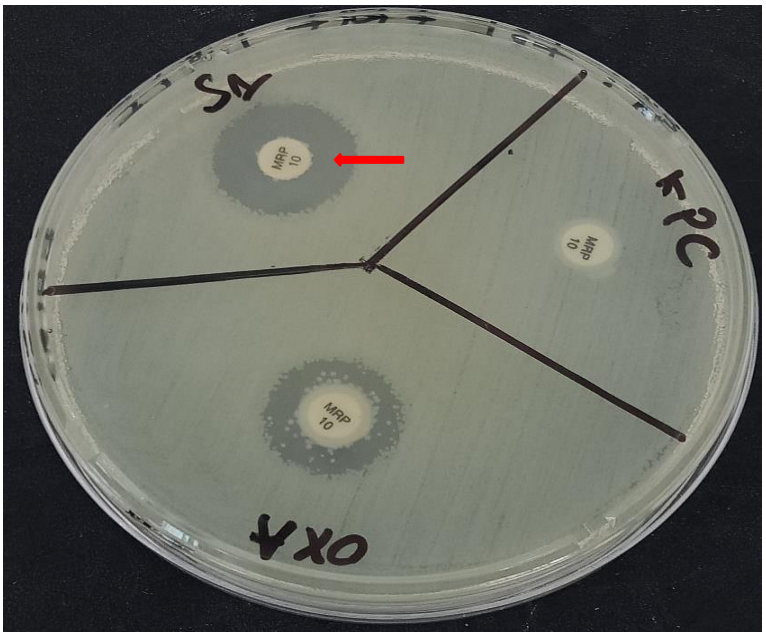

Figure 36: Undetermined mCIM test

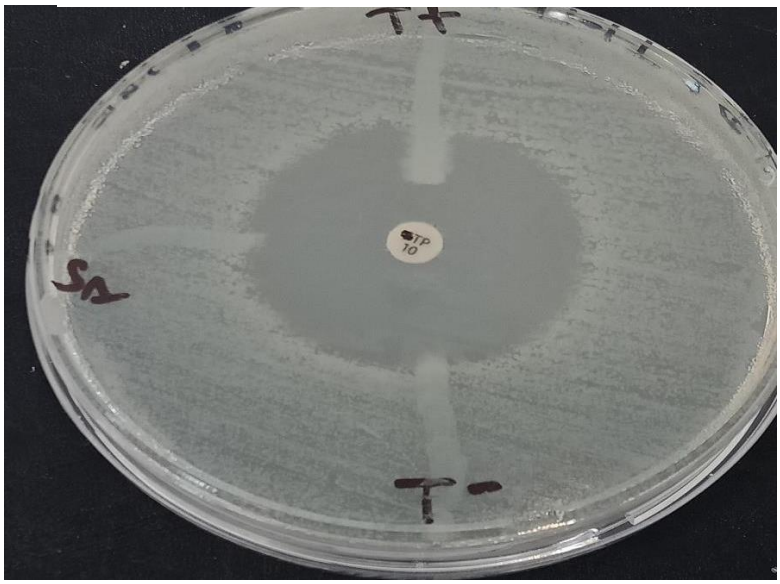

Figure 37: Negative MHT

9. Antimicrobial resistance patterns of IMP^R -GNB strains

Amongst the 14 IMP^R -GNB strains, the highest resistance rates were noticed against trimethoprim-sulfamethoxazole (64%), amoxicillin-clavulanic acid and imipenem (36%) and ciprofloxacin (29%). In contrast, only 2 strains exhibited resistance towards aztreonam, cefotaxime and kanamycin (14%) while only one strain was resistant to cefepime and streptomycin (7% for each). Interestingly, 6 strains were MDR including the CRB-GNB strain that was identified as *K. pneumoniae* using the API 20E system. Moreover, 4 were susceptible to all antibiotics tested (29%).

Table 7: Antimicrobial resistance pattern of IMP^R -CRB strains

Number of strains	Strain code	Antimicrobial resistance pattern	Carbapenemases detection
1	DWW1/IMI/LAC-	AMC-SXT-ATM	-
1	DWW2/IMI/LAC-	FEP-ATM-SXT-S (*)	-
1	DWW2'/IMI/LAC-	SXT	-
1	HWW2/IMI/LAC-	AMC-CTX-SXT-K (*)	-
1	HWW1/IMI/LAC+	SXT-K	-
1	WWTP1/IMI/LAC+H2S+	SXT-IMI-C-CIP (*)	-
1	WWTP1/IMI/LAC-	AMC-SXT-IMI-CIP (*)	-
1	WWTP2/IMI/LAC+	AMC-IMI	Undetermined
1	WWTP1/IMI/LAC+S	AMC-CTX-SXT-IMI-C-CIP (*)	+
1	WWTP2/IMI/LAC+G	IMI-SXT-CIP (*)	-
4	/	Susceptible to all antibiotics	-

DWW: Domestic Wastewater, **HWW:** Hospital Wastewater, **WWTP:** Wastewater treatment plant **AMC:** Amoxicillin- calvulanic acid, **CTX**: cefotaxime, **SXT**: Trimethoprime-sulfamethoxazole, **FEP:** cefepime, **ATM**: Aztreonam, **C:** Chloramphenicol, **IMP:** Imipenem, **CIP:** Ciprofloxacin, **K:** Kanamycin, **LAC+**: lactose-positive GNB, **LAC-**: lactose- negative GNB, **(*):** multi-drug resistant (MDR) phenotype

II- Discussion

Antimicrobial resistance is presently one of the leading causes of death worldwide, and wastewater is regarded as an important driver of resistant bacteria. In this study conducted on 14 wastewater samples collected from different sources in BBA city, 12 were contaminated and 30 CTX^R-GNB strains were recovered (86% of the samples). Our findings are higher than those published by **Hassen *et al*. (2021)** (54%) in wastewater collected in Tunisia. This is likely owing to the huge variation in the total number of samples (only14 samples were assessed in our study against 100 in the Tunisian study).

In WWTPs, elevated isolation frequencies of CTX^R-GNB were noted. Nearly 37% of CTX^R strains were gathered from the main WWTP of BBA and 27% from the second one. This result is not surprising as WWTPs have long been considered hotspots for resistant bacteria, and all wastewaters of BBA city drain into it. As for HWW, the limited number of samples could be the cause of making this source in the second position with 13% while **Anssour *et* al. (2014)** reported a rate of 21%.

In the present study, 37% of the CTX^R-GNB strains were confirmed to be ESBL producers. These values are close to those outlined by **Alouache *et al*. (2014)**, where 40% of the CTX^R strains were found to be ESBL-GNB, while only 28.72% were detected in the study of **Mechai *et al*. (2019)**, and 23.8% in a Tunisian study conducted by **Hassen *et al*. (2020).** The relatively high rates found in our study is a result of the inappropriate usage of antibiotics in both human and veterinary medicines, which leads to the selection of resistant-bacteria. On the other hand, the isolation methods used in the different surveys could also explain these differences.

A variety of pathways can lead to cefotaxime resistance. Enzymatic resistance, ESBLs and Cephalosporinases, for instance, can hydrolyze a wide range of β-lactam antibiotics, including penicillin and cephalosporins. On the other hand, non-enzymatic resistance does not rely on enzymes but may involve other strategies like efflux pumps.

As several studies already reported, including **Dropa *et al*. (2016)**, our study has known a high occurrence of ESBL-E. The predominance of the two resistant *Enterobacteriaceae E.coli* and *K. pneumoniae* highlights their high genomic plasticity and capacity to harbor and transfer the ESBL-encoding genes via the different mobile genetic elements. Additionally, all of the latter were identified by the common synergy test. Conversely, the ESBL-*C.freundii* strain acquired a confirmation procedure via the double-disc test. This may be due to the superior

prevalence of cephalosporinases encoding genes (*bla*$_{AMPc}$) over the ESBL genes (*bla*$_{CTX-M}$) **(Liu *et al*., 2018)**.

All ESBL-E strains were recovered from the WWTPs and HWW samples. These two environments are globally known as relevant reservoirs of resistant bacteria including ESBL-E. Furthermore, the detection of ESBL- producing bacteria in HWW may be a sign of the occurrence of nosocomial infections, as they have been increasingly reported as causative agents worldwide. In general, we can outline that the presence of ESBL-E in wastewater from BBA city constitutes a risk for public health, and consequently, their surveillance in wastewaters is crucial for tackling the antibiotic resistance crisis in the region.

In contrast, no ESBL-E strain was detected in PWW samples collected in Ch'fa region. This could be explained by the antibiotic therapy used in the poultry house during the collection of samples, because of the colibacillosis disease that occurred in the poultry flocks.

No ESBL-producing *Pseudomonas aeruginosa* was revealed in the present study, which is probably due to the high occurrence of efflux pumps as a resistance mechanism to cefotaxime in *Pseudomonas* species **(Khalili *et al*., 2019)**.

As expected, the *bla*$_{CTX-M}$ encoding gene was detected in the 4 selected ESBL-E by PCR essays. These results reinforce the previous background about the global expansion of the blaCTX-M different ecosystems, worldwide. complementary studies including sequencing of the ESBL-E strains and molecular typing are required to better investigate the dissemination and epidemiology of ESBL-E in wastewater of BBA city.

Most of the strains showed a multi-resistant phenotype mainly to: cefotaxime, ceftazidime, aztreonam, trimethoprime-sulfamethoxazole and ciprofloxacin. This is not surprising, given that, most of our isolates exhibited a co-resistance to old drug agents such as β-lactams, sulfonamides and quinolones. Also, it is known that β-lactams are the most used antibiotics worldwide **(Rodriguez-Villalobos & Struelens, 2006)** leading to their presence in wastewater witch are considered reservoirs of MDR bacteria due to the presence of antibiotics that select them and favor their dissemination **(Zagui *et al*., 2020)**. This theory explains the high rate of resistance to β-lactams observed for all samples.

Conversely, all strains were sensitive to meropenem and gentamycin which underscores the significant importance of these antibiotics as an alternative treatment for MDR-GNB infections. These results are not surprising since MRP and GEN are very effective antibiotics against a wide range of GNB **(Bassetti *et al*., 2019; Sharma *et al*., 2024)**.

Isolates of HWW showed high resistance to SXT, K, CTX, CAZ, ATM and AMC. This result is alarming since 87% of isolates were resistant to SXT, a very effective antibiotic against GNB **(Deconinck *et al*., 2019)**.This finding could be explained by frequent antibiotic use since hospitals are settings where antibiotics are heavily employed to treat and prevent infections. Most resistance shown was to antibiotics belonging to β-lactams with 13 resistant strains followed by folate pathway antagonists and aminoglycosides with 7 and 6 strains, respectively.

For GNB isolated from PWW, the high level of resistance noticed towards CIP and SXT is worrying since these 2 antibiotics are very effective against GNB. Moderate resistance (42%-71%) was observed for AMC, C and CTX. Hopefully, low resistance was shown to CAZ and ATM (14%) and no resistance for MRP and GEN (0%). The antibiotic resistance results are logical given the accelerated use of antibiotics in poultry farms, highlighting the crucial need for regulation and control by the competent authorities **(Veloo *et al*., 2022)**. Moreover, the high usage of antibiotics in poultry farms for preventive and/or curative purposes may lead to a strong selective pressure, in addition of a lack of regulation and/or surveillance **(Hedman *et al*., 2020)**.

GNB isolated from both WWTPs showed interesting similarities with complete resistance to CTX (100%), high resistance to SXT, low resistance to C and complete susceptibility to MRP and GEN. The obtained results are similar to those published by other researchers who affirm that diversity of resistance profiles observed throughout the WWTPs is concerning **(Rodríguez *et al*., 2020)**. This result may be attributed to the selection pressure and the very high concentration of waste as it is a mix of domestic, agricultural and industrial wastewater, therefore WWTP can include antimicrobial agents and a variety of bacterial strains **(Karkman *et al*., 2018)**.

In our work, we also investigated the prevalence of CRB-GNB in wastewater in BBA city. The results showed a prevalence of 7% of total IMP^R-GNB strains. This value is lower than the one reported in Batna (79.16%) **(Cherak *et al*., 2021)**, in the United States 80% **(Reinke *et al*., 2020)**, and in China 59.61% **(Zhang *et al*., 2020)**. However, the detection of CRB-E is still worrying since carbapenems (meropenem, imipenem, doripenem, and ertapenem) are used as last resort antibiotics for treating the most severe infections caused by ESBL-E and other MDR bacteria. Excessive carbapenem usage promotes a variety of resistance mechanisms? including carbapenemases that can hydrolyze carbapenems. Carbapenemases production predominates worldwide as the main mechanism of resistance to carbapenems, which represents a significant health risk, as mentioned by **Nasser-Ali *et al*. (2024)**.

During our work, we employed two different techniques to detect carbapenemases. The first technique (mCIM) showed positive result, unlike the second technique (MHT). This discrepancy suggests that the mCIM technique is superior in terms of sensitivity and accuracy, making it a more reliable method for carbapenemase detection **(Shaikh *et al*., 2020)**. Therefore, MHT is no longer considered a reliable phenotypic method for carbapenemase detection; other methods such as the CarbaNP test and the mCIM, are more reliable according to CLSI, and should be further employed for the undetermined strain.

To sum up, we can say that this study revealed the circulation of antimicrobial resistant bacteria (ESBL-E, CRB-E, and MDR-GNB) in wastewater of BBA city. This result is alarming since these bacteria can be easily spread to the environment and be transmitted to human, notably if treatment procedures are not efficient. Thus, we emphasize that wastewater surveillance can be an additional tool for monitoring antimicrobial resistance (AMR) at the population level. In depth surveys should be further designed on more wastewater samples of BBA region, including molecular characterization of ESBL- and/or CRB-GNB strains.

Conclusion

Conclusion

The discovery and use of antibiotics during the early 20th century played a major role in reducing the fatality associated with infections by microorganisms. However, the silent pandemic of antibiotic resistance led to the appearance of MDR-GNB able to resist to a wide range of antibiotics using various mechanisms. Among these mechanisms is the production of β-lactamases able to hydrolyze enzymes of the β-lactams family; two types of enzymes are notable: ESBLs and carbapenemases.

In this study, a total of 14 samples of wastewater from distinct discharge sources (DWW, HWW, PWW and WWTPs) were collected in BBA in order to investigate the prevalence of ESBL-GNB and CRB-GNB, and evaluate their antimicrobial resistance patterns.

Amidst the 14 samples, 12 (86%) harbored CTX^R strains, and 30 CTX^R-GNB strains were recovered, among which 11 (37%) were found to be ESBL-E: *K. pneumoniae* (6 strains), *E. coli* (4 strains), and *Citrobacter freundii* (1 strain). Moreover, the molecular characterization of 4 ESBL-E strains showed the detection of the $bla_{CTX\text{-}M}$ encoding gene. Regarding the antimicrobial resistance patterns, the highest resistance levels were observed against cefotaxime (100%), trimethoprim-sulfamethoxazole (73%) and aztreonam (64%) while complete susceptibility was shown towards gentamicin, imipenem, ertapenem, meropenem, spiramycin, streptomycin, cotrimoxazole and chloramphenicol. We also noted 5 MDR among ESBL-E.

On the other hand, 10 samples (71%) contained IMP^R strains, and 14 IMP^R-GNB strains were collected, among which 1 (7%) was CRB-GNB. Furthermore, in IMP^R-GNB strains, the highest resistance rates were noticed against trimethoprim-sulfamethoxazole (64%), amoxicillin-clavulanic acid and imipenem (36%) and ciprofloxacin (29%) while 4 strains were susceptible to all antibiotics. Six IMP^R-GNB strains displayed a MDR phenotype, including the CRB-E strain identifies as *K. pneumoniae*.

This study revealed the circulation of antimicrobial resistant bacteria (ESBL-E, CRB-E, and MDR-GNB) in wastewater of BBA city. This result is alarming since these bacteria can be easily spread to the environment and then transmitted to human, notably if treatment procedures of wastewater are not efficient. Thus, our findings call for more precautions regarding the anarchic use of antibiotics, which led to the establishment and spread of antibiotic resistance. Raising awareness and establishing strict regulations are crucial. The presence of these antibiotic resistant bacteria in wastewater requires more sophisticated purification systems. Finally, we emphasize that wastewater surveillance can be an additional tool for monitoring

antimicrobial resistance at the population level. New safe biological antimicrobial alternatives are urgently needed.

Bibliographic References

Bibliographic References

Alouache, S., Estepa, V., Messai, Y., Ruiz, E., Torres, C., & Bakour, R. (2014). Characterization of ESBLs and associated quinolone resistance in Escherichia coli and Klebsiella pneumoniae isolates from an urban wastewater treatment plant in Algeria. *Microb Drug Resist*, *20*(1), 30-38. https://doi.org/10.1089/mdr.2012.0264.

Anssour, L., Messai, Y., Derkaoui, M., Alouache, S., Estepa, V., Somalo, S., Torres, C., & Bakour, R. (2014). ESBL, plasmidic AmpC, and associated quinolone resistance determinants in coliforms isolated from hospital effluent: first report of qnrB2, qnrB9, qnrB19, and blaCMY-4 in Algeria. *J Chemother*, *26*(2), 74-79. https://doi.org/10.1179/1973947813y.0000000115.

Asokan, G. V., Ramadhan, T., Ahmed, E., & Sanad, H. (2019). WHO Global Priority Pathogens List: A Bibliometric Analysis of Medline-PubMed for Knowledge Mobilization to Infection Prevention and Control Practices in Bahrain. *Oman Med J*, *34*(3), 184-193. https://doi.org/10.5001/omj.2019.37.

Balan K. Modified Hodge test and remodified Hodge test for carbapenemase detection: Better indicator. Indian J Appl Res. 2013;3:279–80.

Bariz, K., De Mendonça, R., Denis, O., Nonhoff, C., Azzam, A., & Houali, K. (2019). Multidrug resistance of the extended-spectrum beta-lactamase-producing Klebsiella pneumoniae isolated in Tizi-Ouzou (Algeria). *Cellular and Molecular Biology*, *65*(8), 11-17. https://doi.org/10.14715/cmb/2019.65.8.3.

Bassetti, M., Peghin, M., Vena, A., & Giacobbe, D. R. (2019). Treatment of Infections Due to MDR Gram-Negative Bacteria [Review]. *Frontiers in Medicine*, *6*. https://doi.org/10.3389/fmed.2019.00074.

Bauer, A. W., Kirby, W. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol*, *45*(4), 493-496.

Bennett, J. W., & Chung, K.-T. (2001). Alexander Fleming and the discovery of penicillin. In *Advances in Applied Microbiology* (Vol. 49, pp. 163-184). Academic Press. https://doi.org/https://doi.org/10.1016/S0065-2164(01)49013-7.

Brinkac, L., Voorhies, A., Gomez, A., & Nelson, K. E. (2017). The Threat of Antimicrobial Resistance on the Human Microbiome. *Microb Ecol*, *74*(4), 1001-1008. https://doi.org/10.1007/s00248-017-0985-z.

Caliskan-Aydogan, O., & Alocilja, E. C. (2023). A Review of Carbapenem Resistance in Enterobacterales and Its Detection Techniques. *Microorganisms*, *11*(6). https://doi.org/10.3390/microorganisms11061491.

Chavan, D., Khatoon, H., Anokhe, A., & Kalia, V. (2022). Oxidase test: A biochemical method in bacterial identification.

Chenouf, N. S., Carvalho, I., Messaï, C. R., Ruiz-Ripa, L., Mama, O. M., Titouche, Y., Zitouni, A., Hakem, A., & Torres, C. (2021). Extended Spectrum β-Lactamase-Producing Escherichia coli and Klebsiella pneumoniae from Broiler Liver in the Center of Algeria, with Detection of CTX-M-55 and B2/ST131-CTX-M-15 in Escherichia coli. *Microb Drug Resist*, *27*(2), 268-276. https://doi.org/10.1089/mdr.2020.0024.

Cherak, Z., Loucif, L., Moussi, A., Bendjama, E., Benbouza, A., & Rolain, J.-M. (2021). Emergence of Metallo-β-Lactamases and OXA-48 Carbapenemase Producing Gram-Negative Bacteria in Hospital Wastewater in Algeria: A Potential Dissemination Pathway Into the Environment. *Microbial Drug Resistance*, *28*(1), 23-30. https://doi.org/10.1089/mdr.2020.0617.

CLSI (Clinical and Laboratory Standards Institute). 2018. *Performance standards for antimicrobial susceptibility testing*, 28th ed. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI (Clinical and Laboratory Standards Institute). 2021. *Performance standards for antimicrobial susceptibility testing*, 31st ed. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA.

Coico, R. (2006). Gram staining. *Current protocols in microbiology*(1), A. 3C. 1-A. 3C. 2.

Cui, X., Zhang, H., & Du, H. (2019). Carbapenemases in Enterobacteriaceae: Detection and Antimicrobial Therapy. *Front Microbiol*, *10*, 1823. https://doi.org/10.3389/fmicb.2019.01823.

Deconinck, L., Dinh, A., Nich, C., Tritz, T., Matt, M., Senard, O., Bessis, S., Bauer, T., Rottman, M., Salomon, J., Bouchand, F., & Davido, B. (2019). Efficacy of cotrimoxazole (Sulfamethoxazole-Trimethoprim) as a salvage therapy for the treatment of bone and joint infections (BJIs). *PLoS One*, *14*(10), e0224106. https://doi.org/10.1371/journal.pone.0224106.

Dropa, M., Lincopan, N., Balsalobre, L. C., Oliveira, D. E., Moura, R. A., Fernandes, M. R., da Silva, Q. M., Matté, G. R., Sato, M. I., & Matté, M. H. (2016). Genetic background of novel sequence types of CTX-M-8- and CTX-M-15-producing Escherichia coli and Klebsiella pneumoniae from public wastewater treatment plants in São Paulo, Brazil. *Environ Sci Pollut Res Int*, *23*(5), 4953-4958. https://doi.org/10.1007/s11356-016-6079-5.

Effendi, M., & Witaningrum, A. (2021). CASES OF MULTIDRUG RESISTANCE (MDR) AND EXTENDED SPECTRUM BETA-LACTAMASE (ESBL) PRODUCING ESCHERICHIA COLI FROM BROILER CHICKEN IN BLITAR, INDONESIA. *Biochemical and Cellular Archives*, *21*, 1923-1929.

Garibyan, L., & Avashia, N. (2013). Polymerase chain reaction. *J Invest Dermatol*, *133*(3), 1-4. https://doi.org/10.1038/jid.2013.1.

Gharout-Sait, A., Touati, A., Benallaoua, S., Guillard, T., Brasme, L., Madoux, J., & De Champs, C. (2012). CTX-M from community-acquired urinary tract infections in Algeria. *Afr J Microbiol Res*, *6*, 5306-5313.

Hassen, B., Abbassi, M. S., Benlabidi, S., Ruiz-Ripa, L., Mama, O. M., Ibrahim, C., Hassen, A., Hammami, S., & Torres, C. (2020). Genetic characterization of ESBL-producing Escherichia coli and Klebsiella pneumoniae isolated from wastewater and river water in Tunisia: predominance of CTX-M-15 and high genetic diversity. *Environ Sci Pollut Res Int*, *27*(35), 44368-44377. https://doi.org/10.1007/s11356-020-10326-w.

Hassen, B., Abbassi, M. S., Ruiz-Ripa, L., Mama, O. M., Ibrahim, C., Benlabidi, S., Hassen, A., Torres, C., & Hammami, S. (2021). Genetic characterization of extended-spectrum β-lactamase-producing Enterobacteriaceae from a biological industrial wastewater treatment plant in Tunisia with detection of the colistin-resistance mcr-1 gene. *FEMS Microbiol Ecol*, *97*(3). https://doi.org/10.1093/femsec/fiaa231.

Hassen, B., Jouini, A., Elbour, M., Hamrouni, S., & Maaroufi, A. (2020). Detection of Extended-Spectrum-β-Lactamases (ESBL) Producing Enterobacteriaceae from Fish Trapped in the Lagoon Area of Bizerte, Tunisia. *BioMed Research International*, *2020*, 7132812. https://doi.org/10.1155/2020/7132812

Hedman, H. D., Vasco, K. A., & Zhang, L. (2020). A review of antimicrobial resistance in poultry farming within low-resource settings. *Animals*, *10*(8), 1264. https://doi.org/10.1089/mdr.2020.0024.

Jarlier, V., Nicolas, M. H., Fournier, G., & Philippon, A. (1988). Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. *Rev Infect Dis*, *10*(4), 867-878. https://doi.org/10.1093/clinids/10.4.867.

Karkman, A., Do, T. T., Walsh, F., & Virta, M. P. (2018). Antibiotic-resistance genes in waste water. *Trends in microbiology*, *26*(3), 220-228.

Khalili, Y., Yekani, M., Goli, H. R., & Memar, M. Y. (2019). Characterization of carbapenem-resistant but cephalosporin-susceptible Pseudomonas aeruginosa. *Acta Microbiol Immunol Hung*, *66*(4), 529-540. https://doi.org/10.1556/030.66.2019.036.

Knowles, J. R. (1985). Penicillin resistance: the chemistry of .beta.-lactamase inhibition. *Accounts of Chemical Research*, *18*(4), 97-104. https://doi.org/10.1021/ar00112a001.

Korzeniewska, E., Korzeniewska, A., & Harnisz, M. (2013). Antibiotic resistant Escherichia coli in hospital and municipal sewage and their emission to the environment. *Ecotoxicology and Environmental Safety*, *91*, 96-102. https://doi.org/https://doi.org/10.1016/j.ecoenv.2013.01.014.

Kumar, A., & Pal, D. (2018). Antibiotic resistance and wastewater: Correlation, impact and critical human health challenges. *Journal of Environmental Chemical Engineering*, *6*(1), 52-58. https://doi.org/https://doi.org/10.1016/j.jece.2017.11.059.

Labid, A., Gacemi-Kirane, D., Timinouni, M., Amoura, K., & Rolain, J.-M. (2014). High prevalence of extended spectrum beta-lactamase (ESBL) producers in fatal cases of pediatric septicemia among the Enterobacteriaceae in the pediatric hospital of Annaba, Algeria. *African Journal of Microbiology Research*, *8*(9), 947-954.

Lade, H., Jeong, S., Jeon, K., Kim, H. S., Kim, H. S., Song, W., & Kim, J. S. (2023). Evaluation of the BD Phoenix CPO Detect Panel for Detection and Classification of Carbapenemase Producing Enterobacterales. *Antibiotics (Basel)*, *12*(7). https://doi.org/10.3390/antibiotics12071215.

Lee, K., Chong, Y., Shin, H. B., Kim, Y. A., Yong, D., & Yum, J. H. (2001). Modified Hodge and EDTA-disk synergy tests to screen metallo-beta-lactamase-producing strains of Pseudomonas and Acinetobacter species. *Clin Microbiol Infect*, *7*(2), 88-91. https://doi.org/10.1046/j.1469-0691.2001.00204.x.

Liu, L. H., Wang, N. Y., Wu, A. Y., Lin, C. C., Lee, C. M., & Liu, C. P. (2018). Citrobacter freundii bacteremia: Risk factors of mortality and prevalence of resistance genes. *J Microbiol Immunol Infect*, *51*(4), 565-572. https://doi.org/10.1016/j.jmii.2016.08.016.

Marston, H. D., Dixon, D. M., Knisely, J. M., Palmore, T. N., & Fauci, A. S. (2016). Antimicrobial Resistance. *JAMA*, *316*(11), 1193-1204. https://doi.org/10.1001/jama.2016.11764.

Mechai, A., Debabza, M., Thabet, R., Sedira, H., Fadeleddine, S., & Mechai, A. (2019). Occurrence and spread of beta-lactamases-producing Enterobacteriaceae isolated from river receiving treated effluent of wastewater treatment plant. *Desal Water Treatment*, *147*, 156-163.

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

Moran-Gilad, J., Adler, A., Schwartz, D., Navon-Venezia, S., & Carmeli, Y. (2014). Laboratory evaluation of different agar media for isolation of carbapenem-resistant Acinetobacter spp. *Eur J Clin Microbiol Infect Dis*, *33*(11), 1909-1913. https://doi.org/10.1007/s10096-014-2159-y.

Mubarak, Z., Rahmayani, L., Nst, A., & Bunjamin, P. (2017). Comparison of Candida sp. Colonies in Gargling-volume Culture from Subject Wearers of Heat-cured and Self-cured Acrylic Resin Removable Partial Dentures. *World Journal of Dentistry*, *8*, 471-474. https://doi.org/10.5005/jp-journals-10015-1489.

Mullis, K. B. (1990). The unusual origin of the polymerase chain reaction. *Sci Am*, *262*(4), 56-61, 64-55. https://doi.org/10.1038/scientificamerican0490-56.

Nasser-Ali, M.; Aja-Macaya, P.; Conde-Pérez, K.; Trigo-Tasende, N.; Rumbo-Feal, S.; Fernández-González, A.; Bou, G.; Poza, M.; Vallejo, J.A. (2024).Emergence of Carbapenemase Genes in Gram-Negative Bacteria Isolated from the Wastewater Treatment Plant in A Coruña, Spain. *Antibiotics*, *13*, 194. https://doi.org/10.3390/antibiotics13020194.

Nouria, L., Hassaine, H., Robin, F., Richard, B., & Djamel-Eddine, A. (2016). Prevalence and molecular typing of extended-spectrum -lactamases in Escherichia coli, Enterobacter cloacae and Citrobacter freundii isolates from Laghouat Hospital, Algeria. *African Journal of Microbiology Research*, *10*, 1430-1438. https://doi.org/10.5897/AJMR2016.8263.

Oumeima, M., Sebaihia, M., Meryem, B., Amraoui, R., Diene, S., & Rolain, J.-M. (2022). Occurrence and Diversity of Extended-Spectrum β-lactamases in Clinical Isolates of Enterobacteriaceae in A Tertiary Care Hospital in Algeria. *39*, 14.

Pasteur Institute of Algeria (IPA). (2020). Standardisation de l'antibiogramme en Médecine Humaine à l'échelle nationale selon les recommandations de l'OMS. 8th Edition. Algeria.

Rao, M. R., Chandrashaker, P., Mahale, R. P., Shivappa, S. G., Gowda, R. S., & Chitharagi, V. B. (2019). Detection of carbapenemase production in Enterobacteriaceae and Pseudomonas species by carbapenemase Nordmann-Poirel test. *J Lab Physicians*, *11*(2), 107-110. https://doi.org/10.4103/JLP.JLP_132_18.

Reinke, R. A., Quach-Cu, J., Allison, N., Lynch, B., Crisostomo, C., & Padilla, M. (2020). A method to quantify viable carbapenem resistant gram-negative bacteria in treated and untreated wastewater. *Journal of Microbiological Methods*, *179*, 106070. https://doi.org/https://doi.org/10.1016/j.mimet.2020.106070.

Rodriguez Villalobos, H., & Struelens, M. (2006). Résistance bactérienne par β-lactamases à spectre étendu : implications pour le réanimateur. *Réanimation*, *15*. https://doi.org/10.1016/j.reaurg.2006.03.006.

Rodríguez, E. A., Garzón, L. M., Gómez, I. D., & Jiménez, J. N. (2020). Multidrug resistance and diversity of resistance profiles in carbapenem-resistant Gram-negative bacilli throughout a wastewater treatment plant in Colombia. *Journal of Global Antimicrobial Resistance*, *22*, 358-366. https://doi.org/https://doi.org/10.1016/j.jgar.2020.02.033.

Rodriguez-Villalobos, H., & Struelens, M. J. (2006). Résistance bactérienne par β-lactamases à spectre étendu : implications pour le réanimateur. *Réanimation*, *15*(3), 205-213. https://doi.org/https://doi.org/10.1016/j.reaurg.2006.03.006.

Sarangan, P., Dharanidevi, S., Das, N. K., & Raj, S. (2016). Prevalence, Phenotypic Characterization and Antibiotic Susceptibility of Non-Fermentative Gram Negative Bacilli Isolates at a Tertiary Care Centre. *International Journal of Current Microbiology and Applied Sciences*, *5*, 442-454.https://doi.org/10.20546/ijcmas.2016.511.051

Schaffarczyk, L., Noster, J., Stelzer, Y., Sattler, J., Gatermann, S., & Hamprecht, A. (2024). Detection of rare carbapenemases in Enterobacterales—comparison of two colorimetric and three CIM-based carbapenemase assays. *Microbiology Spectrum*, *12*(2), e03015-03023. https://doi.org/doi:10.1128/spectrum.03015-23.

Schultsz, C., & Geerlings, S. (2012). Plasmid-mediated resistance in Enterobacteriaceae: changing landscape and implications for therapy. *Drugs*, *72*(1), 1-16. https://doi.org/10.2165/11597960-000000000-00000.

Shaikh, N., Drego, L., Shetty, A., & Rodrigues, C. (2020). Evaluation of modified carbapenem inactivation method with CARBA NP, MHT and real-time PCR for detection of carbapenemase-producing Enterobacteriaceae. *International Journal of Infectious Diseases*, *101*, 64.

Sharma, E., Chen, Y., Kelso, C., Sivakumar, M., & Jiang, G. (2024). Navigating the environmental impacts and analytical methods of last-resort antibiotics: Colistin and carbapenems. *Soil & Environmental Health*, *2*(1), 100058. https://doi.org/https://doi.org/10.1016/j.seh.2024.100058.

Touati, A., Benallaoua, S., Forte, D., Madoux, J., Brasme, L., & de Champs, C. (2006). First report of CTX-M-15 and CTX-M-3 beta-lactamases among clinical isolates of Enterobacteriaceae in Béjaia, Algeria. *Int J Antimicrob Agents*, *27*(5), 397-402. https://doi.org/10.1016/j.ijantimicag.2005.12.007.

Veloo, Y., Thahir, S. S. A., Rajendiran, S., Hock, L. K., Ahmad, N., Muthu, V., & Shaharudin, R. (2022). Multidrug-Resistant Gram-Negative Bacteria and Extended-Spectrum β-Lactamase-Producing Klebsiella pneumoniae from the Poultry Farm Environment. *Microbiology Spectrum*, *10*(3), e02694- 02621. https://doi.org/doi:10.1128/spectrum.02694-21.

Zagui, G. S., de Andrade, L. N., Moreira, N. C., Silva, T. V., Machado, G. P., da Costa Darini, A. L., & Segura-Muñoz, S. I. (2020). Gram-negative bacteria carrying β-lactamase encoding genes in hospital and urban wastewater in Brazil. *Environmental Monitoring and Assessment*, *192*(6), 376. https://doi.org/10.1007/s10661-020-08319-w.

Zenati, F., Barguigua, A., Nayme, K., Benbelaïd, F., Khadir, A., Bellahsene, C., Bendahou, M., Hafida, H., & Timinouni, M. (2019). Characterization of uropathogenic ESBL-producing Escherichia coli isolated from hospitalized patients in western Algeria. *J Infect Dev Ctries*, *13*(4), 291-302. https://doi.org/10.3855/jidc.10702.

Zhang, L., Ma, X., Luo, L., Hu, N., Duan, J., Tang, Z., Zhong, R., & Li, Y. (2020). The prevalence and characterization of extended-spectrum β-lactamase-and carbapenemase-producing bacteria from hospital sewage, treated effluents and receiving rivers. *International journal of environmental research and public health*, *17*(4), 1183.

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The burden of *Carbapenemase*-producing Enterobacteriaceae (cpe) in Algeria: first report & review

At the *1st Pan Arab Symposium on Parasitology and Infectious Diseases (PASPID24)* on *Mai 18th & 19th, 2024*

Co-authors: *Khouchane Abderaouf, Chenouf Nadia Safia, Messai Chafik Redha*

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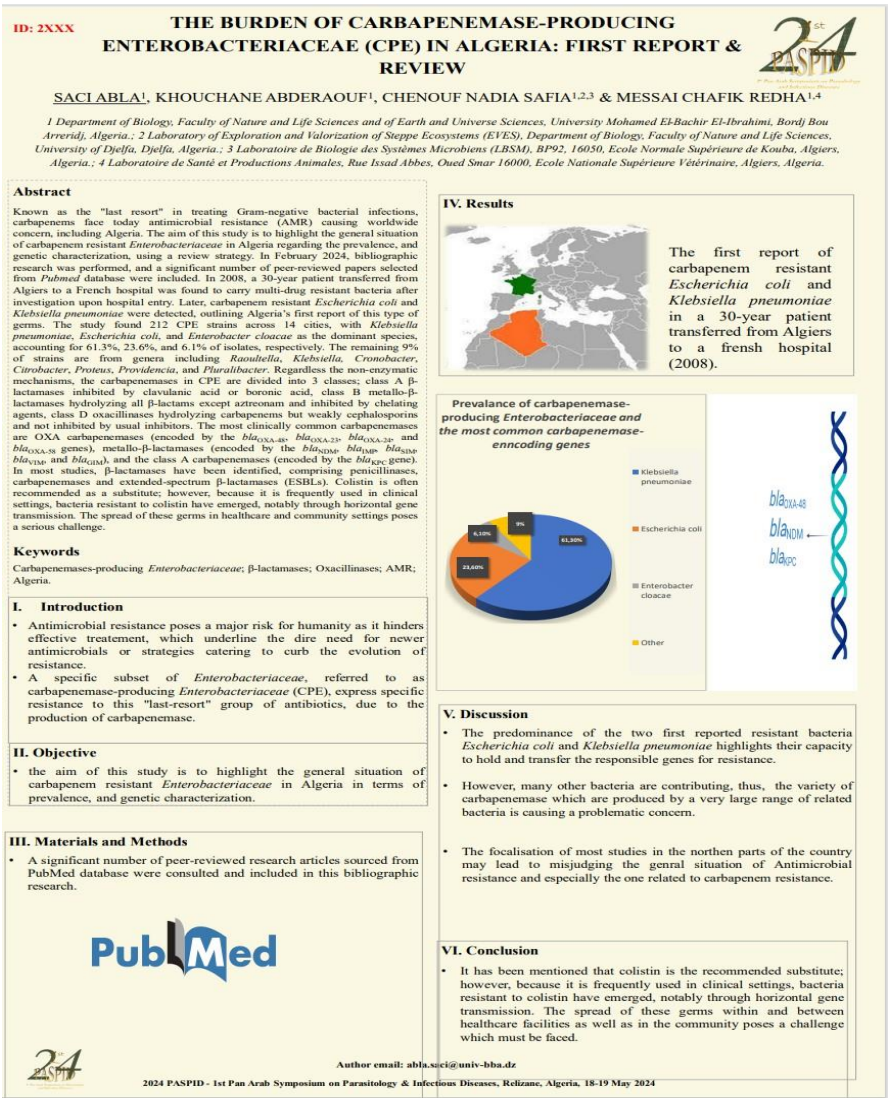

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THE BURDEN OF CARBAPENEMASE-PRODUCING ENTEROBACTERIACEAE (CPE) IN ALGERIA: FIRST REPORT & REVIEW

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Abstract

Known as the "last resort" in treating Gram-negative bacterial infections, carbapenems face today antimicrobial resistance (AMR) causing worldwide concern, including Algeria. The aim of this study is to highlight the general situation of carbapenem resistant *Enterobacteriaceae* in Algeria regarding the prevalence, and genetic characterization, using a review strategy. In February 2024, bibliographic research was performed, and a significant number of peer-reviewed papers selected from *Pubmed* database were included. In 2008, a 30-year patient transferred from Algiers to a French hospital was found to carry multi-drug resistant bacteria after investigation upon hospital entry. Later, carbapenem resistant *Escherichia coli* and *Klebsiella pneumoniae* were detected, outlining Algeria's first report of this type of germs. The study found 212 CPE strains across 14 cities, with *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter cloacae* as the dominant species, accounting for 61.3%, 23.6%, and 6.1% of isolates, respectively. The remaining 9% of strains are from genera including *Raoultella*, *Klebsiella*, *Cronobacter*, *Citrobacter*, *Proteus*, *Providencia*, and *Pluralibacter*. Regardless the non-enzymatic mechanisms, the carbapenemases in CPE are divided into 3 classes; class A β-lactamases inhibited by clavulanic acid or boronic acid, class B metallo-β-lactamases hydrolyzing all β-lactams except aztreonam and inhibited by chelating agents, class D oxacillinases hydrolyzing carbapenems but weakly cephalosporins and not inhibited by usual inhibitors. The most clinically common carbapenemases are OXA carbapenemases (encoded by the bla_{OXA-48}, bla_{OXA-23}, bla_{OXA-24}, and bla_{OXA-58} genes), metallo-β-lactamases (encoded by the bla_{NDM}, bla_{IMP}, bla_{SIM}, bla_{VIM}, and bla_{GIM}), and the class A carbapenemases (encoded by the bla_{KPC} gene). In most studies, β-lactamases have been identified, comprising penicillinases, carbapenemases and extended-spectrum β-lactamases (ESBLs). Colistin is often recommended as a substitute; however, because it is frequently used in clinical settings, bacteria resistant to colistin have emerged, notably through horizontal gene transmission. The spread of these germs in healthcare and community settings poses a serious challenge.

Keywords

Carbapenemases-producing *Enterobacteriaceae*; β-lactamases; Oxacillinases; AMR; Algeria.

I. Introduction

- Antimicrobial resistance poses a major risk for humanity as it hinders effective treatement, which underline the dire need for newer antimicrobials or strategies catering to curb the evolution of resistance.
- A specific subset of *Enterobacteriaceae*, referred to as carbapenemase-producing *Enterobacteriaceae* (CPE), express specific resistance to this "last-resort" group of antibiotics, due to the production of carbapenemase.

II. Objective

- the aim of this study is to highlight the general situation of carbapenem resistant *Enterobacteriaceae* in Algeria in terms of prevalence, and genetic characterization.

III. Materials and Methods

- A significant number of peer-reviewed research articles sourced from PubMed database were consulted and included in this bibliographic research.

PubMed

IV. Results

The first report of carbapenem resistant *Escherichia coli* and *Klebsiella pneumoniae* in a 30-year patient transferred from Algiers to a frensh hospital (2008).

Prevalance of carbapenemase-producing *Enterobacteriaceae and the most common carbapenemase-enncoding genes*

V. Discussion

- The predominance of the two first reported resistant bacteria *Escherichia coli* and *Klebsiella pneumoniae* highlights their capacity to hold and transfer the responsible genes for resistance.
- However, many other bacteria are contributing, thus, the variety of carbapenemase which are produced by a very large range of related bacteria is causing a problematic concern.
- The focalisation of most studies in the northen parts of the country may lead to misjudging the genral situation of Antimicrobial resistance and especially the one related to carbapenem resistance.

VI. Conclusion

- It has been mentioned that colistin is the recommended substitute; however, because it is frequently used in clinical settings, bacteria resistant to colistin have emerged, notably through horizontal gene transmission. The spread of these germs within and between healthcare facilities as well as in the community poses a challenge which must be faced.

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Panorama of extended-spectrum β-lactamases (esbls) in algerian hospitals: prevalence, phenotypic, and genotypic features (2006-2023)

At the *1st Pan Arab Symposium on Parasitology and Infectious Diseases (PASPID24)* on *Mai 18th & 19th, 2024*

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ID: 2XXX **PANORAMA OF EXTENDED-SPECTRUM β-LACTAMASES (ESBLs) IN ALGERIAN HOSPITALS: PREVALENCE, PHENOTYPIC, AND GENOTYPIC FEATURES (2006-2023)**

KHOUCHANE ABDERAOUF[1], SACI ABLA[1], CHENOUF NADIA SAFIA[1,2,3] & MESSAI CHAFIK REDHA[1,4]

[1] Department of Biology, Faculty of Nature and Life Sciences and of Earth and Universe Sciences, University Mohamed El-Bachir El-Ibrahimi, Bordj Bou Arreridj, Algeria.; [2] Laboratory of Exploration and Valorization of Steppe Ecosystems (EVES), Department of Biology, Faculty of Nature and Life Sciences, University of Djelfa, Djelfa, Algeria.; [3] Laboratoire de Biologie des Systèmes Microbiens (LBSM), BP92, 16050, Ecole Normale Supérieure de Kouba, Algiers, Algeria.; [4] Laboratoire de Santé et Productions Animales, Rue Issad Abbes, Oued Smar 16000, Ecole Nationale Supérieure Vétérinaire, Algiers, Algeria.

Abstract

The 1928 discovery of penicillin was revolutionary. However, by the 1940s, penicillin resistance emerged. Today, antimicrobial resistance is a critical health concern. In Algeria, research focuses on Extended-Spectrum β-Lactamases (ESBLs), a remarkable resistance mechanism in Gram-negative bacteria. The aim of this study is to provide an overview of the situation of ESBLs in Algerian hospitals. For this purpose, 26 peer-reviewed research articles published between 2006 and 2023, and mostly sourced from PubMed database were consulted and included. They describe studies conducted on ESBLs in clinical isolates collected from different Algerian hospitals in Algiers, Tlemcen, Bejaia, Oran, Tizi-Ouzou, Annaba, Sidi-Bel-Abbes, Constantine, Boumerdes, Laghouat, Ouargla, and Tebessa. The general scheme of the selected studies relied on the identification of Gram-negative bacterial isolates from hospitals, determination of their susceptibility to antibiotics by the standard disk diffusion method on Muller-Hinton, detection of ESBLs by double disk synergy test, and the identification of ESBL genetic determinants using various techniques, including PCR and sequencing. In broad terms, the results obtained show the identification of 1477 isolates, with a dominance of the species: *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella enterica*, and *Enterobacter cloacae*, with 47.12%, 20.51%, 14.42%, and 6.63% of total isolates respectively, while the remaining 11.32% are represented by species belonging to the genera: *Serratia*, *Proteus*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Morganella*, *Raoultella*, *Kluyvera*, and *Acinetobacter*. The phenotypic detection of ESBL-producing strains demonstrated different levels of multi-drug resistance, with susceptibility to imipenem. At the genetic level, the bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes, which are typically carried by conjugative plasmids and transferred horizontally, are the most frequent ESBL-encoding genes, with a notable predominance of CTX-M-15 ESBL type. Our findings reveal that data from Southern hospitals are still scarce, and more studies should be designed in order to plan a national prevention strategy to limit the ascent of the antimicrobial resistance.

Keywords

Extended-Spectrum β-Lactamases (ESBLs); Algeria; CTX-M-15; Antimicrobial resistance; *Klebsiella pneumoniae*

I. Introduction

The revolutionary discovery of penicillin marked a milestone in medicine, offering unprecedented hope in the fight against bacterial infections. However, within a short span, resistance to this wonder drug began to emerge, foreshadowing the persistent challenge of antimicrobial resistance (AMR). Today, this issue stands as a critical global concern, with Algeria's scientific community actively investigating Extended-Spectrum β-Lactamases (ESBLs), a formidable resistance mechanism found in Gram-negative bacteria.

II. Objective

The objective of this study is to offer a comprehensive overview of the prevalence and characteristics of Extended-Spectrum β-Lactamases (ESBLs) within Algerian hospital settings.

III. Materials and Methods

26 peer-reviewed research articles published between 2006 and 2023, and mostly sourced from PubMed database were consulted and included in this bibliographic reasearch

PubMed

IV. Results

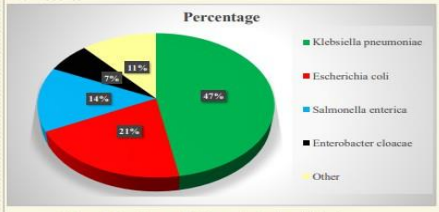

Figure1: Prevalence of ESBL-producing bacterial isolates

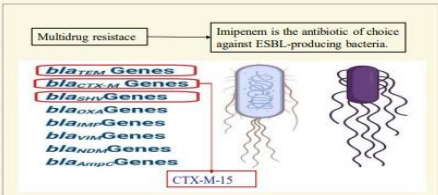

Figure2: Main identified *bla* Genes

V. Discussion

Prevalence and Distribution: The dominance of Klebsiella pneumoniae and Escherichia coli as ESBL-producing organisms underscores their significance in antimicrobial resistance in Algerian hospitals.

Antibiotic Resistance: The observed multi-drug resistance highlights the challenge in treating infections caused by ESBL-producing bacteria, emphasizing the importance of prudent antibiotic use.

Genetic Factors: Identification of ESBL genes and their predominance, particularly CTX-M-15, suggests a need for understanding genetic mechanisms underlying resistance for effective management strategies.

Geographical Discrepancies: Disparities in data availability from Southern hospitals indicate the necessity for broader geographical coverage in surveillance efforts to comprehensively address antimicrobial resistance.

VI. Conclusion

The findings underscore the urgency of implementing a national prevention strategy to mitigate the rising threat of antimicrobial resistance in Algerian healthcare settings.

References

[1] Touati A, Benallaoua S, Forte D, Madoux J, Brasme L, de Champs C. First report of CTX-M-15 and CTX-M-3 beta-lactamases among clinical isolates of Enterobacteriaceae in Bejaia, Algeria. Int J Antimicrob Agents. 2006 May;27(5):397-402. doi: 10.1016/j.ijantimicag.2005.12.007.

[2] Debabza M, Dziri R, Mechai A, Bouguessa A, Klibi N, Ouzari HI. Characterization of carbapenemase-producing Gram-negative bacilli: first report of blaNDM-1 in Enterobacter cloacae. J Infect Dev Ctries. 2023 Sep 30;17(9):1300-1309. doi: 10.3855/jidc.18031.

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Abstract:

This study assesses the prevalence of Extended Spectrum β-Lactamase (ESBL)- and carbapenemase (CRB)-producing Gram-negative bacteria (GNB) from 14 wastewater samples collected in BBA. Overall, 12 samples (86%) harbored CTX^R strains, and 30 CTX^R-GNB strains were recovered, among which 11 (37%) were found to be ESBL-E, and 4 strains harbored the *bla*$_{CTX-M}$ encoding gene. ESBL-E were identified as: *K. pneumoniae* (6 strains), *E. coli* (4 strains), and *Citrobacter freundii* (1 strain). Six multi-drug resistant (MDR) strains were detected among ESBL-E. In addition, 10 samples (71%) contained IMP^R strains, and 14 IMP^R-GNB strains were collected, among which 1 (7%) was CRB-GNB and MDR.

Keywords: ESBL-producing Gram-negative bacteria, Carbapenemase-producing Gram-negative bacteria, *Enterobacteriaceae*, *bla*$_{CTX-M}$, wastewater, BBA.

Résumé:

Cette étude évalue la prévalence des bactéries à Gram-négatif (BGN) productrices de β-lactamases à spectre étendu (BLSE) et de carbapénémases (CRB) à partir de 14 échantillons d'eaux usées collectés dans la ville de BBA. Au total, 12 échantillons (86 %) hébergeaient des souches CTX^R et 30 souches CTX^R-BGN ont été collectées, parmi lesquelles 11 (37 %) se sont révélées BLSE-E et 4 souches hébergeaient le gène codant pour *bla*$_{CTX-M}$. Les BLSE-E ont été identifiées comme suit : *K. pneumoniae* (6 souches), *E. coli* (4 souches) et *Citrobacter freundii* (1 souche). Six souches multirésistantes (MR) ont été détectées parmi les BLSE-E. De plus, 10 échantillons (71 %) contenaient des souches IMP^R et 14 souches IMP^R-GNB ont été collectées, parmi lesquelles 1 (7 %) était CRB-GNB et MR.

Mots-clés: Bactéries à Gram-négatif productrices de BLSEs, bactéries à Gram-negatif productrices de carbapenémases, *Enterobacteriaceae*, *bla*$_{CTX-M}$, eau usée, BBA.

ملخّص:

تقيّم هذه الدّراسة مدى انتشار البكتيريا سالبة الجرام المنتجة لإنزيم البيتالاكتاماز واسع الطيف وأيضا إنزيم الكاربابينيماز من 14 عيّنة من مياه الصرف الصحي تم جمعها في مدينة برج بوعريريج. أوضحت النتائج أنّ 12 عينة (86٪) تحتوي على سلالات مقاومة للسيفوتاكسيم، وتم جمع 30 سلالة منها، من بينها 11 (37٪) صنّفت أنها منتجة لإنزيم البيتالاكتاماز واسع الطيف، كان من بينها 4 سلالات تحتوي على المورّثة *bla*$_{CTX-M}$ ، وقد تم تحديد هذه السلالات على النحو التالي: *K. pneumoniae* (6 سلالات)، *E. coli* (4 سلالات)، و *Citrobacter freundii* (سلالة واحدة). كما تم الكشف عن ست سلالات متعددة المقاومة للمضادات الحيوية من بين هذه السلالات المنتجة للبيتالاكتاماز واسع الطيف. بالإضافة إلى ذلك، احتوت 10 عينات من مياه الصرف (71%) على سلالات مقاومة للاميبينام، وتم جمع 14 سلالة منها، من بينها 1 (7%) كانت منتجة لإنزيم الكاربابينيماز و متعددة المقاومة للمضادات الحيوية.

الكلمات المفتاحية: البكتيريا سالبة الغرام المنتجة للبيتالاكتاماز واسع الطيف، البكتيريا سالبة الغرام المنتجة للكاربابينيماز، البكتيريا المعوية، مياه الصرف الصحي، المورّثة *bla*$_{CTX-M}$ ، برج بوعريريج.