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List of journal publications:

1. **Anfal Kara**, Chiara Massaro, Giovanni M. Giammanco, Rosa Alduina and Naouel Boussoualim. Phylogenetic Diversity, Antibiotic Resistance, and Virulence of *Escherichia coli* Strains from Urinary Tract Infections in Algeria. 2024. *Antibiotics*, 3 (18): 773. https://doi.org/10.3390/antibiotics13080773.
2. **Anfal Kara**, Meriem Elkolli, Naouel Boussoualim. Antibiotic Resistance in *Morganella morganii* Species: Mechanisms, Prevalence and Clinical Implication. 2024. Anti-Infective agents. 22 (5). http://dx.doi.org/10.2174/0122113525288670240408034624.

List of communications:

1. Poster communication. The 3rd Doctorial Scientific Days. 10 February 2022. Setif. Algeria. **Kara A.**, Alduina R. and Boussoualim N. Anti-bioresistance profile of Clinical Strains.
2. Oral communication. The 1st International Day of Natural and Life Sciences (INDNS). 01-02 March 2022. Ouargla. **Kara A.**, Belfihadj F., Boussoualim N. and Elkolli M. Presence of Antibiotic in Food: Survey.
3. Oral communication. The 1st International Day of Natural and Life Sciences (INDNS). 01-02 March 2022. Ouargla. Algeria. Belfihadj F., **Kara A.**, Elkolli M. and Boussoualim N. Role of plants in treating and limiting the spread of Coronavirus infection.
4. Oral communication. The 1st International Conference on Engineering and Applied Natural Sciences. 10-13 May 2022. Konya. Turkey. Belfihadj F., **Kara A.**, Elkolli M. and Boussoualim N. Evaluation of Resistance Rates in *Enterobacteria* Isolated from Broiler Chickens in Setif (Algeria).
5. Oral communication. The 1st International Conference on Engineering and Applied Natural Sciences. 10-13 May 2022. Konya. Turkey. **Kara A.**, Belfihadj F., Elkolli M. and Boussoualim N. Evaluation of Antibioresistance in Isolated Clinical Strains of Urinary Tract Infections in Setif (Algeria).
6. Oral communication. The 3rd International Conference on Applied Engineering and Natural Sciences. 20-23 July 2022. Konya. Turkey. **Kara A.**, Belfihadj F., Elkolli M. and Boussoualim N. Bacteriology Profile and Antibiotic Susceptibility Analyses of Pus Isolates from El-Eulma Hospital (Setif).
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8. Poster communication. The 1st National Seminar on Biotechnology and Macrobian Biodiversity. 26-27 October 2022. Khenchela. Algeria. **Kara A.**, Belfihadj F., Elkolli M. and Boussoualim N. Profile of Anti-Bioresistance of Strains Isolated from Acquired Urinary Tract Infections.
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21. Oral Communication. The 2nd International Seminar on Biodiversity, Environnement and Organized Heath. 09-10 December 2024. Tlemcen. Algeria. **Kara A.**, Massaro C., Giammanco G. M, Alduina R. and Boussoualim N. Detection of Phylogenetic Groups, Antibiotic Resistance Pattern, Biofilm and Hemolysin Production Among Uropathogenic *Escherichia coli* Isolated from Urinary Tract Infections in Sétif, East of Algeria.
22. Poster communication. The 2nd National Seminar of Physics, Chemistry and their Application (NSPCA'25). 17-18 February 2025. Boussoualim N., Krache I., Trabsa H and **Kara A**. Enzymatic Inhibition test of Beta-lactamases by Sedum sediforme.

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الملـخص

تمثل مقاومة المضادات الحيوية واحدة من أهم القضايا العالمية في مجال الصحة حتى اليوم. في هذه الدراسة تم جمع 402 سلالة من سبع مختبرات للتشخيص الطبي وثلاثة مستشفيات عامة في منطقة سطيف على مدى ثلاث سنوات من 2021-2023. أولاً، تم تحديد 326 عينة سريرية مجتمعية و76 عينة مستشفوية، تم عزلها من مختلف العينات، باستخدام اختبارات ميكروبيولوجية وكيميائية حيوية وتم تأكيدها بواسطة النظام API , واسفرت هذه التحليلات عن تحديد 10 أنواع من السلالات تنتمي إلى البكتيريا سالبة الجرام و3 أنواع تنتمي إلى البكتيريا موجبة الجرام. كانت *E. coli* هي الممرض السائد بين البكتيريا سالبة الجرام (50%) بينما كانت *S. aureus* هي الأكثر تواجدا بين البكتيريا موجبة الجرام (7.7%). وجد ان غالبية هذه السلالات كانت في عينات البول المأخوذة من المرضى البالغين. تم إجراء اختبارات حساسية المضادات الحيوية واختبارات التركيز المثبط الأدنى وفقًا لتوصيات الجمعية الفرنسية للأحياء الدقيقة. نسبة كبيرة من السلالات المحددة أظهرت معدلات مقاومة مرتفعة ضد المضادات الحيوية من الخط الأول المستخدمة عادة في علاج مختلف العدوى البكتيرية، بما في ذلك البنسيلينات والسيفالوسبورينات، ولكن تم تسجيل مقاومة كبيرة ضد الإيميبينيم لدى *E. coli* (49.8%)وأنواع *Proteus* (32.1%). وبالمثل، أظهرت التوبراميسين والجنتاميسين فعالية محدودة ضد *P. aeruginosa*، وأنواع *Enterobacter*، وجميع البكتيريا موجبة الجرام تراوحت بين (20% و100%). كانت السلالات المقاومة المتعددة للأدوية MDRتمثل 56% من جميع العزلات المختبرة، تليها السلالات المقاومة الشديدة XDR(26.1%). الأميكاسين كان المضاد الحيوي الأكثر فعالية. من بين عزلات *K. pneumoniae*، تم تصنيف 66.6% منها على أنها *K. pneumoniae* شديدة الفوعة (hvKp) بناءً على اختبار التمدد (اختبار الخيط). في أنواع *S. aureus*، كان 61.3% منها من *S. aureus* المقاومة للميثيسيلين، بينما كان 9.7% منها تظهر النمط التكويني للماكروليد- لينكوساميد- ستريبتوغرامين ب ، و16.1% منها تظهر النمط الظاهري القابل للتحفيز ، كما تم تحديده بواسطة اختبارD.تم اختبار عوامل مختلفة من الفوعة في السلالات الممرضة. حيث تم تقييم تشكيل الأغشية الحيوية في الألواح الدقيقة باستخدام الملون الكريستال البنفسجي، بينما تم تحديد إنتاج الهيموليسين، والبروتياز، والليسيتيناز، والليباز باستخدام أطباق تحتوي على الدم البشري، والحليب الخالي من الدسم، وصفار البيض على التوالي. بشكل عام، كانت 88.1% من السلالات منتجة للأغشية الحيوية، حيث أظهرت الغالبية (64.9%) قدرة منخفضة على إنتاج الأغشية الحيوية. أظهرت السلالات الشديدة المقاومة أعلى نسبة من منتجي الأغشية الحيوية القوية (47.6%). تمت ملاحظة إنتاج الهيموليسين في 55.5% من السلالات، تليها إنتاج البروتياز بنسبة 47.3% وأنشطة الليسيثيناز والليباز (23.1% و10%) .تم إجراء بحث مظهري لإنتاج β-lactamases ذات الطيف الممتد وAmpC، حيث أظهر 32.6% نمط مظهري إيجابي لإنتاج β-lactamases ذات الطيف الممتد و12.7% إيجابي لـ AmpC. بالإضافة إلى ذلك، تم استخدام التوصيف الجزيئي لجينات β-lactamase وجينات الكينولون باستخدام PCR متعدد الطور للتأكيد المظهري. كان bla_{TEM} هو الجين الناشئ من نوعESBL الأكثر انتشارا(53.8%) ، يليه bla_{SHV} (15.9%)، بينما كان $bla_{CMY\ II}$ هو الجين AmpC الذي تم اكتشافه بشكل متكرر (10.4%)، يليه bla_{DHA} (7.1%). كانت جميع هذه الجينات وغيرها من جينات الضراوة محمولة على بلازميدات تتراوح أحجامها بين حوالي 1.5 إلى أكثر من 10 كيلو قاعدة، وتم تحديدها بطريقة PCR. بلغت نسبة عزلات *E. coli* القادرة على حمل جين *qnr* واحد على الأقل 26.7% وكان الجين *qnrB*هو الأكثر انتشارًا بين السلالات الموجبة لـ *qnr* بنسبة 78.3%، يليه *qnrD* بنسبة 26.1%.تم استخدام تقنية PCR من Clermontوآخرين للكشف عن المجموعات النشوء والتطور لبكتيريا *E. coli* البولية المقاومة للإيميبينيم. من بين 86 عزلة سريرية من *E. coli* المقاومة للإيميبينيم، كان المجموعة B2 (48.9%) هي المجموعة الفيلوجينية الأكثر انتشارًا، تليها المجموعات(22.1%) E ، غير المعروفة (12.8%)، A (8.1%) ، وB1 (4.7%) ، بالإضافة إلى Clades I، D، Clade I أوClade II (1.2%) .

الكلمات المفتاحية: مقاومة المضادات الحيوية، البكتيريا متعددة المقاومة، β-lactamase، ESBL، AmpC، جينات *qnr*، المجموعات النشوء والتطور

SUMMARY

Antibiotic resistance (AR) is among the leading global health concerns to date. In our study, 402 strains were collected from 7 medical diagnostic laboratories and 3 government hospitals in Sétif region over three years (2021-2023). First, the identification of 326 clinical community samples and 76 hospitalized samples, isolated from different specimens, was carried out using microbiological and biochemical tests and confirmed by API gallery. This analysis identified 10 species belonging to Gram negative bacteria (GNB) and 3 species belonging to Gram positive bacteria (GPB). *E. coli* was the predominant pathogen among GNB (50%) while *S. aureus* was the most common GPB (7.7%). The majority of these strains were found in urine samples from adult patients. Antibiotic susceptibility tests and MICs were performed according to CA-SFM recommendations. A significant proportion of the identified strains exhibited high resistance rates against first-line antibiotics commonly used in the treatment of various bacterial infections including penicillins and cephalosporins, but a substantial resistance was recorded against imipenem in *E. coli* (49.8%) and *Proteus* species (32.1%). Similarly, tobramycin and gentamycin exhibited limited effectiveness against *P. aeruginosa*, *Enterobacter* spp., (20% to 100%) and all GPB. MDR strains represented 56% of all tested isolates, followed by XDR (26.1%). Amikacin was the most effective antibiotic. Among *K. pneumoniae* isolates, 66.6% were classified as hypervirulent *K. pneumoniae* (hvKp) based on the string test. In *S. aureus* species, 61.3% were methicillin-resistant *S. aureus* (MRSA), while 9.7% exhibited the constitutive macrolide-lincosamide-streptogramin B (CMLSB) phenotype, and 16.1% displayed the inducible MLSB (IMLSB) phenotype, as determined by the D-test. Different virulence factors were tested in our pathogenic strains. Biofilm formation was assessed in microtiter plates using crystal violet dye, while hemolysin, protease, lecithinase, and lipase enzyme production were determined using plates containing human blood, skimmed milk, and egg yolk, respectively. Overall, 88.1% of strains were biofilm producers, with the majority (64.9%) exhibiting a weak ability to produce biofilms. XDR strains exhibited the highest percentage of strong biofilm producers (47.6%). Hemolysin production was found in 55.5% of strains, followed by protease production 47.3% and lecithinase and lipase activities (23.1% and 10%). A phenotypic search for the production of extended-spectrum β-lactamases and AmpC was conducted, 32.6% presented ESBL phenotype and 12.7% were positive AmpC, furthermore, molecular characterization of β-lactamase genes and quinolone genes with Multiplex PCR was used for phenotypic confirmation. bla_{TEM} was the emergence ESBL-type gene (53.8%), followed by bla_{SHV} (15.9%), while $bla_{CMY\ II}$ was the most frequent AmpC detected gene (10.4%) followed by bla_{DHA} (7.1%). All these genes and other virulence genes were carried by plasmids with size ranged from approximately 1.5 to >10 kb profiling by PCR method. 26.7% of *E. coli* strains harbored at least one *qnr* gene. The most frequent was *qnrB* (78.3%), followed by *qnrD* (26.1%). Clermont *et al.* PCR was used for the detection of phylogenetic groups of urinary imipenem-resistant *E. coli*. Of 86 clinical imipenem resistant *E. coli* isolates, the B2 group (48.9%) was the most prevalent phylogroup, followed by groups E (22.1%), unknown (12.8%), A (8.1%), and B1 (4.7%), and Clade I, D, Clade I, or Clade II (1.2%).

Keywords: Antibiotic resistance, Multidrug-resistant bacteria, β-lactamase, ESBL, AmpC, *qnr* genes, phylogenetic groups.

RESUME

La résistance aux antibiotiques (RA) représente parmi les principales préoccupations mondiales en matière de santé à ce jour. Dans notre étude, 402 souches ont été collectées dans 7 laboratoires de diagnostic médical et 3 hôpitaux publiques de la région de Sétif sur une période de trois ans (2021-2023). Tout d'abord, l'identification de 326 échantillons cliniques communautaires et de 76 échantillons hospitalisés, isolés à partir de différents spécimens, a été réalisée à l'aide de tests microbiologiques et biochimiques et confirmée par la galerie API. Cette analyse a identifié 10 espèces appartenant aux bactéries Gram négatif (GNB) et 3 espèces appartenant aux bactéries Gram positif (GPB). *E. coli* était le pathogène prédominant parmi les GNB (50%) tandis que *S. aureus* était le GPB le plus courant (7,7%). La majorité de ces souches ont été trouvées dans des échantillons d'urine provenant de patients adultes. Les tests de sensibilité aux antibiotiques et les CMI ont été réalisés selon les recommandations du CA-SFM. Une proportion significative des souches identifiées a montré des taux de résistance élevés contre les antibiotiques de première ligne couramment utilisés dans le traitement de diverses infections bactériennes, y compris les pénicillines et les céphalosporines, mais une résistance substantielle a été enregistrée contre l'imipénème chez *E. coli* (49,8 %) et les espèces de *Proteus* (32,1 %). De même, la tobramycine et la gentamicine ont montré une efficacité limitée contre *P. aeruginosa*, *Enterobacter* spp., (20% et 100%) et tous les GPB. Les souches MDR représentaient 56 % de tous les isolats testés, suivies par les souches XDR (26,1 %) dont l'amikacine était l'antibiotique le plus efficace. Parmi les isolats de *K. pneumoniae*, 66,6 % ont été classés comme *K. pneumoniae* hypervirulente (hvKp) sur la base du test de filamentation (String test). Dans les espèces de *S. aureus*, 61,3 % étaient des *S. aureus* résistants à la méthicilline (MRSA), tandis que 9,7 % présentaient le phénotype constitutif macrolide-lincosamide-streptogramine B (CMLSB), et 16,1 % affichaient le phénotype MLSB inductible (IMLSB), comme déterminé par le test-D. Différents facteurs de virulence ont été testés dans nos souches pathogènes. La formation de biofilm a été évaluée dans des microplaques en utilisant le crystal violet, tandis que la production d'hémolysine, de protéase, de lécithinase et de lipase a été déterminée en utilisant des boites contenant respectivement du sang humain, du lait écrémé et du jaune d'œuf. Dans l'ensemble, 88,1 % des souches étaient des producteurs de biofilms, la majorité (64,9 %) montrant une faible capacité à produire des biofilms. Les souches XDR ont montré le pourcentage le plus élevé de producteurs de biofilm forts (47,6 %). La production d'hémolysine a été observée dans 55,5 % des souches, suivie par la production de protéase à 47,3 % et les activités de lécithinase et de lipase (23,1 % et 10 %). Une recherche phénotypique de la production de β-lactamases à spectre étendu et d'AmpC a été menée, 32,6 % présentaient un phénotype BLSE et 12,7 % étaient positifs pour AmpC. La caractérisation moléculaire des gènes de β-lactamase et des gènes de quinolone avec PCR multiplex a été utilisée pour la confirmation phénotypique. bla_{TEM} était le gène de type ESBL émergeant (53,8 %), suivi par bla_{SHV} (15,9 %), tandis que $bla_{CMY\ II}$ était le gène AmpC détecté le plus fréquemment (10,4 %), suivi par bla_{DHA} (7,1 %). Tous ces gènes et d'autres gènes de virulence étaient portés par des plasmides dont la taille variait d'environ 1,5 à plus de 10 kb, profilés par la méthode PCR. Un taux de 26,7 % des souches d'*E. coli* pouvait héberger au moins un gène *qnr*. Parmi les souches *qnr*-positives, *qnrB* était le gène de résistance le plus prévalent (78,3 %) suivi de *qnrD* (26,1 %). Le PCR de Clermont *et al.* a été utilisée pour la détection des groupes phylogénétiques d'*E. coli* urinaire résistants à l'imipenème. Parmi les 86 isolats cliniques d'*E. coli* résistants à l'imipenème, le groupe B2 (48,9%) était le phylogroupe le plus prévalent, suivi des groupes E (22,1%), inconnu (12,8%), A (8,1%), et B1 (4,7%), ainsi que des Clades I, D, Clade I, ou Clade II (1,2%).

Mots-clés : Résistance aux antibiotiques, Bactéries multirésistantes, β-lactamase, ESBL, AmpC, gènes *qnr*, groupes phylogénétiques.

Abbreviation's list

-A-	
ABC	ATP-binding cassette
ABC-F proteins	ATP-binding cassette family-F proteins
ADH	Arginine dihydrolase test
AIEC	Adherent-invasive *E. coli*
AMEs	Aminoglycoside modifying enzymes
AmpC	Cephalosporinases
AMR	Antimicrobial resistance
APEC	Avian pathogenic *E. coli*
API	Analytic profile index
API Staph	Analytic profile index for *S. aureus*
API 20E	Analytic profile index for Enterobacteriaceae
AR	Antibiotic resistance
ARB	Antibiotic resistance bacteria
ATCC	American type culture collection
ATP	Adenosine triphosphate
-B-	
BHIB	Brain heart infusion broth
bp	Base pair
-C-	
Ca2+	Calcium cation
CDC	Centers for disease control and prevention
CDT	Combined disk test
CFU/ml	Colony-forming unit per millilitre
CIT	Citrate utisation test
CMLSB	Constitutive macrolide, lincosamide, streptogramin resistance *S. aureus*
CTX- M	Cefotaxime enzyme type
CV	Crystal violet
-D-	
DAEC	Diffusely adherent *E. coli*
DHP	Dihydropteroate
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
-E-	
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylenediaminetetraacetic acid
EF-G	Elongation factor -G
EHEC	Enterohemorrhagic *E. coli*
EIEC	Entero-invasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
Eps	Efflux pumps
EPS	Extracellular polymeric substances
ESBL	Extended spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
EU	European union

EUCAST	European committee on antimicrobial susceptibility testing
ExPEC	Extra-intestinal pathogenic *E. coli*
EYA	Egg yolk agar
-G-	
GEL	Gelatinase test
GNB	Gram-negative bacteria
GPB	Gram-positive bacteria
-H-	
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
HUS	Hemolytic and uraemic syndrome
H_2S	Thiosulfate reductase test
H+	Hydrogen cation
-I-	
IMLSb	Inducible macrolide, lincosamide, streptogramin resistance *S. aureus*
IMP	Imipenemase metallo -β- lactamase
InPEC	Intestinal pathogenic *E. coli*
ISEcp1	Insertion sequences
-K-	
KPC	*Klebsiella pneumoniae* carbapenemase
-L-	
LDC	Lysine decarboxylase
-M-	
MATE	Multidrug and toxic compounds extrusion
MBL	Metallo -β- lactamases
MDR	Multi-drug resistance bacteria
MFS	Major facilitator superfamily
$MgCl_2$	Magnesium chloride
MGE	Mobile genetic element
MHB	Muller-Hinton broth
MHT	Modified hodge test
MIC	Minimal inhibitory concentration
MLSB	Macrolide lincosamide streptogramin resistance *S. aureus*
MLST	Multi-locus sequence typing
MRSA	Methicillin resistant *S. aureus*
MSSA	Methicillin sensitive *S. aureus*
-N-	
NaCl	Sodium chloride
Na+	Sodium cation
NDM	New Delhi metallo-β-lactamase
NMEC	Newborn meningitis *E. coli*
-O-	
OD	Optical density
ODC	Ornithine decarboxylase test
Omps	Outer membrane protein
OXA	Oxacillinases
-P-	
pAmpC	Plasmidic AmpC enzymes
PBP	Penicillin-binding proteins
PBP2a	Penicillin-binding proteins 2a type

PCR	Polymerase chain reaction
pH	Potential of hydrogen
PMF	Proton motive force
PMQR	Plasmid-mediated quinolone resistance
-Q-	
qnr	Quinolone resistance gene
-R-	
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
RND	Resistance nodulation and cell division
-S-	
SHV	Sulfhydryl reagent variable enzyme
SMA	Skimmed milk agar
SMR	Small multidrug resistance
SPM	São Paulo metallo-β-lactamase
STEC	Shiga toxin-producing *E. coli*
-T-	
TAE	Tris-acetate-EDTA buffer
TEM	Temoniera (patient name) enzyme
THF	Tetrahydrofolate
TRPPs	Tetracycline ribosomal protection proteins
TSI	Triple Sugar Iron
TTC	2,3,5-triphenyl-2H-tetrazolium chloride
-U-	
UHT	Ultra-high temperature
UPEC	Uropathogenic *E. coli*
URE	Urease test
UTI	Urinary tract infection
UV	Ultra-violet
-V-	
VIM	Verona integron-encoded metallo-β-lactamase
VP	Voges-Proskauer test
-W-	
WHO	World health organization
-X-	
XDR	Extensively drug resistance bacteria

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1 General introduction

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Over many years, pathogenic bacteria have been a major cause of illness and death. The discovery of penicillin in 1928 rendered many life-threatening infections treatable, providing significant advantages for humans, veterinarians and animal breeders. Nevertheless, since the 1960s, antibiotics have been extensively utilized in sub-lethal doses as growth enhancers for food-producing animals (Muteeb et al., 2023; Zalewska et al., 2021). Also, their massive, excessive and sometimes unjustified use in humans has created a selection pressure on bacterial populations and allows bacteria to defend and adapt in presence of antibiotic via genetic or phenotypic prosses (Bhardwaj et al., 2022; Ahmed et al., 2024).

Antibiotics resistance (AR) has been acknowledged to be one among the top three major public health threats by the World Health Organization (WHO) (Salam et al., 2023). However, the spread of antibiotic resistance bacteria (ARB) has become a critical public health issue worldwide (Zalewska et al., 2021). WHO published a list of the top 12 bacterial families that threaten human health, in this list, bacteria are divided into three priority categories: critical, high, and medium priority, depending on how urgently new antibiotics must be developed to tackle these pathogens (Salam et al., 2023). *Acinetobacter*, *Pseudomonas*, and certain Enterobacteriaceae, including *K. pneumoniae, E. coli*, and *Enterobacter* spp., are among the microorganisms with a critical priority (Mancuso et al., 2021).

In the European Union (EU) alone, it is estimated that AR causes approximately 25,000 deaths annually, while globally, the toll is as high as 700,000 lives lost each year (World Health Organization, 2014). An estimated 1.27 million deaths were attributable to AR infections in 2019 alone, while nearly 5 million deaths were associated with drug-resistant infections, according to a major study published in January 2023 (Salam et al., 2023). This number is estimated to be increased to 10,000,000 per year by 2050, could surpass cancer as a leading cause of mortality worldwide (World Health Organization, 2014).

In the face of the development of bacterial resistance, there are an emergence of multi-drug resistant bacteria (MDR) (Saha and Sarkar, 2021). The severity of infections in hospitalized patients, a rise in immunocompromised individuals, the introduction of resistant pathogens from the community, inconsistent infection control practices, frequent use of antimicrobial prophylaxis, widespread use of broad-spectrum therapies, and high overall antibiotic usage in specific regions, all these factors contributing to increased AR in healthcare settings (Chen et al., 2021).

In community settings, the factors can include: the overuse of antibiotics by both healthcare providers and patients, the administration of antibiotics in numerous fields, patient noncompliance with treatment, longer survival of people with chronic infections, and limited research into new antibiotics (Chen et al., 2021). The food chain may be an additional factor contributing to the AR (Saha and Sarkar, 2021), it can also be affected by factors contributing from pharmaceuticals, inappropriate waste management, trade, and finance, socioeconomic determinants, accumulation of antibiotics in the environment (Chen et al., 2021; Mancuso et al., 2021; Salam et al., 2023). An often-overlooked factor is the role of disinfectant exposure, which can also promote AR and lead to the emergence of MDR (Chen et al., 2021).

Consequently, the reduction of the effectiveness of antibiotic usage in healthcare, decreases the probability of controlling and avoiding diseases that compromise the immune system, such as HIV, cancer, surgical interventions, and diabetes (Abushaheen et al., 2020; Mancuso et al., 2021; Salam et al., 2023). As first-line antimicrobials have become less effective, there is an increasing dependence on second- and third-line treatments, which are frequently more expensive, unsafe, and need longer duration for therapy (Ahmed et al., 2024). Thus, if this situation persists, it may result in a "post-antibiotic era" where common illnesses and minor injuries become the main causes of mortality (O'Neill, 2016; Abushaheen et al., 2020; Tarín-Pelló et al., 2022).

A clear understanding that AR involves humans, animals and environment health, which fall within the One Health concept's (Aslam et al., 2021; Larsson and Flach, 2022), human usage of antibiotics has been linked to resistance in a number of significant human infections that impact different systems of the body. There is currently significant evidence that antibiotic usage in animals is an important contributor to antimicrobial resistance among human infections, in particular, common enteric pathogens such as *Salmonella* spp., *Campylobacter* spp., *Enterococcus* spp., and *E. coli* and in some cases other bacteria that can also be zoonotic like *S. aureus*. Additionally, there is growing worry that bacteria exposed to biocides (such as disinfectants and antiseptics) and heavy metals in animals and environmental niches may co-select for resistance to antimicrobials (McEwen and Collignon, 2018).

In the WHO African region, Algeria has one of the highest proportions of infection-related deaths associated with resistant pathogens, estimated at 48% the largest share within the region (Sartorius et al., 2024). Indeed, over the past years, a significant increase in AR, particularly among Gram-negative bacteria (GNB) was observed (Baba Ahmed-Kazi Tani and Arlet, 2014;

Yagoubat et al., 2017). Some recent surveys reported an increase in resistance strains, with a predominance of extended spectrum β-lactamase (ESBL) Enterobacteriaceae (Zemmour et al., 2021; Khaldi et al., 2022), with $bla_{CTX\text{-}M\ 3}$, $bla_{CTX\text{-}M\ 15}$, bla_{TEM} and bla_{SHV} being the most frequently detected genes (Nabti et al., 2021; Merah-Fergani et al., 2022). These genes are responsible for enzymes frequently being the cause of potentially serious infections in both community and hospital settings. The plasmid-mediated cephalosporinases DHA-1, CMY-2, and CMY-12 have been found (Baba Ahmed-Kazi Tani and Arlet, 2014).

At the regional level, Sétif province have an increase levels of AR genes; especially, $bla_{CTX\text{-}M\ 15}$, $bla_{CTX\text{-}M\ 14}$, bla_{TEM} and bla_{SHV} and also carbapenemase encoding genes $bla_{OXA\text{-}48}$, $bla_{OXA\text{-}48\ like}$, $bla_{OXA\text{-}23}$ and $bla_{OXA\text{-}72}$ (Baba Ahmed-Kazi Tani and Arlet, 2014; Nabti et al., 2021). Resistance genes to quinolones and fluoroquinolones are more recent identification, the most common are the *qnr* determinants like *qnrA*, *qnrB* and *qnrS* (Alouache et al., 2014).

this work is part of a broader research problem focusing on AR in Sétif region. The purposes of this study are:

- The isolation and identification of strains considered as human pathogens from medical analysis laboratories and different hospitals around Sétif province, in the east of Algeria.
- To test their sensitivity to different antibiotics and the phenotypic characterization of different resistance profile and to investigate the relationship between AR profiles and biofilm activity and toxins production including: hemolysin, protease, lipase and lecithinase.
- Followed by a molecular characterization of β-lactamase resistance genes responsible of β-lactams resistance.
- Characterize *qnr* determinants of some strains with plasmid profiling.
- To found links and to understand and follow these resistances and study the most prevalent pathogens and type of resistance distributed in this province. Finally, this study assessed:
- The phylogenetic groups of imipenem-resistant uro-pathogenic *E. coli* (UPEC).

2 Literature Review

Since their discovery, antibiotics designate against bacterial diseases by reducing bacterial capacity to grow, killing bacteria, or inhibiting their multiplication (Abushaheen et al., 2020). However, they are also prescribed for the treatment of non-bacterial infections, such as respiratory infections caused by viruses (Seifert and Schirmer, 2021).

2.1 Principal targets and classes of antibiotics

The fundamental interaction between antibiotics and their targets leads to bacterial cell death by blocking vital cellular activities (Singh, 2023). Antibiotics target five essential parts and processes in a bacterial cell: cell wall, cell membrane, protein synthesis, nucleic acid synthesis and folate synthesis (Fig. 1) (Ghosh et al., 2020; Salam et al., 2023).

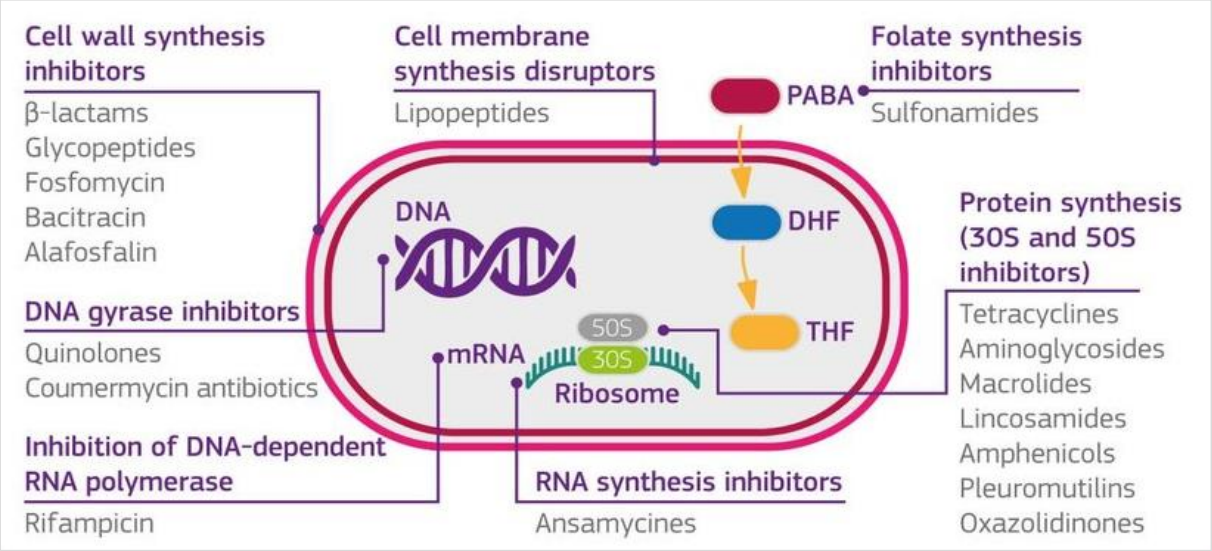


Figure. 1 : Different targets of antibiotics (European Commission. Joint Research Centre., 2018).

By all these processes and based on the specific system or cellular component they target, antibiotics either kill the bacteria (bactericidal antibiotic) or inhibit cellular growth (bacteriostatic antibiotic) (Singh, 2023); although based on different mechanism of action, antibiotics can be subdivided into groups (Mancuso et al., 2021) :

2.1.1 Inhibition of cell wall production

Two main groups of antibiotics use this mechanism: β-lactams and glycopeptide antibiotics (Fig. 2.), in addition of bacitracin and fosfomycin (Bhattacharjee, 2022); by inhibiting the peptidoglycan polymerization, with direct or indirect ways (Baran et al., 2023).

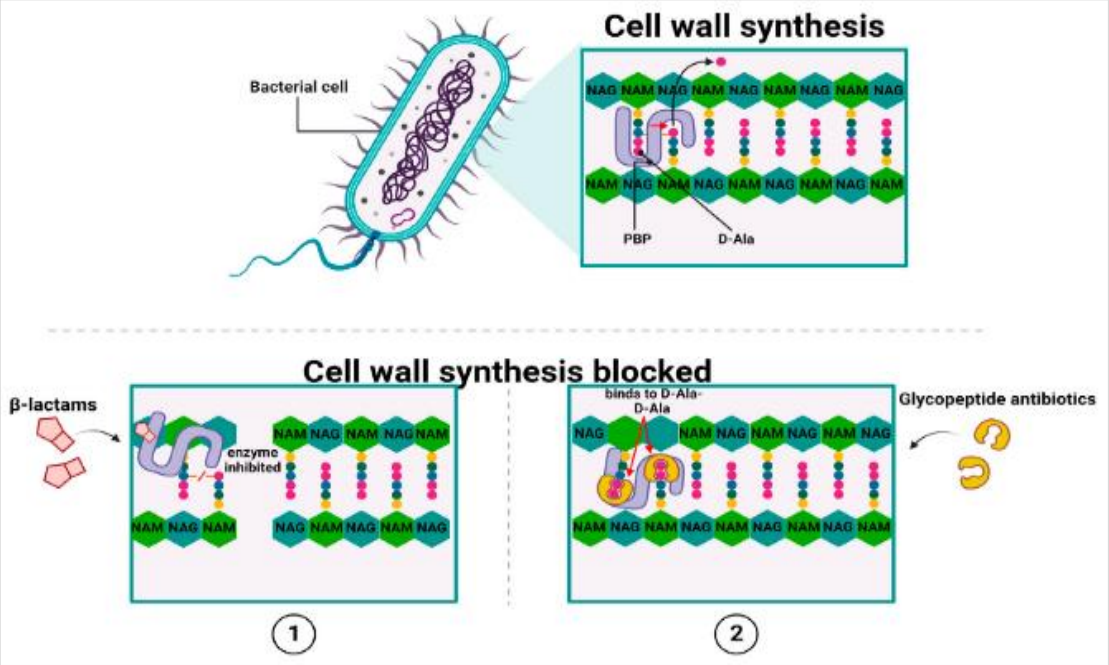


Figure. 2 : Mode of action of β-lactams and glycopeptide (Baran et al., 2023).

2.1.2 Inhibition of cell membrane synthesis

These antibiotics alter bacterial cell membrane integrity and cyclic lipopeptide such as daptomycin (Huang, 2020) and polymyxins (Ayoub Moubareck, 2020).

Ca^{2+}- daptomycin combination generates micelles that reach the bacterial inner membrane, interact with negatively charged phosphatidylglycerol groups and neutralize them. It subsequently integrates into the membrane and initiates oligomerization dependent on phosphatidylglycerol. This results in the creation of membrane channels, leading to compromised membrane integrity (Fig 3. (1)) (Baran et al., 2023). While polymyxins exhibiting polycationic properties, destroys the phosphate groups of lipopolysaccharides in the bacterial membrane, owing to its hydrophilic and lipophilic nature. This interaction leads to an increase

in membrane permeability, resulting in the leakage of intracellular content (Fig. 3. (2)) (Yin et al., 2020; Jian et al., 2021).

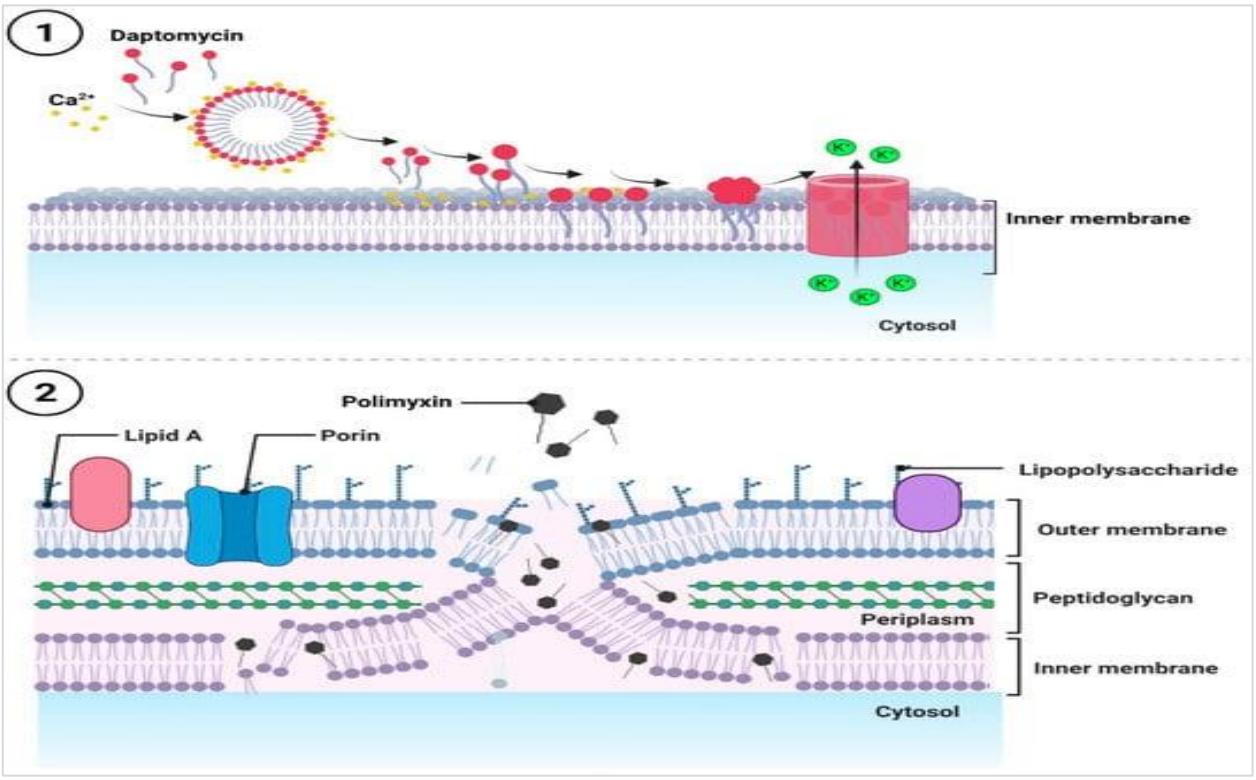


Figure. 3 : Mode of action of daptomycin (1) and polymyxins (2) (Baran et al., 2023).

2.1.3 Inhibition of nucleic acid synthesis

Quinolones, fluoroquinolones and rifamycins were most used in human and animal treatment. Quinolones and fluoroquinolones exert their antibacterial effects by targeting DNA synthesis, specifically through the inhibition of DNA gyrase and DNA topoisomerase IV, enzymes indispensable for the replication and transcription of bacterial DNA (Bush et al., 2020). Rifamycins inhibit the synthesis of bacterial RNA, the antibiotic binds to the β-subunit of RNA polymerase, this binding inhibits the initiation of bacterial DNA transcription as well as the formation of all messenger RNAs, transfer RNAs, and ribosomal RNAs (Fig. 4) (Baran et al., 2023).

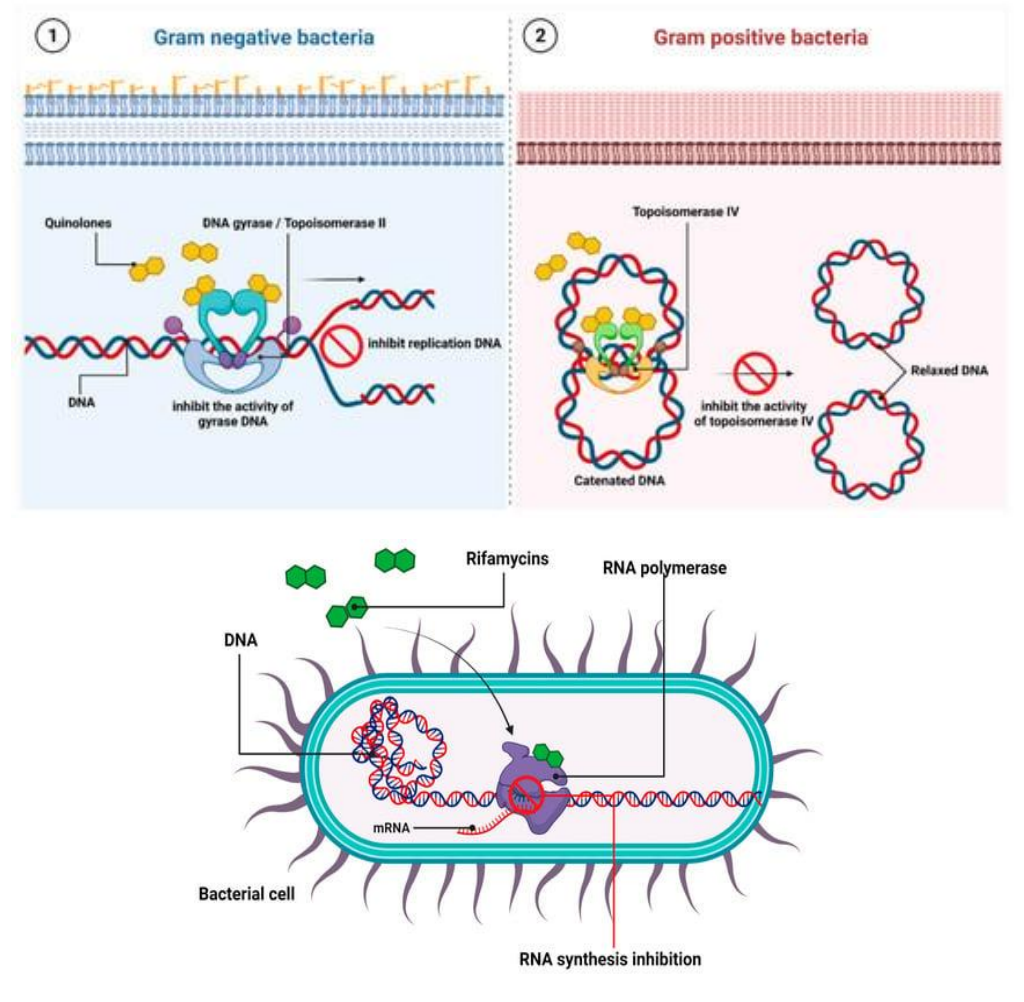


Figure. 4 : Mechanism of action of quinolones and rifamycins (Baran et al., 2023).

2.1.4 Inhibition of protein synthesis

Tetracyclines, aminoglycosides, macrolides, lincosamides, streptogramins B and oxazolidinones inhibit protein synthesis by binding selectively to the 30S or 50S subunits of intracellular ribosomes (Fig. 5) (Mancuso et al., 2021; Baran et al., 2023).

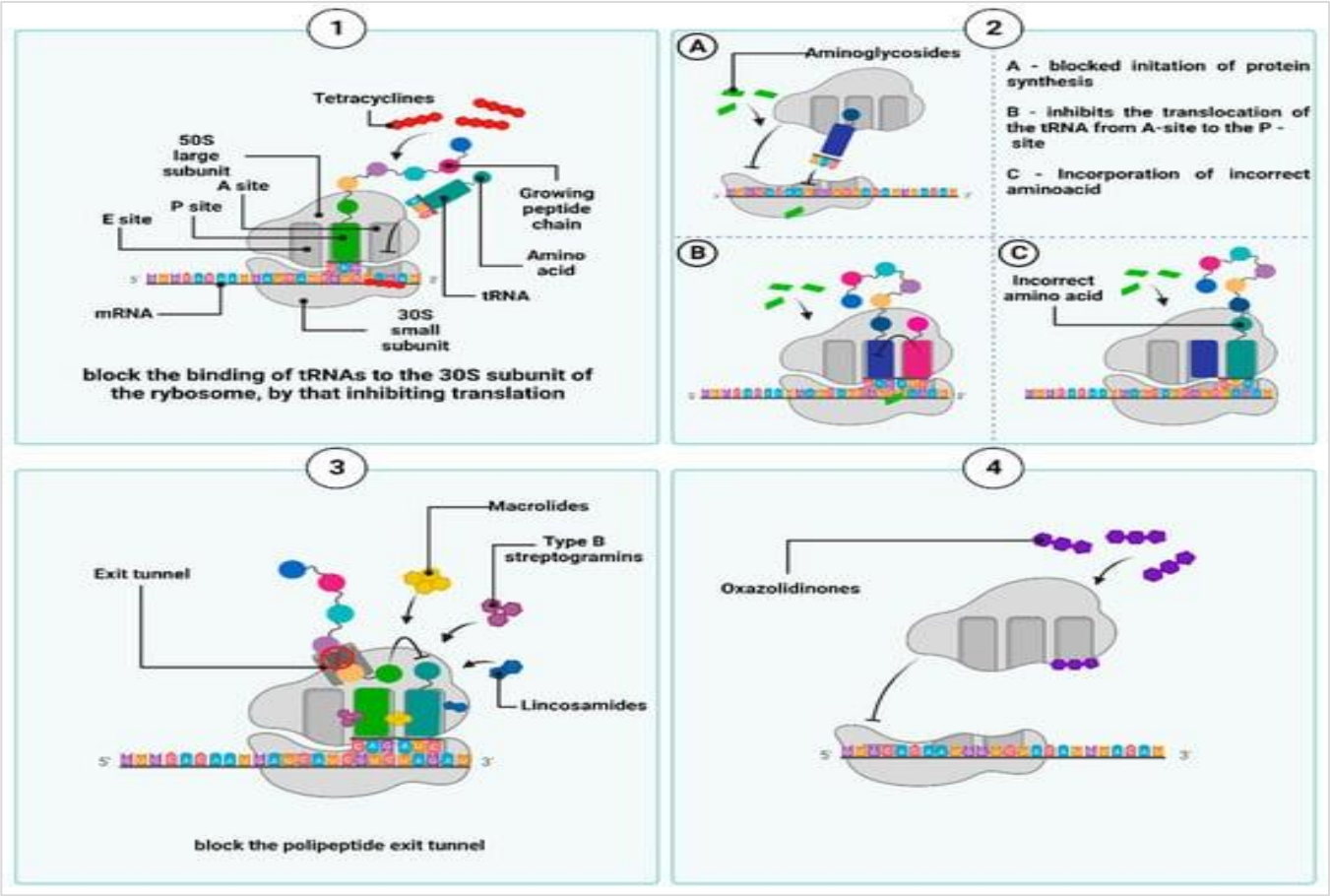


Figure. 5 : Mechanism of action of tetracyclines (1), aminoglycosides (2), macrolides, lincosamides and B streptogramins (3) and oxazolidinones (4) (Baran et al., 2023).

2.1.5 Inhibition of folate synthesis

Bacteria need folic acid for purine and pyrimidine bases synthesis, which are essential for the production of bacterial genetic material. Sulfonamides alone or in combination with trimethoprim were affect the bacterial metabolic pathway especially by inhibition the production of dihydropteroate (DHP) and tetrahydrofolate (THF), which are both essential metabolic stages in the folate pathway (Fig. 6) (Chen et al., 2022).

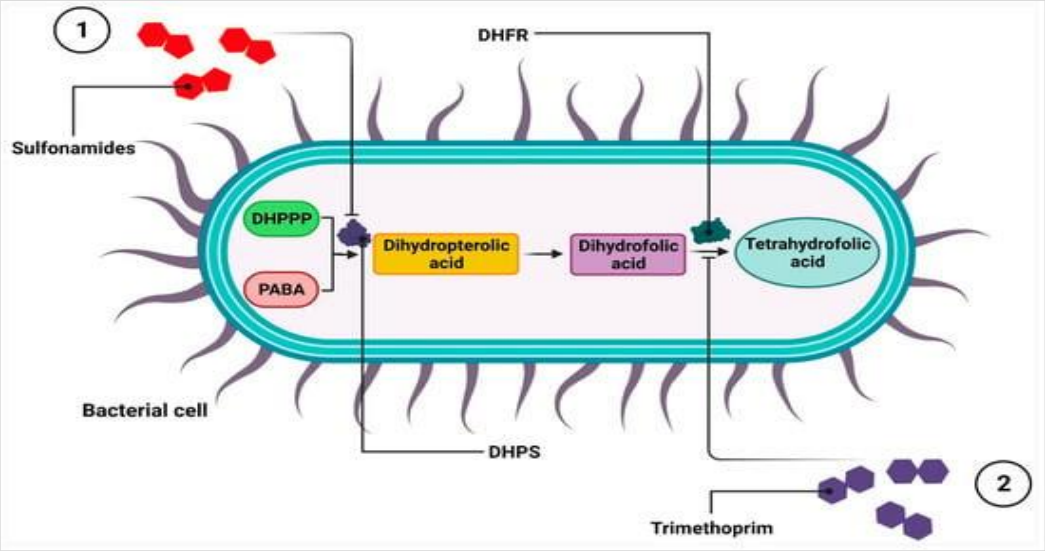


Figure. 6 : Mode of action of sulfonamides (1) trimethoprim (2) (Baran et al., 2023).

2.2 Antibiotic resistance

With their effective performance, antibiotics do not just used for medicinal purposes and in the treatment of human infections of all severity levels (Abushaheen et al., 2020), but they have also been used in agriculture production and animal treatment and industry (Zalewska et al., 2021; Salam et al., 2023). Consequently, it must be examined via a One-Health approach, as human health is fundamentally interconnected with animal health and ecological sustainability (Christaki et al., 2020).

Due to the ever-increasing use and misuse of antibiotics, their effectiveness has deteriorated and bacteria have developed AR (Chen et al., 2021). AR refers to the capacity of microorganisms including bacteria, viruses, fungi, parasite to persist, be alive and continue to grow and proliferate in the presence of lethal antimicrobial agents like antibiotics and antiviruses and antifungal (Chen et al., 2021; Salam et al., 2023). Antibiotics represent the most extensively utilized class of antibacterial agents, making AR the most common form of resistance among all classes of antimicrobials (Salam et al., 2023). So AR can be defined as the capacity of bacteria and other microorganisms to no longer respond to antibiotics that were previously sensitive (Chen et al., 2021).

Since the late of 1950s, AR was already discovered (Fig. 7) (Christaki et al., 2020; Mancuso et al., 2021; Salam et al., 2023). However, for a long time, AR was not a serious worldwide

problem, until 1960s and with the discovery of new classes of antibiotic, which suggested that the problem of AR can be solved (Mancuso et al., 2021). However, resistance developed quickly and AR soon became a major public health concern (Uddin et al., 2021).

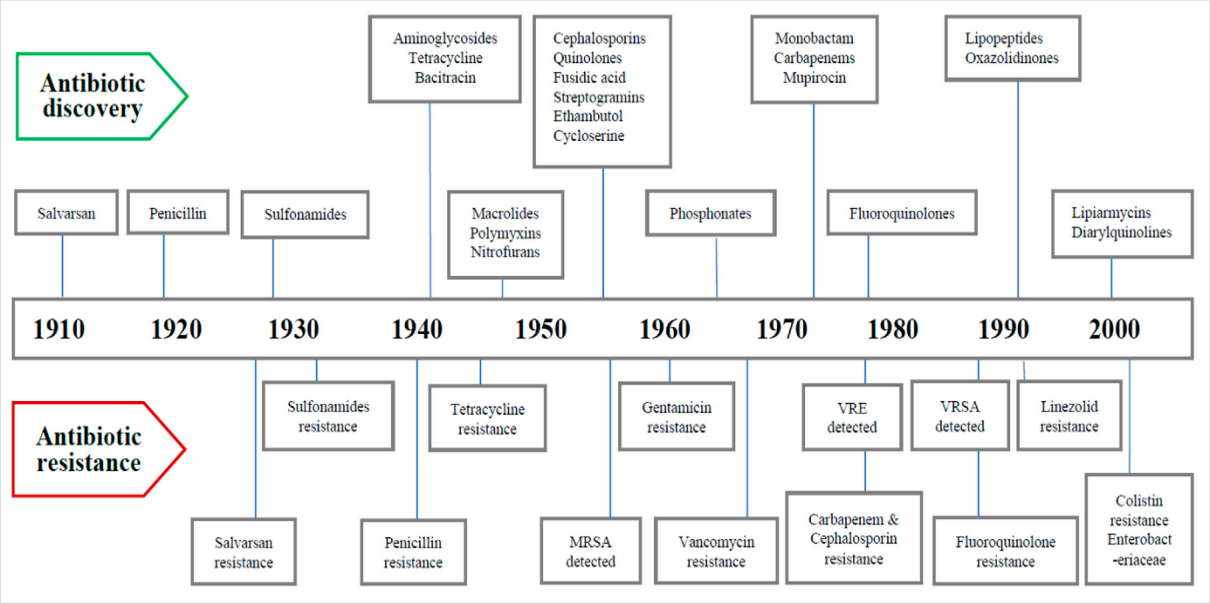


Figure. 7 : Timeline of antibiotic discovery and antibiotic resistance (Salam et al., 2023).

2.3 Antibiotic resistance sources

Bacteria expressed resistance via three forms:

2.3.1 Natural resistance

Natural resistance refers to bacteria's inherent ability to resist specific classes of antibiotic and this resistance conferred by their chromosomal genes, without the need for genetic mutations or the acquisition of external genes (Mancuso et al., 2021; Salam et al., 2023). As result, bacteria occur naturally mechanism for resistance derived from the fundamental structural and/or functional characteristics of the bacteria (Baran et al., 2023; Hasan and AL-Harmoosh, 2020; Jian et al., 2021).

2.3.2 Acquired resistance

This resistance is due to changes in bacterial chromosome, it's not affected by the antibiotic. It was previously susceptible for it (Hasan and AL-Harmoosh, 2020; Naveed et al., 2020), it's originated from the main chromosome or extrachromosomal structures:

- Chromosomal resistance due to mutations occur by certain physical and chemical factors (Hasan and AL-Harmoosh, 2020; Salam et al., 2023),
- Mutant strains are able to transfer the mutation via vertical transmission (Mancuso et al., 2021).
- Extrachromosomal resistance can occur via gaining new exogenous DNA via horizontal gene transfer (HGT) by transformation, transposition, transduction, conjugation and by transmission of plasmids, transposons, integrons… (Fig. 8) (Baran et al., 2023; Hasan and AL-Harmoosh, 2020; Singh, 2023).

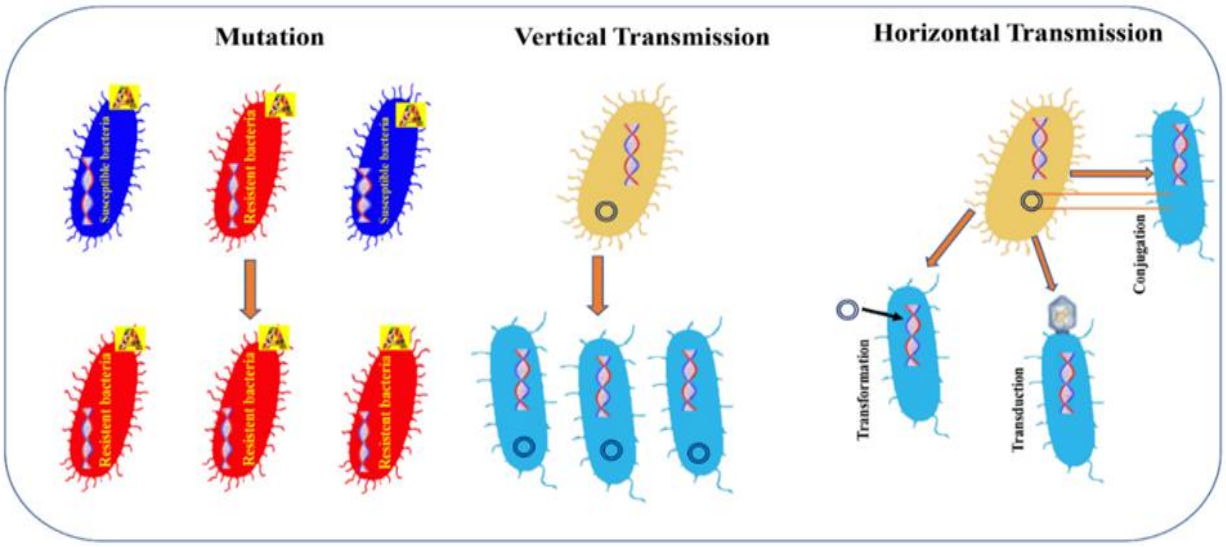


Figure. 8 : Types of spread of acquired resistance (Mancuso et al., 2021).

2.3.3 Adaptive resistance

This resistance was affected by environmental changes, depending on the intensity and duration of selection pressure (Jovanovic et al., 2021; Langendonk et al., 2021). When bacteria are exposed to antibiotic concentrations that are below their minimum levels, with environmental factors such as growth conditions, stress, pH, and ion concentrations, they can develop adaptive resistance, And the best example of this category of resistance is biofilm formation capacity. Unlike intrinsic or acquired resistance, adaptive resistance is typically temporary and reverses once the inducing factors are removed (Salam et al., 2023).

2.4 Mechanisms of antibiotic resistance

Bacteria develop and use diverse resistance strategies as long as antibiotic are administed against them (Cars et al., 2021). Unfortunately, these AR mechanisms have protected them from the effects of these antibiotics (Mancuso et al., 2021). The mechanism of AR is categorized into the following:

2.4.1 Antibiotic inactivation

Bacteria inactivate antibiotic by two different ways: antibiotic destruction or antibiotic alteration and these two mechanisms are enzymatic-dependent mechanisms (Ahmed et al., 2023). Antibiotic destruction lead by hydrolyzation, β-lactamases are the best example for this type of resistance, these enzymes cleave the amide bond of β-lactams ring, rending the antibiotic ineffective (Christaki et al., 2020). Second example of TetX enzymes, which catalyze the oxygen-dependent destruction of tetracyclines (Fig. 9) (Iskandar et al., 2022).

2.4.2 Chemical modification of antibiotic

Bacteria possess the ability to synthesize enzymes that modify antibiotics through the addition of chemical groups, preventing it from binding to their targets. The most common ways bacteria inactivate drugs are by adding phosphoryl, acetyl or adenyl groups. Acetylation is used to inactivate drugs like aminoglycosides, chloramphenicol, streptogramins and fluoroquinolones. Aminoglycosides are also inactivated by adenylation and phosphorylation (Uddin et al., 2021). Enzymes called aminoglycoside modifying enzymes (AMEs) modify the antibiotic's structure (alerts the hydroxyl or amino groups), rendering it inactive, and this is a key example of bacterial resistance through drug modification (Christaki et al., 2020).

2.4.3 Antibiotic target modification

The changes in target sites are bacterial mechanism that consist of: target replacement, alteration and protection (Fig. 9) (Iskandar et al., 2022):

2.4.3.1 Target replacement

Three examples can explain this mechanism:

- Changes in the arrangement of penicillin-binding proteins (PBP) lead to β-lactams resistance (Uddin et al., 2021).
- In *S. aureus*, methicillin resistance was occurred by the acquisition of *mecA* gene, which incorporate and becomes a part of their DNA, this gene is found in mobile genetic element (MGE) called staphylococcal chromosomal cassette (SCC mec); the *mecA* gene produce PBP2a, a specific PBP with low affinity to for β-lactams(Abebe and Birhanu, 2023).
- The acquisition of *van* gene cluster in enterococci by MGEs induces structural modifications in peptidoglycan precursors lead to the replacement of glycopeptide target (Christaki et al., 2020).

2.4.3.2 Target alteration

Target alteration by mutation is a type of resistance witch was observed in drugs that inhibit nucleic acid synthesis like: quinolones and fluoroquinolones, with chromosomal mutation in bacterial DNA gyrase and/or topoisomerase IV (Christaki et al., 2020). These changes reducing and limiting the drug's capacity to bind to certain components (Uddin et al., 2021), while mutations in β-subunit genes of RNA polymerase result to rifamycins resistance (Patel et al., 2023).

The enzymatic alteration was seen in resistance to chloramphenicol, clindamycin and linezolid, it has been attributed to methylation of the 23S rRNA, catalyzed by an enzyme encoded by the *cfr* gene (Tsai et al., 2022) and also in macrolides, lincosamides and streptogramin B. This resistance was conferring by large group of erythromycin ribosomal methylation (erythromycin ribosomal methylase: *erm*) genes (Cars et al., 2021; Christaki et al., 2020).

2.4.3.3 Target protection

This method of resistance was described in both GPB and GNB (Christaki et al., 2020). Several genetic determinants encoding proteins that mediate target site protection have been identified in bacterial chromosomes. This form of resistance is frequently associated with MGEs, which facilitate the transfer of these genes (Ndagi et al., 2020).

These protective proteins safeguard particular antibiotic targets from the inhibitory effects of various antibiotics (Zhang and Cheng, 2022). Examples included: tetracycline ribosomal

protection proteins (TRPPs), which bind to ribosomes and restore their structure, preventing tetracycline from binding (Iskandar et al., 2022; Zhang and Cheng, 2022). Quinolone's protection proteins by functioning as a DNA analogue and decreasing the interaction between bacterial gyrase and topoisomerase IV and DNA (Christaki et al., 2020).

Changing of the target's structure mediated by ABC-F proteins, which are the main contributors to resistance in drugs targeting the 50S ribosomal subunit. These drugs include macrolides, lincomycins, phenols and streptogramins (Zhang and Cheng, 2022) and resistance to fusidic acid by FusB-type protein, which bind to elongation factor G (EF-G), FusB drives EF-G's release from the ribosome. Even in the presence of fusidic acid, weakening the drug's ability to bind to EF-G, and allowing resistance to occur (Tomlinson et al., 2020).

2.4.4 Limiting drug uptake

GNB possess an outer membrane that serves as a permeability barrier for numerous chemical compounds, including antibiotics (Christaki et al., 2020). They are intrinsically less permeable to several antibiotics than GPB (Uddin et al., 2021) because the outer membrane constituted of proteins and lipopolysaccharides, where hydrophilic molecules have difficulty to traverse the lipid layer and require facilitation via porin channels or outer membrane porins (Omps) (Zhang and Cheng, 2022). This explain the intrinsic resistance of some GNB to hydrophilic molecules, such as vancomycin (Fig. 9) (Christaki et al., 2020; Iskandar et al., 2022).

Moreover, alterations in outer membrane permeability may facilitate the emergence of acquired resistance (Christaki et al., 2020). Porins serve as the primary pathway for the introduction of hydrophilic antibiotics through the bacterial outer membrane such as β-lactams, tetracyclines, fluoroquinolones and chloramphenicol (Christaki et al., 2020). Each bacterial type synthesizes distinct porins (OmpF, OmpC and OmpE), the entry of hydrophilic antibiotics will be influenced by the number and the type of expressed Omps and the loss or impairment of one or more Omps contributes to bacterial resistance (Zhang and Cheng, 2022).

Limiting antibiotics uptake may result from mutations that modify the expression, structure or function of porins (Saha and Sarkar, 2021); these mutations can have different affects such as: deletion of porin, changes of the size and conductance or reduced the expression of porins (Christaki et al., 2020). For example, resistance to carbapenems in Enterobacteriaceae will be

emerged even in the absence of carbapenemase enzymes by mutations which decrease porin production (Uddin et al., 2021).

However, in many bacterial strains, this effect is amplified when combined with other resistance mechanisms. Reduced antibiotic uptake from porin changes boosts the impact of co-existing mechanisms like efflux pumps or antibiotic-degrading enzymes, leading to high-level resistance (Christaki et al., 2020; Saha and Sarkar, 2021).

2.4.5 Efflux pumps

One of the typical mechanisms of drug resistance is the drug efflux from bacterial cells to prevent the intracellular accumulation of toxic substances in a way does not involve modification or deterioration (Baran et al., 2023). Efflux pumps (Eps) are among the fastest and most effective resistance mechanisms bacteria deploy in response to antibiotics or toxins. These systems have evolved as a self-defense strategy, preventing the buildup of harmful compounds within bacterial cells by actively pumping them out. These proteins are located in the plasma membrane (Gaurav et al., 2023). Eps transport a wide range of substances from the cytoplasm to protect the cell from antibiotic accumulation, making it less effective. This phenomenon is responsible for MDR (Fig. 9) (Zhang and Cheng, 2022; Muteeb et al., 2023).

Eps are energy-dependent complex bacterial transporter systems that have been identified in both GNB and GPB (Christaki et al., 2020). There are two types of genes that encode antibiotic Eps: acquired and intrinsic (Iskandar et al., 2022). Multidrug efflux mechanisms are usually chromosomally encoded in GNB, contributing to the bacteria's intrinsic resistance (Uddin et al., 2021). In contrast, genes found in plasmids, MGEs, and mutations may increase pump expression or efficiency, often driving clinically acquired resistance (Christaki et al., 2020; Baran et al., 2023).

Therefore, numerous active Eps classes are found in both kinds of bacteria, which are divided into five groups based on several factors, including their composition, the nature of the substrates, their transport and their energy source (Tab. 1) (Ebbensgaard et al., 2020; Iskandar et al., 2022).

Table 1. Five primary families of efflux pump (Jagessar et al., 2020; Drew et al., 2021; Kim et al., 2021; Klenotic et al., 2021; AL-Lami et al., 2022).

Transporter system	Acronym	Nature	Location	Substrate	Transport system
Resistance nodulation and cell division	RND	Multi-proteins transporter	In the entire bacterial cell membrane	antibiotics, heavy metals, toxins	From cytoplasm to the external environment
Major facilitator superfamily	MFS	Single protein transporter	Cytoplasmic membrane	Fluoroquinolones, macrolides, chloramphenicol, linezolid, trimethoprim, tetracycline	From the cytoplasm into the periplasmic space
Multidrug and toxic compounds extrusion	MATE	Multi-proteins transporter	In the entire bacterial cell membrane	Fluoroquinolones and some aminoglycosides	Use the energy from the H^+ or Na^+ ion gradient to extrude the antibiotic from the cytoplasm to the external environment
Small multidrug resistance	SMR	Multi-proteins transporter	In the entire bacterial cell membrane	β-lactams and some aminoglycosides	From cytoplasm to the external environment
ATP-binding cassette	ABC	Multi-proteins transporter	In the entire bacterial cell membrane	Tetracyclines and macrolides	Use the energy generated from ATP hydrolysis for transport antibiotics outside the bacterial cell

According to energy source, ABC classified as "primary active transporters" remove substrates using energy from ATP hydrolysis. In contrast, "secondary active transporters" such as MATE, , RND, SMR and MFS rely on the proton motive force (PMF) to drive the expulsion of sodium and hydrogen ions across the membrane (Zhang and Cheng, 2022).

The RND superfamily is regarded as the primary drug Eps family, playing a key role in conferring drug resistance to various contributing significantly to multidrug resistance in GNB (Baran et al., 2023). While RND is exclusive for GNB; MATE, SMR, MFS and ABC families are found in both GPB and GNB (Ebbensgaard et al., 2020; Iskandar et al., 2022; Zhang and Cheng, 2022).

The classic example for Eps mechanisms were MexAB-OprM in *P. aeruginosa* and AcrAB-TolC in Enterobacteriaceae, both from RND family (Uddin et al., 2021; Zhang and Cheng,

2022). Eps help bacteria regulate their internal environment by removing not only antibiotics but also toxic substances, virulence molecules and heavy metals (Gaurav et al., 2023).

2.4.6 Biofilm

Bacterial biofilm is a unique survival mechanism formed by bacteria that adhere to inert surfaces (Zhang and Cheng, 2022), where they are encased in a self-produced polymer matrix contain polysaccharides, proteins and DNA (Uddin et al., 2021). Biofilm formation can lead to resistance to antibiotics, this is due to the fact that bacterial cells can become more resistant to antimicrobials when they develop biofilms and are subsequently encased in a complex matrix, which limits the penetration of antibiotics, which prevents drug penetration at bactericidal concentrations (Fig. 9) (Abebe, 2020). Biofilms are particularly problematic in medical settings, especially on indwelling devices such as urinary catheters, where they serve as persistent sources of infection that are difficult to eradicate (Donlan, 2001)

Biofilms exhibit the ability to tolerate antimicrobial agents at concentrations 10-1000 times greater than what is needed to inactivate genetically identical planktonic bacteria. This increased resistance in biofilm-associated microorganisms is attributed to several suggested factors (Dincer et al., 2020; Zhang and Cheng, 2022):

- Polymeric matrix, which is capable of inhibiting antibiotic diffusion.
- Combination of antibiotics with a polymeric matrix that diminishes their efficacy.
- Enzyme-mediated resistance.
- Alterations in metabolic activity inside the biofilm.
- In term of communities, the interaction within the community lead to augmentation of members ability to resist antibiotics, antibiotics tolerance and protection of sensitive members caused by high rate of mutation and genetic changes which allows them to develop defense mechanisms (Sharma et al., 2023).

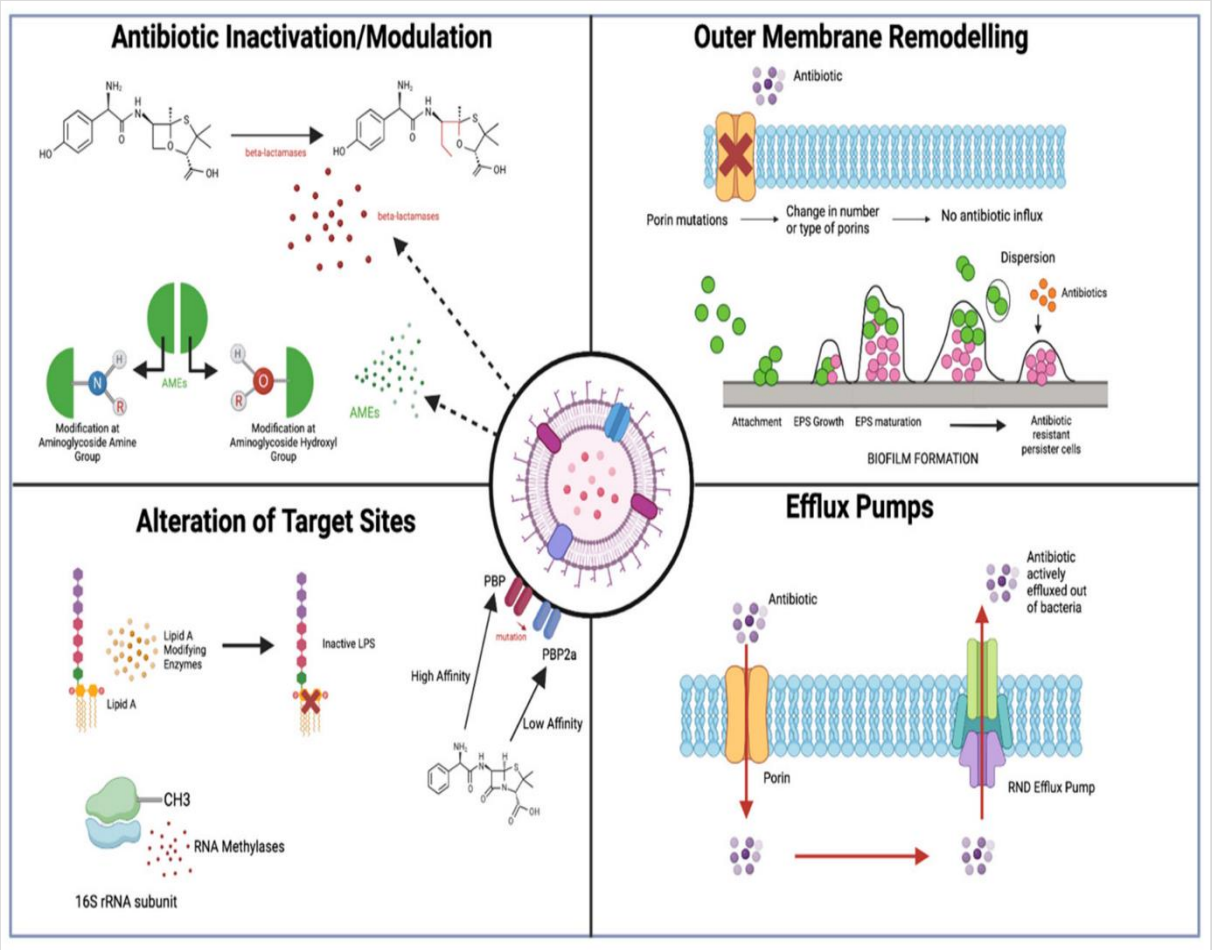


Figure. 9 : Common mechanism of antibiotic resistance (Gauba and Rahman, 2023).

2.5 Resistance for β-lactam antibiotics

β-lactam antibiotics are among the safest and most effective antimicrobial agents available (Watkins and Bonomo, 2016), this group represents a broad class of antimicrobial agents, encompassing penicillins, cephalosporins, monobactams, and carbapenems (Fig. 10) (Hasan and AL-Harmoosh, 2020; Schaenzer and Wright, 2020). The most prevalent resistance mechanism against these antibiotics involves the production of β-lactamase enzymes (Muteeb et al., 2023).

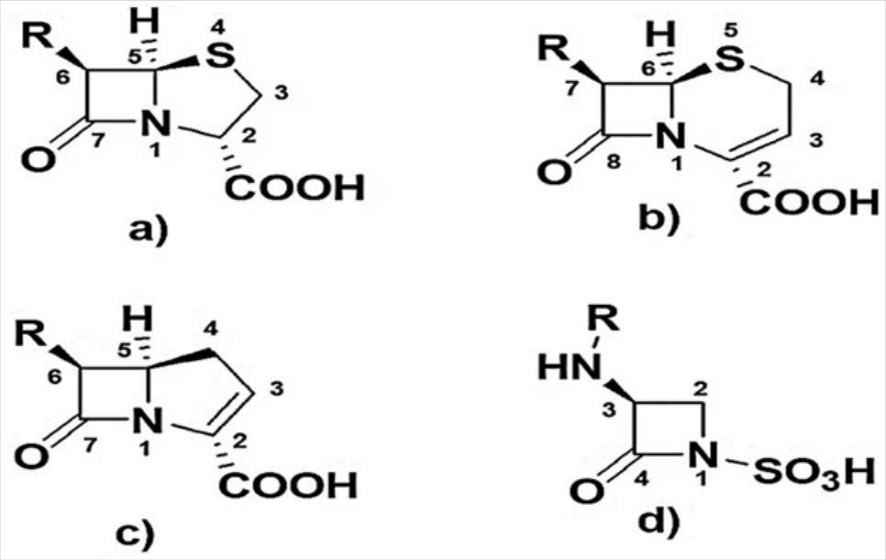


Figure. 10 : β-lactam antibiotics: a) penicillin, b) cephalosporin, c) carbapenem, d) monobactam (Alfei and Schito, 2022).

The Centers for Disease Control and Prevention (CDC) and the WHO have identified β-lactamase-producing GNB as some of the most serious or critical global health threats (Watkins and Bonomo, 2016). These enzymes neutralize the bactericidal activity of β-lactams by hydrolyzing the amide bond within the β-lactam ring. They can hydrolyze β-lactam antibiotics before they reach their intended targets, such as the PBPs, further contributing to the inactivation of the antibiotics and rendering it ineffective (Fig.11) (Munita and Arias, 2016; Ding et al., 2021).

However, genes encoding β-lactamase enzymes are conventionally designated as *bla*, followed by the specific enzyme variant (ex: bla_{TEM}). These genes may be located on the bacterial chromosome or within MGEs as part of the accessory genome, facilitating their HGT. Additionally, they are often associated with integrons, further promoting their dissemination. The expression of these genes can either be constitutive or inducible, depending on whether an external signal is required to activate their transcription (Munita and Arias, 2016).

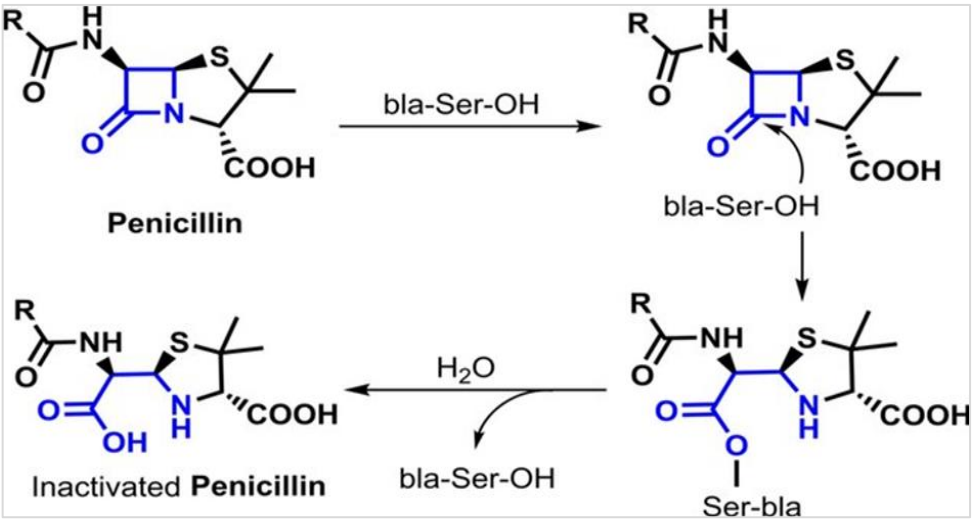


Figure. 11 : Hydrolysis mechanism for penicillin with β-lactamase (Ding et al., 2021).

Two main classification schemes have been proposed based on two major attributes of the physiologically important enzymes: structure and function. Structural classes were first defined by Ambler in 1980 (Ambler classification) categorizes β-lactamases based on their amino acid sequence identity, dividing them into four distinct groups: A, B, C, and D (Watkins and Bonomo, 2016). Bush-Jacoby classification system organizes β-lactamases into four primary categories, each comprising several subgroups, based on their biochemical function, with a particular focus on substrate specificity (Fig. 12) (Bush and Bradford, 2020).

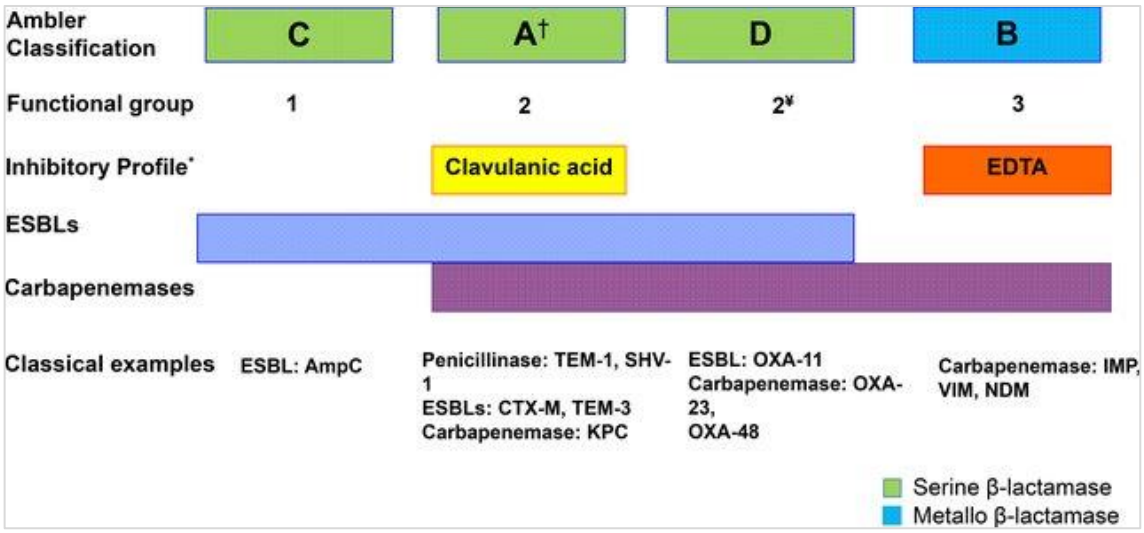


Figure. 12 : β-lactamase classification: Ambler classification (molecular classification), Bush-Jacoby classification (Functional groups), † Class A enzymes include penicillinases, ESBLs, and carbapenemases. ¥ Ambler class D enzymes belong to the functional group/subgroup 2d (Munita and Arias, 2016).

2.5.1 Class A β-lactamases

This class was characterized by the presence of a serine residue in their catalytic site and use amino acids as their active centers, a characteristic it shares with enzymes in classes C and D (Munita and Arias, 2016; Ding et al., 2021). They are most inhibited by clavulanic acid or tazobactam and exhibit activity against monobactams while lacking efficacy against cephamycins (cefoxitin and cefotetan) and confer resistance for both GNB and GPB (Hasan and AL-Harmoosh, 2020).

This class includes a diverse range of enzymes with varying catalytic activities, including penicillinases (TEM-1 and SHV-1, which hydrolyze only penicillins), ESBL such as CTX-M, and carbapenemases like KPC (*K. pneumoniae* carbapenemase) (Hasan and AL-Harmoosh, 2020; Carcione et al., 2021).

2.5.1.1 TEM β-lactamases

TEM enzyme plays a role in producing ESBL and penicillinase characters, capable of hydrolyze β-lactam antibiotics such as penicillins and few cephalosporins, this can render these antibiotics ineffective over the course of treatment, leading to an increase of resistance (Carcione et al., 2021; Effendi et al., 2022). The majority of β-lactamase TEM enzymes are originated from parent enzymes such as TEM-1 and TEM-2, this evolution is attributed to point mutations occurring within the active sites of the TEM sequence, altering their substrate specificity and resistance profiles (Ejaz et al., 2021; Islam et al., 2023).

TEM type enzymes are encoding by bla_{TEM} genes which reside on bacterial plasmids, facilitating their rapid HTG both within and across bacterial species (Effendi et al., 2022). It's the most common plasmid mediated class A β-lactamases in GNB, including Enterobacteriaceae, *P. aeruginosa*. To date, approximately 140 variants of TEM-type enzymes have been identified (http://www.lahey.org/Studies/other), resulting from single amino acid substitutions (Carcione et al., 2021).

2.5.1.2 SHV β-lactamases

SHV enzyme are the mutant form of SHV-1 parent enzyme which was identified in the 1970s in *E. coli* and was commonly found in *K. pneumoniae* and shares 68% of its amino acids with

TEM-1 (Liakopoulos et al., 2016; Ejaz et al., 2021; Islam et al., 2023; Shakil et al., 2023), named for their response to sulfhydryl variable reagents and represent a significant group of resistance enzymes with more than 60 variants reported capable of hydrolyzing aztreonam and third generation cephalosporins. However, they remain susceptible to inhibition by clavulanic acid and are ineffective against cephamycins, fourth generation cephalosporins and carbapenems (Saravanan et al., 2018; Carcione et al., 2021).

The majority bla_{SHV} encoding genes confer the ESBL phenotype in various members of bacteria like Enterobacteriaceae and *P. aeruginosa* (Bush and Bradford, 2020; Carcione et al., 2021).

2.5.1.3 CTX-M β-lactamases

CTX-M β-lactamases were first identified in the 1980s (Bush and Bradford, 2020), they show greater significance against cefotaxime and ceftriaxone when compared with ceftazidime or cefepime (Carcione et al., 2021). Notably, these enzymes are effectively inhibited by all commercially available β-lactamase inhibitors, including newer agents such as avibactam and vaborbactam. They are originated from the chromosome of environmental rarely pathogenic. These enzymes are the examples of the plasmid-mediated acquisition of β-lactamase genes (Blair et al., 2015), because $bla_{CTX\text{-}M}$ genes have been identified in association with insertion sequences (ISEcp1) and with transposable elements which lead to be captured by a broad range of conjugative plasmids or phage-like sequences that act like as vehicles for their dissemination. They have 40% of similarity with TEM and SHV enzymes (Munita and Arias, 2016).

CTX-M β-lactamases are frequently identified in *E. coli* and *K. pneumoniae* but are also detected in other Enterobacteriaceae species, including typhoidal and nontyphoidal *Salmonella* spp., *Enterobacter* spp., *Citrobacter freundii*, *Shigella* spp. and *Serratia marcescens*. Additionally, they have been found in various species of non-fermenting bacteria (Saravanan et al., 2018; Bush and Bradford, 2020).

In the late 1980s and 1990s, CTX-M-type enzymes emerged and replaced TEM and SHV mutants, which were the first two ESBL variants identified. Over time, they became the predominant group of AR determinants among ESBL (Jovanovic et al., 2021).

2.5.1.4 Epidemiology of ESBL β-lactamases

ESBL pose a significant global challenge, affecting both hospitalized and community settings. The prevalence of ESBL-producing organisms varies widely across different healthcare institutions, countries, and continents.

Among European nations, the highest prevalence rate was detected in Greece (27.4%), and the lowest rate was observed in Netherlands and Germany (2% and 2.6%, respectively). The United States showed a relatively low prevalence, with approximately 3% of Enterobacteriaceae encoding ESBL (Nagshetty et al., 2021).

Moreover, In Africa, Egypt has the highest rate (38.5%) (Nagshetty et al., 2021). In Algeria, several studies conducted in hospital and community settings indicate the prevalence of ESBL producer Enterobacteriaceae ranges were found between 16.4%-99%. Among these, Class A ESBLs were the most commonly identified (Saravanan et al., 2018), several ESBL variants were identified in Algeria $bla_{CTX\text{-}M\ 3}$, $bla_{CTX\text{-}M\ 15}$, $bla_{SHV\ 12}$, $bla_{CTX\text{-}M\ 14}$, $bla_{CTX\text{-}M\ 28}$, $bla_{SHV\ 98}$, $bla_{SHV\ 99}$, $bla_{SHV\ 100}$, $bla_{TEM\ 4}$, $bla_{TEM\ 48}$, $bla_{TEM\ 188}$, $bla_{SHV\ 11}$, $bla_{SHV\ 12}$, $bla_{SHV\ 28}$, $bla_{SHV\ 110}$, $bla_{TEM\ 1}$, $bla_{CTX\text{-}M\ 1}$ (Baba Ahmed-Kazi Tani and Arlet, 2014), the presence of plasmid-encoded AmpC (pAmpC) was also observed (Saravanan et al., 2018).

2.5.2 Class B β-lactamases

Referred to metallo-β-lactamases (MBL), employ a metal ion (often zinc) as a cofactor for the nucleophilic attack on the β-lactam ring, in place of a serine residue (Oelschlaeger et al., 2023), these enzymes were encoded by genes typically situated within the chromosomes of nonpathogenic bacteria (Boyd et al., 2020).

Their activity is inhibited by ion-chelating agents like EDTA (Fig. 12); however, they exhibit efficacy against a broad spectrum of β-lactams, MBL are resistant to inhibition by clavulanic acid or tazobactam and although they effectively hydrolyze cephamycins. Aztreonam is often an ineffective substrate (Munita and Arias, 2016; Hasan and AL-Harmoosh, 2020; Nagshetty et al., 2021). In 1990, with the discovery of new MBL like: IMP and VIM in clinical strains of Enterobacteriaceae, *Pseudomonas* species and *Acinetobacter* spp., caused a major change in the antimicrobial resistance landscape; because, genes encoding for MBL enzymes have been

found in pathogenic bacteria as part of its accessory genome, which strongly suggests that HGT as a way of spread (Munita and Arias, 2016).

The most clinically important metallo-carbpemase belong to 4 families: IMP (Imipenemases), VIM (Verona Integron-encoded Metallo-β-lactamase), SPM (São Paulo Metallo-β-lactamase), and NDM (New Delhi Metallo-β-lactamase) (Boyd et al., 2020).

2.5.3 Class C β-lactamases

This class provides resistance to all penicillins and cephalosporins, including cephamycins, although cefepime is usually not effectively targeted by these enzymes. They generally do not hydrolyze aztreonam effectively and are not effected by clavulanic acid inhibition (Carcione et al., 2021).

The most clinically significant enzyme in this class is AmpC, a cephalosporinase predominantly encoded chromosomally (Hasan and AL-Harmoosh, 2020). However, the bla_{AmpC} gene has also been identified on plasmids, facilitating its HGT. The chromosomal expression of bla_{AmpC} genes is a property of *Enterobacter cloacae*, *E. aerogenes*, *P. aeruginosa*, *Providencia* species, *C. freundii*, *S. marcescens* and *Morganella morganii*. On the other hand, the bla_{AmpC} gene is not present in the core genome of the classic examples of *Proteus mirabilis*, *P. vulgaris*, *E. coli* and *Klebsiella* species (Munita and Arias, 2016).

The most commonly reported plasmid-mediated AmpC (pAmpC) β-lactamase genotypes include ACC, DHA, CMY, FOX, MOX, EBC and CIT. Among these, CMY-2-like enzymes are the most prevalent in clinical isolates of Enterobacteriaceae. Although DHA-like β-lactamases have also shown significant dissemination, these enzymes exhibit a resistance profile similar to the overproduction of chromosomal AmpC β-lactamases, conferring resistance to nearly all β-lactam antibiotics except carbapenems and cefepime (Peymani et al., 2016).

2.5.4 Class D β-lactamases (OXA-type enzymes)

This class Include a diverse group of enzymes that were initially distinguished from class A penicillinases due to their ability to hydrolyze oxacillin and their poor inhibition by clavulanic acid (Munita and Arias, 2016; Saravanan et al., 2018; Hasan and AL-Harmoosh, 2020). They

are entirely differs from TEM and SHV enzymes and showing 20% of similarity with the other members of ESBL family (Saravanan et al., 2018).

Several variants of OXA-type enzymes have been described such as enzymes have the capacity to degrade third generation cephalosporins (OXA-11) and carbapenems (OXA-23), resist to clavulanic acid (OXA-48 and OXA-48 like), hydrolyze cefepime (OXA-1) (Nagshetty et al., 2021), resistance to cefotaxime and cefepime (OXA-17) (Munita and Arias, 2016; Bush and Bradford, 2020; Hasan and AL-Harmoosh, 2020; Carcione et al., 2021). They have been identified in many clinical species, including *E. coli*, *Enterobacter* spp., *K. pneumoniae*, *P. aeruginosa* and also produced in GPB such as *S. aureus* (Munita and Arias, 2016; Hasan and AL-Harmoosh, 2020).

in Algeria, their role and relationship with *Acinetobacter baumannii* has become increasingly concerning. Local surveillance has demonstrated that $bla_{OXA\text{-}23\text{-}like}$ is highly prevalent, detected in approximately 40–70% of imipenem-resistant *A. baumannii* clinical isolates across multiple regions including western Algeria, Constantine, and Batna (Mesli et al., 2013). In addition, $bla_{OXA\text{-}24\text{-}like}$ has emerged in a high proportion of these isolates up to 63% in some recent Batna hospitals and occasionally co-expressed alongside $bla_{OXA\text{-}23}$ (Bouali et al., 2025).

2.6 Plasmid-mediated Quinolone Resistance (PMQR)

Recent studies have indicated that quinolone (Fig. 13) resistance is not solely chromosomally encoded but can also be mediated by MGE, such as plasmids. PMQR is primarily associated with the *qnr* genes, which encode proteins belonging to the small-pentapeptide repeat family. These proteins protect critical bacterial enzymes, DNA gyrase and topoisomerase IV, from the inhibitory effects of quinolone compounds (Fig. 9) (Rezazadeh et al., 2016; Hasan and AL-Harmoosh, 2020).

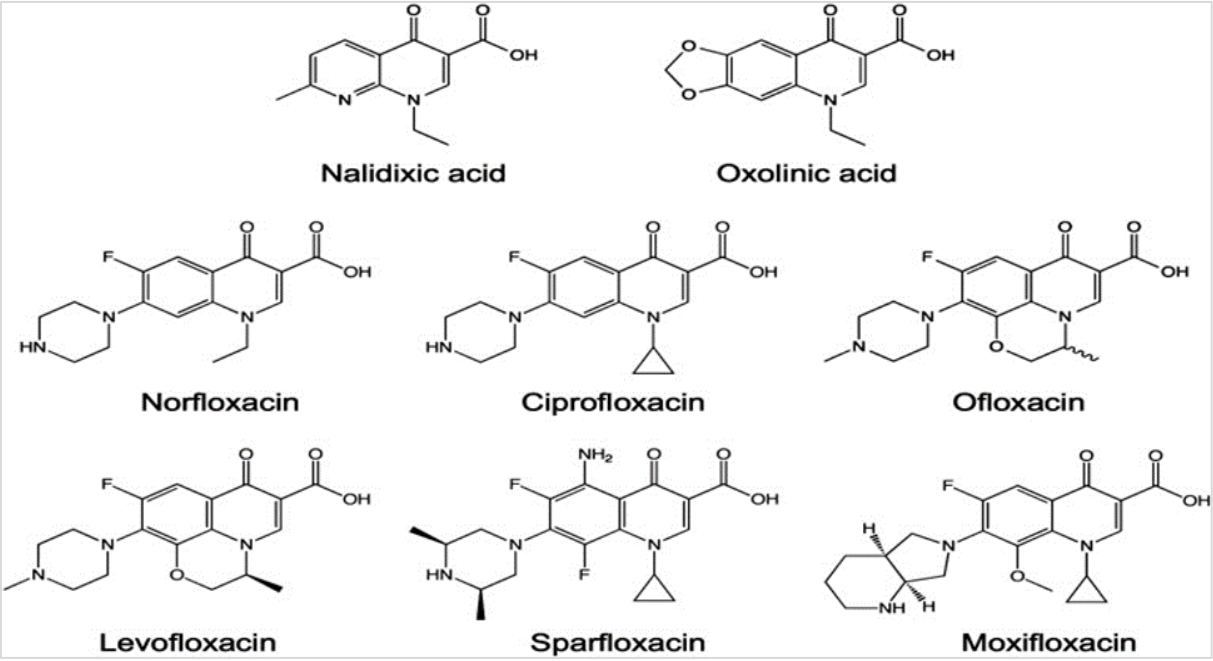


Figure. 13 : Quinolone structures (Aldred et al., 2014).

The first PMQR gene was identified in a *K. pneumoniae* isolate from Birmingham (Alabama) in 1994, that contain plasmid confer for resistance to ciprofloxacin (Nourozi et al., 2020; Miranda et al., 2022). *qnr* genes are typically located within multi-resistance plasmids that also contain additional resistance determinants like β-lactamase genes including: ESBL genes, AmpC genes and carbapenem genes (Hooper and Jacoby, 2015).

Different *qnr* genes have been identified in pathogenic and non-pathogenic bacterial strains from diverse regions worldwide include: *qnrA*, *qnrS*, *qnrB*, *qnrC*, and *qnrD*, as well as more recently discovered variants such as *qnrVC* and *qnrT*. The first *qnrB* was found in *K. pneumoniae* which code for Qnr protein with 214 amino acid, wihle *qnrC* was identified in *P. mirabilis*, *qnrD* in *S. enterica*, *qnrS* in *Shigella flexneri* 2b and the *qnrVC* from *V. cholerae* (Rodríguez-Martínez et al., 2016; Yanat et al., 2017; Amereh et al., 2023; Abdelrahim et al., 2024).

The *qnr* genes alleles differ by 35% or more in their sequences, table 2 presents the nucleotide and amino acid sequence identities of the *qnr* alleles, the highest similarity is observed between the *qnrB* and *qnrE* alleles, with 75.81% and 85.98% identity at the nucleotide and amino acid levels, respectively. Followed by *qnrC* and *qnrVC* alleles, which show 68.65% and 73.85% identity, respectively. Conversely, the *qnrS* allele shows the lowest amino acid similarities

when compared to the *qnrE*, *qnrD* and *qnrB* alleles, exhibiting approximately 36%, 39%, and 40% identity, respectively (Yanat et al., 2017; Miranda et al., 2022).

Table 2. Percentages of the similarity of nucleotide and amino acid sequences of the *qnr* genes (Miranda et al., 2022).

Genes	Percentage of nucleotide/Amino acid similarity (%)						
	qnrA	***qnrB***	***qnrC***	***qnrD***	***qnrE***	***qnrS***	***qnrVC***
qnrA	100/100	46.33/41.59	59.36/64.22	47.91/46.26	48.99/42.06	58.75/59.63	60.43/61.93
qnrB		100/100	48.78/42.99	62.79/64.49	75.81/85.98	48.32/39.72	50/42.99
qnrC			100/100	49.77/44.39	46.98/42.52	60.73/60.09	68.65/73.85
qnrD				100/100	63.10/65.89	46.51/39.25	50.70/43.93
qnrE					100/100	48.37/35.98	49.61/42.06
qnrS						100/100	63.93/64.68
qnrVC							100/100

qnrA gene has been identified on plasmid in *Aeromonas punctata* and *Vibrio fluvialis*. It is an unusual member of the *qnr* family that differentiates oneself from the plasmid-mediated *qnr* genes due to the presence of the attC site, a feature typical of integron cassettes (Yanat et al., 2017), this gene encodes a 218-amino acid QnrA protein (Al-Rafyai et al., 2021) is one of the resistance genes that has not been commonly found in *E. coli* (Tageldin et al., 2023).

2.7 Phylogenetic groups of *E. coli*

Phenotypic assays, including the identification of serogroups in *E. coli* and metabolic assays, play a central role in bacterial classification. While these characteristics serve as valuable markers, they are relatively imprecise indicators that offer only limited insights (Robins-Browne et al., 2016).

PCR and sequencing techniques enable the phylogenetic classification (categorizing of organisms into groups within large phylogenetic entity) within the species of bacteria by elucidating evolutionary relationships among genera, species, and strains (Bhattacharjee, 2022; Denamur et al., 2021). Comparative analyses of 5S and 16S ribosomal RNA sequences have indicated that *Escherichia* and *Salmonella* originated from a common ancestor approximately 120-160 million years ago, coinciding with the emergence of mammals (Hu et al., 2010). Conversely, genomic DNA sequence analysis of *Escherichia* and *Shigella* has revealed a high level of sequence similarity, despite their historical classification into separate genera within the family Enterobacteriaceae (Pettengill et al., 2016).

The genus *Escherichia* is now composed of three nomen species (*E. albertii*, *E. fergusonii* and *E. coli*) and five *Escherichia* clades labelled I–V (Denamur et al., 2021). In the early 1980s, Whittam *et al.* identified a genetic substructure within *E. coli*, a discovery later validated by subsequent research. This work revealed that *E. coli* strains are categorized into four primary phylogenetic groups (A, B1, B2, and D) (Hyun et al., 2021), with non-random distribution based on their source of isolation.

The clinical significance of these findings prompted Clermont *et al.* (2000) to develop and validate a simpler, faster, and more effective method for assigning *E. coli* isolates to their respective phylogenetic groups, compared to existing approaches (Clermont et al., 2000). Clermont *et al.* designed a multiplex PCR assay targeting three genetic markers: the *chuA* gene (required for heme transport in enterohemorrhagic *E. coli* O157:H7), the *yjaA* gene (identified in the genomic sequence of *E. coli* K-12 with an as-yet-unknown function), and a 14.9 Kb DNA fragment, *TspE4.C2*, which encodes a lipase closely associated with strains causing neonatal meningitis (Clermont et al., 2000). The method's accuracy was validated by multi-locus sequence typing (MLST), with only a small percentage of misclassifications reported.

Subsequent research, spanning from 2000 to the present, has expanded the understanding of *E. coli* phylogenetics through MLST and, to a lesser extent, whole genome sequencing. These analyses have identified additional phylogenetic groups, including groups E and F (a sister group to B2) and group C (related to group B1, but distinct from it). Furthermore, Walk and colleagues uncovered cryptic lineages that are genetically distinct but phenotypically identical to *E. coli* (Walk et al., 2009). Among these is the clade I, which is now considered a phylogenetic group within *E. coli* (Okuno et al., 2023).

Currently, eight phylogenetic groupings of *E. coli* are acknowledged: A, B1, B2, C, D, E, F, and clade I. Consequently, Clermont *et al.* updated the original methodology, preserving the same target genes, altering the primer sequences that identify them, and incorporating an additional gene, *arpA*, which is found in all *E. coli* strains except those belonging to phylogroups B2 and F, as well as in the cryptic clades II, III, IV, and V, and in *E. albertii* and *E. fergusonii*. The final gene acts as an internal control to assess DNA quality and is utilized to differentiate phylogroup F from phylogroup D (Fig. 14) (Clermont et al., 2013).

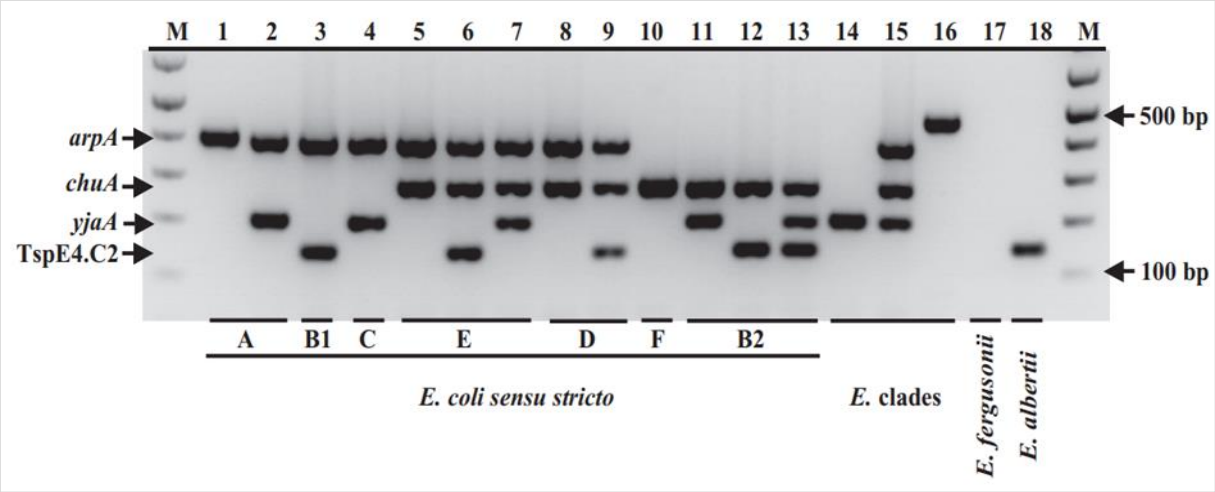


Figure. 14 : PCR profiles that can be observed using the Clermont *et al.* (2013) method. On the left are indicated the amplified gene targets and the respective expected bands; the last well on the right shows the 100 bp DNA ladder. The band pattern obtained allows to attribute the phylogenetic group (A, B1, C, E, D, F, B2, E. clade I) to the isolated strains (Clermont et al., 2013).

To date, the definition of *E. coli* pathovars is based on various factors, including the target organ (urinary tract and uropathogenic *E. coli* (UPEC)), the infected host (bird and avian pathogenic *E. coli* (APEC)), the association with specific organs and hosts (cerebro-spinal fluid in newborns and newborn meningitis *E. coli* (NMEC)), the relationship with the targeted organs, the presence of particular genes or virulence in an animal model (extra-intestinal pathogenic *E. coli* (ExPEC)), the type of pathology caused by the strains (diarrhea and intestinal pathogenic *E. coli* (InPEC)); the presence of a specific gene or genes combinations (Shiga-toxin encoding *stx* gene and Shiga toxin-producing *E. coli* (STEC))… with phylogenetic relationships (Tab. 3) (Denamur et al., 2021; Kara et al., 2024).

Table 3. Main characteristics of the more commonly *E. coli* pathotypes (Denamur et al., 2021).

Pathotype	**Definition basis**	**Main strain host**	**Strain phylogenetic background**
ExPEC	Non-intestinal infection, specific genes, animal model	Human, domestic mammals, birds	B2, D, C, F
UPEC	Isolated form urine	Human, domestic mammals	B2, D
NMEC	Isolated from cerebrospinal fluid of neonates	Human	B2, F
Pneumonia-associated *E. coli*	Isolated from lung	Human	B2
APEC	Isolated from birds	Poultry	B2, C
InPEC	Diarrhoeal disease	Human, domestic mammals	All phylogroups
STEC and/or EHEC	*stx* genes	Human, cattle, sheep	E, B1
EPEC	Attaching and effacing lesions on intestinal epithelial cells	Human, domestic mammals	A, B1, B2, E
ETEC	Heat-stable and heat-labile enterotoxins	Human, pig, cattle	A, B1, C, E

EIEC	Colonocyte invasion	Strictly human	A, B1, E
EAEC	Aggregative adhesion on enterocytes	Human, domestic mammals	A, B1, B2, D
DAEC	Diffuse adhesion on enterocytes	Human	All phylogroups
AIEC	Adhesion and invasion of intestinal epithelial cells	Human	All phylogroups, majority of B2
Hybrid InPEC	EHEC and EAEC characteristics	Human	B1
Hybrid InPEC–ExPEC	HUS and septicemia	Human, cattle	A

3 Material and Methods

3.1 Place of study

This study was conducted in several geographical locations around Sétif province in the northeastern of Algeria; the collection were distributed on seven medical diagnostic laboratories, two laboratories from the southwest of the province (Aïn Oulmène city), one from the extreme north (Bouandas city), two laboratories from the capital (Sétif city), two laboratories from the north (Tizi N'Bechar city and Bougaa city) and three government hospitals: Ain Oulmène and El-Eulma hospitals, Sétif university hospital (Fig. 15).

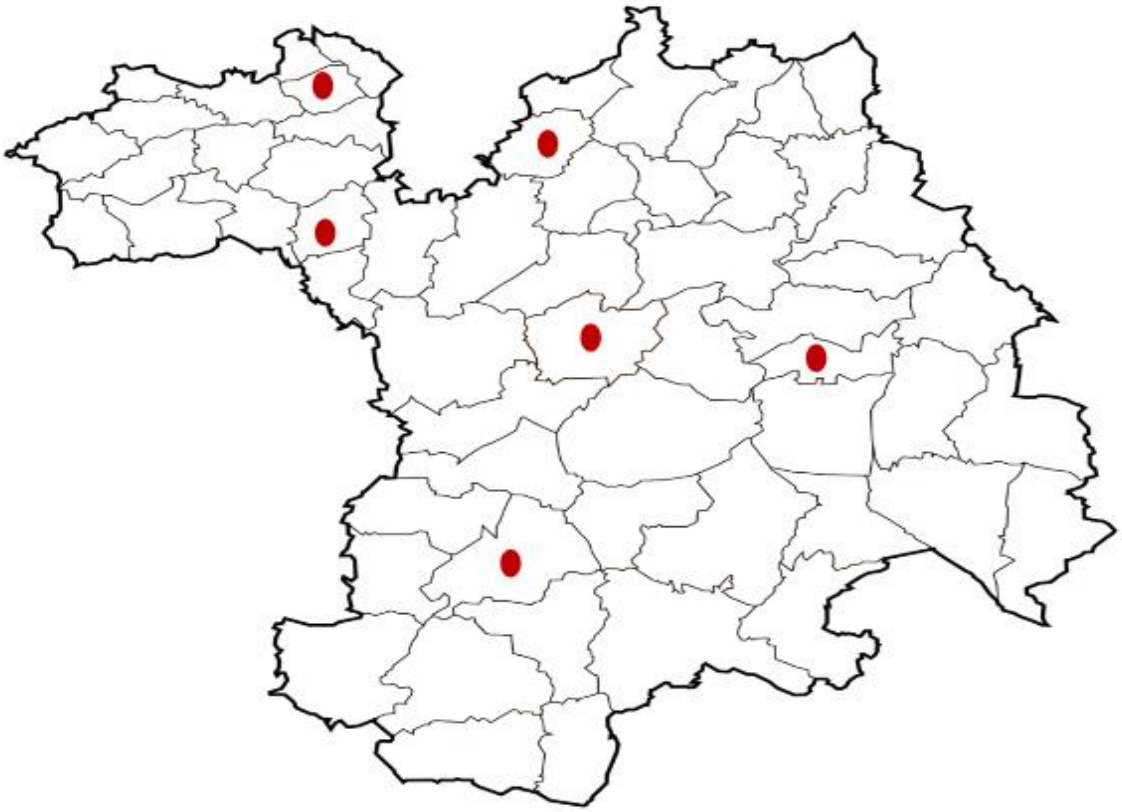


Figure. 15 : Geographical carte of samples locations (https://images.app.goo.gl/iRKfjtiQKChNpz1e7).

3.2 Chemical reagents, antibiotics and media

The media used in this study included Nutrient agar (TM-Media, Delhi, India; Oxoid, Milano, Italy), Hektoen agar, Chapman agar, MacConkey agar, Nutrient broth, Mueller-Hinton agar, and Mueller-Hinton broth (all from TM-Media, Delhi, India), as well as blood base agar (BioScan Industrie, Sétif, Algeria).

Classical biochemical identification tests were obtained from BioScan Industrie (Sétif, Algeria), while the API 20E and API Staph systems were sourced from BioMérieux (France).

Antibiotics were acquired from three different laboratories HiMedia Laboratories (Mumbai, India), BioMaxima (Lublin, Poland), and BioScan Industrie (Sétif, Algeria).

Additional microbiological reagents, including peptone, agar, and Brain Heart Infusion Broth (BHIB), were supplied by Liofilchem (Abruzzo, Italy).

Chemical reagents such as DMSO, phosphate buffer, EDTA, yeast extract, glucose, crystal violet, ethanol, and methanol were purchased from Sigma-Aldrich. Sterile UHT non-fat milk was obtained from Soummam (Béjaïa, Algeria).

For PCR procedures, agarose gel was sourced from PanReac AppliChem (Milano, Italy); DNA marker (GeneRuler™) from Thermo Fisher Scientific (MA, USA), buffer, dNTPs, $MgCl_2$, and DreamTaq polymerase enzyme were from Thermo Fisher Scientific (MA, USA); and additional reagents, including ethidium bromide, TAE buffer, blue bromophenol (Sigma-Aldrich), and RNase (Invitrogen), were used.

3.3 Isolation of bacterial strains

Over a period of three years (2021–2023), 402 patients out of 450 from the previously defined care territories were selected for this study. The samples originated from different specimens: urine, pus, blood, vaginal swab and bladder catheter.

- Inclusion criteria: 1) isolates from all clinical samples, 2) patients from all ages and both sexes, 3) isolates showing decreasing sensitivity to at least one family of used antibiotics were included in this study.
- Exclusion criteria: 1) incomplete clinical or laboratory data, 2) samples that did not establish quality control or sterility criteria, 4) samples doesn’t respect temperature and duration of conservation before analysis were excluded from this study.

- Information about patients (age, gender, nature of sample, date of sample collection and type of patient) was collected and recorded in the microbiology laboratory's sample register after patient consent.

All samples were collected under aseptic precautions, and cultured on various non-selective and selective media (Annex 1), including nutrient agar, Hektoen agar, MacConkey agar Chapman agar and nutrient broth and incubated at 37°C for 24 h. Urine samples were cultured using the calibrated loop technique, pus samples were directly streaked onto the specific media described in annex 1, blood and vaginal swab samples were directly culturing in the covenant media; while for samples collected from medical devices, such as catheters, were vortexed in sterile saline to dislodge adherent microorganisms, and the suspension was plated on specific media (Annex 1).

3.4 Identification of bacterial strains

All isolates were identified based on their microscopic examination and colony morphology, Gram staining, motility and biochemical tests: indole production, mannitol, citrate utilization, glucose, sucrose, lactose fermentation in TSI agar, catalase, oxidase, urease and gas production (Annex 1). The primary identification was confirmed by API 20E biochemical gallery for non-oxidative bacteria and API Staph gallery for the research of *S. aureus* strains. The identified strains were preserved in nutrient broth containing 30% of sterile glycerol.

3.4.1 Identification with API gallery

The primary identification was confirmed by API 20E biochemical gallery for non-oxidative bacteria especially for the differentiation of members of Enterobacteriaceae family and API Staph gallery for the research of *S. aureus* strains. The identified strains were preserved in nutrient broth containing 30% of sterile glycerol.

From pure culture of 18 to 24 h, one colony was used to prepare 0.5 McFarland standard solution in saline solution (0.9%). The bacterial suspension was used to rehydrate each of the wells and the strips were incubated. For CET, VP, GEL the microtubes should fill up with bacterial suspension, and for ADH, LDC, ODC, H2S, URE compartments anaerobic conditions should be created by adding sterile oil. The gallery was incubated at 37°C for 18-24 h.

During incubation, metabolic activity causes color changes that were either spontaneous or upon the addition of reagents. All positive and negative test results were compiled to create a profile number, which was then compared with profile numbers in a commercial codebook (or online) to identify the bacterial species (Annex 2). *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were the positive control.

3.5 Phenotypic study

3.5.1 Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion method was employed to assess susceptibility in Muller-Hinton's agar plates (Hudzicki, 2009). Susceptibility testing was conducted for 28 antimicrobial drugs (Tab. 4).

From young culture of 18h to 24h in non-selective agar media, a bacterial inoculum was prepared in saline solution of 0.9% of NaCl equivalent to the 0.5 McFarland standard (OD= 0.08 - 0.13 at 625 nm, $\approx 10^8$ CFU/ml).

The bacterial solution (should be inoculated within 15 min of inoculum preparation) was inoculated on the surface of Muller Hinton agar plates using swabs, the swab was moved across the whole surface of the agar plate three times, the plate was rotated about 60° after each streak to ensure the distribution of the inoculum. The antibiotic disks were applicated and placed on agar surface of the plates using sterile forceps, each disk must be pressed down to ensure the disk-agar contact. The plates were incubated from 18 to 24 h at 37°C. Each bacterial isolate was processed in duplicate to ensure the reliability and reproducibility of the results.

After incubation, and using a ruler, the different inhibition zones were measured, and compared with the critical diameter published on (The European Committee on Antimicrobial Susceptibility Testing, 2022), to interpretate the resistance profile of the strains: resistant (R) if the inhibition zone diameter was less than the defined critical diameter, sensitive (S) if it was equal to or greater than the susceptibility point, intermediate (I) when the diameter fell between the resistant and sensitive cutoffs, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as quality control.

After interpreting the resistance profile of strains, resistance classification was performed using acronyms (R, MDR, XDR); R is defined as strains that resist to fewer than three classes of antibiotics; MDR was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories. Extensively drug resistant (XDR) was defined as nonsusceptibility to at least one agent in all but two or fewer antimicrobial categories (one or two antimicrobial categories) (Basak et al., 2016).

Table 4. Antibiotic tested list.

Family	Antibiotic	Acronym	Disk charge (µg)
Penicillins	Amoxicillin	AMX	25
	Ticarcillin	TC	75
	Piperacillin	PRL	30
	Oxacillin	OX	10
Penicillins + inhibitor	Amoxicillin + clavulanic acid	AMC	30
	Ticarcillin + clavulanic acid	TCC	85
	Piperacillin + tazobactam	TPZ	110
1st generation cephalosporins	Cefalexin	CN	30
2nd generation cephalosporins	Cefoxitin	FOX	30
3rd generation cephalosporins	Cefixime	CFM	5
	Ceftazidime	CAZ	30
	Cefotaxime	CTX	30
4th generation cephalosporins	Cefepime	FEP	30
Carbapenems	Imipenem	IMP	10
Aminoglycosides	Gentamicin	GEN	10
	Amikacin	AK	30
	Tobramycin	TOB	10
	Kanamycin	K	30
Quinolones	Nalidixic acid	NA	30
Fluoroquinolones	Ciprofloxacin	CIP	5
	Ofloxacin	OF	5
	Levofloxacin	LE	5
Phenicolates	Chloramphenicol	C	30
Monobactams	Aztreonam	ATM	30
Sulfamids	Trimethoprim/sulfamethoxazole	SXT	25
Nitrofurans	Nitrofurantoin	NIT	300
Lincosamides	Clindamycin	CD	2
Macrolides	Erythromycin	E	15
Tetracycline	Doxycyclin	DO	30
Phosphonic	Fosfomycin	FF	50
Polymyxin	Colistin	CS	10

3.5.2 Minimal Inhibitory Concentration (MIC)

MIC concentration of an antibiotic is defined as the minimum concentration (mg/l) that inhibit visible bacterial growth under defined growth conditions. For all susceptible strains MIC

concentration was determined through broth microdilution method as described in (The European Committee on Antimicrobial Susceptibility Testing, 2022).

To determine MIC by dilution method, antibiotics solutions should be prepared, antibiotics need to be dissolved first to create a stock solution, then diluted to reach the desired starting concentration. Sterile water serves as both a solvent and diluter for most of antibiotics, including β-lactams, fluoroquinolones, and aminoglycosides. Some require alcohol as a solvent, including macrolides, chloramphenicol, and rifampicin; others require a phosphate buffer or dimethyl sulfoxide (DMSO). Stock solutions must include double dilutions of antibiotics, with the concentration range selected for testing varying based on the specific antibiotic (Tab. 5). These concentrations should be determined in accordance with the MIC breakpoints established in (The European Committee on Antimicrobial Susceptibility Testing, 2022).

The selected strains were cultured on non-selective agar media, after an incubation period of about 18 to 24 h and confirming that all the isolates were indeed pure and viable, bacterial suspension adjusted at 0.5 McFarland was prepared in saline solution (0.9% NaCl); after turbidity adjustment, the bacterial suspension should be used within 15 min.

96-well microtiter plate was used, 100 µl of Muller-Hinton broth (MHB) was distributed along the wells, 100 µl of antibiotic solution was added in the first well, a sequence of geometric ratio 2 dilutions (1/2-fold dilution) was performed in MHB using the stock solution from the first well, this involved moving 100 µl from one well to the next, up to the last well, with the 100 µl in the last well being discarded. 100 µl of the inoculum previously diluted at ratio 1/100 or about 10^6 CFU/ml was added to each well, two wells were servers as negative control (200 µl of sterile MHB) and positive control (MHB + bacterial suspension), the microtiter plates were incubated at 37°C for 18 to 24 h.

After incubation, 20 µl of 2,3,5-triphenyl-2H-tetrazolium chloride (Filter sterilized solution: TTC) at 0.05% was added to each well. for the detection of bacterial growth based on color changing.

Table 5. MIC dilution range of antibacterial agents used in the susceptibility test of isolates.

Antibiotics	**MIC dilution range (mg/l)**	**Antibiotics**	**MIC dilution range (mg/l)**
Amoxicillin	2 - 32	Gentamycin	1 - 16
Piperacillin	4 - 64	Tobramycin	0.25- 4
Cefalexin	8 - 128	Nalidixic acid	8 - 128

Cefixime	1 - 16	Ciprofloxacin	0.125 - 2
Ceftazidime	0.25 - 4	Ofloxacin	0.125 - 2
Cefotaxime	0.25 - 4	Chloramphenicol	2 - 32
Imipenem	0.25 - 4	Aztreonam	0.125 - 2
Trimethoprim/sulfamethoxazole	2 - 32		

3.5.3 Phenotypic identification of ESBL producing bacteria

All strains were screened for ESBL production using Kirby Bauer disk diffusion method during the antibiogram technic. Isolates which demonstrate resistant to one or more third generation cephalosporins, were screened as ESBL production. All screened isolated were examined by using phenotypic confirmatory test or synergy test (a synergy between a third-generation cephalosporin, a fourth-generation cephalosporin or aztreonam, and an association containing clavulanic acid). Following the criteria established by (The European Committee on Antimicrobial Susceptibility Testing, 2022) the confirmation of ESBL production was carried out by disk diffusion method on Muller Hinton agar and incubation at 37°C for 24 h, ESBL production was confirmed when the inhibition zone around cefotaxime (30µg), ceftazidime (30µg), cefepime (30µg), or aztreonam (30µg) antibiotics disks was enhanced on the side of amoxicillin + clavulanic acid central disk, resulting in characteristically shaped zone referred to as “champagne cork” (Fig. 16) (Kettani Halabi et al., 2021).

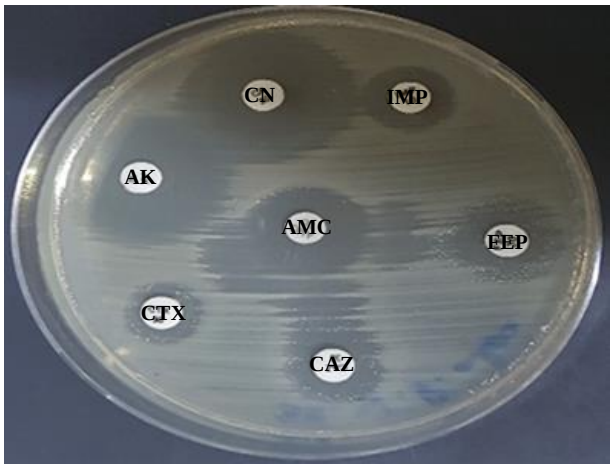


Figure. 16 : Phenotypic detection of ESBL strains.

3.5.4 Phenotypic detection of AmpC strains

AmpC screening was conducted when isolates showing resistance to cefoxitin (FOX-30 µg) during antibiogram method, according to (The European Committee on Antimicrobial

Susceptibility Testing, 2022) standards the critical diameter of cefoxitin was 18 mm (>18 mm: sensitive strain, <18 mm: resistant strains = phenotypically positive AmpC strains) (Fig. 17) (Powell et al., 2020; Tekele et al., 2020).

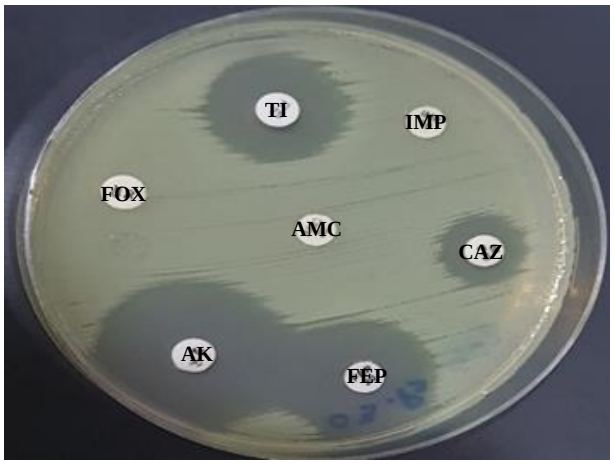


Figure. 17 : Isolates with decreased diameter against cefoxitin (CX-30 µg).

3.5.5 Phenotypic detection of carbapenemases

3.5.5.1 Modified Hodge test (MHT)

Hodge modified test is for objective of carbapenemases phenotypic detection, all strains that showed reduced sensitivity against carbapenem, were selected for phenotypic detection of carbapenemases tests. A suspension of *E. coli* ATCC 25922 was prepared and adjusted at 0.5 McFarland standard, using cotton swab the suspension was inoculated evenly on the surface of Muller-Hinton agar plates, the plates were allowed stand 15 min at room temperature for drying. Next, a 10-µg imipenem disk was placed in the center of the plate, carbapenem-resistant strains from the overnight culture plates were streaking heavily in a straight line from the edge of the disk to the periphery of the plate, the plates were incubated at 37°C for 24 h. After incubation, the clover leaf like appearance between the test streaks near the disk was taken as positive MHT test (Ramana et al., 2013; Aminul et al., 2021).

Quality control of the following organisms MHT positive *K. pneumoniae* ATCC 1705 and MHT negative *K. pneumoniae* ATCC 1706 (Fig. 18).

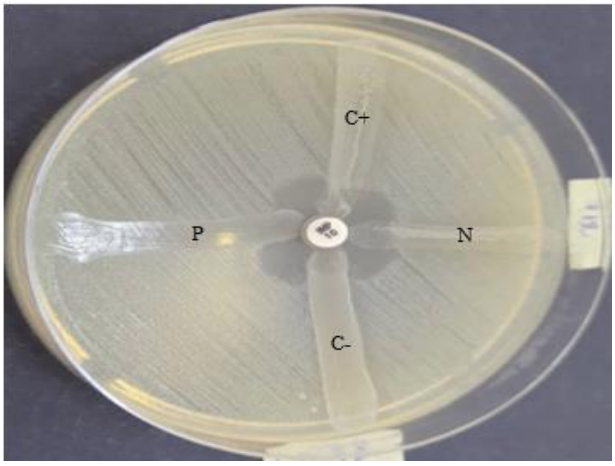


Figure. 18 : Modified Hodge test (C-: negative control, C+: positive control, P: positive MHT strain, N: negative MHT strain).

3.5.5.2 Imipenem (IMP)-EDTA combined disk test

The EDTA was used in this test as inhibitor of imipenemase activity, an adjusted solution of tested organism was prepared (0.5 McFarland) and inoculated onto plate with Muller Hinton agar. Two disks of imipenem were placed 20 mm apart from the center, 10 µl of EDTA solution (0.5M) were added to one of them (Combination imipenem-EDTA), after incubation from 16 to 18 h at 35°C, the inhibition zones of imipenem and imipenem-EDTA disks were measured and compared. The inhibition zone of imipenem + EDTA ≥ 5 mm than that of imipenem alone confirmed the MBL production, *P. aeruginosa* ATCC 27853 was used as control organism (Fig. 19) (Deshmukh et al., 2011; Radhika et al., 2022; Shrestha et al., 2023).

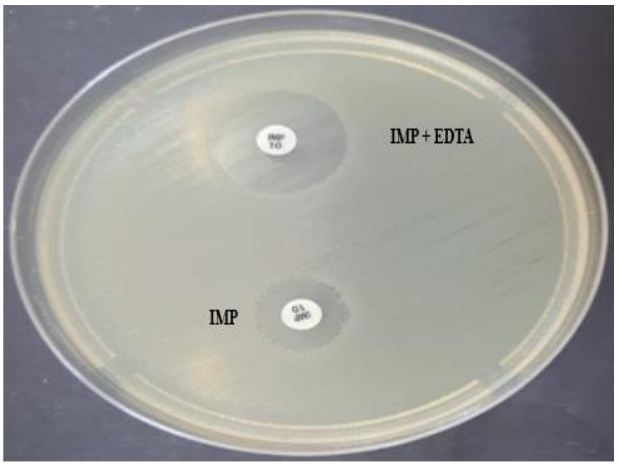


Figure. 19 : Imipenem-EDTA combined disk test for phenotypic detection of MBL strains.

3.5.6 Strig test for hypervirulent *K. pneumoniae*

The string test was performed as previously described (Eisenmenger et al., 2021). *K. pneumoniae* isolates were cultured on nutrient agar plates and incubated overnight at 37 °C. A standard inoculating loop was used to gently touch and lift the colonies. A positive result was indicated by the formation of a mucoid string longer than 5 mm, observed visually (Fig. 20).

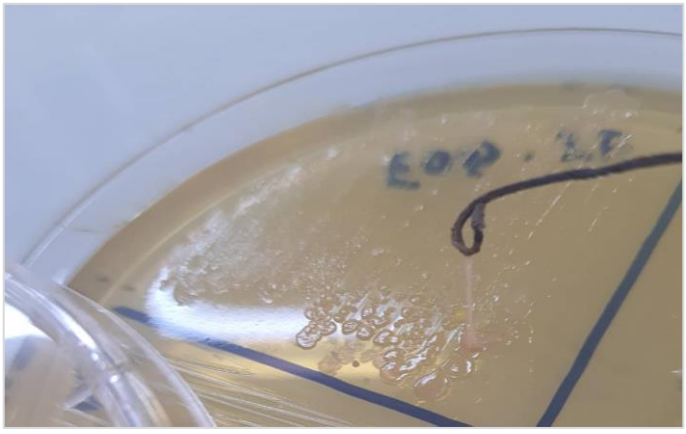


Figure. 20 : Mucoviscous string test for hypervirulent *K. pneumoniae.*

3.5.7 Phenotypic resistance in *S. aureus*

3.5.7.1 Phenotypic detection of resistance to methicillin (MRSA)

The resistance of *S. aureus* to isoxazolyl-penicillins (oxacillin) is investigated in our study using a cefoxitin disk (30 µg) under standard conditions of the staphylococcal antibiogram (in Mueller Hinton medium with a 0.5 MacFarland inoculum and incubation for 18-24 h). The inhibition zone was measured following incubation at 35°C for 16–18 h. Zone size was interpreted according to (The European Committee on Antimicrobial Susceptibility Testing, 2022) criteria. Isolates with an inhibition zone ≤ 22 were classified as MRSA, while isolates with an inhibition zone ≥ 22 were classified as MSSA (methicillin sensitive *S. aureus*), *S. aureus* ATCC 25923 was used as quality control.

3.5.7.2 Phenotypic detection of inducible MLSB resistance (D-Zone Test)

The D-zone test is simple test used to macrolide lincosamide streptogramin B resistance (MLSB) *S. aureus*, by disk diffusion method, the test consist briefly, two antibiotics disks erythromycin (macrolide) and clindamycin (lincosamide derivative) are placed adjacent to each

other over the Mueller Hinton agar medium inoculated with the test isolate. The growth of the organism up to the edges of the disc indicating that the organism having constitutive MLSB (CMLSB), flattening of the clindamycin zone (D test positive) near the erythromycin disc (resistant) indicating the presence of inducible MLSB (IMLSb) (Fig. 21), MSB phenotype if isolate was resistant to erythromycin and susceptible to clindamycin with negative D test, L phenotype when organism was resistant to clindamycin and susceptible to erythromycin and S phenotype if isolate was susceptible to both erythromycin and clindamycin, respectively. *S. aureus* ATCC 25923 was used as quality control (Shrestha and Rana, 2014).

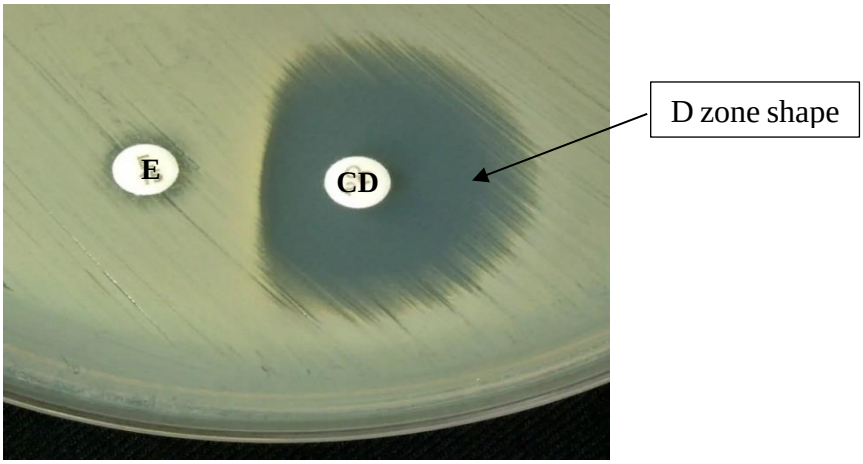


Figure. 21 : D- zone test for *S. aureus* strains.

3.6 Biofilm production assay

The biofilm is a collection of microbial cells that attached to surface enclosed in extracellular polysaccharide; it displays a crucial role in increasing of AR and host immune defense, it's an important virulence factors of some microorganism pathogens. Biofilm formation ability was performed according to the microtiter plate assay, essentially as described by O'Toole and Kolter, (1998); Stepanović *et al.*, (2007) and Türkel *et al.*, (2018).

After incubation, the medium was discarded and the wells were washed three times with distilled water to eliminate any planktonic cells, adherent cells were fixed by adding 150 µl of methanol for 20 min. Biofilms were stained by 0.2% crystal violet (w/v) solution for 15 min at room temperature.

Crystal violet was removed and each well was rinsed with sterile distilled water and plates were allowed to air dray. The biofilms were solubilized by 150 µl of ethanol 95% and absorbance at

570 nm was measured by ELISA reader (BioTek, El Dorado Hills, CA, USA). Sterile broth was served as a negative control and *E. coli* ATCC 25922 was used as the control organism (Fig. 22B). The interpretation of the results divided into:

- No biofilm production: OD sample ≤ OD control.
- Weak biofilm production: OD control < OD sample < 2 x OD control.
- Moderate biofilm production: 2 x OD control < OD sample < 4 x OD control.
- Strong biofilm production: 4 x OD control < OD sample.

(A) (B)

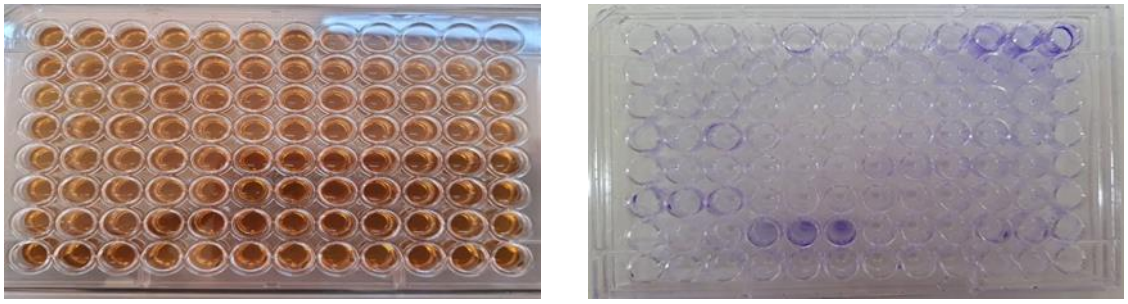


Figure. 22 : Biofilm formation assay using CV staining (A: 96-well microtiter plate before staning and incubation, B: 96-well microtiter plate after staining with CV).

3.7 Hemolysin production

The hemolysin production was determined using 5% human blood agar. The isolates were grown on BHIB, then a blood agar was prepared by adding 5% of human blood to blood agar base at 45°C-50°C. The tested isolates were streaked on blood agar plates and incubated at 37°C for 18-24 h.

The organisms were classified as either α, β or γ-hemolytic. Complete lysis of red blood cells shown by clear zone around the colonies was taken as β-hemolysis (Fig. 23A). The presence of a halo (greenish coloration: reduction of hemoglobin of the red blood cells to methemoglobin) around the bacterial growth was taken as α-hemolysis (partial hemolysis) (Fig. 23B), while γ-hemolysis was documented when there was normal growth without changes in the culture medium (no hemolysis) (Fig. 23C) (Mogrovejo-Arias et al., 2020; Tula et al., 2023).

(A) (B) (C)

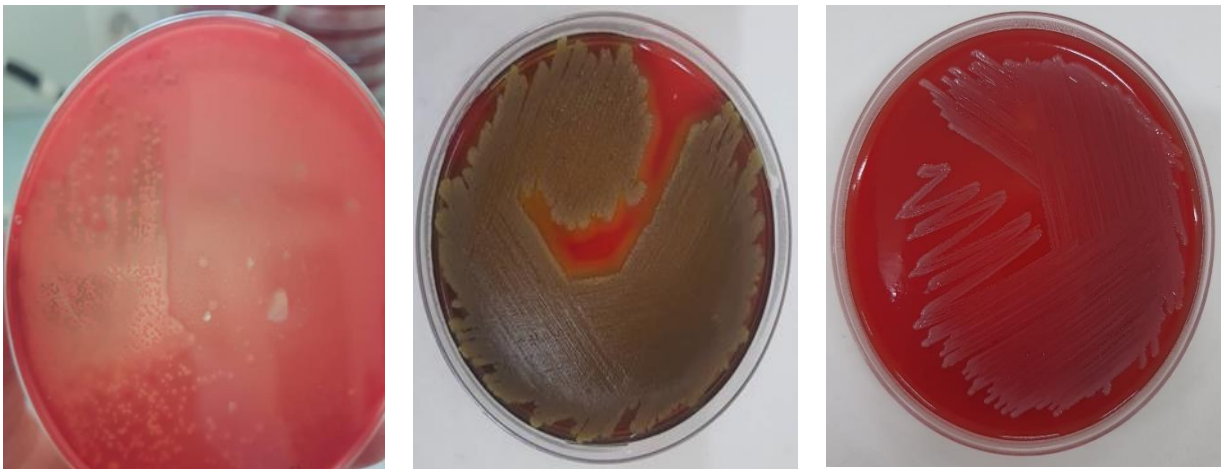


Figure. 23 : Detection of hemolysin production (A: β-hemolysis, B: α-hemolysis, C: γ-hemolysis).

3.8 Protease production

The Protease production is one of important virulence factors, which increase the pathogenicity of microorganisms. Proteolytic activity was detected using skimmed milk agar plates, the plates were prepared for primary screening, to prepare skimmed milk agar using: peptone, yeast extract and agar, the mixture was sterilized by autoclaving and 100 ml/l sterile UHT non-fat milk was added to the base in temperature of (45°C- 50°C). The tested strains were inoculated by swab streaking on the surface of the plates and incubated at 37°C for 24h. The presence of a clear zone around the bacterial colonies was considered indicative of protease positivity. *E. coli* ATCC 25922 was used as negative control and *P. aeruginosa* ATCC 27853 was used as positive control (Fig. 24) (Riffel and Brandelli, 2006; Hammood Abed Al Doori et al., 2020).

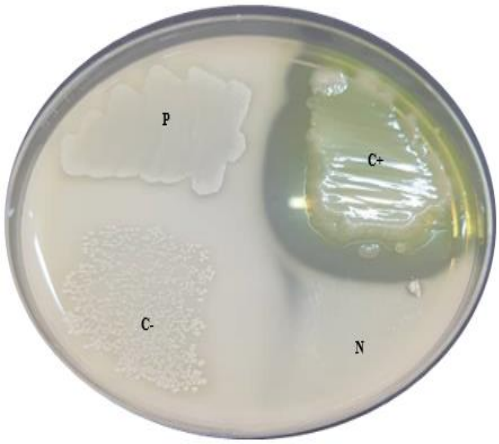


Figure. 24 : Protease production test on skimmed milk agar (SMA) (C+: positive control, C-: negative control, P: positive protease production, N: negative protease production).

3.9 Lecithinase and lipase production

For the detection of lecithinase and lipase production, the strains were cultured on egg yolk agar (EYA) and incubated at 37°C for 24h, non-selective agar was prepared and sterilized and an emulsion of egg yolk (egg yolk + sterile saline solution) was added to nutrient agar (45°C-50°C), the strains were streaked on EYA.

Lecithinase production is found by the emergence of halos around the colonies. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as negative control for lecithinase production,

The lipase enzyme is also inferred through the appearance of iridescent sheen on the surface of the colony, the incubation of the plates for the detection of lipase production can be delayed for 5 days to one week at the same temperature.

P. aeruginosa ATCC 27853 and *S. aureus* ATCC 29213 as positive and negative controls, respectively for the lipase detection (Fig. 25) (Hammood Abed AlDoori et al., 2020).

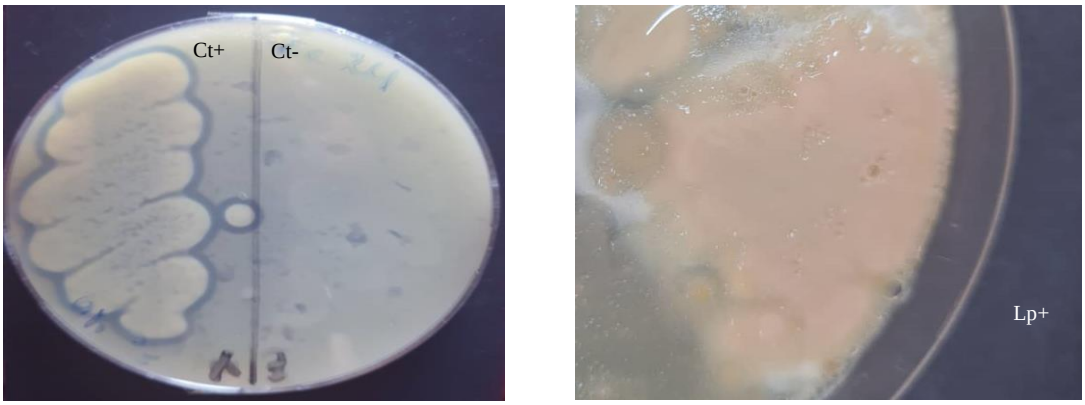


Figure. 25 : Detection of lecithinase and lipase production on egg yolk agar (EYA) (Ct+: licethinase positive, Ct-: licethinase negative, Lp+: lipase positive).

3.10 Molecular study of gene's resistance

3.10.1 Total DNA extraction

DNA was extracted from strains using the Direct PCR of intact bacteria (Colony PCR) method described previously (Woodman et al., 2016). Briefly, after growing strains in nutrient agar overnight, 1-2 colonies were dissolved in an Eppendorf tube with 100 µl of distilled sterile water. The mixtures were vortexed for 10 sec and then incubated at 99°C for 15 min in a block heater (Sparks, USA). Supernatants were collected after centrifugation at 10000 ×g for 10 min and pellets were discarded. A 1% agarose gel electrophoresis was conducted to evaluate the quality of DNA, in addition, NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, MA, USA) was used to assess purity and concentration of extracted DNA.

3.10.2 PCR for detection of 16s DNA

The bacterial 16s ribosomal gene (approximately 1500 bp) was amplified to evaluate the amplifiability of the previously extracted genomic DNA using primers F1 and R12 described in (Coy et al., 2014) (Tab. 6). Mix PCR was prepared for one sample, 2.5 µl of Dream Taq buffer (10x) + 0.5 µl of dNTP (10 µM) + 0.5 µl of $MgCl_2$ + 0.5 µl of forward and rivers primers (100 pmol/ µl) + 0.125 µl of Taq polymerase enzyme (5 U/µl) was mixed with 2 µl of extract DNA and filled with to 25 µl total volume of distilled sterile water and vortexed for 10 sec (Fig. 26a).

Table 6. Primers used for the 16s gene.

Target gene	Primer ID	Sequence	Amplification size (bp)
16s rDNA gene	F1 R12	5'-GAGTTTGATCCTGGCTCAG-3' 5'-ACGGCTACCTTGTTCAGACT-3'	1500

After preparing PCR Mix, negative control was preparing by mixing all the Mix PCR compound without DNA, and a previously amplifiable DNA was used as positive control; all PCR mix, negative and positive controls were placed in thermocycler (Biometra, Germany) following the conditions cycle of amplification in table 7.

An electrophoresis (Bio-Rad, Bd Raymond Poincaré, Belgium) of 1.5% agarose gel was performed for PCR products, by applauding 5 µl of PCR product and 1 µl of blue bromophenol and 3 µl of DNA maker. After migration, agarose gel was incubated in TAE 1x with 3 µl of ethidium bromide (0.5%) for 12 min. The final results were reading under UV lamp (Fig. 26b).

Table 7. Cycles conditions of PCR amplification.

Step	Temperature (°C)	Time	
Initial denaturation	95°C	5 min	
Denaturation	95°C	30 sec	× 35 cycles
Primers annealing	56°C	30 sec	
extension	72°C	1 min	
Final extension	72°C	5 min	

(a) (b)

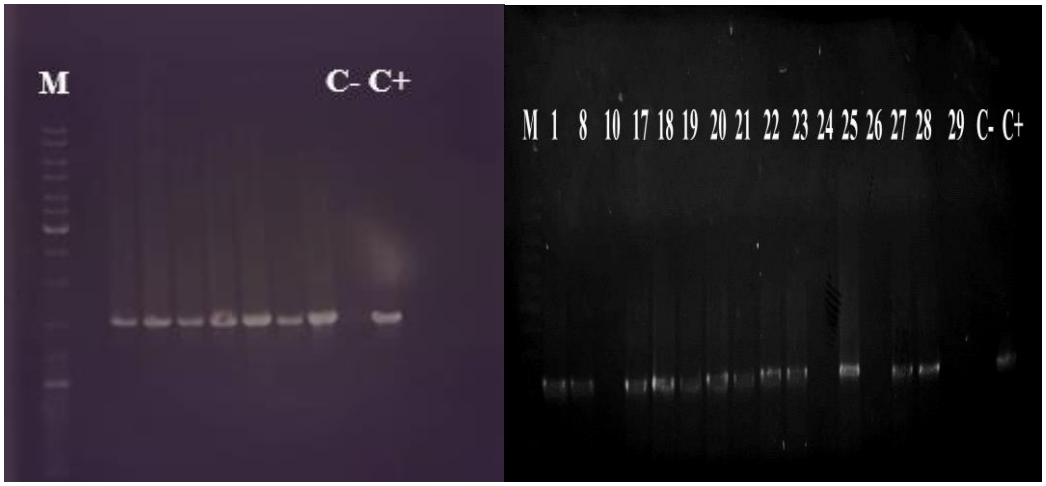


Figure. 26 : Agarose gel after PCR assay (a: total DNA, b: 16s ribosomal DNA, M: marker, 1-29: strains, C-: negative control, C+: positive control)

3.10.3 Multiplex PCR for detection of resistance genes

3.10.3.1 Molecular detection of β-lactamase

Two multiplex PCRs were prescribed by Kim *et al.*, (2009) and used for the detection of β-lactamase genes :

- The first multiplex assay (Set I) was designed to detect TEM, SHV, CTX-M IV group, and OXA β-lactamase encoding genes (Tab. 8).
- The second assay (Set II) was designed to detect CTX-M I group, CTX-M II group, CMY II, and DHA encoding genes (Tab. 8).

Both PCR reactions were conducted under the same conditions, reactions were performed in a final volume of 25 µl containing 2 µl of template DNA, 2.5 µl reaction buffer, 0.5 µl of dNTPs, 0.5 µl of each primer, and 0.125 µl of Taq polymerase.

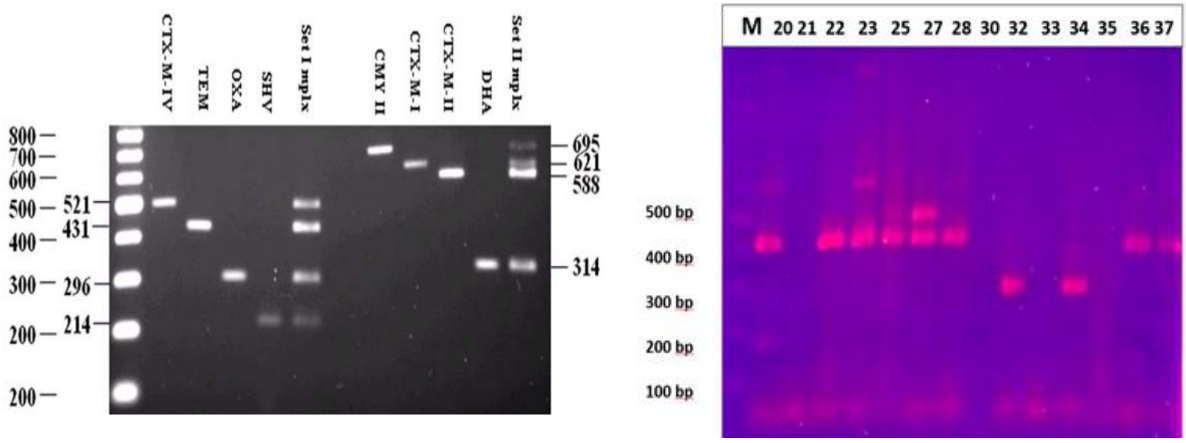
Both assays were conducted with the same cycling conditions: denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 61°C for 1 min and 72°C for 1 min, ending with final extension of 72°C for 5 min. After PCR amplification, 3 µl of each reaction was loaded onto 2% agarose gel for electrophoresis.

The PCR products were electrophoresed for 30-40 min at 100 V in 1× TEA buffer. DNA was stained with ethidium bromide and the gels were imaged under UV light. The sizes of PCR amplicons were determined by a comparing them with molecular weight size markers (Fig. 27 a,b).

Table 8. Primers used in multiplex PCR for the detection of β-lactamase genes.

Assay	Primers	Primer's sequence (5'_3')	Size (bp)
SET I	**CTX-M IV F**	GACAAAGAGAGTGCAACGGATG	501
	CTX-M IV R	TCAGTGCGATCCAGACGAAA	
	TEM F	AGTGCTGCCATAACCATGAGTG	431
	TEM R	CTGACTCCCC GTCGTGTAGATA	
	OXA F	ATTATCTACAGCAGCGCCAGTG	296
	OXA R	TGCATCCACGTCTTTGGTG	
	SHV F	GATGAACGCTTTCCCATGATG	214
	SHV R	CGCTGTTATCGCTCATGGTAA	
SET II	**CMY II F**	AGCGATCCGGTCACGAAATA	695
	CMY II R	CCCGTTTTATG CACCCATGA	

CTX-M I F	TCCAGAATAAGGAATCCCATGG	621
CTX-M I R	TGCTTTACCCAGCGTCAGAT	
CTX-M II F	ACCGCCGATAATTCGCAGAT	588
CTX-M II R	GATATCGTTGGTGGTGCCATAA	
DHA F	GTGGTGGACAGCACCATTAAA	314
DHA R	CCTGCGGTATAGGTAGCCAGAT	



(a) (b)

Figure. 27 : Amplification profile of multiplex PCR for β-lactamase genes (a: SET I and SET II in kim *et al.* comparison, b: SET II gel).

3.10.3.2 Multiplex PCR for the detection of *qnr* genes

The presence of *qnr* genes *qnrA, qnrB, qnrC, qnrD* and *qnrS* was detected used multiplex PCR based on methods described by Zhao *et al.*, 2020. Amplification was carried out with the previously thermal cycling profile (of β-lactamase multiplex PCR), each primer had a specific annealing temperature (Tab. 9).

Table 9. Primes used for the amplification of *qnr* genes.

Gene	Sequence (5'_3')	Tm (°C)	Size (bp)
qnrA F	ATTTCTCACGCCAGGATTTG	55	516
qnrA R	TGCCAGGCACAGATCTTGAC		
qnrB F	CGACCTKAGCGGCACTGAAT	55	515
qnrB R	GAGCAACGAYGCCTGGTAGYTG		
qnrC F	GGGTTGTACATTTATTGAATC	50	446
qnrC R	TCCACTTTACGAGGTTCT		
qnrD F	CGAGATCAATTTACGGGGAATA	50	581
qnrD R	AACAAGCTGAAGCGCCTG		
qnrS F	GACGTGCTAACTTGCGTGAT	56	118
qnrS R	TGGCATTGTTGGAAACTTG		

Tm: Annealing temperature

3.10.3.3 Plasmid profiling

Plasmid DNA was extracted by alkaline lysis method. Briefly, from an overnight liquid culture and after centrifugation at 13 000 g for 6 min, the supernatant was discarded and the pellet was resuspen[22]ded in 100 µl of solution 1 (Glucose 50 Mm, Tris-HCl 25 Mm, EDTA 10 Mm at pH=8).

The tubes were placed on ice and add 200 µl of freshly prepared solution 2 (NaOH 10N 1 ml, SDS 10% 0.5 ml, make up the solution to 100 ml and store at 4°C). The tubes were closed and inverted 7-9 times, then placed on ice. 150 µl of solution 3 was added (Glacial acetic acid 11.5 ml, potassium acetate 5M 60 ml, make up the solution to 100 ml and store at 4°C). After second centrifugation, the supernatants were transferred to a new Eppendorf tubes and 1 ml of 100% ethanol was added and mixed by inversion.

Next centrifugation, the pellets were washed with 400-500 µl of 70% ethanol and re-centrifuged for 6 min at 13 000 g. Inverted onto absorbent paper to allow the ethanol to drain, then the pellets were dried in a speed vacuum for 20 min.

All the pellets were resuspended in 50 µl of H_2O and 1 µl of RNase and incubated at 37°C for 10 min. The extracted DNA was separated using 1% agarose gel electrophoresis at 100V for more than 30 min (Fig. 28).

Plasmidic DNA appear as one or multiple bands with small molecular size compared with chromosomal DNA, which is much larger and usually appear as unique and very large band near the well.

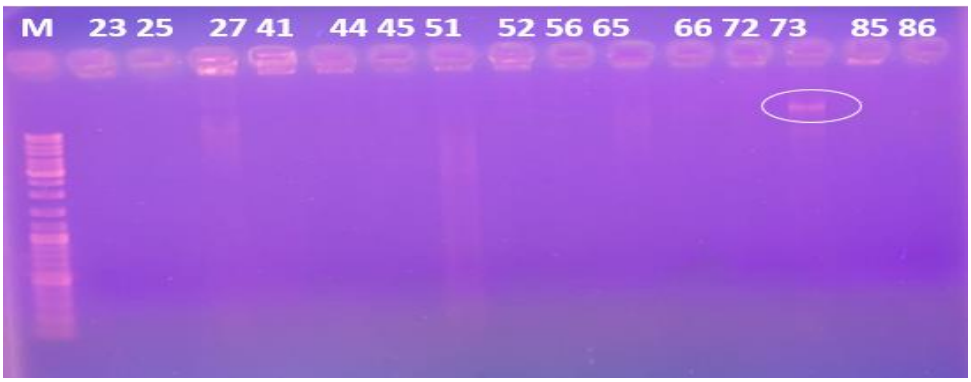


Figure. 28 : Separation of plasmid DNA on agarose gel stained with ethidium bromide (M: gene marker).

3.10.4 Phylogenetic grouping of *E. coli* strains by quadruplex PCR

The "Clermont method" (described in Clermont, 2013) was used to characterize the *E. coli* strains within the seven phylogroups (A, B1, B2, C, D, E, F). PCR assays for assignment phylogenetic groups were carried out using DreamTaq DNA polymerase with phylogenetic primers (*arpA*, *chuA*, *yjaA*, *TspE4.C2*) and cycle conditions (modification in primers annealing temperature) reported in table 10. The amplicons obtained were detected on with electrophoretic run (100 V in TAE 1x) on 1.5% agarose gel under UV light (Fig. 29).

Table 10. List of primers with annealing temperatures (the amounts of primer used were 20 pmol for phylogenetics grouping primers, except for *AceK.f* (40 pmol), *ArpA1.r* (40 pmol)).

Target gene	Primer ID	Primer sequences	Amplification size (bp)	Tm	Reference
chuA	chuA.1b chuA.2	F: 5′-ATGGTACCGGACGAACCAAC-3′ R: 5′-GCCGCCAGTACCAAAGACA-3′	288	59	(Clermont et al., 2013)
yjaA	yjaA.1b yjaA.2b	F: 5′-CAAACGTGAAGTGTCAGGAG-3′ R: 5′-AATGCGTTCCTCAACCTGTG-3′	211	59	(Clermont et al., 2013)
TspE4.C2	TspE4C2. 1b TspE4C2.2b	F: 5′-CACTATTCGTAAGGTCATCC-3′ R: 5′-AGTTTATCGCTGCGGGTCGC-3′	152	59	(Clermont et al., 2013)
arpA	AceK.f ArpA1.r	F: 5′-AACGCTATTCGCCAGCTTGC-3′ R: 5′-TCTCCCCATACCGTACGCTA-3′	400	59	(Clermont et al., 2000, 2004)
arpA	ArpAgpE.f ArpAgpE.r	F: 5′-AACGCTATTCGCCAGCTTGC-3′ R: 5′-TCTCCCCATACCGTACGCTA-3′	301	57	(Lescat et al., 2013)
trpA	trpAgpC.1 trpAgpC.2	F: 5′-AGTTTTATGCCCAGTGCGAG-3′ R: 5′-TCTGCGCCGGTCACGCCC-3′	219	57	(Lescat et al., 2013)
trpA	trpBA.f trpBA.r	5′-CGGCGATAAAGACATCTTCAC-3′ 5′-GCAACGCGGCCTGGCGGAAG-3′	489	57	(Clermont et al., 2008)

Tm: Temperature.

Finally, the assignment of all clinical strains into phylogroups was performed using the scheme proposed by Clemont *et al.* reported in table 11. Briefly, Phylogroups were assigned to each strain based on the presence or absence of four genes (*arpA*/*chuA*/*yjaA*/*TspE4.C2*) evaluated with quadruplex PCR.

Using specific primers C and E, further PCR were conducted to differentiate strains in A or C, D or E and E or Clade I. C-specific/*arpA* (301bp) was used to differentiate strains in A or C group, while E-specific/*trpA* (219bp) were used to differentiate strains in D or E and E or Clade I groups, the presence of amplicon classified strains in C and E groups, respectively. While the absence of amplicon classified strains in C, D, Clade I groups, respectively.

Table 11. Method applied to assign the phylogroup to each *E. coli* strain.

QUADRUPLEX PCR					
***arpA* (400 bp)**	***chuA* (288 bp)**	***yjaA* (211 bp)**	***TspE4.C2* (152 bp)**	**Phylo-group**	**Next step**
+	-	-	-	**A**	
+	-	-	+	**B1**	
-	+	-	-	**F**	
-	+	+	-	**B2**	
-	+	+	+	**B2**	
-	+	-	+	**B2**	
+	-	+	-	**A or C**	**distinguish with C-specific primer *arpA* (301 bp)**
+	+	-	-	**D or E**	**distinguish with E-specific primer *trpA* (219 bp)**
+	+	-	+	**D or E**	
+	+	+	-	**E or clade I**	
-	-	+	-	**Clade I or II**	
-	-	-	+	**Unknow**	
-	-	+	+	**Unknow**	
+	-	+	+	**Unknow**	
+	+	+	+	**Unknow**	
-	-	-	-	**Unknow**	

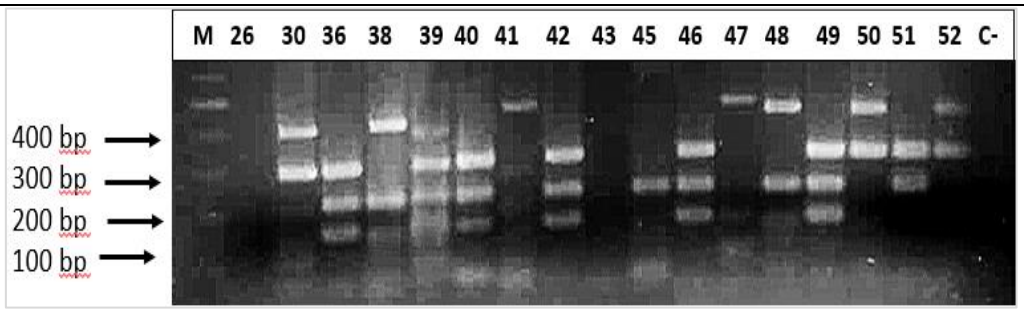


Figure. 29 : Quadruplex polymerase chain reaction amplification of phylogenetic genes.

3.11 Statistical analysis

All data were collected and organized using Excel (Microsoft office 16), statistical analysis was performed using the Statistical Package for the Social Sciences software, SPSS (version 27.0). Categories were compared using the chi-square test. Graphs or chart were used to show the prevalence and distribution of the isolated bacteria against gender (male and female), age groups (children, adults and elderly), specimens (urine, pus, vaginal swab, blood, catheters) species, resistance profiles and virulence factors. In addition, a frequency table expressed in percentage and absolute numbers was used to display the susceptibility patterns, distribution of resistance strains, distribution of genes….. Correlation test was used to establish relationship between different factors. ANOVA and Tukey tests were used for analyzed the continuous variable (OD), while means independent comparison test confer the comparison between two

means, the significance level was set at $p < 0.05$ with 95% of confidence that the observed results were statistically significant, $p < 0.01$ indicate a 99% of confidence level.

4 Results and discussion

During the work period that spanned from May 2021 to May 2023 at three government hospitals and seven medical private laboratories, a screening of 402 bacterial strains was conducted to achieve the objective of our study.

4.1 Demographic Characteristics

The clinical analysis of the samples showed that 326 (81.09%) isolates collected from community setting. While 76 (18.91%) isolates were collected from hospital environment, the highest percentage (36.8%) originated from the infectious diseases unit (IND), and the lowest percentage (1.3%) obtained from the hemodialysis unit (HDM) (Fig. 30).

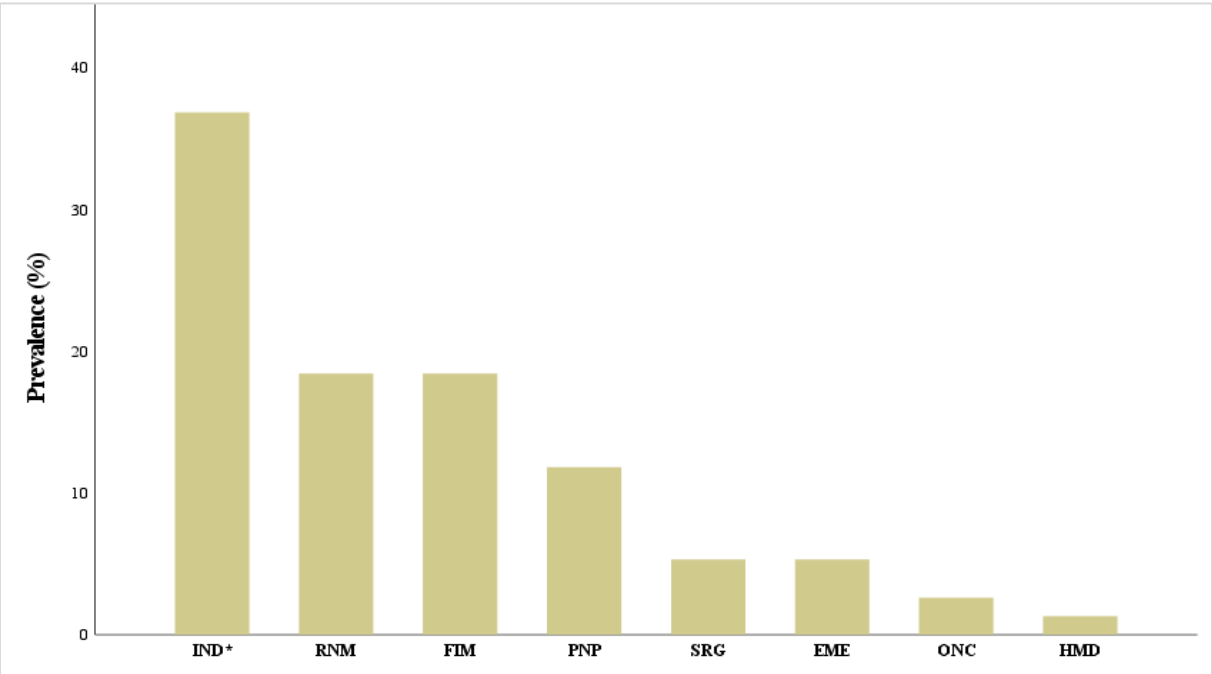


Figure. 30 : Distribution of samples according to infection category and hospital unit (IND: infectious diseases, RNM: reanimation, FIM: female internal medicine, PNP: pneumophtisiology, SRG: surgery, EME: emergency, ONC: oncology, HMD: hemodialysis, *: p <0.01).

A total of 402 clinically suspected patients for bacterial infections were included in this study. The majority of study participants were females 266 (66.2%), confirmed by a 99% of confidence showed by the statistical analysis (p<0.01), with a male-to-female ratio of 1/4. The mean age of participants was 40.8 ±24.5 years ranged between 1 day and 96 years. The majority (67.9%) of patients were adults, while 17.2% were children's patients and 14.9% belongs to elderly group. The main samples were isolated from urine (83.6%, p<0.0001) followed by pus (10.4%), vaginal swab (2%) and 1% were isolated from thoracic drainage and bladder catheter (Tab. 12).

Table 12. Characteristics of patients including in this study.

Characteristics		**Total (%)**
Gender	Male	136 (33.8)
	Female	266 (66.2)
Age group	Children (1d- 18y)	69 (17.2)
	Adult (19y- 64y)	273 (67.9)
	Elderly (≥ 65y)	60 (14.9)
Nature of sample	Urine	336 (83.6)
	Pus	42 (10.4)
	Vaginal swab	8 (2)
	Central catheter	2 (0.5)
	Thoracic drainage	4 (1)
	Blood	3 (0.7)
	Femoral catheter	2 (0.5)
	Tracheal tube	1 (0.2)
	Bladder catheter	4 (1)

From urinary samples adult were more susceptible to develop UTI especially female (50%), also in pus samples female adult were more prevalent (50%), while vaginal swab was exclusively found in adult female. Males were more susceptible to occur bacterial infections originated from central catheter (100%), thoracic drainage (75%), tracheal tube (100%) and bladder catheter (75%) (Tab. 13).

Table 13. Nature of samples according to demographic characteristics: age groups and gender.

		Age groups n (%)					
		Children		**Adult**		**Elderly**	
		Male	**Female**	**Male**	**Female**	**Male**	**Female**
Nature of sample	**Urine**	31 (9.2)	33 (9.8)	54 (16.1)	168 (50)	26 (7.7)	24 (7.1)
	Pus	4 (9.5)	0	8 (19)	21 (50)	1 (2.4)	8 (19)
	Vaginal swab	0	0	0	8 (100)	0	0
	Central catheter	0	0	2 (100)	0	0	0
	Thoracic drainage	0	0	3 (75)	0	1 (25)	0
	Blood	1 (33.3)	0	1 (33.3)	1 (33.3)	0	0
	Femoral catheter	0	0	0	2 (100)	0	0
	Tracheal tube	0	0	1 (100)	0	0	0
	Bladder catheter	0	0	3 (75)	1 (25)	0	0

The results obtained shows that the most of bacterial infections in hospital setting were found in infectious diseases unit, these results align with the definition of infectious diseases unit as a hotspot for bacterial infections due to various factors such as prolonged hospital stays, invasive procedures, and the use of immunosuppressive therapies (Peleg and Hooper, 2010).

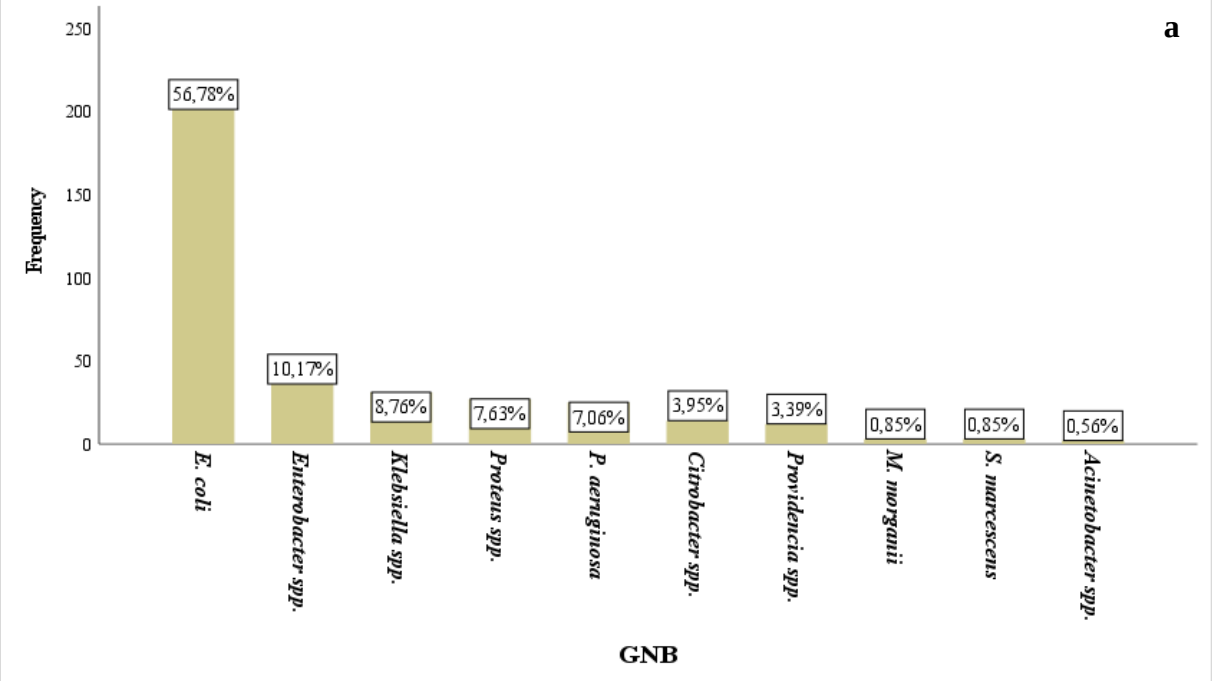
this study revealed that adult patients were more susceptible to develop bacterial infections especially UTIs, which is in accordance with Assefa *et al.*, (2022) in Ethiopia, where 52.6% of bacterial infections were reported among adult patients aged ≥ 36 years. Another research was

conducted in COVID-19 phase report that the majority of patients with bacterial infections were above 60 years (Mutua et al., 2023). The biological immune system's diminished response with age and its weakening in adults can explain this observation (Díaz et al., 2020).

Adult females (50%) were more at risk to occur bacterial UTIs compared to males (16.1%) in our study, these results are consistent with findings of Nzalie *et al.*, (2016), who reported similar gender disparities in UTI prevalence with a percentage of (83.6%), Kiiru *et al.*, (2023) report also in their document that female were more likely to have UTIs compared to males. Recent studies show that women face a 50 times greater risk of developing UTIs than men (Aslam et al., 2020; Islam et al., 2022; Konwar et al., 2022), the increased risk among females may be influenced by factors including: the shorter urethra female anatomy, sexual activity, pregnancy, diabetes and menopause (Aslam et al., 2020; Lee et al., 2020; Czajkowski et al., 2021).

4.2 Bacterial strains identification

In the current study, 88.1% of isolates were GNB, while 11.9% were GPB. Among GNB, *E. coli* was the predominant species 56.78% (p<0.01) followed by *Enterobacter* spp. 10.17%, *Klebsiella* spp. 8.76%, *Proteus* spp. 7.63%, *P. aeruginosa* 7.06%, *Citrobacter* spp. 3.95% and *Providencia* spp. 3.39%. On the other hand, inside GPB, *Staphylococcus* spp. were the predominant organisms (p<0.01) with a percentage of 81.25% followed by *Streptococcus* spp. 12.5% and finally *Enterococcus* spp. 6.3% (Fig. 31).



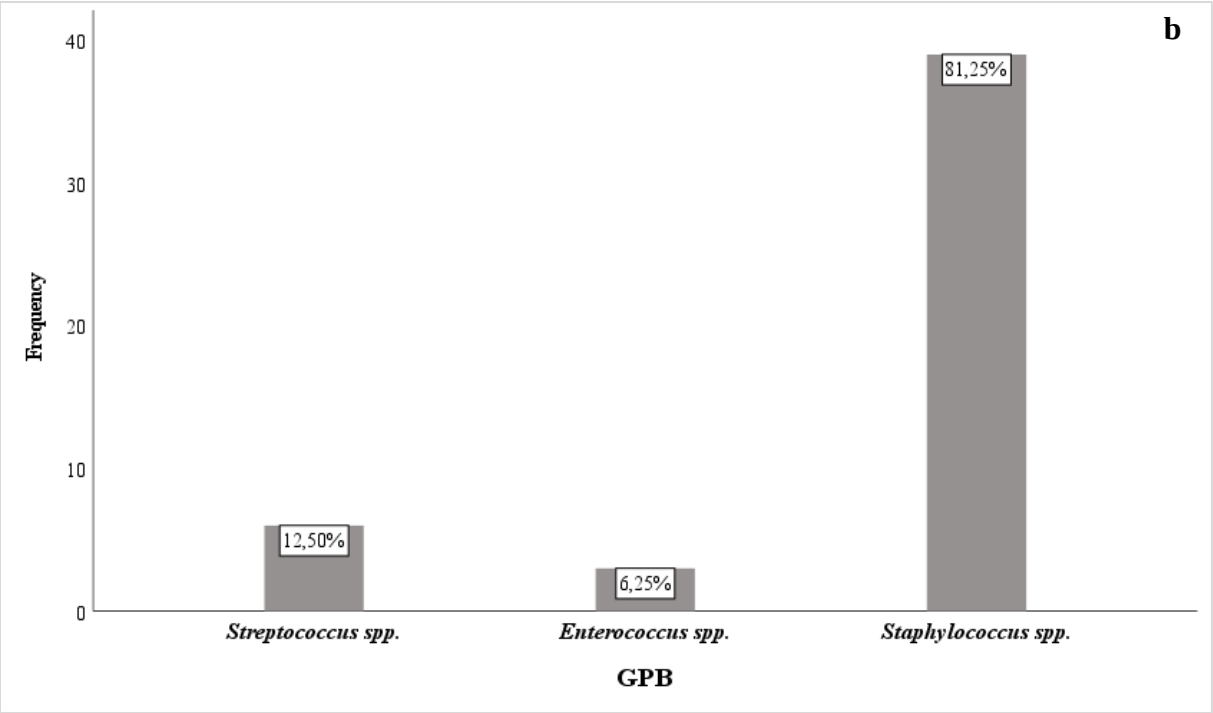


Figure. 31 : Prevalence of bacterial isolates (**a:** GNB, **b:** GPB).

After identification of isolate species, *E. coli* and *S. aureus* were the most identified organisms 50%, 7.7%, *klebsiella* sp., *P. aeruginosa* and *P. mirabilis* have showed an important number among identified organisms 6.2%, 6.2% and 5.7%, respectively (Tab. 14). A significantly higher number of females had an infection with both GNB and GPB (p<0.001), exception *K. oxytoca* which found exclusively in male patients in both children and adult group.

Table 14. Prevalence of identified microorganism among the clinical samples by age groups and gender.

Species (n, %)	Age groups n (%)					
	Children		Adult		Elderly	
	M	F	M	F	M	F
***E. cloacae* (n=10, 2.5)**	1 (10)	1 (10)	1 (10)	5 (50)	1 (10)	1 (10)
***E. aerogenes* (n=16, 4)**	2 (12.5)	0	3 (18.75)	8 (50)	1 (6.25)	2 (12.5)
***Enterobacter* sp. (n=10, 2.5)**	0	1 (10)	3 (30)	4 (40)	0	2 (20)
***P. mirabilis* (n=23, 5.7)**	3 (13)	4 (17.4)	4 (17.4)	8 (34.8)	2 (8.7)	2 (8.7)
***P. vulgaris* (n=4, 1)**	0	0	0	1 (25)	3 (75)	0
***E. coli* (n=201, 50)**	18 (9)	19 (9.5)	34 (16.9)	104 (51.7)	10 (5)	16 (7.9)
***Providencia alcalifaciens* (n=9, 2.2)**	2 (22.2)	0	0	6 (66.7)	1 (11.1)	0
***Providencia stuartii* (n=2, 0.5)**	0	0	1 (50)	1 (50)	0	0
***Providencia* sp. (n=1, 0.2)**	0	0	0	1 (100)	0	0
***C. diversus* (n=8, 2)**	1 (12.5)	2 (25)	0	5 (62.5)	0	0
***C. freundii* (n=6, 1.5)**	0	2 (33.3)	1 (16.7)	3 (50)	0	0
***P. aeruginosa* (n=25, 6.2)**	2 (8)	2 (8)	4 (16)	11 (44)	3 (12)	3 (12)
***K. pneumoniae* (n=3, 0.7)**	1 (33.3)	0	1 (33.3)	1 (33.3)	0	0
***K. oxytoca* (n=3, 0.7)**	1 (33.3)	0	2 (66.7)	0	0	0

***Klebsiella* sp. (n=25, 6.2)**	3 (12)	0	3 (12)	16 (64)	1 (4)	2 (8)
***M. morganii* (n=3, 0.7)**	0	0	2 (66.7)	1 (33.3)	0	0
***Acinetobacter* sp. (n=2, 0.5)**	0	0	0	2 (100)	0	0
***Serratia marcescens* (n=3, 0.7)**	0	0	0	2 (66.7)	0	1 (33.3)
***Streptococcus* sp. (n=6, 1.5)**	0	0	4 (66.7)	2 (33.3)	0	0
***Enterococcus* sp. (n=3, 0.7)**	0	0	0	1 (33.3)	1(33.3)	1(33.3)
***S. aureus* (n=31, 7.7)**	2 (6.5)	0	4 (12.9)	18 (58)	5 (16.1)	2 (6.5)
CoNS (n=4, 1)	0	1 (25)	3 (75)	0	0	0
***Staphylococcus* sp. (n=4, 1)**	0	1 (25)	2 (50)	1 (25)	0	0

M: male, F: female, CoNS: Coagulase negative *Staphylococcus*.

There was a strong correlation between species and the nature of samples ($p<0.01$). The majority of identified strains were originated from urine, where *E. coli* was the most found species (52.38%). Important numbers of strains were originated from pus specimen (*P. aeruginosa*, *Acinetobacter* sp., *Serratia marcescens*). The second source of *K. pneumoniae* species after urine was blood, which was also a second source of *C. freundii* (Fig. 32).

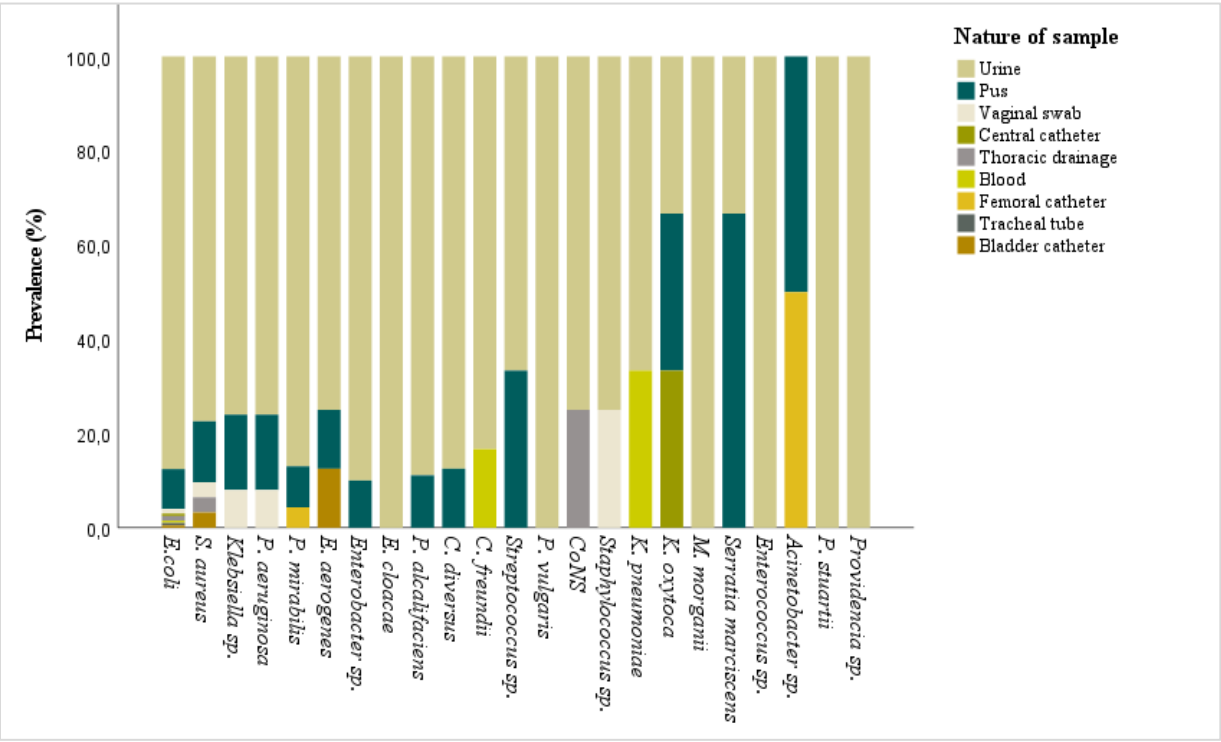


Figure. 32 : Distribution of isolates by specimen sources.

The bacterial identification of strains in our study showed that *E. coli* was the predominant species, accounting for the majority of isolated pathogens, this predominance can be attributed to the high proportion of samples derived from urine. Also, *E. coli* was identified as the leading pathogen in urine samples (52.38%), these finding align with previous studies, such as Bereanu *et al.*, (2024), which similarly reported *E. coli* as the primary organism in urine cultures. The

same percent was found by Islam *et al.*, (2022) study, which found that *E. coli* was the predominant organism in their urine bacterial samples (51.6%). *E. coli* was the most prevalent uropathogen in the both studies by Akrami *et al.*, (2020) and Al Benwan and Jamal, (2022), with prevalence rates of 54.8% and 67.3%, respectively. The ability of this germ to adhere to host uroepithelial cells via fimbriae and produce virulence factors, such as toxins contributes to its dominance in urine and its capacity to cause UTIs (Terlizzi et al., 2017; Kim et al., 2022).

4.3 Antibiotic susceptibility study

4.3.1 Trend of antibiotic resistance by year of isolation

Over the three-year period of collection, a general variation (increase/decrease) in resistance levels was observed across several antibiotic classes. However, the degree and direction of change varied significantly across specific antibiotics and bacterial strains. For penicillins higher rates were recorded in 2022 and 2023 (87.1% and 71.4%, respectively). Also, for the combination β-lactams/inhibitor similar resistance rates were recorded over the three years, with an advantage for the year 2022 (74.2%) (Fig. 33).

Resistance to cephalosporins increased significatively ($p<0.001$) across the three years especially in 2023, where the increase rate was 57% from 2022 to 2023. The resistance to carbapenem was relatively high in 2022 (77.4%, $p=0.001$) compared with the other years (Fig. 33).

The same rate of resistance was recorded in 2021 and 2023 (21%, $p<0.001$) for aminoglycosides while the low rate was found in 2022 (12.9%). By 2023, an increase resistance levels (39.4%, 24.9% and 39.8%) were recorded in quinolones, phenicolates and monobactams, respectively (Fig. 33).

Regarding sulfamids, the high rate was recorded in 2021 (58%), while for phosphonic family, a significant high rate was found in 2022 (48.4%, $p=0.039$) and the rate declined in 2023 (Fig. 33).

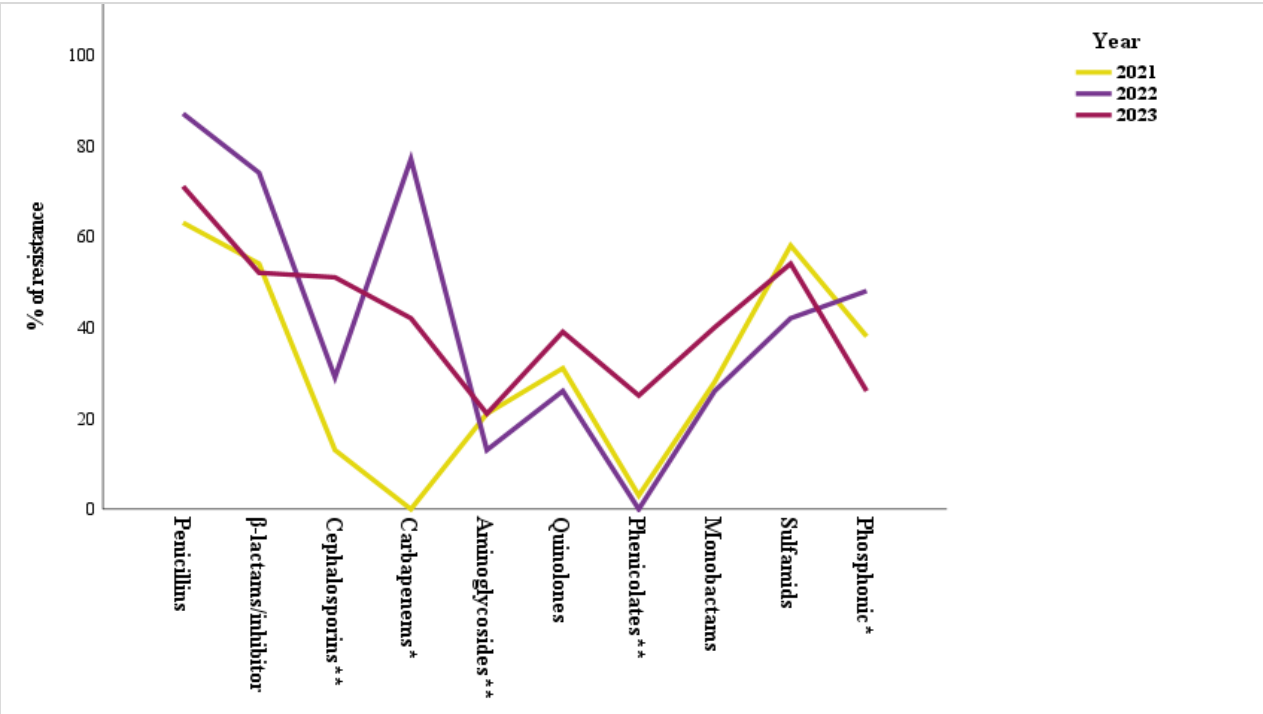


Figure. 33 : Trend of resistance to various antibiotics in 2021, 2022 and 2023 (*: statistical significance $p<0.05$, **: $p<0.001$).

The study report data on the prevalence of antibiotic patterns among the most common pathogen bacteria from 2021 to 2023, similar tendencies were reported from different locations in Africa, notably in Uganda (Namubiru et al., 2024); resistance spikes were found particularly against commonly-used antibiotics such as β-lactams, cephalosporins, carbapenems and monobactams. The documented increase in resistance among several antibiotic classes, impacting both GPB and GNB, highlights a diminishing range of practical therapeutic alternatives, this development presents a significant risk to patient care, potentially leading to extended diseases, worsening infections, and increased mortality rates.

4.3.2 Distribution of resistance by source of sample

The examination of AR rates among several sample types including: urine, pus, vaginal swabs, blood, and medical devices revealed distinct patterns indicative of microbial diversity.

Resistance rates in urine samples were significatively higher for penicillins and polymyxin (73.2%, 57.1% ($p<0.001$), respectively). Pus samples demonstrated a significant elevated resistance rate to cephalosporins and lincosamides (73.8%, 38.1%, $p=0.009$, respectively). However, a significant level of resistance to combination β-lactams/inhibitor, cephalosporins, aminoglycosides and quinolones were found in medical devices species (61.5%, 76.9%, $p=0.015$ and 53.8%, 76.9%, $p<0.001$, respectively) (Fig. 34).

Blood cultures revealed concerning resistance level to monobactams (100%. $p<0.001$), while phenicolates and sulfamids record statistical levels in medical devices species (69.2%, $p<0.001$, 84.6%, $p=0.008$, respectively), for the other families no significative difference was found through the sources (Fig. 34).

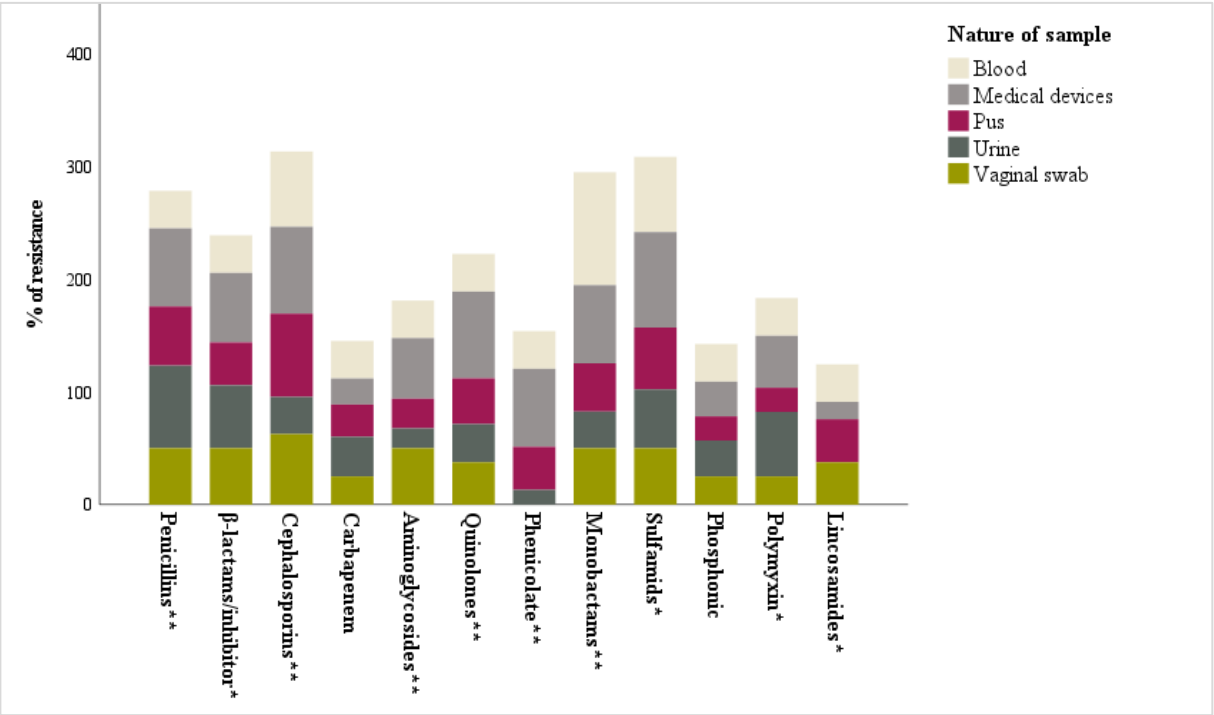


Figure. 34 : Prevalence of antibiotic resistance by nature of sample (*: statistical significance $p<0.05$, **: $p<0.001$).

The finding of our study reveals important rates of resistance across various sample types, the significantly high resistance rates to penicillins and polymyxins in urine samples are particularly concerning aligns with global trend: USA (Dunne et al., 2022), Saudi Arabia (Ahmed et al., 2019). These findings suggest that commonly prescribed antibiotics for UTIs may be losing their efficacy, likely due to overuse or misuse. The resistance to polymyxins, often considered a last-resort therapy, raises further concerns about treatment options for MDR uropathogens.

The elevated resistance rates observed for cephalosporins and lincosamides in pus samples were in accordance with trends in Rwanda (Ntirenganya et al., 2015) and Saudi Arabia (AlBahrani et al., 2024).

Resistance to combination β-lactams/inhibitor, cephalosporins, aminoglycosides, and quinolones among medical device pathogens is a significant concern. These pathogens are often implicated in healthcare-associated infections, which are challenging to treat due to biofilm

formation and persistent exposure to antibiotics (Mishra et al., 2024). The high resistance levels observed align with a research in Italy (Folliero et al., 2021).

The complete resistance to monobactams (100%) in blood cultures represent a significant health concern which reported also in China (Zhang et al., 2015), aztreonam is β-lactam antibiotics effective primarily against GNB and this observed resistance implies that our blood pathogens have either acquired resistance genes or have intrinsic mechanisms that render monobactam ineffective.

4.3.3 Distribution of resistance by species

4.3.3.1 Enterobacter species

The antibiotic resistance profile of species included in this study were illustrated in figure 35, among GNB, *Enterobacter* spp. showed a high rate of resistance (p<0.01) for amoxicillin, colistin (88.9%, 79.5%), ticarcillin + clavulanic acid (83.3%) and piperacillin (80.6%) and also for trimethoprim/sulfamethoxazole and fosfomycin (69.4% and 52.8%). The rate of resistance to different generations of cephalosporins ranged between 50% to 61.1%; the most effective antibiotics against *Enterobacter* spp. were imipenem and amikacin (19.4% and 8.3%, respectively).

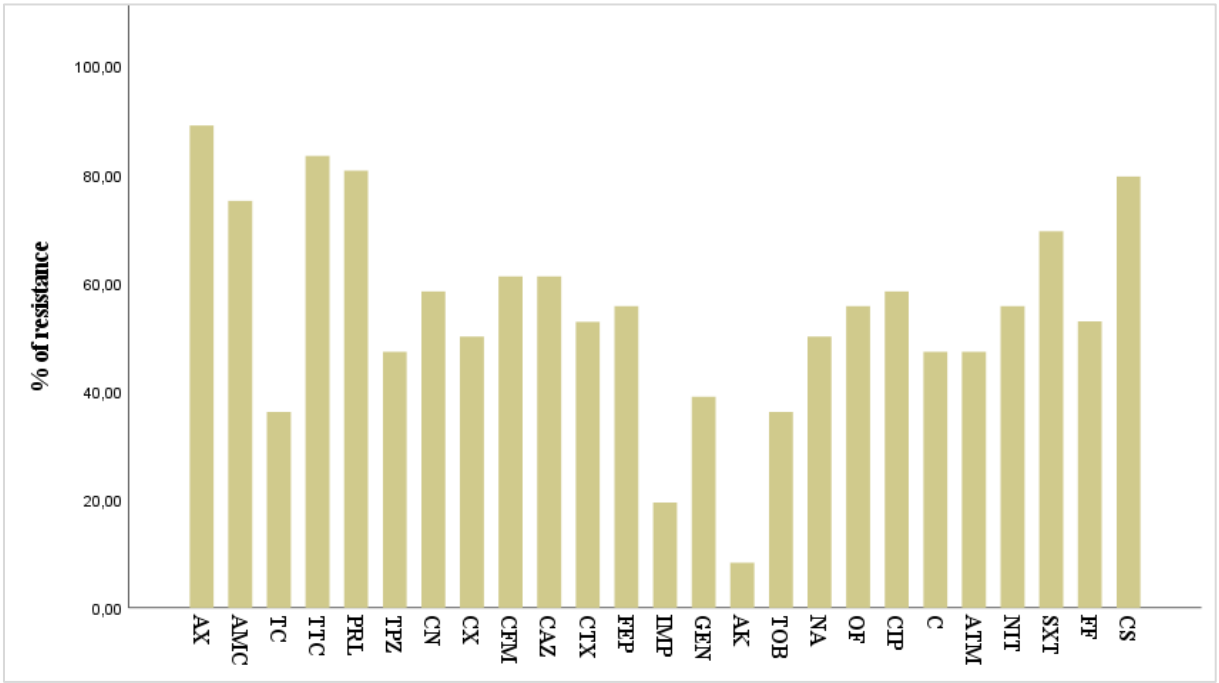


Figure. 35 : Resistance rates of *Enterobacter* species.

Enterobacter spp. are significant opportunistic pathogens implicated in various healthcare associated infections. Our findings are similar with previous studies conducted in Bangladesh and in India, which reported the same resistance profiles to β-lactams and cephalosporins (Mishra et al., 2020; Haque et al., 2023). Therefore, the resistance of *Enterobacter* spp. to amoxicillin, first-generation cephalosporins and cefoxitin was resulted to intrinsically resistance due to the presence of constitutive AmpC β-lactamase (Liu et al., 2022).

However, the prevalence of imipenem-resistant *Enterobacter* species in our study matches with what Haque *et al.,* (2023) found, which was also 20%. But it was lower than what was reported by Mishra *et al.,* (2020), they found a percentage of 30%. Regional factors can be the cause of this difference.

The report of colistin and fosfomycin resistance was higher inside our *Enterobacter* spp. than the studies conducted by Mishra *et al.* with 50% for colistin. In Haque *et al.* study, 40% for fosfomycin and 20% for colistin. The WHO report *Enterobacter* spp. as being naturally susceptible to colistin but heterogenic resistance was observed in several *Enterobacter* species (Doijad et al., 2023). The emergence use of colistin as a last resort for the treatment might be the reason for increasing this type of resistance.

4.3.3.2 E. coli

In our study, a total of 201 *E. coli* isolates were tested, revealing high resistance rates to commonly used antibiotics ($p<0.01$) such as the first-line antibiotics: amoxicillin (82.6%), piperacillin (77.6%), ticarcillin + clavulanic acid (72.1%), a moderate resistance levels were showed for the combination piperacillin + tazobactam (32.8%) and also for cephalosporins (between 26.9% for cefotaxime to 39.3% for ceftazidime), trimethoprim/sulfamethoxazole (51.2%), fosfomycin (40.3%) and nitrofurantoin (35.8%) (Fig. 36).
For the second line, *E. coli* showed a moderate resistance to quinolones family (33.8%-39.8%). In last-resort antibiotics, 49.8% of *E. coli* isolates exhibited a resistance for imipenem, while a high rate of resistance was observed in colistin case (55.2%). Notably, aminoglycosides showed the best performance against this pathogen, with low resistance rates were noticed for amikacin (13.9%) and chloramphenicol (13.4%).

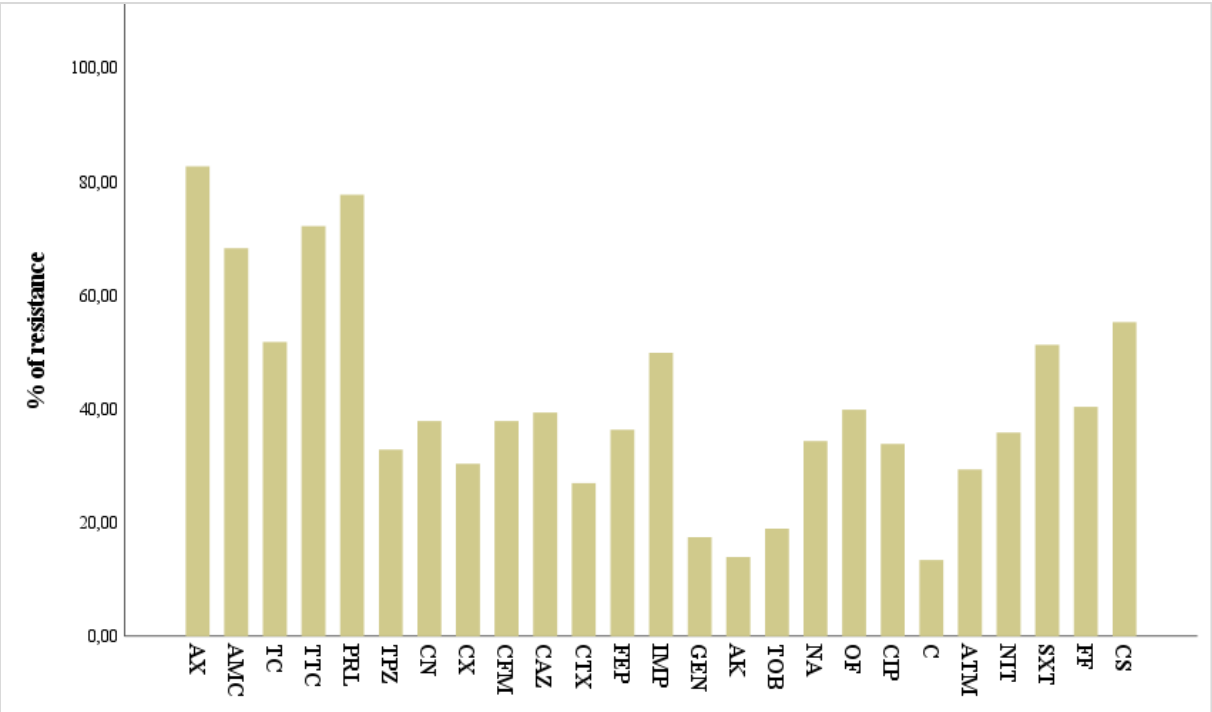


Figure. 36 : Resistance rates of *E. coli*.

In the current study, *E. coli* showed differences in AR patterns, it was highly resistance to the first-line treatment including amoxicillin, piperacillin and ticarcillin + clavulanic acid, same rates was observed in Marwa and Ibrahim, (2024) study in Iraq, which demonstrated a significant resistance level to piperacillin. A higher resistance rate was observed for ticarcillin + clavulanic acid (89.09%) and piperacillin (70.09%) in Shahid and Yousif study, (2022).

In our study, moderate resistance to cephalosporins was observed, consistent with findings from previous studies conducted in Thailand and Poland, which also reported moderate resistance to various classes of cephalosporins (Tewawong et al., 2020; Kot et al., 2021).

It was observed that the rate of resistance of *E. coli* to trimethoprim/sulfamethoxazole was significant high in our results, which aligns with previous studies reporting similar resistance rates (53.3%, 54.3%, 63%) (Nouri et al., 2020; Tewawong et al., 2020; Nji et al., 2021). Nitrofurantoin, colistin and fosfomycin are mainly used in treatment of several *E. coli* infections, our tested *E. coli* showed a high resistance to colistin, same results was reported by Mahmoud *et al.*, (2022) in Egypt, they found that the rate of colistin resistance was 42.9%. This increasing rate in north Africain countries may be due the dissemination of *mrc-1* gene among *E. coli* strains which has been reported as epidemiological alert by WHO (Carmona-Cartaya et al., 2022).

Similar rates were showed against fosfomycin and nitrofurantoin in other studies (34.35% and 46% for fosfomycin, 29% for nitrofurantoin only) in Kanaujia e*t al.*, (2020) and Ejaz *et al.*, (2021) studies. Our results reveal a higher resistance rate to nitrofurantoin compared to a meta-analysis of 14 studies across 8 countries, which analyzed 1,888 *E. coli* strains and reported a resistance rate of 20% (Bunduki et al., 2021). These results can be explained by the fact that antibiotics are highly effective in treating UTIs, particularly due to their rapid oral absorption (Sorlozano-Puerto et al., 2020).

For carbapenems, a rate of 49.8% was exhibited against imipenem which is in accordance with Nasif *et al.*, (2023); Marwa and Ibrahim, (2024) studies in Iraq and Egypt (30% and 30.9%, respectively). A moderate resistance was showed for quinolones family, in agreement with results obtained by Kot *et al.*, (2021); Nji *et al.*, (2021) and Bunduki *et al.*, (2021) studies (28.6%, 30% and 49.4%, respectively).

4.3.3.3 Klebsiella and Proteus species

The resistance profiles of *Klebsiella* and *Proteus* species revealed significant patterns of MDR, the highest resistance was observed against amoxicillin (100%, 96.8% for *Proteus* spp. and *Klebsiella* spp., respectively). Ticarcillin, piperacillin and ticarcillin + clavulanic acid showed a high rate of resistance ranged between 60.7% to 67.9%. The amoxicillin + clavulanic acid noticed a significant level of resistance ($p<0.001$) in *Proteus* species (85.7%). Piperacillin + tazobactam represented the most effective β-lactam/inhibitor antibiotic among these two species, a moderate to high level of resistance exhibited by the previous species against cephalosporins with cefotaxime being the most effective one (28.6% for *Proteus* spp. and 19.3% for *Klebsiella* spp.) (Fig. 37).

For carbapenem, *Proteus* species have a significate rate of resistance 32.1%, while for *Klebsiella* spp. it showed a good efficacity 16.1%. Aminoglycosides improved the best performance for these two species, amikacin was the best aminoglycoside for *Proteus* spp. (3.6%).

While for *Klebsiella* spp., gentamicin and tobramycin were the best (9.7% for both). *Klebsiella* spp. showed a high sensitivity for chloramphenicol (12.9%); while trimethoprim/sulfamethoxazole showed a low efficacity for *Proteus* spp. (67.9%) and inversely for *Klebsiella* spp. (32.2%).

Klebsiella and *Proteus* isolates exhibited substantial resistance for nitrofurantoin and colistin, fosfomycin showed a lower resistance (17.9%) in *Proteus* spp. comparatively with *Klebsiella* spp. which showed a high resistance (45.2%) (Fig. 37).

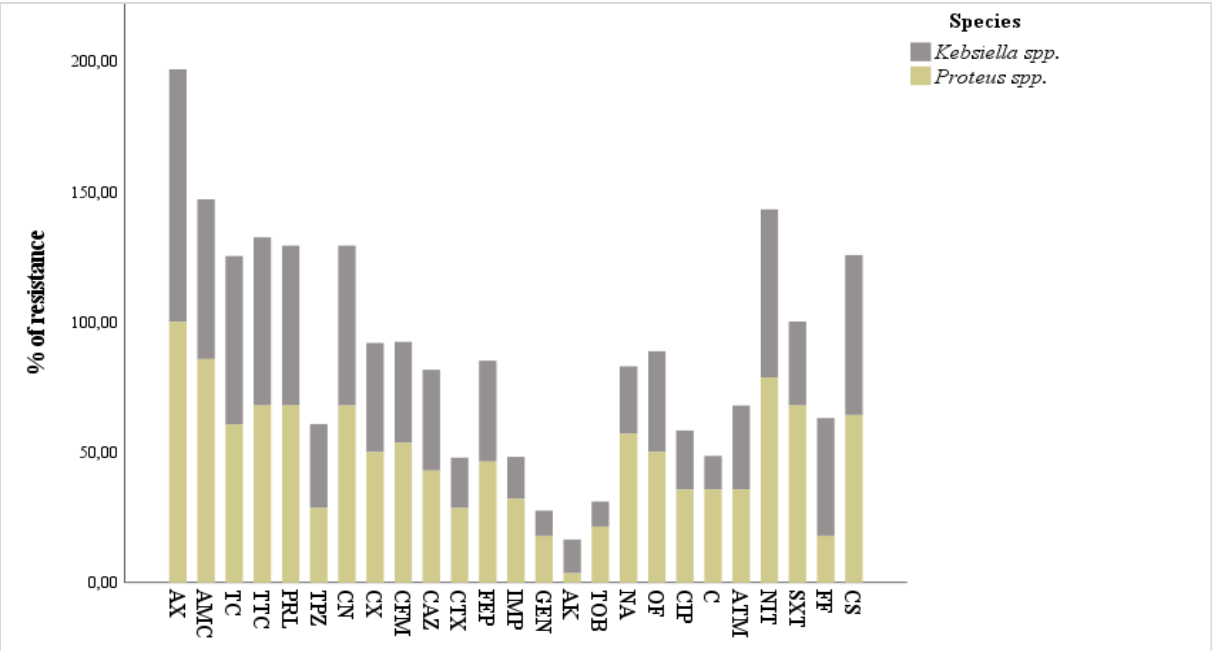


Figure. 37 : Resistance rates of *Klebsiella* and *Proteus* species.

Proteus spp. and *Klebsiella* spp. have showed a strong resistance to several antibiotics, especially to amoxicillin, similar resistance rates have been reported in several studies: in Ethiopia (Gebremeskel et al., 2023) and in Nigeria (Osei Sekyere and Reta, 2020).

A high rate was also showed for ticarcillin, piperacillin and the combination ticarcillin + clavulanic acid, similar rates were found in Saudi Arabia, Iraq and Iran (Al-Zalabani et al., 2020; Thabit et al., 2020; Vaez et al., 2022). A significant resistance level to amoxicillin/clavulanic acid (75%) was observed in Thabit *et al.*, (2020) study among *Proteus* species which in accordance with our finding (85.7%). These high resistance rates could be explained by the overuse of this class of antibiotics, which is considered the safest class for the majority of bacterial infections.

Piperacillin + tazobactam showed a good efficacity among β-lactam/inhibitor family for both species, this finding is similar to a study in Iraq (3.33%) for *Klebsiella* spp. (Hussein et al., 2024) and (13.3%) for *Proteus* spp. in Namibia (Haindongo et al., 2022).

Proteus isolates showed a significant rate of resistance to imipenem similarly to that of 35.3% reported in Italy (Facciolà et al., 2022), contrary *Klebsiella* spp. showed a good sensitivity, similar to that of 13.2%-20% reported in Iraq (Jalil and Al Atbee, 2022; Jwair et al., 2023). The resistance rate to trimethoprim/sulfamethoxazole among *Klebsiella* species was 36.84% in Spain and 36.89% in Bangladesh (Ballén et al., 2021; Salam et al., 2024) which aligns closely with our results.

Significant resistance was observed for nitrofurantoin and colistin, consistent with a rate of 85% for nitrofurantoin in *Klebsiella* spp. in Nigeria, 67.6% and 83.3% for *klebsiella* and *Proteus* species, respectively, in Saudi Arabia (Al-Zalabani et al., 2020; Osei Sekyere and Reta, 2020). In contrast, fosfomycin demonstrated lower resistance, with a rate of 17.9% in *Klebsiella* spp., comparable to the 24.56% reported in Iraq (Hussein et al., 2024).

Regarding aminoglycosides, resistance to amikacin is due to cell wall permeability, it showed a high efficacity for *Proteus* spp. in several studies, (2.6%) in China (Mo et al., 2022) and (2.5%) in Indonesia (Suhartono et al., 2022) which is align with our results. While gentamicin and tobramycin had the best performance against *Klebsiella* spp. in our study which confirmed in Iran and Saudi Arabia (Jafari-Sales, 2020; Alsubaie et al., 2023).

4.3.3.4 P. aeruginosa

Among *P. aeruginosa* isolates, the resistance rates varied significantly across the tested antibiotics. Cefalexin showed a higher resistance rate of 92% ($p<0.001$), followed by nitrofurantoin of 88%, cefoxitin and amoxicillin (84% for both). 80% of *P. aeruginosa* isolates showed resistance to amoxicillin + clavulanic acid and trimethoprim/sulfamethoxazole.

A considerable resistance was showed also for gentamicin (76%) and chloramphenicol (60%), a moderate to higher resistance was showed against quinolones (40%-48%). Among cephalosporins, cefotaxime showed a high efficacity (19%), similar to imipenem and amikacin, with only 12% of isolates exhibited resistance (Fig. 38).

The findings of our study provide critical insights into the AR patterns of *P. aeruginosa*, substantial resistance rates were demonstrated (92.9%) against cefalexin in Iran (Gharavi et al., 2021) with was in accordance with our results. The high rate presented for nitrofurantoin, is consistent with a study in Nigeria with a rate of 92% among 39 clinical *P. aeruginosa* (Ugwuanyi et al., 2021). A high level of resistance was found among *P. aeruginosa* isolates for cefoxitin and nitrofurantoin in Saudi Arabia (Ahmad et al., 2020).

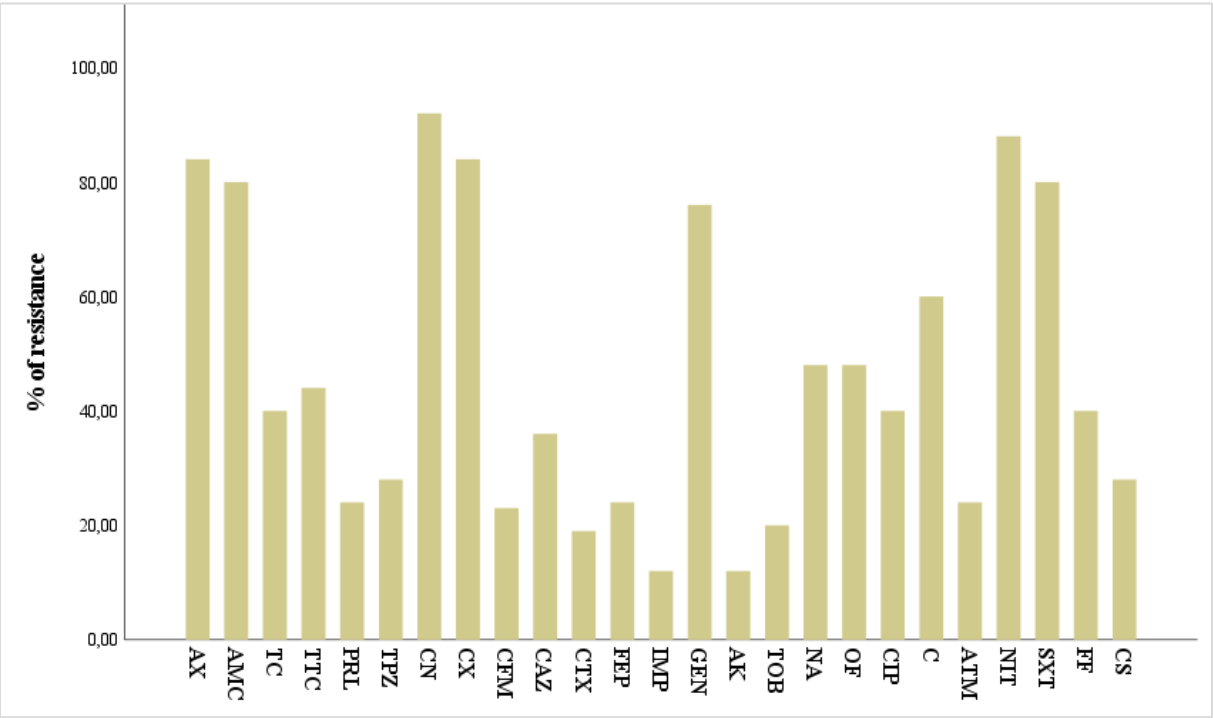


Figure. 38 : Resistance rates of *P. aeruginosa*.

A significant level of resistance was observed in 110 *P. aeruginosa* in Nigeria for amoxicillin and cefoxitin (Akingbade et al., 2012) which accord with our study, the reason for higher resistance to β-lactams and cephalosporins could be attributed to the non-permeability of these antibiotics through the plasma membrane (Alaboudi and Aljwaid, 2024). Between all cephalosporins tested, cefotaxime showed a good efficacity, same results (15.4%) were reported in Brazil (Ribeiro et al., 2020).

A rate ranged between 74.1% to 84.2% was observed during five years of study on 729 *P. aeruginosa* for amoxicillin + clavulanic acid in Ethiopia (Araya et al., 2023) and (89% and 75.41%) for trimethoprim/sulfamethoxazole in Somalia and Ethiopia, respectively (Mohamed et al., 2022; Asmare et al., 2024) which is similar to our findings.

P. aeruginosa showed a lower sensitivity to gentamicin (76%), similarly to those reported in Iran (64.7%) (Zahedi Bialvaei et al., 2021) and in Ethiopia (62.2%) (Tsigereda Asamenew et al., 2023), a lower sensitivity was also showed against chloramphenicol (60%), a concordance results was reported in Chad (69%) (Ahmat et al., 2023).

Our work showed that imipenem and amikacin exhibited the best performance on *P. aeruginosa* among all tested antibiotics; likewise, other studies confirm our results (Ullah et al., 2019; Roulová et al., 2022; Araya et al., 2023). The resistance to amikacin could be associated with the production of the AMEs (Khabipova et al., 2022), regarding the resistance to imipenem, The European Centre for Disease Prevention and Control's annual report indicates that 18.7% of *P. aeruginosa* isolates were resistant to carbapenems (ECDC, 2020).

Alongside the natural antibiotic resistance in *P. aeruginosa*, the misuse and overuse of antibiotics have resulted in the emergence of MDR. Moreover, this bacterium has developed resistance to antibiotics via the HTG of resistance genes from resistant strains and other bacterial species (Thuo et al., 2019).

4.3.3.5 Staphylococcus species

The AR profile of the frequently isolated GPB showed a high rate of resistance ($p<0.001$) to oxacillin (89.7%), erythromycin (82.1%), clindamycin and cefixime (76.9%), ceftazidime and kanamycin (74.3%), cefalexin and cefepime (71.8%), cefoxitin and aztreonam (69.2%), followed by nalidixic acid and doxycycline (56.4%), nitrofurantoin (51.3%) and 48.7% for trimethoprim/sulfamethoxazole. *Staphylococcus* spp. showed a notable resistance for fosfomycin (41%) and levofloxacin (35.9%), while a lower resistance was showed in amikacin and chloramphenicol (28.3%) and 17.9% for imipenem (Fig. 39).

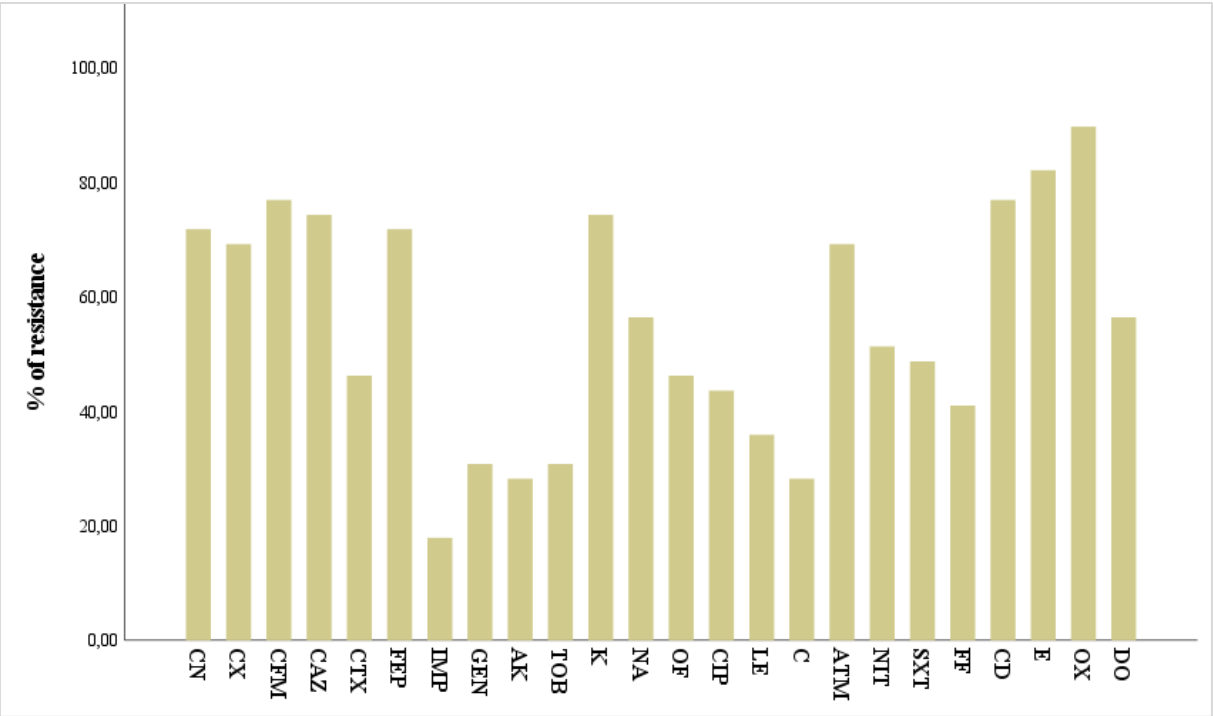


Figure. 39 : Resistance rates of *Staphylococcus* species.

This study underscores the growing prevalence of AR in *Staphylococcus* species, same rates were found in Vietnam for erythromycin (82.82%) and clindamycin (82.32%) (An et al., 2024). Likewise, 75.6% for erythromycin in China (Zhang et al., 2022). A high rate of resistance was found for cefoxitin (75%) in Nepal (Gurung et al., 2020), clindamycin (87.5%) and erythromycin (81.9%) and cefoxitin (61.7%) in Italy (Petrillo et al., 2021). Similarly, *Staphylococcus* species isolated in Egypt showed a higher levels including: erythromycin (83.98%), cefoxitin (76.52%) and clindamycin (52.1% - 60.2%) (Fahim, 2021) which align with our findings.

Accordance rates of resistance were reported for oxacillin (74.2%) and trimethoprim/sulfamethoxazole (58.1%) in Italy (Folliero et al., 2021), the same rate also was found in Bangladesh for trimethoprim/sulfamethoxazole (53.33%) (Majumder et al., 2022), and in Egypt for nitrofurantoin (40%) and doxycycline (43%) (Fahim, 2021), (57.1%) for doxycycline in Ethiopia (Birru et al., 2021).

Our *Staphylococcus* spp. showed a lower resistance to amikacin, chloramphenicol and imipenem, in agreement with 25% reported to chloramphenicol in Iran (Hashemzadeh et al., 2021) and with 90.6% were resistant for oxacillin in Peru (Suaréz-Del-Aguila et al., 2020) and also with 98% of sensitivity for imipenem reported in Kenya (Gitau et al., 2018), a high

sensitivity was showed also to amikacin for urinary *Staphylococcus* spp. in Iraq (Mohamed, 2023).

The variations in antibiotic susceptibility patterns inside all tested species might be due to the frequent and varied use of these antimicrobials in treating bacterial infections in different geographical areas.

Additionally, the increase in AR among different bacterial types may be attributed to their misuse, storage, purchase without a physician's prescription, and the inappropriate prescription of these antibiotics for treating every infection. All resistance rates of all isolated species are summarized in table 15.

Table 15. Antibiotic resistance among species.

	***Enterobacter* spp.**	***E. coli***	***Proteus* spp.**	***Providencia* spp.**	***Citrobacter* spp.**	***P. aeruginosa***	***M. morganii***	***Klebsiella* spp.**	***S. marcescens***	***Acinetobacter* spp.**	***Streptococcus* spp.**	***Enterococcus* spp.**	***Staphylococcus* spp.**
AX	88.9	82.6	100	81.8	92.9	84	66.7	96.8	66.7	50	40	75	NT
AMC	75	68.2	85.7	72.7	71.4	80	66.7	61.3	66.7	50	20	75	NT
TC	36.1	51.7	60.7	63.6	42.9	40	33.3	64.5	33.3	50	0	NT	NT
TTC	83.3	72.1	67.9	63.6	50	44	66.7	64.5	33.3	50	20	75	NT
PRL	80.6	77.6	67.9	81.8	50	24	66.7	61.3	0	50	20	75	NT
TPZ	47.2	32.8	28.6	18.2	35.7	28	0	32.2	0	0	NT	NT	NT
CN	58.3	37.8	67.9	54.5	50	92	66.7	61.3	66.7	50	60	75	71.8
FOX	50	30.3	50	27.2	35.7	84	33.3	41.9	66.7	50	100	100	69.2
CFM	61.1	37.8	53.6	45.4	42.9	23	66.7	38.7	33.3	100	100	100	76.9
CAZ	61.1	39.3	42.9	36.3	50	36	33.3	38.7	0	100	40	75	74.3
CTX	52.7	26.9	28.6	36.3	35.7	19	0	19.3	0	50	80	25	46.2
FEP	55.6	36.3	46.4	36.3	42.9	24	0	38.7	66.7	50	40	50	71.8
IMP	19.4	49.8	32.1	18.2	14.3	12	33.3	16.1	0	0	0	0	17.9
GEN	38.9	17.4	17.9	9.1	7.1	76	0	9.7	33.3	50	60	100	30.8
AK	8.3	13.9	3.6	9.1	7.1	12	0	12.9	0	0	20	25	28.2
TOB	36.1	18.9	21.4	0	17.6	20	33.3	9.7	0	50	60	75	30.8
K	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	80	100	74.3
NA	50	34.3	57.1	36.3	28.6	48	0	25.8	0	100	60	25	56.4
OF	55.6	39.8	50	36.3	50	48	66.7	38.7	0	50	40	25	46.2
CIP	58.3	33.8	35.7	36.3	35.7	40	66.7	22.6	0	50	60	25	43.6
LE	NT	NT	NT	NT	NT	NT	NT	NT	NT	100	80	50	35.9
C	47.2	13.4	35.7	18.2	28.6	60	0	12.9	0	50	40	0	28.2
ATM	47.2	29.3	35.7	18.2	50	24	33.3	32.2	0	100	60	25	69.2
NIT	55.6	35.8	78.6	27.2	57.1	88	33.3	64.5	100	100	60	75	51.3
SXT	69.4	51.2	67.9	36.3	50	80	66.7	32.2	0	50	60	75	48.7
FF	52.8	40.3	17.9	36.4	57.1	40	100	45.2	66.7	0	40	100	41
CS	79.5	55.2	64.3	45.5	57.1	28	66.7	61.3	33.3	50	40	50	NT
CD	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	100	75	76.9
E	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	80	100	82.1
OX	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	80	100	89.7
DO	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	80	75	56.4

NT: non tested.

4.3.4 MIC concentration

The secondary analysis assessed the resistance prevalence using the MIC breakpoint values published in EUCAST recommendations, most sensitive tested strains showed their capacity to tolerated the critical concentration; excepted for the following antibiotics: ceftazidime (p<0.001), cefotaxime, imipenem, tobramycin (p<0.001), nalidixic acid (p<0.001) and aztreonam (p<0.001) (Fig. 40); for imipenem all isolates were susceptible at the recommended breakpoint, while the previous antibiotics showed good efficacity at the EUCAST critical breakpoints.

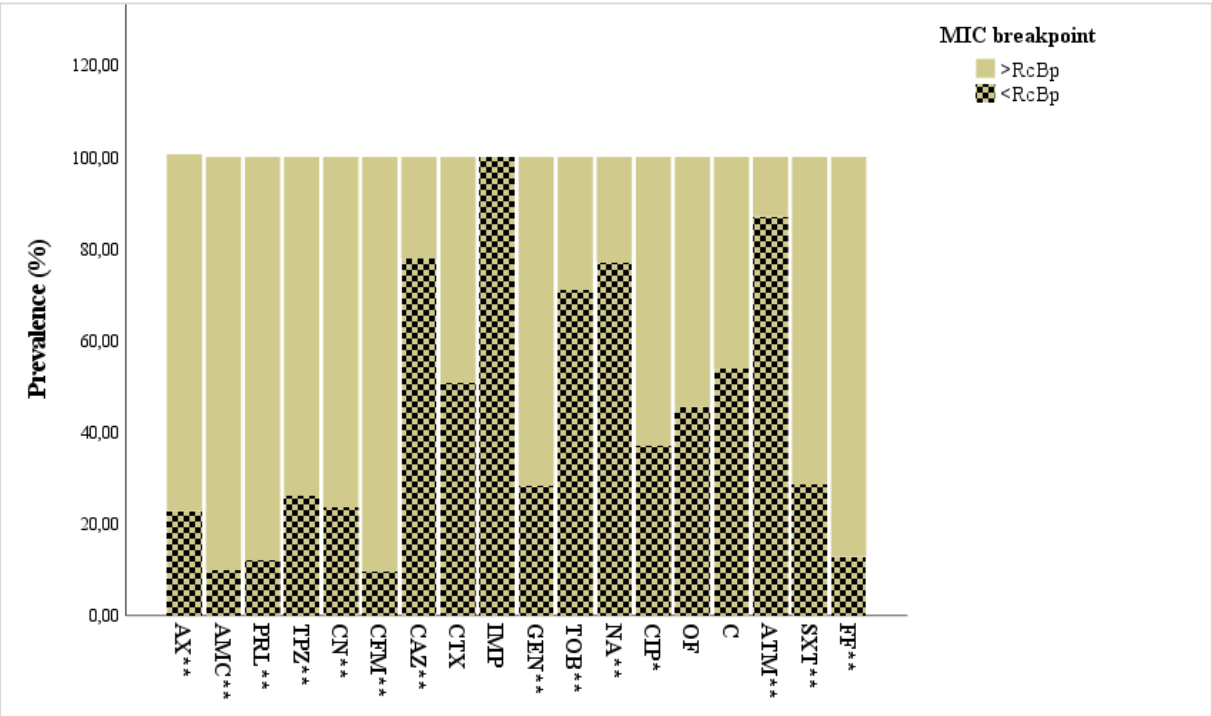


Figure. 40 : Minimum inhibitory concentration (MIC) for the most important antibiotics of isolates (RcBp: resistant clinical breakpoint, *: statistical significance p<0.05, **: p<0.001).

4.4 Phenotypic resistance

4.4.1 Antibiotic resistance profiles

The data presented in the chart highlights trends in the percentages of R, MDR, and XDR across the isolation years. During the three years of isolation MDR strains recorded in higher percent compared with R and XDR (p<0.001). In the R category, there is a fluctuating pattern with a decline from 22% in 2020 to 12.5% in 2021, followed by a slight increase to 17% in 2022.

In contrast, the MDR category showed a consistent decrease over the three years, dropping from 66% in 2020 to 62.5% in 2021 and further to 51.5% in 2022. On the other hand, the XDR category exhibited a significant and consistent upward trend (p=0.011), rising from 12% in 2020 to 25% in 2021, and further to 31.5% in 2022 (Fig. 41),

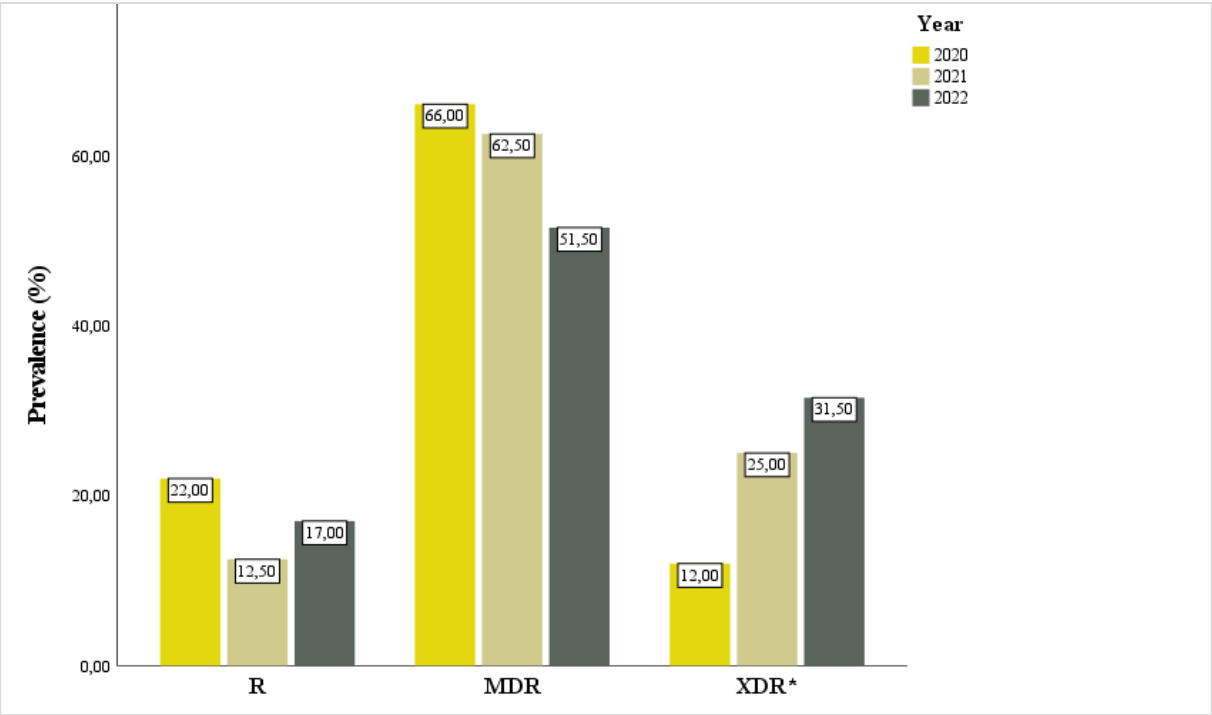


Figure. 41 : Trends in R, MDR, and XDR percentages from 2020 to 2022 (*: statistical significance p<0.05).

The observed trends in the chart provide critical insights into the resistance profile over the three-year period from 2020 to 2022. The fluctuating pattern in R percentages, with a decline in 2021 followed by a slight increase in 2022, may suggest variable efficacy in preventive measures during COVID-19 period.

Meanwhile, the higher percentages in MDR during the same period, could be explained by the widespread use of broad-spectrum antibiotics in COVID-19 treatment (Bentivegna et al., 2021). While the decline percentages in 2022 and 2023 could represent a proof for the impact of improved management strategies. However, the increasing trend in XDR percentages is particularly concerning, as it indicates the emergence and spread of highly resistant strains particularly following the COVID-19 pandemic, which is in accordance with other study (Lagadinou et al., 2024).

MDR strains represented 56% of all tested isolates, followed by XDR (26.1%) and R (17.9%). The analysis of gender distribution across these resistance profiles revealed significant differences; among individuals with R profile, males accounted for 23.6%, while females

represented a significantly larger proportion at 76.4%. In the MDR group, males constituted 32.9%, whereas females were 67.1%. For XDR, the male proportion increased to 42.9%, with females making up the remaining 57.1%. There is a strong correlation between resistance profile and gender distribution (p=0.007) (Tab. 16).

The distribution of phenotypic resistance categories across age groups revealed that adult represent the dominant group across all categories (Tab. 16). Regarding the nature of sample, all the resistance profiles were dominant in urine samples, while in pus samples there is an increasing trend of resistance profile from R to XDR. A minimal proportion of MDR and XDR recorded in vaginal isolates. Medical devices and blood samples showed a rising proportion of XDR (Tab. 16). *E. coli* demonstrated particularly high frequencies in the R (51.4%), MDR (56.4%) and XDR (35.2%) profiles, *P. aeruginosa* and *Klebsiella* spp. showed a high proportion in MDR isolates. Rare species like *Providencia* spp., *M. morganii*, and *S. marcescens* exhibit lower frequencies but demonstrate resistance trends. *Staphylococcus* spp. exhibited a strong presence in XDR (24.8%) followed by *Enterobacter* spp. (13.3%) (Tab. 16).

Table 16. Demographical and species distribution of R, MDR and XDR.

		Resistance profile n (%)			Correlation (p value)
		R (72; 17.9)	**MDR (225; 56)**	**XDR (105; 26.1)**	
Gender	**Male**	17 (23.6)	74 (32.9)	45 (42.9)	0.007
	Female	**55 (76.4) ***	**151 (67.1) ***	60 (57.1)	
Age groups	**Children**	15 (20.8)	43 (19.1)	11 (10.5)	0.035
	Adult	**50 (69.4) ***	**147 (65.3) ***	**76 (72.4) ***	
	Elderly	7 (9.7)	35 (15.6)	18 (17.1)	
Nature of sample	**Urine**	**67 (93) ***	**199 (88.4) ***	**70 (66.7) ***	<0.001
	Pus	4 (5.6)	17 (7.6)	21 (20)	
	Vaginal swab	0	4 (1.8)	4 (3.8)	
	Medical devices	1 (1.4)	4 (1.8)	8 (7.6)	
	Blood	0	1 (0.4)	2 (1.9)	
Species	***Enterobacter* spp.**	7 (9.6)	15 (6.8)	14 (13.3)	<0.001
	E. coli	**37 (51.4) ***	**127 (56.4) ***	37 (35.2)	
	***Proteus* spp.**	5 (6.9)	14 (6.2)	9 (8.6)	
	***Providencia* spp.**	4 (5.6)	6 (2.7)	1 (1)	
	***Citrobacter* spp.**	4 (5.6)	7 (3.1)	3 (2.9)	
	P. aeruginosa	1 (1.4)	20 (8.9)	4 (3.8)	
	M. morganii	0	2 (0.9)	1 (1)	
	***Klebsiella* spp.**	9 (12.5)	19 (8.4)	3 (2.9)	
	S. marcescens	2 (2.8)	1 (0.4)	0	
	***Acinetobacter* spp.**	1 (1.4)	0	1 (1)	
	***Streptococcus* spp.**	0	1 (0.4)	4 (3.8)	
	***Enterococcus* spp.**	0	2 (0.9)	2 (1.9)	
	***Staphylococcus* spp.**	2 (2.8)	11 (4.9)	26 (24.8)	

*: statistical significance p<0.001, R: strains that resist less than three classes of antibiotics, MDR: strains that resist three or more classes of antibiotics, XDR: strains susceptible to one or fwer classes of antibiotics.

The results of this study determine a strong association between distribution of phenotypic resistance and gender and age (most of R, MDR and XDR being found in females and adult group) which is in accordance with a study conducted in Nepal (Sah et al., 2023). Gender and age among the risk factors that influence the distribution of resistance strains (Dias et al., 2022).

Urine samples were the most frequent source of resistant isolates across all profiles. This dominance was in accordance with other results reported in Nepal (Shilpakar et al., 2021; Rajbhandari et al., 2024) and in Congo (Irenge et al., 2024), various factors contribute to this prevalence including: the high incidence of UTIs and the frequent use of antibiotics for their treatment.

E. coli emerges as the most dominant species across all resistance categories, particularly in MDR and XDR profiles. This finding aligns with studies conducted in Bangladesh(Runa et al., 2019), Nepal (Sah et al., 2023; Rajbhandari et al., 2024) and a cross study conducted in east Africa: Kenya, Tanzania, Uganda (Maldonado-Barragán et al., 2024), that identifying *E. coli* as a leading MDR pathogen. Similarly, *Klebsiella* spp. demonstrates significant representation in MDR profiles in Ethiopia (Hailemariam et al., 2021) which is consistent with our findings.

Staphylococcus spp., predominantly contributing to the XDR category, similar findings were reported in Nepal (Sah et al., 2023) and Gabon (Mouanga-Ndzime et al., 2023), that highlighting *Staphylococcus* species including: MRSA as a global concern due to its resistance to multiple antibiotic classes.

4.4.2 Phenotypic detection of carbapenemases

Concerning phenotypic detection of carbapenem resistance, 33.8% (136/402) of positive screened isolates (that showed decreasing sensitivity to carbapenem by disc diffusion) were selected to confirmatory test (MHT). 86% were MHT positive, all positive MHT strains were screened for combined disk test (CDT), the results record 37.6% were positive MBL strains confirmed by CDT, where the most detected positive MBL organism was *E. coli* (56.8%), followed by *P. mirabilis* (11.4%), *K. pneumoniae* and *P. aeruginosa* (9.1%) (Tab. 17).

Table 17. Phenotypic resistance of isolates.

Phenotype resistance	**n (%)**
Positive MHT	117 (86)
Negative MHT	19 (14)
Positive MBL	44 (37.6)
Negative MBL	73 (62.4)

MHT: modified Hodge test, MBL: Metallo-β-lactamase.

These finding were correlated with other studies that reported a high rate of MHT-positive strains: 100% in Algeria (Bourafa et al., 2018), 75% in Ghana and Bangladesh (Dwomoh et al., 2022; Munny et al., 2023), 71.4% in Sri Lanka (Jayathilaka et al., 2024) and 79.72% in Nepal (Hamal et al., 2023). For MBL positive strains, similar rate was found in Bangladesh (Aminul et al., 2021).

While phenotypic methods like MHT and CDT provide valuable insights, they require integration with molecular tools for comprehensive resistance profiling and to address the limitations in detecting specific resistance mechanisms or non-MBL carbapenemase types.

4.4.3 Phenotypic detection of hvKp, MRSA and MLSB resistant strains

Hypervirulent *K. pneumoniae* (hvKp) is an emerging pathotype that is more virulent than classical *K. pneumoniae*. Out of 3 strains of *K. pneumoniae* tested in our study, 66.7% (2/3) were hvKp, these two strains were originated from both community and hospitalized patients, and presented a significant resistance profile with one was MDR and the other was XDR, both were found in females with UTI (Tab. 18).

From thirty-one *S. aureus*, (61.3%) were reported as MRSA strains, while 38.7% were identified as methicillin-sensitive (MSSA) strains, MRSA strains were distributed in 57.9% XDR and 36.8% MDR.

The phenotypic identification of macrolide lincosamide streptogramin B (MLSB) of *S. aureus* in our study showed that among 45.2% of erythromycin resistant *S. aureus* (MSB), 9.7% was constitutive macrolide lincosamide streptogramin B (CMLSB) and inducible macrolide lincosamide streptogramin B (IMLSB) represent 16.1%. 6.5% of isolates resistant to clindamycin only and 25.8% were susceptible to both; among MRSA strains 6.5% were CMLSB while 12.9% were IMLSB (Tab. 18).

Table 18. Phenotypic resistance of isolates.

Phenotype resistance	**n (%)**
hvKp	2 (66.7)
MRSA	19 (61.3)
MSSA	12 (38.7)
CMLSB	3 (9.7)
IMLSB	5 (16.1)
MSB	14 (41.9)
L	2 (6.5)
S	8 (25.8)

hvKp: hypervirulent *K. pneumoniae*, MRSA: Methicillin Resistant *S. aureus*, MSSA: Methicillin Sensitive *S. aureus*, CMLSB: Constitutive Macrolide Lincosamides Streptogramin B Resistant *S. aureus*, IMLSB: Inducible Macrolide Lincosamides Streptogramin B Resistant *S. aureus*, MSB: *S. aureus* resistant to erythromycin and susceptible to clindamycin with negative D test, L: *S. aureus* resistant to clindamycin and susceptible to erythromycin, S: *S. aureus* susceptible to clindamycin and erythromycin.

The frequency of hvKp in our study was consistent with 62.5% found in Egypt (Mohammed et al., 2024) and 51% in Iraq (Jassim et al., 2023), these hvKp exhibited a high resistance profile. hvKp harbors plasmids containing genes that encode numerous virulence factors, including a protective capsule, fimbriae, lipopolysaccharides and siderophores, as well as resistance to heavy metals and antibiotics (Loaiza et al., 2023; Dingiswayo et al., 2024).

The study revealed a high prevalence of MRSA among *S. aureus* isolates, with 57.9% of MRSA strains classified as XDR. These findings are consistent with reports from Egypt, where MRSA accounted for 55.3% of *S. aureus* isolates, while MSSA represented 44.7% (Ajlan et al., 2022). Similarly, another study in Egypt documented an MRSA prevalence of 65.2%, and data from Nigeria reported MRSA and MSSA rates of 67.46% and 32.3%, respectively (Yahaya et al., 2022). However, our results are significantly higher than those reported from Tanzania, where MRSA prevalence was documented at 49.3% (Juma et al., 2024). These disparities likely reflect variations in antibiotic stewardship programs and infection control practices across different regions.

The extensive use of MLSB antibiotics in *Staphylococcus* infections lead to the emergence of *S. aureus* resistant to MLSB, the same rate of MSB resistance (45.2%) was reported in Egypt (Kishk et al., 2020) and the predominance of IMLSB in our study aligns with findings from similar studies: India (Modukuru et al., 2021), Nigeria (Yahaya et al., 2022) and in Nepal (Gurung et al., 2020), which call to a higher level of attention in the prescription of macrolides. Lower prevalence of CMLSB *S. aureus* reported in our study can be attributed to the rational

prescription and usage of macrolides, both within community and hospital settings which has not favored the predominance of hyper-resistant strains and molecular types.

4.5 Biofilm formation ability

Biofilm formation assays were carried out in a microtiter plate, using CV staining. The results indicated that the majority of tested strains were biofilm producers (88.1%, $p<0.001$) while non producers represented a low frequency (11.9%). Of 88.1% positive biofilm producers, 64.9% were weak producers, 17.9% have moderate production and only 5.2% formed biofilm strongly.

The distribution of biofilm producers across age groups revealed that no significant difference was observed between the OD mean in the three categories, but the weak production profile was the predominant profile in all groups (Fig. 42).

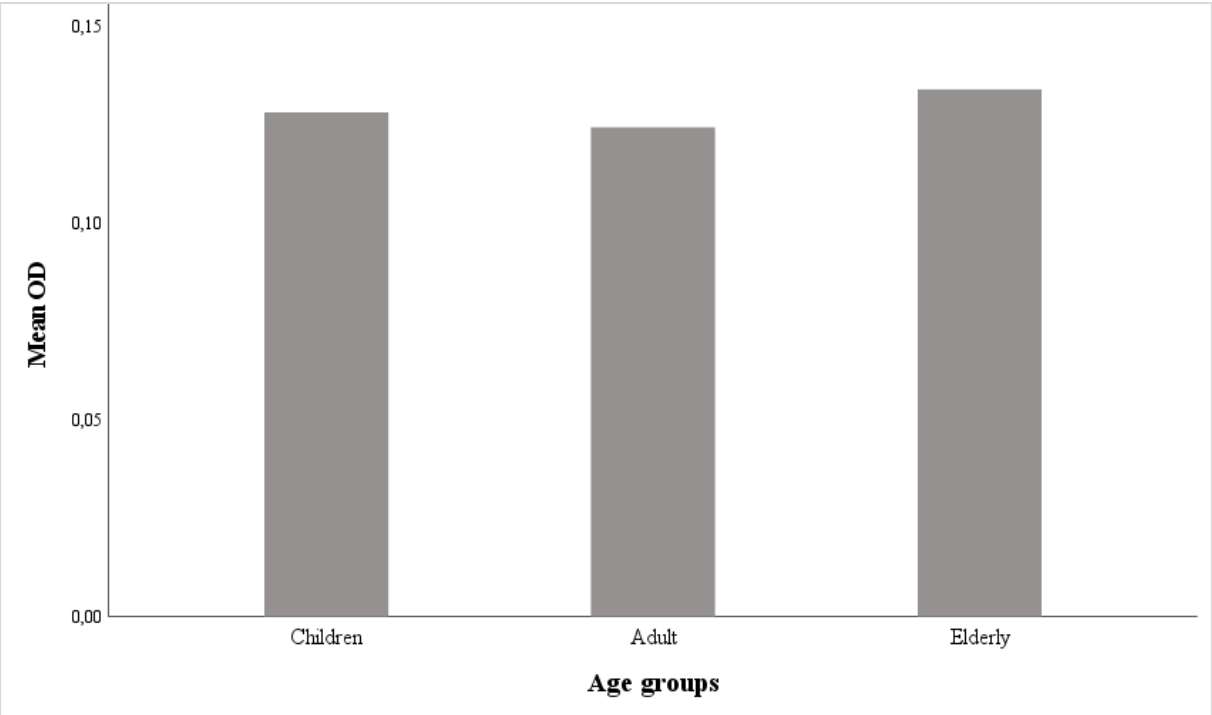


Figure. 42 : Difference in OD mean among age groups.

These results are consistent with other studies reporting high biofilm formation capabilities among clinical isolates. For instance, Asaad *et al.*, (2021) observed biofilm production in 70.1% of their tested strains. Similarly, Nirwati *et al.*, (2019) reported biofilm formation in approximately 85.6% of isolates and Syaiful *et al.*, (2023) observed 87.5% of biofilm producers, Namuq *et al.*, (2019) suggested a percentage of 98% of biofilm forming strains. The high

prevalence of biofilm production in clinical samples could be explained by different factors including: the exchange of genetic materials and virulence genes, the use of disinfectants and antibiotics in hospital and community settings, which creates selective pressure, allowing biofilm-producing strains to thrive and outcompete non-biofilm producers.

However, some studies showed variability in biofilm formation rates. For example, Kulayta *et al.*, (2024) found a lower percentage (62.2%) of biofilm producers when testing isolates from wounds, the observed variations in prevalence could be influenced by the non-uniformity in the sample size, study period, demographic characteristics of study participants, region, applied methodology.

Our data indicated a significant (p<0.001) portion of the population exhibited low production capabilities, similar to that reported in Haghighifar *et al.*, (2021) study, which found a predominance of weak producers and to what reported in Shadkam *et al.*, (2021) study.

4.5.1 Biofilm production among specimens

Among different clinical specimens, there are significant difference in frequency distribution (p=0.023), with the most biofilm producers were found in urine (293/402), but by mean OD comparison, no difference was found between the specimens (Fig. 43).

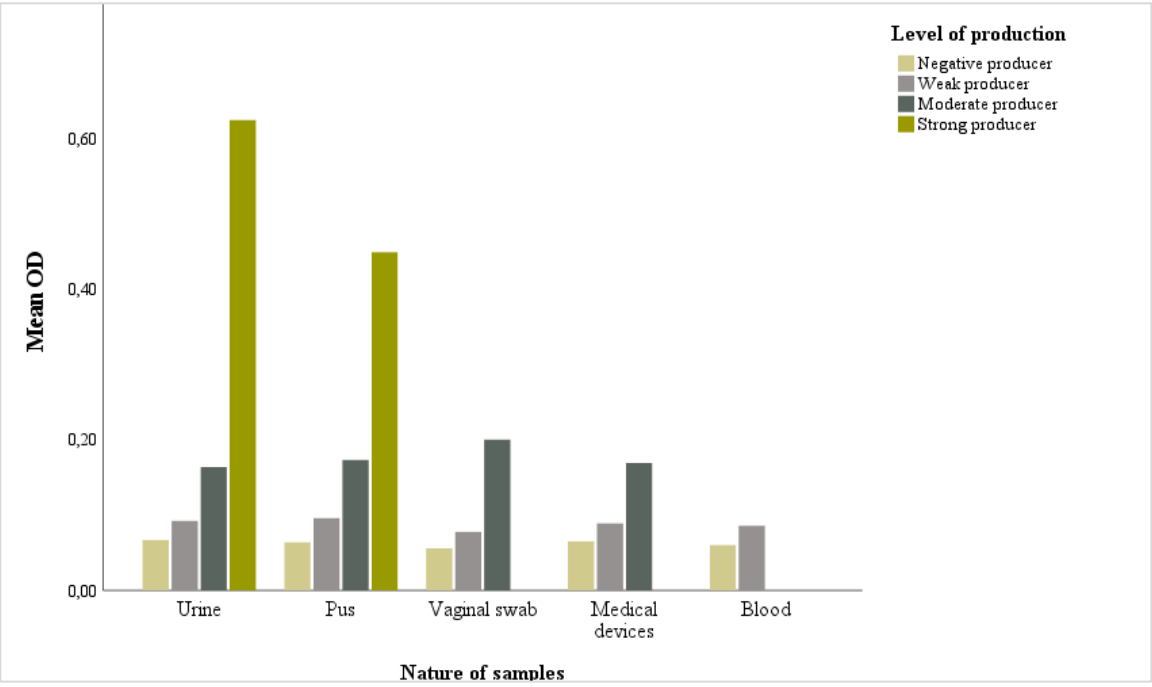


Figure. 43 : Difference in OD mean among specimens.

This comparison confirmed that the weakly production is the predominant profile in all types of samples 61.9%, 7.3%, 1.7%, 2.3% and 0.6% in urine, pus, vaginal swab, medical devices and blood, respectively. The most elevated rate of moderate production was found in urine, pus and medical devices while strong production was found only in urine and pus specimen types (Tab. 19).

Table 19. Biofilm formation in different specimens.

Sample type	Biofilm formation strength			
	No production (%)	**Weak (%)**	**Moderate (%)**	**Strong (%)**
Urine	43 (89.6)	219 (61.9)	61 (17.2)	13 (3.7)
Pus	2 (4.1)	26 (7.3)	6 (1.7)	8 (2.2)
Vaginal swab	1 (2.1)	6 (1.7)	1 (0.3)	0
Medical devices	1 (2.1)	8 (2.3)	4 (1.1)	0
Blood	1 (2.1)	2 (0.6)	0	0

The urine samples exhibited the highest proportion of biofilm producers, because the majority of our strains were originated from urine samples, these results indicating the strong association of biofilm formation with UTIs, this aligns with findings from Ethiopia (Gebremariam et al., 2024), who reported a similar dominance of biofilm producers in urine specimens.

The predominance of weak biofilm production across all sample types underscores a consistent trend observed in other studies, such as, Karimi *et al.*, (2021) who noted that weak producers dominate clinical isolates.

The moderate biofilm production rates were elevated in urine, pus, and medical devices. This observation aligns with findings of Ashwath *et al.*, (2022), who also found moderate and strong production rates to be associated with wound infections, and with Folliero *et al.*, (2021), who found a high rate of moderate producers isolates in medical devices pathogens, potentially due to higher selective pressures in these environments.

4.5.2 Biofilm production among species

The biofilm formation ability across bacterial species showed critical results, in GNB, *E. cloacae* showed the high rates of strong biofilm production (20%), followed by *P. mirabilis* (13%), *C. diversus* (12.5%), (12%) *Klebsiella* species and (10%) for *Enterobacter* sp. *Streptococcus* sp. showed the highest level of strong biofilm formation among GPB (Fig. 44).

Moderate biofilm production was observed in high proportions in *Providencia* sp. (100%), *K. oxytoca* (66.7%) and (50%) for *C. diversus* and *Acinetobacter* sp. For GPB, *Enterococcus* sp. recorded the high proportion (33.3%).

A predominant number of bacterial species exhibit weak biofilm production, total species (100%) of *P. stuartii*, *C. freundii* and *Staphylococcus* sp. were weak biofilm producers, a prevalence rate of 80% was observed in *Enterobacter* sp. while a range between 60% to 70% was showed for *E. aerogenes*, *E. cloacae*, *P. mirabilis*, *P. vulgaris*, *E. coli*, *P. aeruginosa*, *Klebsiella* sp., *M. morganii*, *Serratia marcescens*, *Streptococcus* sp., *Enterococcus* sp., *S. aureus* and CoNS (Fig. 44).

Some species demonstrated their inability to produce biofilm, among these: *P. aeruginosa* (36%), *K. pneumonia* (33.3%) and *P. alcalifaciens* (22.2%).

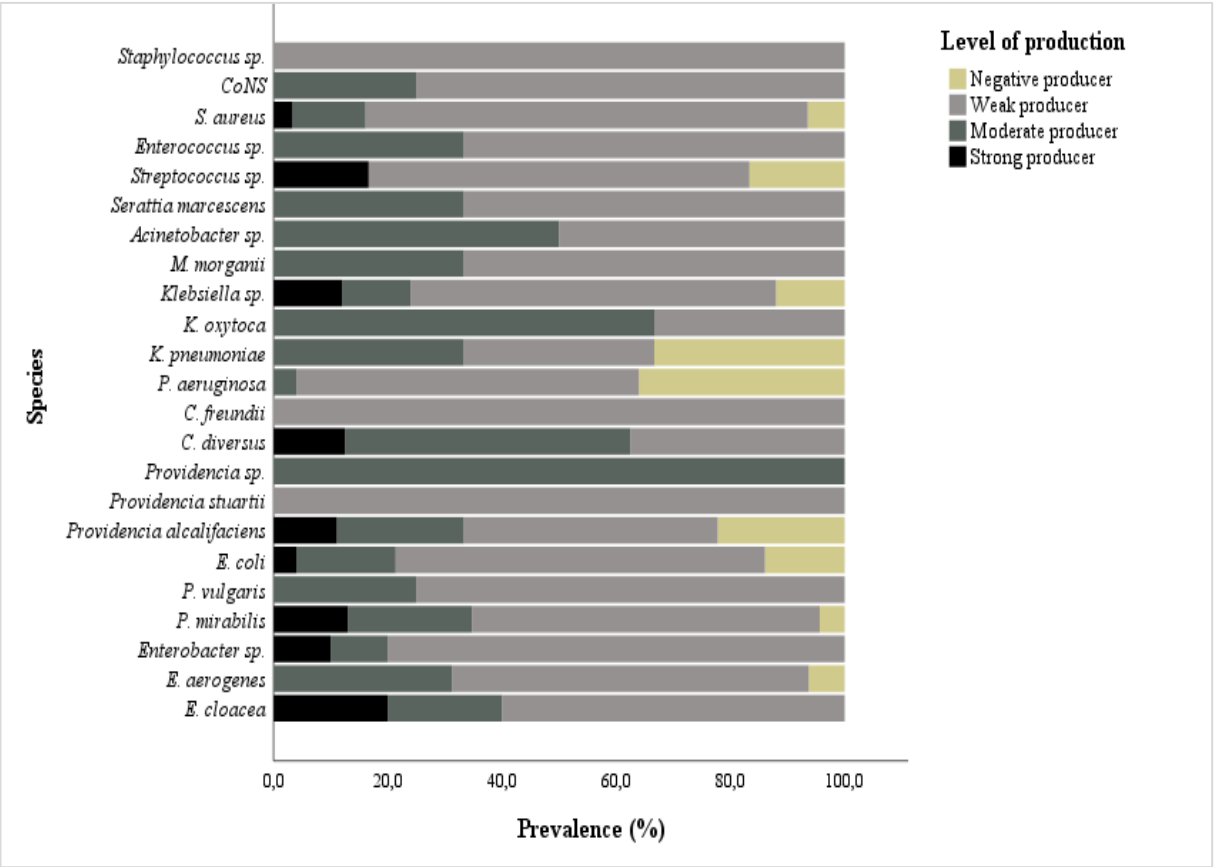


Figure. 44 : Biofilm formation among species.

The findings of this study revealed notable differences in biofilm formation across bacterial species. Among *Enterobacter* species, *E. cloacae* and *Enterobacter* sp. showed significant levels of strong biofilm production (20% and 10%, respectively). These results align with (Liu et al., 2022; Misra et al., 2022) studies highlighting *Enterobacter* species as robust biofilm formers, particularly in clinical and environmental settings.

E. coli and *P. mirabilis* showed a variability in biofilm production which infected by several genetic and environmental factors, these findings supported in previous study by (Folliero et al., 2021). The study of Garousi *et al.*, (2022) and Alshaikh e*t al.*, (2024) showed that the high

proportion of biofilm production in *E. coli* was recorded in weak ability, which align with our results. A high proportion of weak biofilm producers was reported also in the study of Ramadan *et al.*, (2021).

Interestingly, *P. aeruginosa* showed the largest percentage of non-biofilm producers (36%). This result contrasts with findings of Karami *et al.*, (2020). Compared to other studies, *P. aeruginosa* showed a strong ability to produce biofilm (Davarzani et al., 2021; Ratajczak et al., 2021), this deviation may reflect differences in isolate origin or environmental conditions.

Enterococcus sp. showed significant capability of moderate production, followed by CoNS species and *S. aureus* (25%), similar levels were found in Ethiopia and Iran (Ghazvinian et al., 2024; Kulayta et al., 2024).

4.5.3 Correlation between antibiotic resistance and biofilm formation

The table 20 demonstrates a clear correlation between biofilm strength and antibiotic resistance profile among tested isolates. A significant correlation was found between biofilm producers isolates and resistance to amoxicillin (p=0.0025), resistant-strains to amoxicillin + clavulanic acid and tazobactam + piperacillin showed a high ability to produce moderate biofilms.

Among the cephalosporins, cefalexin, cefoxitin and cefotaxime-resistant strains showed a high capability to produce moderate biofilms compared with sensitive strains. In contrast, imipenem-resistant strains exhibited high resistance (69.9%) in weak biofilms, but this rate diminished in moderate and strong biofilms (Tab. 20).

Strains resistant to quinolones were more associated with strong biofilm production especially nalidixic acid, which showed notable resistance across moderate and strong biofilms. Similar trends were observed for levofloxacin, ciprofloxacin and ofloxacin.

The evaluation of chloramphenicol against biofilms reveals that strains resistant to this antibiotic were more strongly associated with moderate and strong biofilm production. Resistance rates were 54.3% in weak biofilms, increasing to 30% in moderate biofilms and 7.1% in strong biofilms. Correspondingly, susceptibility decreased significantly with biofilm strength, with susceptibility rates of 68.2% in weak biofilms, dropping to 13.3% in moderate biofilms and 3.3% in strong biofilms.

Positive correlation was found between biofilm formation and polymyxins (p=0.002) with a strong association with colistin-resistance (p=0.003). Resistance rates were 68.6% in weak biofilms, 17.1% in moderate biofilms, and 5.7% in strong biofilms compared with high susceptibility across biofilm types, with 52.6% in weak biofilms, decreasing to 10.5% in moderate biofilms, and 0% in strong biofilms, demonstrating its reduced effectiveness as biofilms matured.

Table 20. Correlation between biofilm formation and antibiotic resistance.

Antibiotic		Biofilm strength			Antibiotic		Biofilm strength		
		Weak	Moderate	Strong			Weak	Moderate	Strong
AX	**R%**	65.7	18.9	4.4	**TOB**	**R%**	60.2	19,3	8
	S%	58.2	16.4	3.6		**S%**	66.2	17.5	4.5
AMC	**R%**	65.4	19.8	3.8	**K**	**R%**	61.5	18.9	9.8
	S%	63.8	13.8	7.7		**S%**	71.7	13	8.7
TC	**R%**	68.3	17.5	1.1	**NA**	**R%**	55.5	19.7	8.8
	S%	53.3	20	3.3		**S%**	69.4	17.8	3.9
TTC	**R%**	68	17	3.6	**OFX**	**R%**	65.3	19.1	5.2
	S%	60.9	20.3	2.9		**S%**	65	17.7	5.4
PRL	**R%**	68.6	16.7	3.9	**CIP**	**R%**	64.6	21.8	4.1
	S%	59.3	22	2,2		**S%**	63.5	16.7	5.9
TPZ	**R%**	59.1	20.5	0	**LE**	**R%**	50	22.2	22.2
	S%	63.9	18.8	2.8		**S%**	100	0	0
CN	**R%**	59.8	20.6	6.7	**C**	**R%**	54.3	30	7.1
	S%	70	15.3	3.7		**S%**	68.2	13.3	3.3
FOX	**R%**	57.3	23.4	7	**ATM**	**R%**	64.3	17.5	6.3
	S%	70.5	14.3	3.6		**S%**	66.2	19.2	4.1
CFM	**R%**	63.2	17.1	6.2	**NIT**	**R%**	63.7	22.3	3.8
	S%	65.3	19.4	4.1		**S%**	65.8	11.7	5.4
CAZ	**R%**	66.4	15.3	6.9	**SXT**	**R%**	65.6	17.2	6
	S%	64.2	20.8	2.5		**S%**	64.5	18	4.4
CTX	**R%**	63.8	19	5.7	**FF**	**R%**	62.1	18.5	5.6
	S%	67	16.3	4.4		**S%**	61.6	21.2	6.1
FEP	**R%**	65.9	15.5	5.4	**CS**	**R%**	68.6	17.1	5.7
	S%	64.6	20.2	3		**S%**	52.6	10.5	0
IMP	**R%**	69.9	14.7	4.4	**CD**	**R%**	52.5	20	17.5
	S%	63	19.6	6		**S%**	71.4	0	14.3
GEN	**R%**	60.2	20.4	5.1	**E**	**R%**	51.3	17.9	17.9
	S%	66.3	17.2	5.3		**S%**	85.7	0	14.3
AK	**R%**	71.1	13.3	4,4	**OX**	**R%**	55	15	20
	S%	64	17.8	5		**S%**	100	0	0
DO	**R%**	36.4	22.7	22.7					
	S%	75	8.3	16.7					

R: resistance, S: sensitive, AX: amoxicillin, AMC: amoxicillin clavulanic acid, TC: ticarcillin, TTC: ticarcillin + clavulanic acid, PRL: piperacillin, TPZ: piperacillin + tazobactam, CN: cefalexin, FOX: cefoxitin, CFM: cefixime, CAZ: ceftazidime, CTX: cefotaxime, FEP: cefepime, IMP: imipenem, GEN: gentamicin, AK: amikacin, DO: doxycyclin, TOB: tobramycin, K: kanamycin, NA: nalidixic acid, OFX: ofloxacin, CIP: ciprofloxacin, LE: levofloxacin, C: chloramphenicol, ATM: aztreonam, NIT: nitrofurantoin, SXT: trimethoprim/sulfamethoxazole, FF: fosfomycin, CS: colistin, CD: clindamycin, E: erythromycin, OX: oxacillin.

The finding of the current study highlight the correlation between biofilm formation and AR, which supported by the results of meta-analysis study, that reported 82.35% of studies showed a positive correlation between biofilm and AR (Garousi et al., 2022).

Our study found that amoxicillin-resistant strains were more likely to produce biofilm, consistent with previous studies by Qi *et al.*, (2016); Kadhim and Jameel, (2024) indicating that amoxicillin-resistant strains were dependent on AR as biofilm-forming strains to survival.

All strong biofilm producing isolates showed a high rate of resistance to cephalosporins, align with Folliero *et al.* who noted that all biofilm forming strains isolated from medical devices showed a high rate of resistance to cephalosporins, while the rate diminished to 50% in carbapenem, this also confirmed in Qi *et al.*, who reported that biofilm-forming isolates were less frequently resistant to imipenem, indicating the efficacity and great effect of this antibiotics to treat biofilm forming and resistant pathogens.

Strong biofilm forming strains showed a significant level of resistant against nalidixic acid, our findings are in agreement with a study in Uganda (Katongole et al., 2020), India (Karigoudar et al., 2019) and China (Sun et al., 2020), might be due to the introduction of quinolones as antibiotics of choice in human health treatment.

Positive strong correlation was found between colistin-resistant strains and biofilm formation in our study; however, this align with a study found that 78% of colistin-resistant isolates were identified as strong biofilm producers (Ozer et al., 2019), this can be due to the exposition to colistin that can induce resistance mechanisms in bacteria and expression of more resistance genes, which lead to increased biofilm formation (Park et al., 2021).

4.5.4 Comparison of biofilm production among resistant strains

The table 21 presents a comparative evaluation of biofilm formation capabilities (weak, moderate and strong categories) measured as optical density (OD) and percentages across phenotypic groups, including R, MDR, XDR, MSSA, MRSA, CMLSB and IMLSB.

Table 21. Comparison in biofilm production capacity among different phenotypic resistance profiles.

	Phenotypic profile						
	R	**MDR**	**XDR**	**MSSA**	**MRSA**	**CMLSB**	**IMLSB**
OD (mean ±SD)	0.11±0.07	0.12±0.1	0.16±0.25*	0.13±0.1	0.16±0.17*	0.17±0.08	0.21±0.16*
Weak (%)	17.2	57.9	24.9	55.6	44.4	6.3	18.8
Moderate (%)	19.4	54.2	26.4	0	100**	11.1	22.2
Strong (%)	9.5	42.9	47.6**	40	60	20*	0

*: statistical significance $p<0.05$, **: statistical significance $p<0.001$, R: strains that resist less than three classes of antibiotics, MDR: strains that resist three or more classes of antibiotics, XDR: strains susceptible to one or fwer classes of antibiotics,

MSSA: methicillin sensitive *S. aureus*, MRSA: Methicillin resistant *S. aureus*, CMLSB: Constitive Macrolide Lincosamide Streptogramine B resistant *S. aureus*, IMLSB: Inducible Macrolide Lincosamide Streptogramine B resistant *S. aureus*.

Among resistance profile, the mean OD values reveal a significant difference, with the highest value recorded among XDR strains (p=0.032), the majority of biofilm forming strains were found in MDR and XDR groups, with XDR exhibited the highest percentage of strong biofilm producers (47.6%), significantly greater than other phenotypic groups.

With *S. aureus* strains, MRSA showed a high mean OD (p=0.038) compared to MSSA strains. MSSA were predominant in weak biofilm formation (55.6%), while MRSA strains displayed a significant production of moderate and strong biofilms (p<0.001).

High capacity to produce biofilm was observed in IMLSB, which confirmed by a significant difference in OD mean compared with CMLSB (p=0.027); IMLSB stains demonstrated a high capacity to produce weak and moderate biofilms. In opposite, no strong production of biofilm was observed in IMLSB group; while CMLSB showed the high level (20%, p=0.017) of strong biofilm producers among MSLB isolates.

The results of this study demonstrate a significant relationship between resistance phenotypes and biofilm production capabilities. Strains with more advanced resistance profiles, such as MDR and XDR exhibited high capacity to produce moderate and strong biofilms. These findings are consistent with previous studies by Karigoudar *et al.*, (2019); Folliero *et al.*, (2021) and Qian *et al.*, (2022), who reported that MDR strains have greater capacity to moderate and strong biofilm production, due to the overexpression of resistance genes associated with biofilm production.

Similarly, MRSA strains noticed the higher OD mean with maximum production of moderate and strong biofilms compared to MSSA in this study. This is in agreement with Omidi *et al.*, 2020; Taşkin Dalgiç *et al.*, 2022 and Pokhrel *et al.*, (2024), who highlighted MRSA as resistant-strong biofilm forming isolate due to its isolation from clinical specimens.

Gaire and colleges, (2021) found that MRSA produced more moderate biofilms than MSSA but strong production was found exclusively in MSSA. Biofilm production is influenced by various elements, including environmental conditions, nutrition availability, geographical origin, specimen kinds, surface adhesion properties, and the organism's genetic composition.

In terms of MLSB resistance, CMLSB strains exhibited a greater propensity for strong biofilm formation compared to IMLSB strains, which primarily produced weak biofilms and moderate biofilms. This consistent with the studies of Manandhar *et al.*, (2021) and Aniba *et al.*, (2024) as their ability to grow in biofilms, these pathogens exhibited drug tolerance to broad-spectrum

of antibiotics. In contrast, IMLSB were identified as strong biofilm producers in the study by Mohamed *et al.*, (2020).This disparity may be attributable to differences in the bacterial strains, strain origin and assay conditions.

4.6 Characterization of the enzymatic activities

The Data represented in chart 45 show the percentage of bacterial isolates produced the four enzymes: hemolysin, protease, lecithinase and lipase. Hemolysin activity had the highest production (55.5%, p=0.028) followed by protease production 47.3%, lecithinase and lipase activities (23.1% and 10%, respectively).

Regarding the hemolysin activity, the majority of isolates were non-hemolysin producers 44.5% (p<0.001) followed by β-hemolysin 28.9% and α-hemolysin 26.6%. A strong negative correlation was recorded in hemolysin and lecithinase production; a negative significance was showed also between protease activity and lecithinase and lipase production.

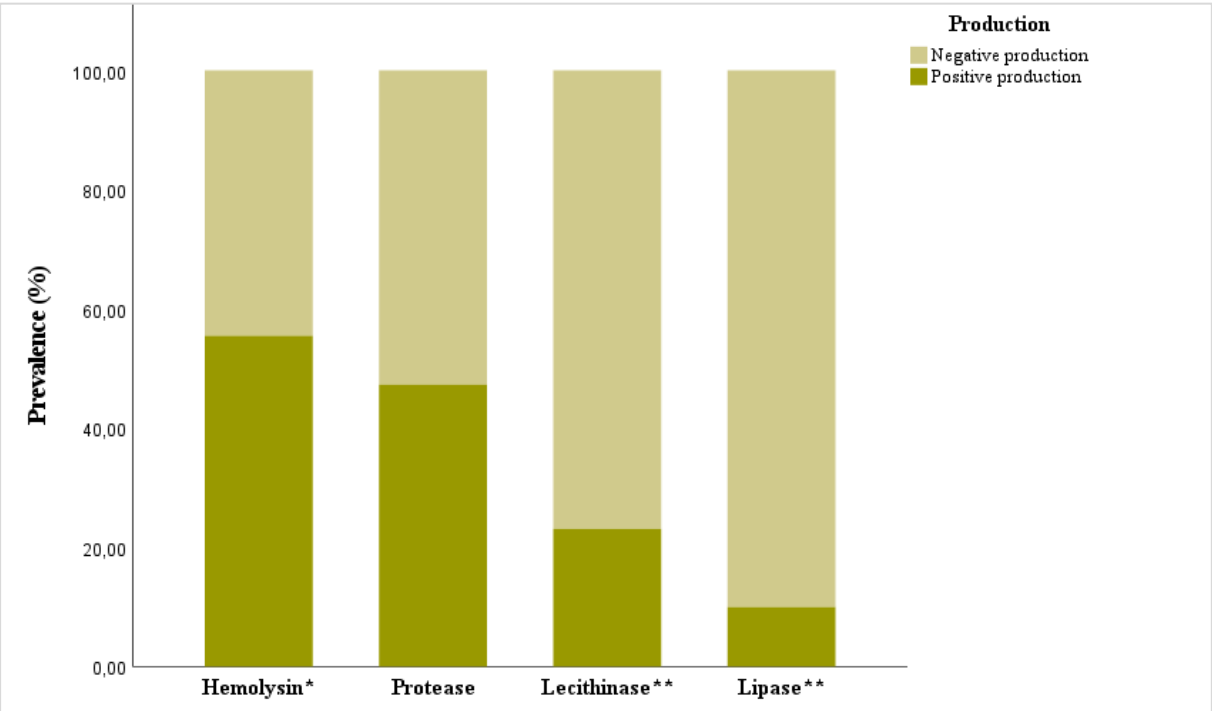


Figure. 45 : Percentage to bacterial isolate produced hemolysin, protease, lecithinase and lipase. (Comparison between positive and negative production was performed with *: statistical significance p<0.05, **: statistical significance p<0.001, positive comparison was found in hemolysin and negative comparison was found in lecithinase and lipase production).

Virulence refers to the extent of pathogenicity demonstrated by various pathogens and serves as a criterion to distinguish between pathogenic and non-pathogenic strains. The degree of

virulence depends on several virulence factors. In this study, the most notable finding was the production of hemolysin, observed in 55.5% of isolates. This rate aligns with the results reported by Fakruddin *et al.*, (2013) study. Hemolytic expression is always connected to the expression of a key virulence factor.

Meanwhile, the second highest virulence factor produced in pathogens was protease observed in 47.3% of isolates. Bacterial proteases are recognized as virulence factors in a number of infectious diseases due to their cell and tissue damaging effects, similar findings were reported in the study by Aqel *et al.*, (2023).

Low percentage of lecithinase production was reported in Dougnon *et al.*, (2021) study. The lowest percentage of virulence factors in the current study was recorded at 23.1% and 10% for both lecithinase and lipase. The lecithinase is a type of enzyme called phospholipases, characterized by its ability to destroy tissues by breaking down the phosphoryl (lecithin) in the cell membrane.

Similarly, the tested strains showed a reduced percentage of lipase production, which has a significant role in several biological processes, it catalyzes the hydrolysis of water-insoluble free fatty acid and glycerol. The results of our study are similar to those of (Ali et al., 2015), in which they found that the tested strains showed a production of 10.16% of lipase.

4.6.1 Enzymatic activities among species

The distribution of virulence factors production among species was evaluated and presented in chart 46, hemolysin production is prominently observed in the majority of GNB: *E. cloacae, Enterobacter* sp., *E. coli, P. alcalifaciens, P. aeruginosa, K. pneumoniae, Enterococcus* sp. and *S. aureus*. The protease production was found in higher levels in species like: *P. stuartii*, *E. coli*, *M. morganii*, *S. marcescens*, *Staphylococcus* sp. and *S. aureus*. Lecithinase and lipase were exhibited in high levels by *Enterobacter* sp., *E. coli, C. freundii, P. aeruginosa*, all *klebsiella* species, *S. aureus* and CoNS.

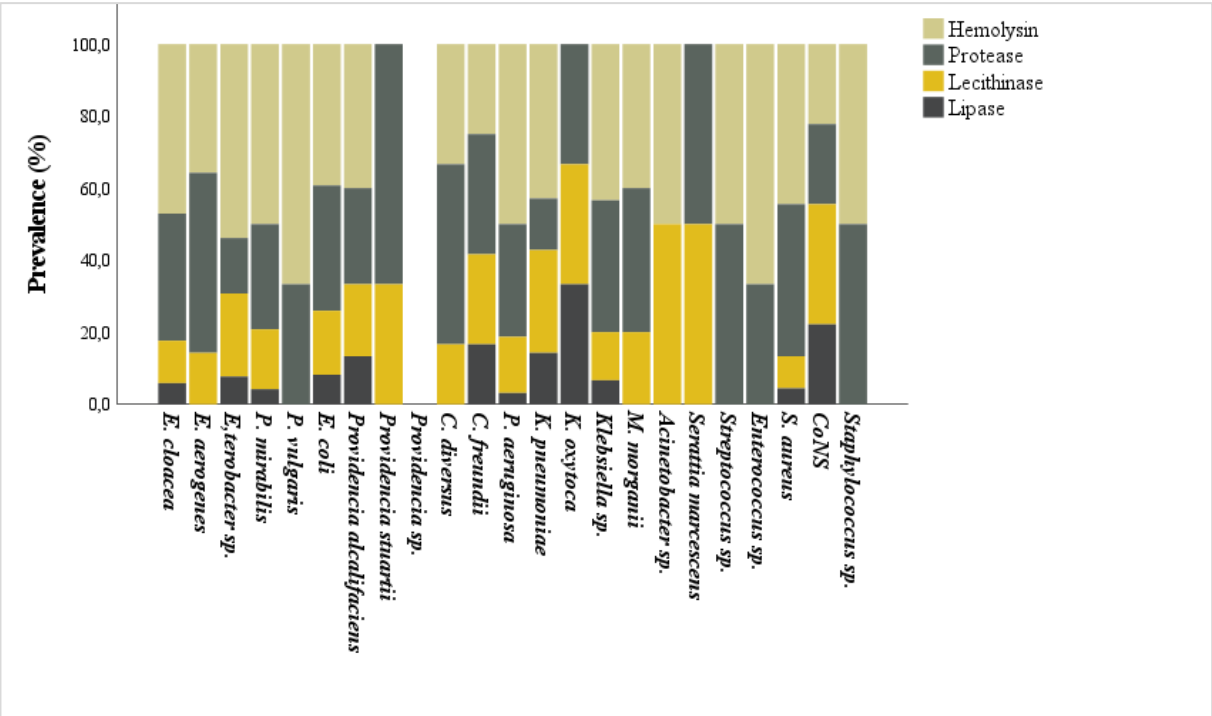


Figure. 46 : The ability of bacterial isolates to produce virulence factors.

It was found that *K. pneumoniae* produced hemolysin, lecithinase and lipase genes, while *E. cloacae* produced hemolysin, lipase and protease genes in Aldoori *et al.*, (2020) study.

K. pneumoniae in Trușcă *et al.*, (2023) study was more likely to produce lecithinase and lipase, while *P. aeruginosa* showed high production of hemolysin, lecithinase and protease. 32% of *E. coli* exhibited hemolytic activity in Kumar M *et al.*, (2022).

P. aeruginosa presented as the highest lipase (69.23%) and protease (76.92%) producer, followed by *S. aureus* (lipase 62.16% and protease 70.27%) in Rahman *et al.*, (2024) study, which is in agreement with our findings.

The presence of several virulence factors in bacteria indicated distinct infection methods, potentially augmenting their capacity to induce diseases in humans and other organisms.

4.7 Association between virulence factors and biofilm and resistance profiles

The relationship between virulence factors tested: hemolysin, protease, lecithinase and lipase across resistance profile and biofilm production was presented in table 22. Statistical analysis showed strong positive correlation between lipase production and fosfomycin (p=0.01), a second correlation was observed in resistant-clindamycin isolates that produce more hemolysin

and lecithinase (p=0.044 and p=0.008, respectively), erythromycin resistant strains positively correlated with lecithinase production (p=0.023).

The statistical analysis results were confirmed by the distribution of virulence factors production among different resistance profiles, where the highest production of hemolysin showed in XDR category (28.7%) compared with negative producers. MDR strains demonstrated a higher rate of protease production (61.6%) compared with negative ones, but lower level of production was observed among producer strains compared to positive strains.

Lecithinase producers have similar XDR percentages (26.9%) to negative producers (25.9%), with slightly increased MDR levels in positive producers (60.2% vs. 54.7%). Regarding lipase production, MDR strains showed a high level of production 60% compared to non-producer strains (55.5%) (Tab. 22).

Positive producers of hemolysin have a higher proportion of moderate (15.7%) and strong (5.8%) biofilm production compared to negative producers (20.7% and 4.5%, respectively), showing a link between hemolysin activity and biofilm formation. While, protease-producers demonstrated higher rates of strong biofilm production (6.3%) compared to negative producers (4.2%), also for moderate biofilm production (20.5% vs. 15.6%); reinforcing the role of protease in biofilm development.

Similarly, lecithinase producers have a greater prevalence of strong biofilm production (6.5%) than negative producers (4.9%), moderate biofilm producers showed considerable level of lecithinase production compared with negative producers (20.4% vs. 17.2%).

Lipase production showed the highest proportion of strong biofilm production (7.5%), with moderate biofilm rates (32.5%) also surpassing those of negative producers (16.3%). Among all enzymes, strong biofilm producers showed a high capability to produce different virulence factors, also for moderate biofilm producers, which showed a high rates of enzymes production expect for hemolysin.

Table 22. Association between virulence factors, antimicrobial resistance profiles, and biofilm production in bacterial isolates.

VF		Resistance profile			Biofilm production			
		R	**MDR**	**XDR**	**Negative**	**Weak**	**Moderate**	**Strong**
Hemolysin	**N (%)**	16.8	60.3	22.9	11.7	63.1	20.7	4.5
	P (%)	18.8	52.5	28.7	12.1	66.4	15.7	5.8
Protease	**N (%)**	20.3	50.9	28.8	13.7	66.5	15.6	4.2
	P (%)	15.3	61.6	23.2	10	63.2	20.5	6.3
Lecithinase	**N (%)**	19.4	54.7	25.9	12.3	65.7	17.2	4.9
	P (%)	12.9	60.2	26.9	10.8	62.4	20.4	6.5

Lipase	**N (%)**	18	55.5	26.5	12.7	66	16.3	5
	P (%)	17.5	60	22.5	5	55	32.5	7.5

VF: virulence factors, N: negative production, P: positive production, R: strains that resist less than three classes of antibiotics, MDR: strains that resist three or more classes of antibiotics, XDR: strains susceptible to one or fwer classes of antibiotics

The production of hemolysin, protease, lecithinase and lipase was correlated with higher levels of AR, particularly with MDR and XDR profiles, these findings align with studies like those of Dhahir and Mutter, (2023) which demonstrated that AR profiles had strong correlation with virulence factors. Similarly, Gunjal and Gunjal, (2024) noted that hemolysin-positive *E. coli* strains exhibited higher MDR rates (93.55%), comparable to positive- hemolysin in non-MDR strains (6.45%).

Hemolysin and protease production was higher in MDR and XDR *P. mirabilis* isolates compared to non-MDR strains in Elhoshi *et al.,* (2023); the same for MDR *P. aeruginosa*, which showed an increased protease production compared with susceptible *P. aeruginosa* (Naik et al., 2021)

However, lecithinase production was significatively higher in MDR *P. aeruginosa* compared to non-MDR strains in Ghanem *et al.*, (2023) study. Lipase and protease-producing bacteria revealed higher resistance to selected antibiotics than non-producers in Rahman *et al.*, (2024) study. A significant association was found in XDR profile of *P. aeruginosa* and the production of virulence factors in Truşcă *et al.,* (2023).

Our data showed that positive virulence factors production correlated with enhanced biofilm production, with lipase-positive strains showed the highest proportion of strong biofilm producers (7.5%). This observation is consistent with findings by Şahin, (2019) which highlighted the presence of evident relationship between biofilm formation with lipase activity among *S. aureus*. These results suggest that the formation of lipase and biofilm, may function together in pathogenic strains.

4.8 Molecular detection of β-lactamase encoding genes

Phenotypic analysis of β-lactamase producing strains reveals that a total of 182 strains were screened as positive β-lactamase-producing strains, with 32.6% presented ESBL phenotype and 12.7% were cefoxitin screened positive (AmpC). Similar rates were found in Mozambique, where 32.6% were ESBL strains and 10.8% were AmpC strains (Estaleva et al., 2021). The

prevalence of ESBL in Sri Lanka was reported at 36.2% (Kumudunie et al., 2020) while the prevalence of AmpC in Nepal was 12.6% (Dhungana et al., 2019), in Algeria the rate was ranged between 23% to 55% (Benyagoub et al., 2021). This wide variation might be due to differences in study population, type of specimen, sample size and the extent of antibiotic use.

There was a statistically significant agreement between phenotypic and genotypic results in the detection of β-lactamase producing strains (p<0.001). The overall prevalence rates of β-lactamase producing strains in our study was as follows: out of 124 (68.1%) positive-PCR isolates, the majority were *E. coli* strains with a percentage of 55.6% (p<0.001) followed by *Klebsiella* species (12.9%), *Enterobacter* spp. (9.7%) and *Staphylococcus* species (8.9%). This distribution of strains was found in urine (78.2%) while 12.1% of positive β-lactamase producing strains were found in pus, and 5.6% in different medical devices.

Out of 182 positive screened strains, and among 65.9% of isolates confirmed as ESBL strains, bla_{TEM} was the most prevalent gene, detected in nearly 53.8% of the isolates, followed by bla_{SHV} with a detection rate of around 15.9%. The $bla_{CTX\text{-}M\ II}$, bla_{OXA} and $bla_{CTX\text{-}M\ I}$ were detected at similar levels (9.9%, 9.3% and 8.8%, respectively), while the lowest rate was recorded for $bla_{CTX\text{-}M\ IV}$ (6.6%). $bla_{CMY\ II}$ was the most frequent AmpC detected gene (10.4%) followed by bla_{DHA} (7.1%) in total of 17% confirmed as positive producing AmpC isolates (Fig. 47).

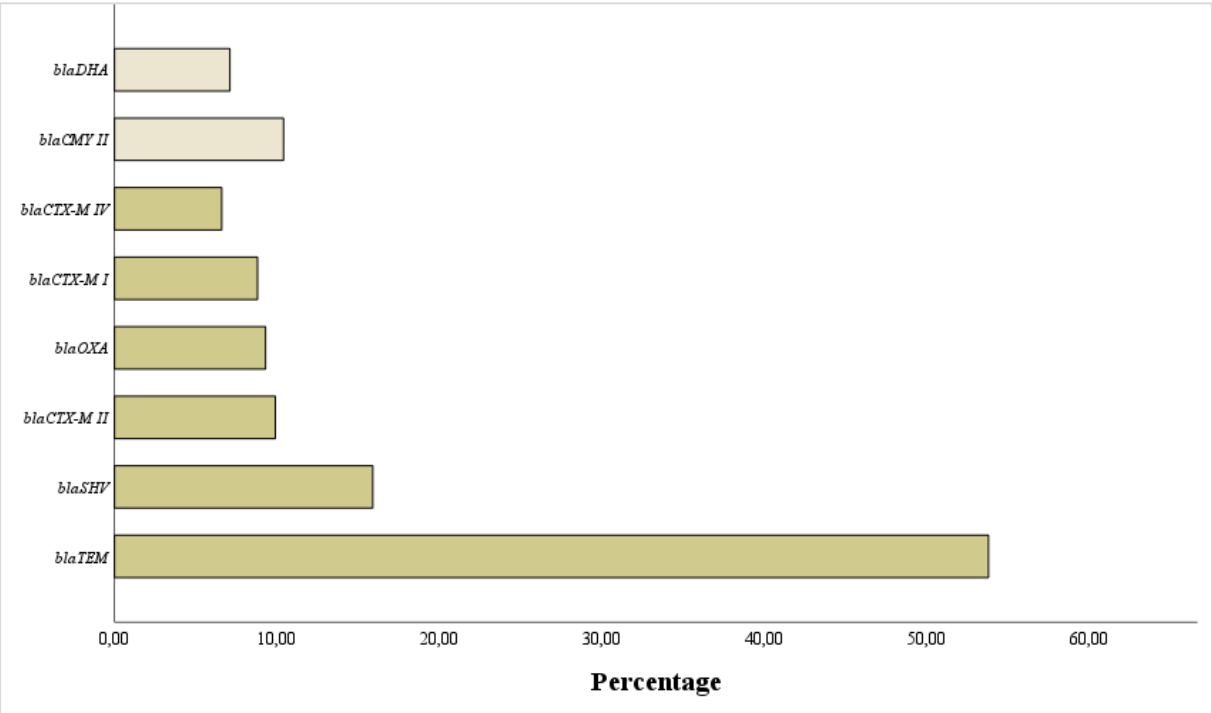


Figure. 47 : Prevalence of detected β-lactamase genes among isolates.

The dominance of *bla*$_{TEM}$ as ESBL gene was reported in several studies, first of all in Algeria (Yahiaoui et al., 2015) and it was the major ESBL gene observed in Hadizadeh *et al.*, (2017) study. In a study on 4083 clinical isolates by Armin *et al.*, (2020) *bla*$_{TEM}$ was the most common ESBL gene found in phenotypic positive strains (255/4083). The *bla*$_{TEM}$ gene was most detected in 42% of isolates in Abdar *et al.*, (2019) study, the prevalent ESBL genotype of *bla*$_{TEM}$ was (55%, 86%) in Ugbo *et al.*, (2020) and Dirar *et al.*, (2020) studies respectively. TEM and SHV types have mostly been found in clinical samples conferring AR (Aabed et al., 2021). The prevalence of plasmid mediated AmpC genes was previously reported in Algeria with a rate of 29.4% (Bougouizi et al., 2024), 5.71% (Khaldi et al., 2022). The *bla*$_{CMY\ II}$ was the most AmpC prevalent gene in Algeria (1.6%) (Iabadene et al., 2009), as well as in Iran (47.4%) (Fallah et al., 2020) in Egypt (32%%) and in Nigeria (Onanuga et al., 2019).

In all screened strains, the majority of positive phenotypic ESBL and AmpC recorded the absence of β-lactamase genes (34.1% and 83% for ESBL and AmpC, respectively). 37.4% of ESBL strains expressed the genotype by one ESBL gene, 20.9% by two genes, 10% by three genes and only 2.2% expressed the ESBL genotype by four *bla* genes, 16.5% of positive screened confirmed the AmpC phenotype by one gene (Tab. 23). *bla*$_{TEM}$/*bla*$_{CTX\text{-}M\ II}$ was the most prevalent combination in ESBL genotype (17.3%) followed by *bla*$_{TEM}$ /*bla*$_{SHV}$ and *bla*$_{TEM}$ /*bla*$_{CTX\text{-}M\ I}$ (15.4% for both) and *bla*$_{TEM}$/*bla*$_{CTX\text{-}M\ IV}$ (9.6%). 13.7% of screened isolates presented ESBL/AmpC genotype.

Table 23. Prevalence of different genotype.

Genotype	n	(%)	Genotype	n	%
No ESBL gene	62	34.1	**Four ESBL gene**	4	2.2
One ESBL gene	68	37.4	**No AmpC gene**	151	83
Two ESBL genes	38	20.9	**One AmpC gene**	30	16.5
Three ESBL genes	10	5.5	**Two AmpC genes**	1	0.5

The most common ESBL-encoding gene combination identified in this study was *bla*$_{TEM}$/*bla*$_{CTX\ M\ I}$, our findings are comparable with those in India reported a predominance of the combination *bla*$_{TEM}$ /*bla*$_{CTX\ M\ I}$ (22.72%) (Khan et al., 2019) as well as in Nigeria, where it was reported at 20.8% among UPEC isolates (Adekanmbi et al., 2020).

An important observation is that many positive ESBL strains were also AmpC positive, this was in accordance with Khalifa *et al.*, (2021) study. The presence of more than one genotype in some of the isolates means that the ESBL producing strains may be related to a complex

antimicrobial resistance. TEM gene is a broad spectrum β-lactamase that is always combined with CTX-M on the same plasmid (Elsafi, 2020).

4.8.1 Distribution of β-lactamase genes among specimens and species

The bar chart in figure 48 represents the prevalence of β-lactamase genes across different clinical specimens; *bla*$_{TEM}$ was the most prevalent gene across all sources, it dominated in blood, urine, vaginal swabs and pus, confirming its widespread presence in various infections.

bla$_{SHV}$ and *bla*$_{CTX\text{-}M}$ genes are distributed across multiple sources, *bla*$_{SHV}$ appeared notably in pus, vaginal swabs, and medical devices, while *bla*$_{CTX\text{-}M\ I}$ and *bla*$_{CTX\text{-}M\ II}$ were detected in urine, pus, and vaginal swabs, suggesting their role in UTIs and gynecological infections. The *bla*$_{OXA}$ was significantly present in urine, pus, and medical devices. On other hand, *bla*$_{CMY\ II}$ and *bla*$_{DHA}$ detected at lower levels, especially in urine and pus.

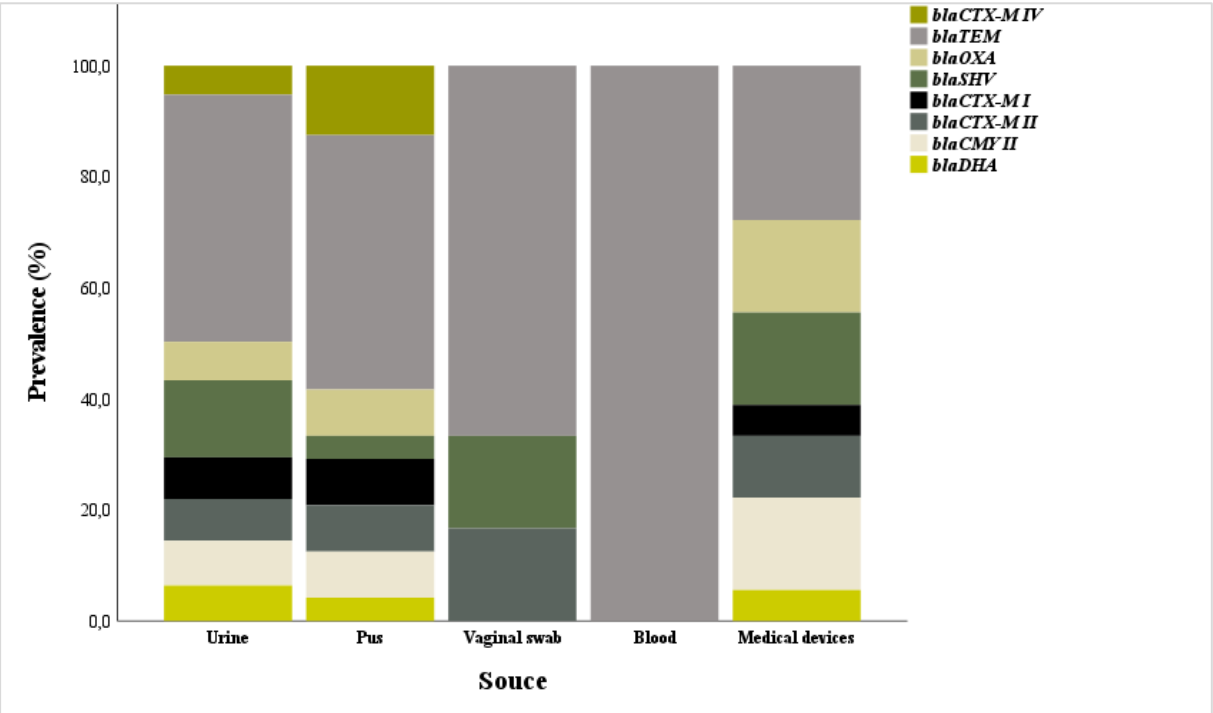


Figure. 48 : Prevalence of β-lactamase genes based on clinical specimens.

The widespread presence of the *bla*$_{TEM}$ gene across different infection types with coexistence of other β-lactamase genes have reported in several studies, it's the most frequented gene across all the type of isolates (sputum, urine, wound, blood, tracheal aspirate and eye swab) while *bla*$_{CTX\text{-}M}$ was found among wound, sputum, blood and urine in Saudi Arabia (Ibrahim et al., 2021).

The table 24 illustrate the distribution of β-lactamase genes among bacterial isolates, regarding ESBL encoding genes: $bla_{CTX\text{-}M}$ gene types were most frequently found in *E. coli* and *Klebsiella* species especially $bla_{CTX\text{-}M\ IV}$ and $bla_{CTX\text{-}M\ I}$. *Citrobacter* and *Enterococcus* species showed a considerable rate of $bla_{CTX\text{-}M\ IV}$ production (8.3%). A strong positive correlation was found between bla_{TEM} and *E. coli* (p<0.001), with *E. coli* being the predominant producer (60.2%). *Enterobacter* and *Klebsiella* species exhibited similar production levels of bla_{TEM} (10.2% each), followed by *Staphylococcus* spp. (9.2%). Similar levels were recorded in bla_{OXA} case. bla_{SHV} is highly characterized of *Klebsiella* spp. (34.5%) compared with other species, followed by *E. coli* (31%) and *P. aeruginosa* (10.3%).

AmpC β-lactamase genes were predominantly detected in *E. coli* (57.9% for $bla_{CMY\ II}$, 61.5% for bla_{DHA}). *Enterobacter* spp. and *Klebsiella* spp. displayed moderate prevalence of $bla_{CMY\ II}$ (21.1% and 10.5%, respectively) and bla_{DHA} (7.7% and 15.4%, respectively). Interestingly, *P. mirabilis*, *P. aeruginosa*, and *Acinetobacter* spp. exhibited minimal AmpC gene presence, by *P. mirabilis* and *Acinetobacter* spp. produced 5.3% of $bla_{CMY\ II}$, while *Citrobacter* and *Acinetobacter* species produced 7.7% of bla_{DHA}.

Table 24. Prevalence of β-lactamase genes among bacterial isolates.

Species	**β-lactamase genes n (%)**							
	ESBL genes						**AmpC genes**	
	$bla_{CTX\text{-}M\ IV}$	bla_{TEM}	bla_{OXA}	bla_{SHV}	$bla_{CTX\text{-}M\ I}$	$bla_{CTX\text{-}M\ II}$	$bla_{CMY\ II}$	bla_{DHA}
E. coli	5 (41.7)	59 (60.2)	7 (41.2)	9 (31)	11 (68.8)	9 (50)	11 (57.9)	8 (61.5)
***Enterobacter* spp.**	2 (16.7)	10 (10.2)	2 (11.8)	2 (6.9)	1 (6.3)	5 (27.8)	4 (21.1)	1 (7.7)
***Klebsiella* spp.**	3 (25)	10 (10.2)	5 (29.4)	10 (34.5)	2 (12.5)	2 (11.1)	2 (10.5)	2 (15.4)
P. mirabilis	0	3 (3.1)	1 (5.9)	2 (6.2)	0	1 (5.6)	1 (5.3)	0
P. aeruginosa	0	4 (4.1)	0	3 (10.3)	0	0	0	0
***Staphylococcus* spp.**	0	9 (9.2)	1 (5.9)	2 (6.9)	1 (6.3)	1 (5.6)	0	0
***Citrobacter* spp.**	1 (8.3)	1 (1)	0	0	0	0	0	1 (7.7)
***Acinetobacter* spp.**	0	1 (1)	1 (5.9)	1 (3.4)	1 (6.3)	0	1 (5.3)	1 (7.7)
***Streptococcus* spp.**	0	1 (1)	0	0	0	0	0	0
***Enterococcus* spp.**	1 (8.3)	0	0	0	0	0	0	0
S. marcescens	0	0	0	0	0	0	0	0

ESBL and AmpC β-lactamases are the prevalent enzymes generated by GNB, which are their principal mechanisms of resistance to all generations of cephalosporins (Tekele et al.,

2020).The analyze of ESBL-encoding genes indicated that ESBL production in *E. coli, P. mirabilis*, *Enterobacter* spp., *Staphylococcus* spp. and *Citrobacter* spp. was harbored by bla_{TEM} (Ojdana et al., 2014; Hadizadeh et al., 2017; Pishtiwan and Khalil Mustafa, 2019; Hosu et al., 2021; Ibrahim et al., 2021; Aso Bakr and Khanda Abdullateef, 2022; Dong et al., 2023).

The bla_{TEM} was the most ESBL gene detected in *E. coli*, *Klebsiella* species, *P. mirabilis* and *Enterobacter* species in Algeria, Sudan and Sri Lanka (Yahiaoui et al., 2015; Perera et al., 2022; Dirar et al., 2020), which were in accordance with our findings.

SHV ESBL-type was most prevalent in *Klebsiella* spp., this dominance was supported by several researches: it was the most detected gene among *Klebsiella* species in Algeria (Yaici et al., 2017) and seven countries: United States, Taiwan, Australia, South Africa, Turkey, Belgium, and Argentina, documented by Paterson *et al.*, (2003) and in India (Ahmed et al., 2014) indicating its role in pathogenicity and resistance of *Klebsiella* species in hospital and community settings.

Previous study reported that $bla_{CMY\ II}$ was the most common AmpC encoding gene found in *E. coli*, *P. mirabilis* and *Enterobacter* spp., in Iran (Fallah et al., 2020; Rizi et al., 2020) and in Algeria (Iabadene et al., 2009).

4.8.2 Correlation of β-lactamase genes with antibiotic resistance

The correlation between the distribution of β-lactamase genes and AR with comparison of most significant resistance rate was showed in figure 49. The presence of *bla* genes allowed the differentiation of three genotypes: ESBL genotype (possessed ESBL gene), AmpC genotype (possessed AmpC gene) and ESBL/AmpC genotype (presence of both ESBL and AmpC genes). The three genotype profiles showed high resistance to amoxicillin, ticarcillin, ticarcillin + clavulanic acid and imipenem, with ESBL/AmpC being the dominant profile (100% for both AX and TC, 85,7% for TCC).

In case of cephalosporins, ESBL/AmpC profile showed a high resistance level for first and second generation cephalosporins (cefalexin and cefoxitin) also for ceftazidime, cefotaxime and cefepime compared with ESBL and AmpC profiles (78.6%, 64.3%, 60.7%, 57.1% and 78.6%, respectively), while AmpC profile had a great resistance for cefixime (75%), but in contrast cefotaxime and cefepime have the best effect on this category with 0% resistance rate.

For β-lactams combined with an inhibitor, the resistance rate decreased with the addition of clavulanic acid. Specifically, the resistance rates against amoxicillin were 87.8% for ESBL producers, 75% for AmpC producers, and 100% for ESBL/AmpC co-producers. However, with the administration of clavulanic acid, these rates dropped to 61.1% for ESBL producers, 50% for AmpC producers, and 85.7% for ESBL/AmpC co-producers. And it was 77.8%, 50% and 100% respectively against ticarcillin, to become 68.9%, 50% and 85.7% in the presence of clavulanic acid. On the other hand, the tazobactam inhibitor demonstrated the most significant impact, reducing the resistance rate by approximately 50% (Fig. 49).

AmpC genes harboring strains showed a high resistance level for monobactam (75%) followed by ESBL/AmpC profile (51.7%). The efficacity of aminoglycosides family showed in gentamycin for AmpC genes harboring isolates (0%) and amikacin for ESBL/AmpC positive strains (14.3%) while for ESBL genes harboring isolates similar rates were observed (23.3% to 25.6%). ESBL strains had significant resistance to both colistin and fosfomycin and the combination trimethoprim/sulfamethoxazole with levofloxacin had the maximal impact (4.4%).

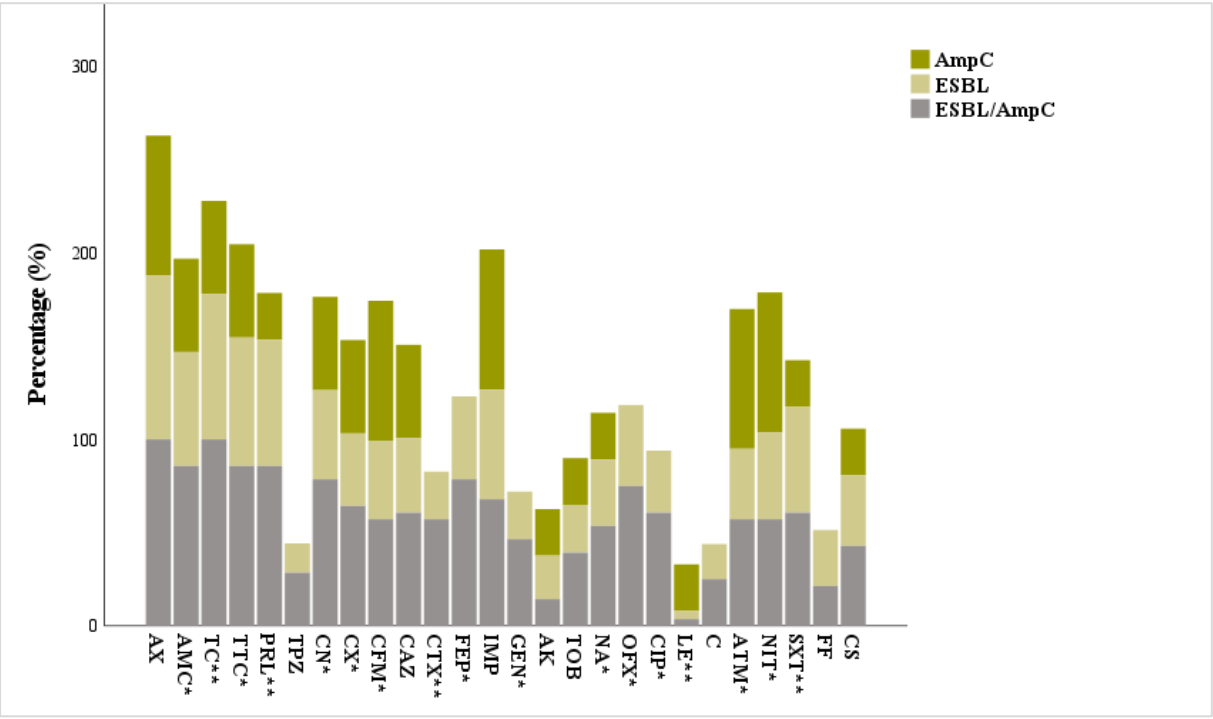


Figure. 49 : Relationship between antibiotic resistance and β-lactamase production (* significant: $p < 0.05$, ** significant: $p < 0.01$).

Our results reveals that the ESBL/AmpC profile was the most dominant, exhibiting high resistance rates to β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems.

These findings align with previous studies reporting the broad-spectrum resistance conferred by the co-occurrence of ESBL and AmpC genes (Lee et al., 2020; Marzah et al., 2024).

In contrast, the AmpC profile exhibited lower resistance to certain cephalosporins, such as cefotaxime and cefepime, with a resistance rate of 0%. These findings align with previous studies in Malaysia, where AmpC-positive strains also demonstrated reduced resistance to cefotaxime (Mohd Khari et al., 2016).

Similarly, Hoellinger *et al.*, (2023) confirmed in their study that cefepime was more effective against AmpC-producing Enterobacterales compared to carbapenems. Additionally, high sensitivity to cefotaxime was observed in AmpC-producing *K. pneumoniae* in Taiwan (Lin et al., 2016).

ESBL profile displayed resistance to several classes of antibiotics with moderate resistance rates to aminoglycosides. These results are congruent with several studies reported that ESBL producing bacteria showed a resistance to several antibiotics: aminoglycoside, fluoroquinolone, tetracycline, chloramphenicol, and trimethoprim/sulfamethoxazole (Nasehi et al., 2010; Hadizadeh et al., 2017; Wibisono et al., 2020; Mirkalantari et al., 2020; Ibrahim et al., 2021; Habibzadeh et al., 2022).
The addition of β-lactamase inhibitors, such as clavulanic acid and tazobactam, significantly reduced resistance rates, with tazobactam showed the most pronounced effect (≈50% reduction). Same results were reported in Sadeghi *et al.*, (2022) study, where the resistance rate in ESBL *E. coli* was reduced from 69.7% in amoxicillin to 40.8% in amoxicillin + clavulanic acid.

Lower resistance rates were observed for amoxicillin + clavulanic acid and piperacillin + tazobactam in ESBL/AmpC *Klebsiella* species (5.2% and 18.5%, respectively) in (Watanabe et al., 2024) study. All these results confirm that the use of tazobactam or clavulanic acid in conjunction with β-lactams leads to a notable decrease in resistance rates.

4.8.3 Association between β-lactamase genes and virulence factors

The correlation between the prevalence of β-lactamase genes with virulence factors was presented in table 25. Weak biofilm formation was predominant across all *bla* genes, ranging from 58.3% ($bla_{CTX\text{-}M\ IV}$) to 94.4% ($bla_{CTX\text{-}M\ II}$). The highest weak biofilm percentage is seen in $bla_{CTX\text{-}M\ II}$ (94.4%), moderate biofilm formation was generally low, with the highest percentage

in *bla*$_{CTX\text{-}M\ I}$ (18.7%), while *bla*$_{CTX\text{-}M\ II}$ and *bla*$_{CMY\ II}$ showed no moderate biofilm formation at all. Positive strong association was found in biofilm formation and *bla*$_{TEM}$ (p=0.049) with *bla*$_{TEM}$ being the only gene with any significant presence (2%). Regarding odds ratio, *bla*$_{CTX\text{-}M\ II}$ (OR = 2.8) has the strongest association with weak biofilm formation, *bla*$_{OXA}$ (OR = 2.6) and *bla*$_{SHV}$ (OR = 2.3) also show potential positive associations.

Non-hemolysin production (γ-hemolysin) was the most prevalent hemolysin type across all strains, particularly associated with strains carrying the *bla*$_{DHA}$ gene (84.6%). α-hemolysin ranging from 6.2% (*bla*$_{CTX\text{-}M\ I}$) to 29.4% (*bla*$_{OXA}$). While for β-hemolysin, *bla*$_{CTX\text{-}M\ I}$ positive isolates were the most produced strains (43.8%), *bla*$_{OXA}$ showed the highest OR for α and β-hemolysin (1.2). *bla*$_{DHA}$ show the lowest OR (0.1), with very low 95% CI (0.03-0.6).

Protease activity is an important virulence factor influencing bacterial pathogenicity. Protease-positive isolates was most frequent in *bla*$_{SHV}$ (69%) followed by *bla*$_{OXA}$ (64.7%) and *bla*$_{CTX\text{-}M\ IV}$ (58.3%), *bla*$_{CTX\text{-}M\ I}$ has the lowest proportion of protease-positive isolates (25%). The *bla*$_{SHV}$ has the highest OR (2.9), indicating a strong positive association with protease production.

Lecithinase activity is generally low across all groups. However, the *bla*$_{OXA}$ gene was detected in 23.6% of cases, and the *bla*$_{SHV}$ gene in 20.7% of cases, showing the highest positivity rates among the tested groups. Lipase activity followed a similar trend, with low positivity across all genes, the highest frequency was in *bla*$_{OXA}$ (17.6%).

Table 25. Association between β-lactamase genes and virulence factors in bacterial isolates.

	VF	%							
		***bla*$_{CTX\text{-}M\ IV}$**	***bla*$_{TEM}$**	***bla*$_{OXA}$**	***bla*$_{SHV}$**	***bla*$_{CTX\text{-}M\ I}$**	***bla*$_{CTX\text{-}M\ II}$**	***bla*$_{CMY\ II}$**	***bla*$_{DHA}$**
Biofilm	**Negative**	25	11.2	5.9	6.9	12.5	5.6	10.5	7.7
	Weak	58.3	78.6	88.2	75.9	68.8	94.4	89.5	84.6
	Moderate	16.7	8.2	5.9	17.2	18.7	0	0	7.7
	Strong	0	2	0	0	0	0	0	0
	OR	0.4	1.4	2.6	2.3	1.1	2.8	1.3	1.9
	95% IC	0.1-1.7	0.6-3.4	0.3-20.5	0.5-10.2	0.2-5	0.3-21.9	0.3-6.1	0.2-15.2
Hemolysin	**γ-hemolysin**	50	51	41.2	51.7	50	55.5	52.6	84.6
	α-hemolysin	25	16.3	29.4	27.6	6.2	27.8	26.3	7.7
	β-hemolysin	25	32.7	29.4	20.7	43.8	16.7	21.1	7.7
	OR	0.8	0.6	1.2	0.7	0.8	0.6	0.7	0.1
	95% IC	0.2-2.5	0.3-1	0.4-3.2	0.3-1.6	0.3-2.2	0.2-1.6	0.3-1.8	0.03-0.6
Protease	**Negative**	41.7	59.2	35.3	31	75	66.7	63.2	61.5
	Positive	58.3	40.8	64.7	69	25	33.3	36.8	38.5
	OR	1.6	0.5	2.1	2.9	0.3	0.5	0.6	0.7
	95% IC	0.5-5.2	0.3-1	0.8-6.1	1.2-6.7	0.1-1.1	0.2-1.4	0.2-1.6	0.2-2.1
Lec	**Negative**	100	85.7	76.7	79.3	87.5	94.4	94.7	84.6

	Positive	0	14.3	23.6	20.7	12.5	5.6	5.3	15.4
	OR	0.9	0.3	1	0.8	0.4	0.2	0.2	0.6
	95% IC	0.9-1	0.2-0.7	0.3-3.2	0.3-2.2	0.1-2	0.02-1.3	0.02-1.2	0.1-2.7
Lipase	**Negative**	100	91.8	82.4	96.6	93.8	94.4	94.7	92.3
	Positive	0	8.2	17.6	3.4	6.2	5.6	5.3	7.7
	OR	0.9	0.4	1.4	0.2	0.4	0.3	0.3	0.5
	95% IC	0.9-1	0.1-0.9	0.4-5.2	0.03-1.5	0.05-3.1	0.04-2.7	0.04-2.5	0.06-4.1

OR: odds ratio, CI: Confidence interval (OD ranged between: OR=0, OR>1 and OR<1).

The results of our study identified that 86.8% of *bla* genes harboring isolates produce biofilm, Subramanian *et al.*, (2012) found that ESBL producing isolates had a higher ability to form biofilm in comparison with non-ESBL isolates; a similar association was found in Neupane *et al.*, (2016) study, where *E. coli* ESBL strains had more ability to produce biofilm that non-ESBL strains.

Meta-analysis study conducted by Keikha and Karbalaei in (2023) demonstrated that ESBL producing clinical isolates have more potential capacity to produce biofilm comparing with non ESBL stains. In Maheshwari *et al.* in (2016) study also, ESBL producing bacteria exhibited varying levels of biofilm formation.

Positive correlation was detected between the presence of $bla_{CTX\text{-}M\ I}$, $bla_{CTX\ M\ II}$, bla_{SHV}, bla_{OXA} and bla_{TEM} with biofilm production ability in our study, a significant correlation between bla_{CTX} and bla_{SHV} β-lactamase type and biofilm producing in *K. pneumoniae* was confirmed in Hamam *et al.*, (2019) study. Giedraitiene *et al.*, (2022) confirmed also the association between $bla_{CTX\ M}$ β-lactamase type and biofilm formation. Heydari and Eftekhar, (2015) confirmed that there was an association between the presence of two or three β-lactamases genes and strong biofilms in *P. aeruginosa*.

β-lactamases *P. mirabilis* were statistically better expressed in biofilm formation in Nucleo *et al.*, (2010) study. Biofilms are known to protect bacteria from antibiotics and immune responses, contributing to chronic and recurrent infections. The strong association between *bla* genes and biofilm activity suggests that these genes may be critical in promoting biofilm-related pathogenicity because these biofilms provide a favorable environment for the exchange of virulence factors and resistance genes, and the simultaneous expression of several virulence genes may promote the creation of new resistance determinants, aggravating the control and treatment of bacterial infection disease (Subramanian et al., 2012; Maheshwari et al., 2016; Surgers et al., 2019).

Among all examined strains, 55.5% of β-lactamase producing isolates demonstrated hemolytic activity. Our results were higher comparing to findings of Lopes *et al.*, (2018) study in Brazil (44.9%) and of Al Zoubi *et al.*, (2020) in the USA, where only 24.5% of the ESBL-generating isolates exhibited hemolytic activity. Sample size, isolate source and geographical location can explain this difference.

Our findings demonstrated a significant positive correlation in $bla_{CTX\text{-}M\ I}$, bla_{TEM} and bla_{OXA} positive strains, with bla_{OXA} harboring strains exhibited the highest odds ratio for the presence of hemolysin enzyme. These results were confirmed in Abd El-Baky *et al.*, (2020) study, which found that $bla_{CTX\ M\ IV}$ showed a significant positive association with *hlyA* (hemolysin gene) in uropathogenic *E. coli*, bla_{OXA} also associated with *hlyA* in *E. coli* isolated from wound samples. A study by Escudeiro *et al.*, (2019) confirmed that there are an association between AR genes and virulence genes in bacterial clones, a positive strong correlation was observed between the acquisition of new AR genes and new virulence genes. The widespread of virulence factors in association with resistance genes can be explained by the acquisition of hybrid plasmids that carry virulence genes in association with AR genes, and the selection of these plasmids by the antibiotics result to the selection of some virulence characteristics (Shankar et al., 2022).

Protease significantly contributes to host immune evasion, invasiveness, and tissue damage. it was observed in 47.8% of screened isolates in our study. 60.7% of ESBL *K. pneumoniae* produced protease enzyme in Mirbag *et al.*, (2024), protease gene was found in association with bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ type in *K. pneumoniae* isolates (Ndiaye et al., 2023).

4.9 Phylogenetic grouping of *E. coli* strains

The strain *E. coli*, GNB frequently located in the gastrointestinal tract of humans and several animals (Bozorgomid et al., 2023) and one of the most important and prevalent species of the *Escherichia* genus in veterinary and medicinal contexts, accounting for around 80–90% of infections (Bozorgomid et al., 2023).

Among all identified *E. coli*, 86 imipenem-resistant *E. coli* were selected to phylogenetic grouping and quinolones detection. The eighty-six unique strains of *E. coli* were isolated only from urine specimens (UPEC), from both hospitalized and non-hospitalized patients of all age groups with a diagnosis of UTI.

Based on the quadruplex PCR assay, phylogenetic analysis of *E. coli* isolates showed that they mainly belonged to phylogroup B2 (48.9%) and E (22.1%) (statistically significant, $p<0.0001$), unknown group were also prevalent with a great frequency (12.8%), the other phylogroups were prevalent as followed: A (8.1%), B1 (4.7%), and D, Clade I, Clade I or Clade II (1.2% for each one), no strain belonging to the phylogroup F was found (Fig. 50**)**. However, the statistical analyses did not reveal any significant correlation between the gender, the age of the patient, and the phylogenetic groups ($p=0.578$ and 0.171, respectively).

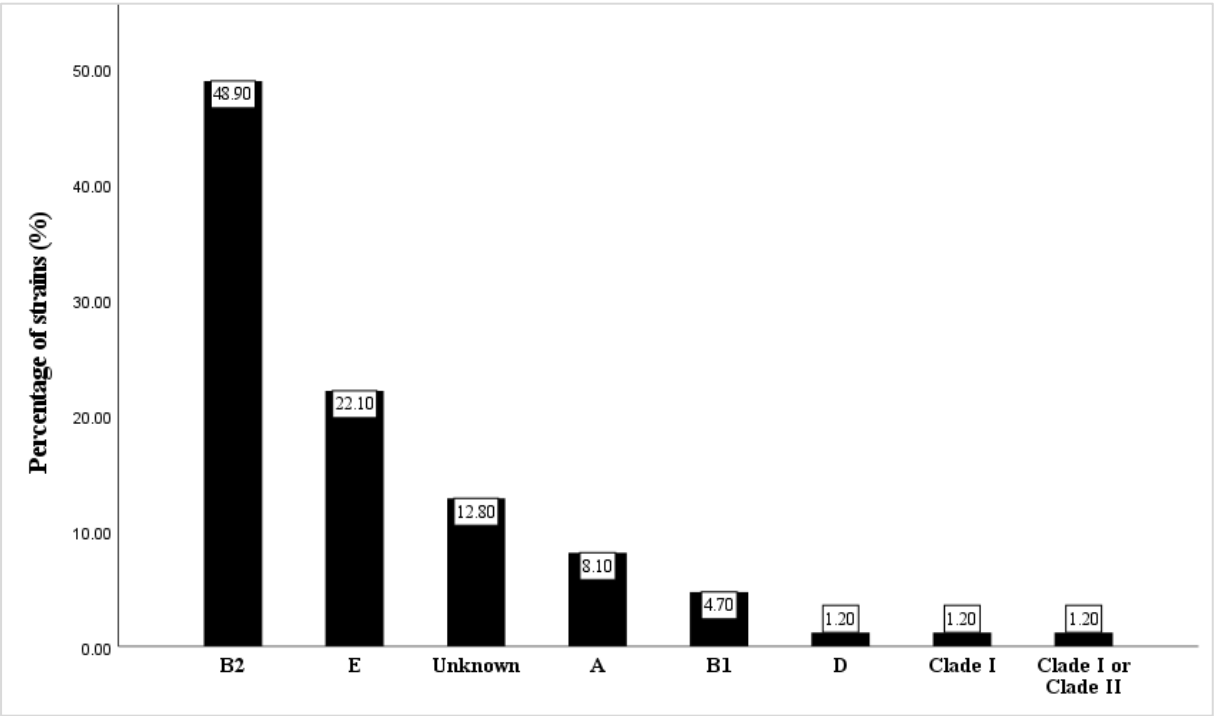


Figure. 50 : Distribution of the seven phylogenetic groups (A, B1, B2, D, E, Unknown, Clade I, and Clade I or Clade II).

Most strains causing extraintestinal infections are predominantly categorized into B2 and D groups (Halaji et al., 2022), while commensal isolates are categorized into groups A and B1 (Saralaya et al., 2015). Our findings align with numerous studies that have identified B2 group strains as the dominant type in UTIs. In Iraq and from 150 urine strains, 58% belonged to phylogroup B2 followed by phylogroup A (22%) (Mohammed et al., 2022). In a study on 105 *E. coli* isolates from Slovenian patients with bacteremia of urinary tract origin showed that 51% belonged to group B2, 20% to group D, 15% to group A, and 13% to the B1 group (Rijavec et al., 2008).

Another study on 190 urinary *E. coli* isolates in Colombia showed that 46.8% of the isolates belonged to group B2 (Baldiris-Avila et al., 2020). In a study on 228 UPEC in Egypt, 64.6% of

the isolates belonged to phylogroup B2 (Elsayed Gawad et al., 2018). Similarly in a study on 113 UPEC isolates in Iran, 44.2% of the strains were classified into group B2, 31% into group D, 20.4% into group A, and 4.4% into group B1 (Bakhtiari et al., 2020).

Similarly, studies in Uganda (Katongole et al., 2020), South Africa (Alfinete et al., 2022), Iraq (Allami et al., 2022), India (Agarwal et al., 2013; Saralaya et al., 2015), Iran (Moez et al., 2020) and Egypt (Hassuna et al., 2020; Farahat et al., 2021), demonstrated that most UPEC isolates from UTIs belonged to the B2 group.

Among *E. coli* phylogenetic groups, the B2 phylogroup is believed to be more important than others. This phylogroup is associated with a high evolution of virulence capacity and characteristics, which may cause the spread and persistence of extraintestinal infections representing, therefore, a major public health concern (Mansouri et al., 2022; Hogins et al., 2023).

Our methodology did not allow the classification of a minor percentage of *E. coli* isolates (12.8%). This latter result can be dependent on the recombination of different or rare phylogroups resulting from the combination of the presence and absence of certain genes, as suggested by Boroumand *et al.*, (2021). Phylogenetic group E also had a high prevalence among our strains, as found in a very recent study (Gunathilaka et al., 2024). However, it should be noted that variations in the source of bacterial isolation, host health state, geographic locations, and genetic variables can affect the distributions and proportions of phylogenetic groupings.

4.9.1 Correlation of phylogenetic groups with antibiotic resistance and virulence factors

The correlation between phylogenetic groups and AR was illustrated in figure 51, with the most significatively higher sensitivity profile (resistance, sensible and intermediate) was presented in the graph. The 86 *E. coli* isolates showed different resistance profiles. Apart from the resistance to imipenem, the highest resistance percentage was significantly (p<0.0001) observed against β-lactam antibiotics: amoxicillin (86%) and ticarcillin (82.6%), followed by piperacillin (73.3%). The association of the β-lactamase inhibitor tazobactam significantly reduced the resistance to piperacillin from 73% to 19%. However, the addition of the β-lactamase inhibitor: clavulanic acid did not significantly reduce the resistant strains to

amoxicillin (from 86% for amoxicillin to 62% for amoxicillin + clavulanic acid) and ticarcillin (from 83% for ticarcillin to 76% for ticarcillin + clavulanic acid).

The lowest percentage of resistance was exhibited towards cephalosporin antibiotics (19.8-38.4%). However, almost all isolates were resistant to nitrofurantoin (84.9%) and trimethoprim/sulfamethoxazole (51.2%). The resistance rate of isolates against fosfomycin was 27.9% and to colistin 38.4%, and lower percentages of nalidixic acid (37.2%), ofloxacin (36%), aztreonam (31.4%) and ciprofloxacin (32.6%) resistant strains were found. Most of isolates were susceptible to chloramphenicol, gentamycin, amikacin, and tobramycin (86.1%, 81.4%, 83.7%, and 76.8%, respectively).

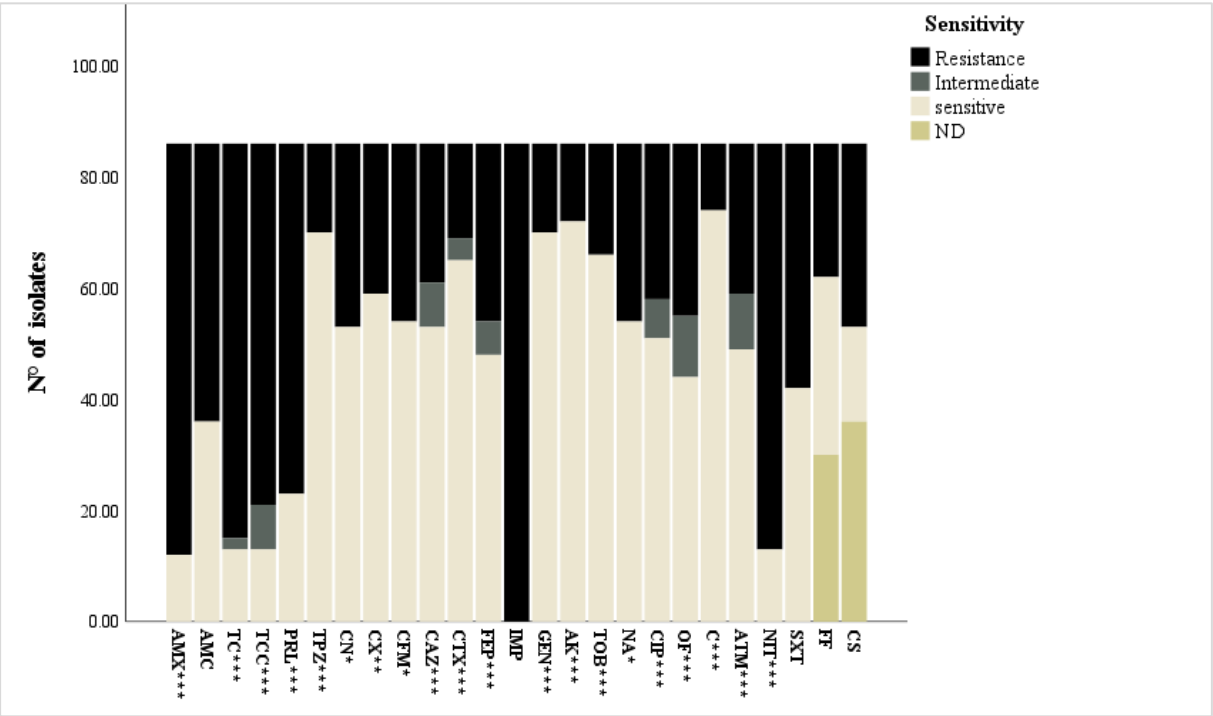


Figure. 51 : Antibiotic resistance profile of *E. coli* isolates (*: p<0.05; **: p<0.01; ***: p<0.0001; ND: non detected).

Our study was focused on imipenem-resistant *E. coli*, since carbapenems are frequently used in hospital setting as first line drugs in the empirical treatment of several bacterial infections in Algeria, the 86 imipenem-resistant isolates displayed a high percentage of resistance to penicillins, similar to other studies in Uganda and Mongolia, respectively (Kabugo et al., 2017; Munkhdelger et al., 2017). Nitrofurantoin and trimethoprim/sulfamethoxazole are recommended as the first-line therapy, while β-lactams and fluoroquinolones are used as alternative agents in UTI therapy (Tewawong et al., 2020; Zhou et al., 2023), this justifies the maximum rates observed against these antibiotics.

The evidence that a lower percentage of isolates was resistant to aminoglycosides (16.2-23.6%), as reported in other studies (Naziri et al., 2020), carried out in Iran (16.7% and 21.8%) could be explained by the limited use of this antibiotic in UTI treatment in developing countries. These results indicate a worrying trend of increased resistance to first-line treatments.

Furthermore, 70% and 23% of the examined strains were MDR and XDR strains, respectively. The prevalence of MDR and XDR strains was very significant (p<0.0001) in the adult group. According to phylogenetic groups, the prevalence of MDR strains was higher in phylogroup B1, unknown, and E. In contrast, only a few strains (7%) were resistant to less than three classes of antibiotics (Tab. 26).

Table 26. Distribution of phenotypic resistance profiles and association with gender, age, clinical status, phylogroups, ESBL production, and hemolytic activity.

	R (n=6, 7%)	**MDR (n=60, 70%)**	**XDR (n= 20, 23%)**	**Total (n=86,100%)**
Gender				
M	3 (3%)	26 (30%)	6 (7%)	**35 (41%)**
F	3 (3%)	34 (40%)	14 (16%)	**51 (59%)**
Age				
Adulte	3 (3%)	37 (43%)	16 (19%)	**56 (65%)**
Children	2 (2%)	18 (21%)	2 (2%)	**22 (26%)**
Elderly	1 (1%)	5 (6%)	2 (2%)	**8 (9%)**
Clinical statuts				
In Patient	0	10 (11%)	1 (1%)	**11 (13%)**
Out-Patient	6 (7%)	50 (58%)	19 (21%)	**75 (87%)**
Phylogroups				
A	1 (1%)	4 (5%)	2 (2%)	**7 (8%)**
B1	0	4 (5%)	0	**4 (5%)**
B2	4 (5%)	27 (31%)	11 (13%)	**42 (49%)**
Clade I	0	0	1	**1 (1%)**
Clade I or II	0	1 (1%)	0	**1 (1%)**
D	0	1 (1%)	0	**1 (1%)**
E	0	14 (16%)	5 (6%)	**19 (22%)**
Unknown	1 (1%)	9 (10%)	1 (1%)	**11 (13%)**
ESBL production				
No	5 (6%)	33 (38%)	6 (7%)	**44 (51%)**
Yes	1 (1%)	27 (31%)	14 (16%)	**42 (49%)**
Hemolysin activity				
γ-hemolysin	2 (2%)	32 (37%)	8 (9%)	**42 (49%)**
α-hemolysin	1 (1%)	9 (10%)	9 (10%)	**19 (22%)**
β-hemolysin	3 (3%)	19 (22%)	3 (3%)	**25 (29%)**

R: strains that resist less than three classes of antibiotics, MDR: strains that resist three or more classes of antibiotics, XDR: strains susceptible to one or fwer classes of antibiotics

The production of ESBL was one of the main mechanisms by which bacteria resist to antibiotics. Out of the isolates tested, 49% were found to be ESBL positive while 51% were

ESBL negative (Tab. 26). However, most of the strains tested did not present hemolytic activity (49%) while others showed α-hemolysin (22%) and β-hemolysin activity (29%). ESBL producing isolates and also strains that produced hemolysin (57.89% for α-hemolysin, and 48% for β-hemolysin) were preferentially observed in phylogenetic group B2 more than the other phylogroups.

Our finding is consistent with several studies (Boroumand et al., 2021; Halaji et al., 2022) confirmed that B2 groups were more resistant than the other phylogenetic groups. This can be explained by the fact that this phylogroup has a greater ability to exhibit characteristics associated with AR (AR genes), the coexistence of some virulence factors, followed by the acquisition of resistance (Rijavec et al., 2008). Conversely, numerous investigations have demonstrated that phylogroup B2 is more vulnerable than the others (Iran, Taiwan) (Norouzian et al., 2019; Wang et al., 2023). Social and environmental conditions and the therapy profile of patients may explain this difference.

Most of our B2 strains were ESBL and MDR, similar to studies conducted in Egypt and Sri Lanka, featuring 65.17% and 60.3% of MDR strains, respectively (Kadry et al., 2020; Gunathilaka et al., 2024). In Kenya, where the most of ESBL strains belonged to B2 group (Muriuki et al., 2022), this high similarity in percentage of MDR and ESBL may be due to the similar inappropriate use of antibiotics and poor healthcare infrastructure and management in these developing countries.

According to biofilm formation assay results, most of the tested clinical strains were weak biofilm producers (71% with $p<0.0001$), while other strains did not produce biofilm at all (19%), moderate (9%) and strong biofilm (1%) producers were in the minority. Biofilm-forming strains were mostly found in the phylogroup B2, although they were mostly weak producers (30%), while the only strong biofilm-forming strain belonged to phylogroup E (n = 1, 1.1%).

In the present study, the production of biofilm and AR were analyzed in figure 52. It was observed that strains possessing resistance to multiple classes of drugs (XDR) exhibited weak biofilm production (75%), or moderate (5%), or were completely unable to produce biofilm (20%).

Regarding MDR, most tested isolates had a weak production of biofilm (72%). Furthermore, 50% of R strains were not able to form biofilm or produced only weak biofilm (50%). Several

previous studies showed that the most of biofilm-forming strains belonged to phylogenetic group B2 (Soto et al., 2007; Javed et al., 2021), virulence factors, toxin proteins, multi-drug resistance, and ESBL increased in UPEC and is connected to phylogroup B2 (Matinfar et al., 2021).

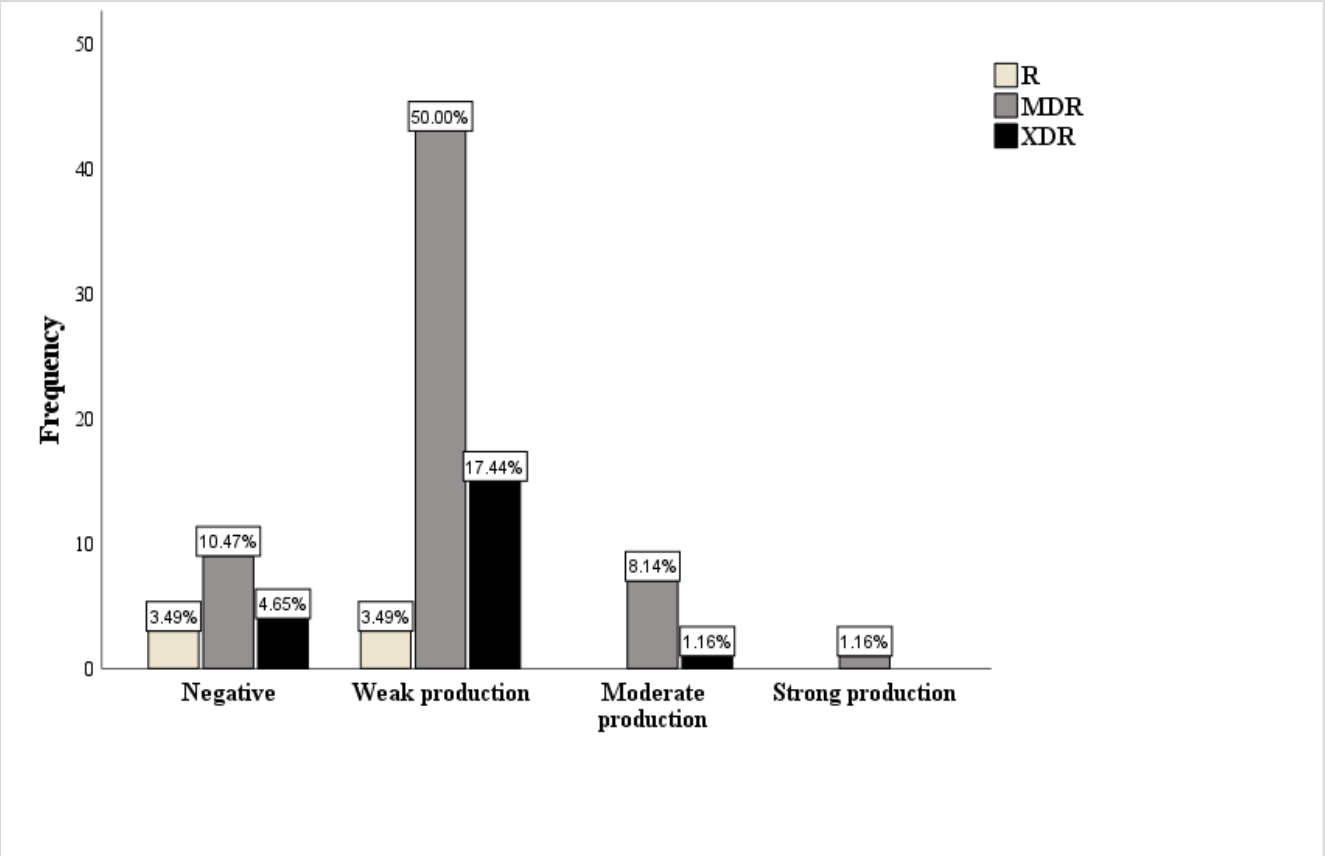


Figure. 52 : Absolute frequencies of R, MDR and XDR strains among non-producers, weak, moderate, and strong biofilm producers.

4.9.2 Distribution of resistance genes among phylogenetic groups

4.9.2.1 β-lactamase genes

Among 86 *E. coli* isolates, bla_{TEM} was the most frequent gene (62.8%) followed by bla_{CMYII} (12.8%), while bla_{SHV}, $bla_{CTX\text{-}M\ I}$ and $bla_{CTX\text{-}M\ II}$ distributed with similar level (10.5%). Across phylogenetic groups, B2 phylogroup showed a high variability of *bla* genes production, it's the primary reservoir for almost all tested *bla*-type genes (Tab. 27).

The bla_{TEM} gene was predominant in B2 and E phylogroups, bla_{OXA} was strongly related with B2 (83.3%), this phylogroup produced bla_{SHV}, $bla_{CTX\text{-}M\ I}$ and $bla_{CTX\text{-}M\ II}$ genes with similar rates

(55.6%). The second producer of $bla_{CTX\text{-}M\ I}$ was E group, $bla_{CMY\ II}$ and bla_{DHA} spread among all phylogenetic groups. The E group being the second significant phylotype, while A, B1 and D have minimal resistance gene presence (Tab. 27).

Table 27. Prevalence and distribution pattern of β-lactamase genes among phylotypes.

Variables	**no. of isolates in each phylotype (%)**							
Single gene	**A**	**B1**	**B2**	**Clade I**	**Clade I or II**	**D**	**E**	**Unknown**
***bla*$_{CTX\text{-}M\ IV}$ (n=3)**	0	0	1 (33.3)	0	0	0	1 (33.3)	1 (33.3)
***bla*$_{TEM}$ (n=54)**	3 (5.5)	1 (1.9)	27 (50)	0	0	1 (1.9)	15 (27.7)	7 (13)
***bla*$_{OXA}$ (n=6)**	1 (16.7)	0	5 (83.3)	0	0	0	0	0
***bla*$_{SHV}$ (n=9)**	1 (11.1)	1 (11.1)	5 (55.6)	0	0	0	1 (11.1)	1 (11.1)
***bla*$_{CTX\text{-}M\ I}$ (n=9)**	0	0	5 (55.6)	0	0	0	4 (44.4)	0
***bla*$_{CTX\text{-}M\ II}$ (n=9)**	1 (11.1)	1 (11.1)	5 (55.6)	0	0	0	0	2 (22.2)
***bla*$_{CMY\ II}$ (n=11)**	1 (9.1)	1 (9.1)	5 (45.5)	0	0	0	1 (9.1)	3 (27.2)
***bla*$_{DHA}$ (n=7)**	1 (14.3)	1 (14.3)	3 (42.8)	0	0	0	1 (14.3)	1 (14.3)

The phylogenetic groups of *E. coli* didn't express their resistance with single genes only, but diverse gene combinations are present in multiple phylotypes, with B1, B2, and E showing the most variation (Fig. 53) and with bla_{TEM}/$bla_{CTX\text{-}M\ I}$ was the most prevalent combination in B2 and E groups.

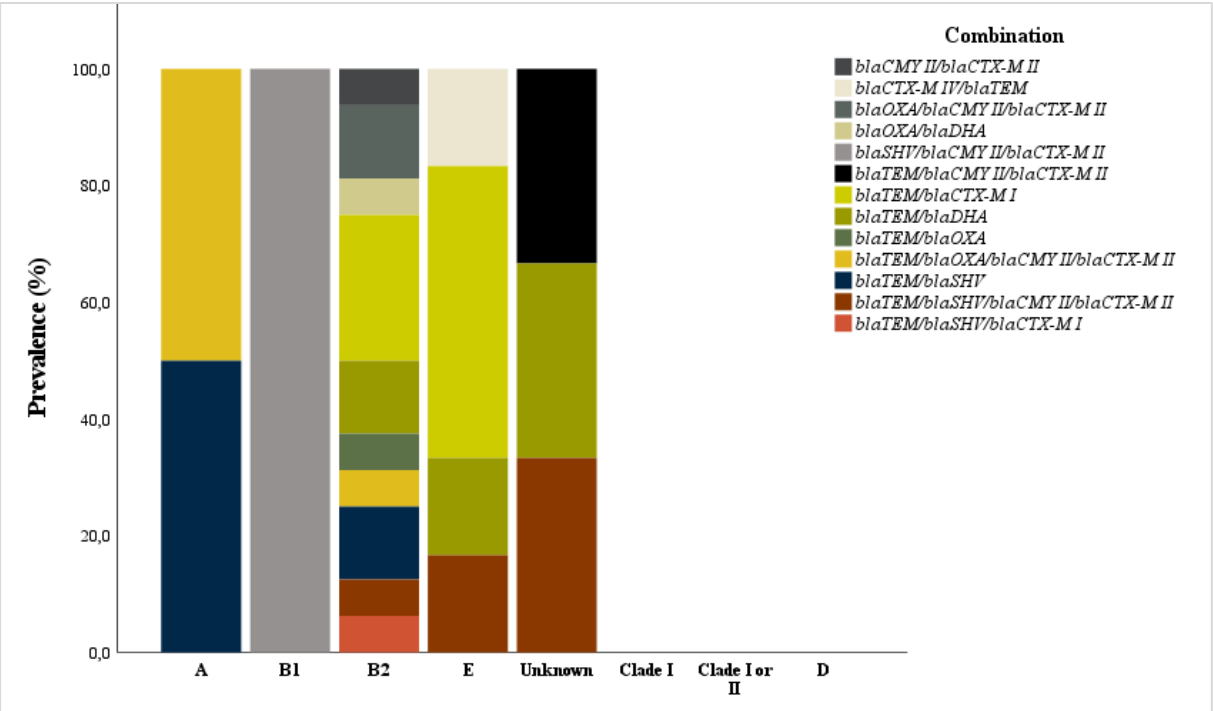


Figure. 53 : Distribution of β-lactamase gene combinations among phylogroups.

These findings were consistent with other studies showed that B2 phylogroup is major reservoir of β-lactamase genes. Afsharikhah *et al.*, (2023) demonstrated in their study that bla_{TEM} and $bla_{CTX\text{-}M}$ genes were predominant among the strains that mostly belonged to the pathogenic phylotypes B2 and D. Another study in Egypt reported that the carriage of ESBL genes in UPEC phylogroups, with ESBL genes being more frequent in phylogroup B2 and D than in other phylogroups (El Maghraby et al., 2024).

4.9.2.2 Distribution of qnr genes

The rate of 26.7% of *E. coli* strains can harbored at least one *qnr* gene. Among *qnr* positive strains, *qnrB* was the most prevalent resistant gene (78.3%, $p<0.01$) followed by *qnrD* (26.1%), *qnrA* and *qnrS* (13%) and finally *qnrC* (4.3%). These results were in accordance with what was reported (30.2%) in Boroumand *et al.*, (2021) study, *qnrB* being the most dominant gene in several studies in Togo (Salah et al., 2019), Nigeria (Nsofor et al., 2021), because PMQR genes are mobile, they may migrate across plasmids or use MGE to spread from one species to another (Nsofor et al., 2021).

The distribution of *qnr* genes across phylogenetic groups was presented in table 28, B2 phylogroup was the primary reservoir of *qnr* genes, with highest prevalence of *qnrB* (77.8%), *qnrD* (66.8%) and *qnrA* (66.7%). B1 group showed limited presence of *qnr* genes, only *qnrD* (16.6%) and *qnrS* (33.3%) were detected. E phylotype harbored various *qnr* variants: *qnrA* (33.3%), *qnrB* and *qnrD* (16.6%), it's the only expressor of *qnrC*. Unclassified class harbored *qnrS* (33.3%) and *qnrB* (5.5%). No *qnr* genes were detected in Clade I, Clade I or II, or D. Generally, *qnr* genes was found in association with ExPEC, specifically with B2 and D groups (Al-Rafyai et al., 2021).

The concomitant presence of two *qnr* genes was detected (Tab. 28), *qnrB* + *qnrD* was the most prevalent combination found exclusively in B2 group, supporting the mobility character of *qnrB* gene.

Table 28. Distribution of *qnr* genes among phylogenetic groups of *E. coli*.

	n (%)							
	A	**B1**	**B2**	**Clade I**	**Clade I or II**	**D**	**E**	**Unknown**
qnrA	0	0	2 (66.7)	0	0	0	1 (33.3)	0
qnrB	0	0	14 (77.8)	0	0	0	3 (16.7)	1 (5.5)
qnrC	0	0	0	0	0	0	1 (100)	0
qnrD	0	1 (16.6)	4 (66.8)	0	0	0	1 (16.6)	0
qnrS	0	1 (33.3)	1 (33.3)	0	0	0	0	1 (33.3)

qnrA* + *qnrB	0	0	1 (100)	0	0	0	0	0
qnrA* + *qnrC	0	0	0	0	0	0	1 (100)	0
qnrA* + *qnrS	0	0	1 (100)	0	0	0	0	0
qnrB* + *qnrD	0	0	4 (100)	0	0	0	0	0

4.9.2.3 Association of quinolone antibiotics resistance and qnr genes

The table 29 presents the relationship between quinolone AR and the distribution of *qnr* genes. Comparison test was used to distinct the difference in quinolones resistance between positive and negative *qnr* strains, *qnrA* harbored strains showed completely resistance to ofloxacin. For nalidixic acid and ciprofloxacin, *qnrA*-negative strains were more resistant compared with *qnrA*-positive strains. *qnrB* positive isolates exhibited higher resistance to all quinolones.

The *qnrC*-positive isolates showed 100% resistance across all quinolones**,** *qnrD*-negative isolates showed high resistance levels for nalidixic acid and ofloxacin (72.5% and 70%, respectively) compared with positive strains, which express good resistance in ciprofloxacin case (66.7%). The *qnrS*-positive strains showed relatively lower resistance to nalidixic acid (33.3%) and ciprofloxacin (33.3%) and ofloxacin (66.7%) compared with negative isolates which stilled exhibited significant resistance (Tab. 29).

Table 29. Distribution of *qnr* genes in relation with quinolone resistance.

	NA	**CIP**	**OF**
***qnrA*-positive**	66.7%	33.3%	100% *
***qnrA*-negative**	72.1%	65.1% **	67.4%
***qnrB*-positive**	77.8%	66.7%	77.8%
***qnrB*-negative**	67.9%	60.7%	64.3%
***qnrC*-positive**	100% *	100% **	100% *
***qnrC*-negative**	71.1%	62.2%	68.9%
***qnrD*-positive**	66.7%	66.7%	66.7%
***qnrD*-negative**	72.5%	62.5%	70%
***qnrS*-positive**	33.3%	33.3%	66.7%
***qnrS*-negative**	74.4% ***	65.1% **	69.8%

*: statistical signification $p<0.05$, **: statistical signification $p<0.01$, ***: statistical signification $p<0.001$.

The correlation between *qnr* genes and quinolone AR was confirmed in various studies, Boroumand *et al.,* (2021) reported that there was a significant relationship between *qnr* variants and nalidixic acid and ciprofloxacin, Nsofor *et al.,* (2021) reported that isolates encoding *qnr* genes showed more resistance to pefloxacin, ciprofloxacin, sparfloxacin, levofloxacin, nalidixic

acid, ofloxacin and moxifloxacin, the same observations were confirmed in Salah *et al.*, (2019) study. These results confirmed the crucial role of these genes in quinolones resistance.

The presence of resistance in *qnr*-negative strains indicates that other resistance mechanisms may be contributing to quinolone resistance, which conferring by other genes such as *AAC (6')-Ib* gene that causing drug modification or genes for quinolone efflux pumps (*QepA* and *OqxAB*) that enhanced efflux of drugs (Das et al., 2022).

4.9.2.4 Correlation of qnr genes and β-lactamase genes

The distribution of *qnr* genes among bacterial isolates carrying different β-lactamase genes (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{SHV}$, *bla*$_{CMY}$, *bla*$_{DHA}$) presents in table 30, the *qnrA* gene was frequently observed in *bla*$_{CTX-M}$ harboring isolates (6.3%, 5.6% for *bla*$_{CTX-M\ I}$ and *bla*$_{CTX-M\ II}$ positive isolates, respectively). The *qnrB* gene was presented across all β-lactamase groups except *bla*$_{CTX-M\ IV}$, the highest prevalence was found in *bla*$_{DHA}$ (23.8%), followed by *bla*$_{OXA}$ (17.6%) and *bla*$_{CTX-M\ II}$ (16.7%).

The *qnrC* gene was absent in all β-lactamase genes, *qnrD* was associated with *bla*$_{DHA}$ (15.4) in our study, it was presented in some groups *bla*$_{CTX-M\ II}$ (11.1%), *bla*$_{CMY\ II}$ (10.5%), *bla*$_{OXA}$ (5.9%) and *bla*$_{TEM}$ (2%). The *qnrS* gene was highly detected in *bla*$_{CTX-M\ II}$ (11.1%) followed by *bla*$_{CMY\ II}$ (10.5%).

Table 30. Distribution of *qnr* genes among different β-lactamase encoding genes.

	n (%)				
	qnrA	***qnrB***	***qnrC***	***qnrD***	***qnrS***
***bla*$_{CTX-M\ IV}$** (n=12)	0	0	0	0	1 (8.3)
***bla*$_{TEM}$** (n=98)	2 (2)	13 (13.3)	0	2 (2)	2 (2)
***bla*$_{OXA}$** (n=17)	0	3 (17.6)	0	1 (5.9)	0
***bla*$_{SHV}$** (n=29)	1 (3.4)	2 (6.9)	0	0	2 (6.9)
***bla*$_{CTX-M\ I}$** (n=16)	1 (6.3)	2 (12.5)	0	0	0
***bla*$_{CTX-M\ II}$** (n=18)	1 (5.6)	3 (16.7)	0	2 (11.1)	2 (11.1)
***bla*$_{CMY\ II}$** (n=19)	1 (5.3)	3 (15.8)	0	2 (10.5)	2 (10.5)
***bla*$_{DHA}$** (n=13)	0	3 (23.8)	0	2 (15.4)	0

A strong association between *qnr* genes and β-lactamase-producing isolates, with *qnrB* being the most prevalent, particularly in *bla*$_{TEM}$ (73.7%), *bla*$_{OXA}$ (33.3%), and *bla*$_{CTX-M}$ (60%) was found in Das *et al.*, (2022) and Juraschek *et al.*, (2022) studies. The *qnr* genes were highly found in ESBL-positive strains, especially with *bla*$_{CTX-M\ I}$ in Liu *et al.*, (2016); Salah *et al.*, (2019); Amin *et al.*, (2021) and Carey *et al.*, (2022). A significant association was found

between *qnrB* and ESBL production in Abdel Salam *et al.*, (2019), these results aligns with our findings.

The correlation matrix represented in figure 54 with color-coded values ranging between -1 and 1 showed strong positive correlation between *qnrA* and *qnrC* presence (r=0.571, p<0.001), moderate positive correlation was found between *qnrA* and *qnrS* (0.309, p=0.004). A weak positive correlation was recorded in *qnrA* with bla_{SHV}, $bla_{CMY\ II}$, $bla_{CTX\text{-}M\ I}$ and $bla_{CTX\text{-}M\ II}$ (≈0.1).

Moderate positive correlation was recorded between *qnrB* and *qnrD* (0.42, p<0.001), while weak positive correlation was found in *qnrB* with bla_{TEM} (0.1), bla_{OXA} (0.196), $bla_{CTX\text{-}M\ II}$ (0.142) and bla_{DHA} (0.16).

Regarding *qnrD*, weak to moderate positive correlation was showed with $bla_{CTX\text{-}M\ II}$ and bla_{DHA} (0.205 and 0.252, p=0.05, 0.019 respectively). Good results were showed for *qnrS*, where moderate positive correlations were found with $bla_{CTX\text{-}M\ IV}$, $bla_{CTX\text{-}M\ II}$, bla_{SHV}, $bla_{CMY\ II}$ (0.307 to 0.349, p ranged from 0.001 to 0.004). Inside β-lactamase genes, strong positive correlation was noticed between $bla_{CMY\ II}$ and bla_{OXA} with $bla_{CTX\text{-}M\ II}$ (0.893 and 0.503, respectively, p<0.001).

	qnrA	*qnrB*	*qnrC*	*qnrD*	*qnrS*	*blaCTX M IV*	*blaTEM*	*blaOXA*	*blaSHV*	*blaCMY II*	*blaCTX M I*	*blaCTX M II*	*blaDHA*	R
qnrA														
qnrB	0,058													
qnrC	0,571	-0,056												**1**
qnrD	-0,052	0,420	-0,030											**0,2**
qnrS	0,309	-0,098	-0,021	-0,052										**0,2**
blaCTX M IV	-0,036	-0,098	-0,021	-0,052	0,309									**0,2**
blaTEM	0,015	0,100	-0,141	-0,167	0,015	0,015								**0,2**
blaOXA	-0,052	0,196	-0,030	0,104	-0,052	-0,052	-0,072							**0**
blaSHV	0,142	0,011	-0,037	-0,094	0,349	0,142	0,027	-0,094						**-0,2**
blaCMY II	0,117	0,060	-0,042	0,168	0,307	-0,073	-0,065	0,442	0,324					**-0,4**
blaCTX M I	0,142	0,011	-0,037	-0,094	-0,065	-0,065	0,263	-0,094	0,131	-0,017				**-0,6**
blaCTX M II	0,142	0,104	-0,037	0,205	0,349	-0,065	-0,051	0,503	0,255	0,893	-0,117			**-0,8**
blaDHA	-0,057	0,160	-0,032	0,252	-0,057	-0,057	-0,035	0,085	-0,102	-0,114	-0,102	-0,102		**-1**

Figure. 54 : Correlation matrix of the presence of *qnr* genes with β-lactamase encoding genes in *E. coli* (Green indicated positive correlation. Blue indicated significant negative correlation. R indicated correlation coefficient).

The *qnrS* gene was positively associated with $bla_{CTX\text{-}M\ I}$ while *qnrB* was positively correlated with bla_{TEM} in Amin *et al.* study, strong positive correlation was documented between *qnrS* with bla_{TEM} and $bla_{CTX\text{-}M}$ in Li *et al.*, (2014) study. The *qnrS* gene was found in association with bla_{TEM} and $bla_{CTX\text{-}M}$ and the combination *qnrS* + *qnrB* was frequently associated with bla_{TEM} and $bla_{CTX\text{-}M\ I}$ in Salah *et al.* study. The same combination was demonstrated in Algeria

(Alouache et al., 2014). Kimera *et al.*, (2024) reported in their study that the combination $bla_{CTX\text{-}M}$ and *qnrS* was most frequent.

These findings raise the hypothesis that the *qnr* genes detected in this study could also have the same plasmid location with β-lactamase genes, especially in *qnrS* and $bla_{CTX\text{-}M}$ and bla_{TEM}. The second reason of this co-occurrence is that strains with $bla_{CTX\text{-}M}$ or bla_{TEM} are more likely to carry *qnrS* gene. This co-existence contributed to the development and spread of plasmids transmission, these plasmids conferring fluoroquinolone and β-lactam resistance in *E. coli*.

4.10 Plasmid profiling and antibiotic resistance

The 183 screened strains, that selected for molecular study, were subjected to plasmid analysis. The results revealed that 20.3% out selected strains possessed plasmids with size ranged from approximately 1.5 to >10 kb. Out of 37 strains that contained plasmid, 67.6% (25/37) harbored single plasmid, while 13.5% contained more than one plasmid. The most common plasmid of size approximately 8 kb, the majority of detected plasmids were found in *E. coli* strains 91.9% (p<0.001), followed by 5.4% in *Enterobacter* species and 2.7% in *P. aeruginosa*.

The relationship between AR and plasmid carriage was presented in figure 55, comparison of resistance rates among different antibiotics in positive-plasmid isolates was evaluated. Plasmid harboring isolates showed high resistance score for imipenem, amoxicillin, ticarcillin, ticarcillin + clavulanic acid, piperacillin and amoxicillin + clavulanic acid (83.8%, 75.7%, 67.6%, 64.9%, 62.2% and 54.1%, respectively), a good rate of resistance was showed also against trimethoprim/ sulfamethoxazole (51.4%).

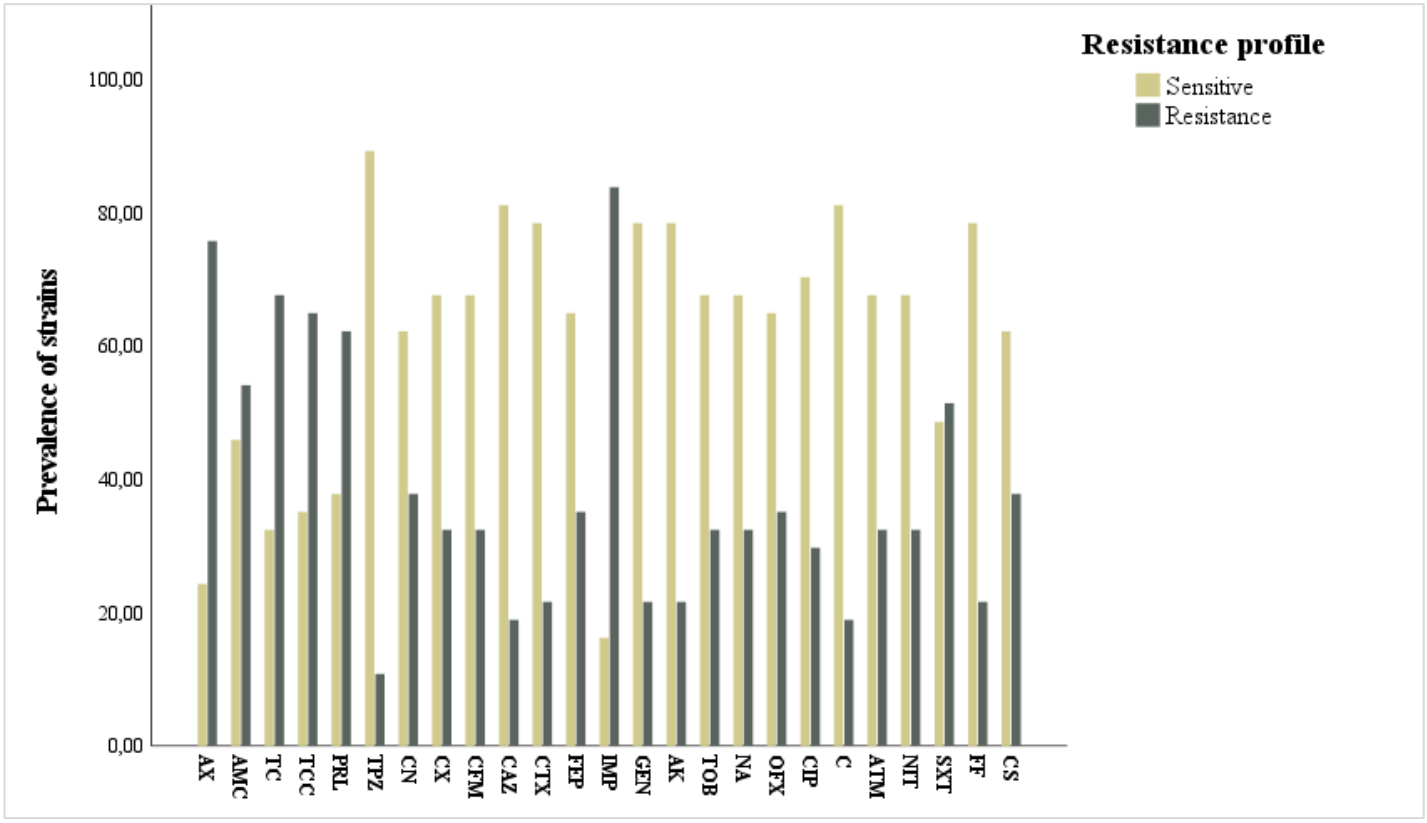


Figure. 55 : Antibiotic resistance among plasmid harboring isolates.

Enterobacteriaceae are recognized for possessing plasmids in several copies of diverse sizes (Al-Shamarti and Mohsin, 2019). The findings from the present study are in agreement with previous studies on the carriage of plasmids by members of Enterobacteriaceae especially *E. coli* with different size (Thapa Shrestha et al., 2020; Uzeh et al., 2021).

There was a correlation between resistance to amoxicillin, ticarcillin, piperacillin, ticarcillin + clavulanic acid and imipenem and the carriage of plasmids, that was also reported in Talukder *et al.*, (2021) study, who found that positive plasmid *P. aeruginosa* were resistant to amoxicillin, ampicillin, cloxacillin, cotrimoxazole, erythromycin and tetracycline. HGT of plasmid-encoded resistance genes is the predominant method for the acquisition of AR, and plasmid-encoded AR includes the majority of clinically important antibiotic classes currently in use.

The distribution of AR among isolates suggests a strong correlation between plasmid presence and multidrug resistance. A highest number of plasmid harboring isolates (n=6) exhibited resistance to 8 antibiotics, indicating that the most of positive plasmid isolates possessed MDR character. The presence of isolates resistant to 17 or more antibiotics suggested that plasmid carriage related with XDR character (Fig. 56).

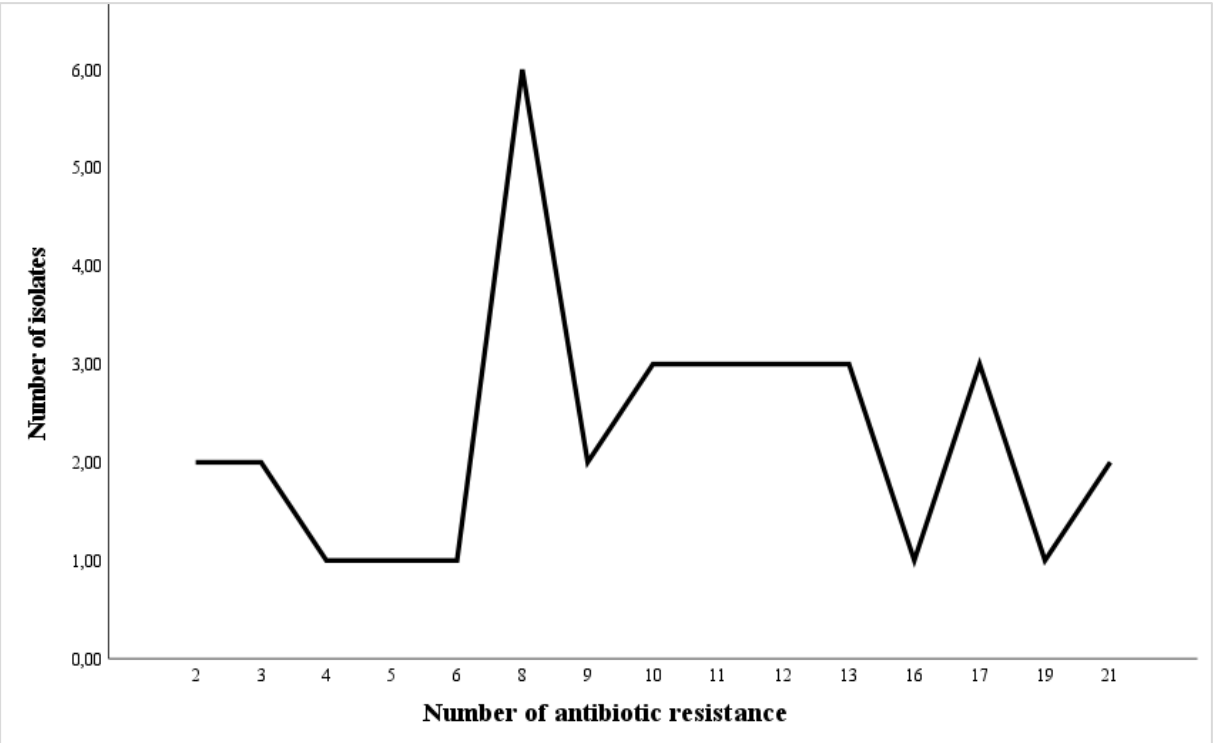


Figure. 56 : Distribution of multidrug resistance among plasmid-harboring bacterial isolates.

There was a correlation between plasmid profiles among the isolated bacteria and their multiple drug resistance in the present study, which is in accordance with the findings of Uzeh *et al.* and Thapa Shrestha *et al.*, thus suggesting the carriage of plasmids may be associated with the resistance profiles. Plasmids are regarded as the primary vectors in the acquisition and dissemination of multi-resistant traits, either phenotypically or genotypically.

However, our study reported that there was MDR and XDR profiles in our bacterial isolates without the carriage of plasmids, indicating that the latter's resistance was chromosomally borne in these strains, in contrast, the containing plasmid resistant strains acquired their plasmids (Okoye et al., 2022).

A percentage of 67.6% of plasmid containing isolates showed ESBL profile with acquisition of one or more *bla* genes, and 18.9% possessed AmpC β-lactamase genes. While for *qnr* genes, 24.3% of positive plasmid isolates have at least one *qnr* gene.

4.10.1 Presence of resistance genes in plasmid harboring isolates

The table below reveals high prevalence and presence of β-lactamase genes and quinolone genes, all resistance genes were present in variant rates with predominance of bla_{TEM} (56.8%). All the subtypes of $bla_{CTX\text{-}M}$ were present in similar percent (10.8%), the same rate was recorded for AmpC genes ($bla_{CMY\ II}$ and bla_{DHA}). Between all *qnr* genes, *qnrB* was the most presented quinolone gene in plasmid harboring isolates (16.2%) followed by *qnrA* and *qnrD* (5.4%) and finally *qnrC* and *qnrS* (2.7%). 18.9% of plasmid containing isolates showed coexistence of *bla* and *qnr* genes.

Table 31. Distribution of antibiotic resistance genes among plasmid harboring isolates.

Gene	n (%)	Gene	n (%)
$\boldsymbol{bla_{CTX\text{-}M\ IV}}$	4 (10.8)	$\boldsymbol{bla_{DHA}}$	4 (10.8)
$\boldsymbol{bla_{TEM}}$	21 (56.8)	***qnrA***	2 (5.4)
$\boldsymbol{bla_{OXA}}$	3 (8.1)	***qnrB***	6 (16.2)
$\boldsymbol{bla_{SHV}}$	1 (2.7)	***qnrC***	1 (2.7)
$\boldsymbol{bla_{CTX\text{-}M\ I}}$	3 (8.1)	***qnrD***	2 (5.4)
$\boldsymbol{bla_{CTX\text{-}M\ II}}$	4 (10.8)	***qnrS***	1 (2.7)
$\boldsymbol{bla_{CMY\ II}}$	4 (10.8)		

It has been reported that AR genes, which encode resistance to multiple antibiotic classes such as fluoroquinolones, aminoglycosides, and β-lactams, are carried on plasmids and can be transmitted from bacteria to bacteria through HGT within bacterial communities.

Many papers reported the production of β-lactamase genes with isolates possessed plasmids (Thapa Shrestha et al., 2020; Negeri et al., 2023), this can be explained that the resistance to β-lactams and quinolones in these strains was originated by antimicrobial plasmids that contains the resistance genes. Resistance to other antibiotic classes may result from the potential dissemination of additional clinically significant resistant genes, including those that confer resistance to trimethoprim/sulfamethoxazole, ciprofloxacin, amoxicillin + clavulanic acid, gentamicin, amikacin, and cefoxitin (Negeri et al., 2023).

Plasmid analysis in Li *et al.*, (2019) study showed that plasmid carrying isolates contained also $bla_{CTX\text{-}M}$, bla_{TEM} and *qnrS*. The presence of resistance genes in plasmids lead to the spread of

multi-resistance. The detection of *bla* genes in plasmid harboring isolates indicates that these germs can easily transfer ESBL and AmpC genes to other pathogens. Also, the presence of *qnr* genes lead to rapid spread of quinolones resistance even in the absence of these antibiotics.

4.10.2 Relationship of plasmids with virulence factors

The prevalence of different virulence factors in harboring plasmid and non plasmid strains was presented in figure 57. Biofilm formation remained the most prevalent virulence factor, with a slightly higher occurrence in plasmid-containing strains (89.2%) compared to non-plasmid strains (86.2%). Similar prevalence was recorded for hemolysin production in both groups (55.9% vs. 54.1%), protease production was negatively correlated with plasmid containing (p=0.004) where the high prevalence was found in non plasmid strains (53.1%), the same trends were showed in lecithinase production (p=0.04). Lipase production was less prevalent with noticeable reductions in plasmid-harboring strains (15.9% to 5.4%).

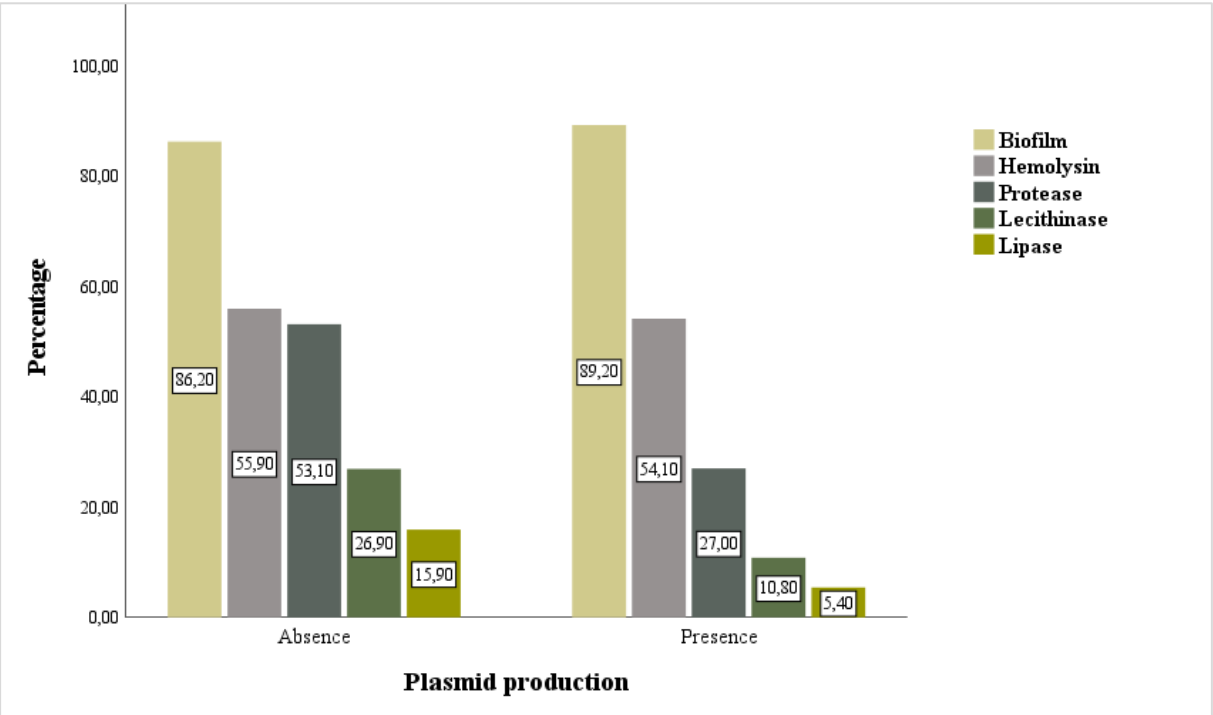


Figure. 57 : Prevalence of virulence factors in plasmid containing and non plasmid strains.

The statistical analysis of virulence factor prevalence in plasmid-harboring and non-plasmid bacterial strains indicating that there is no statistically significant association between plasmid presence and the biofilm and hemolysin production but minor variations in prevalence rates were observed across the two categories. The same observations were found by Qi *et al.*, (2016),

where no differences in biofilm formation were observed among strains with different plasmid profiles.

Meanwhile, several papers reported that the presence of plasmids was associated with AR and biofilm formation, genes encoded proteins and virulence factors are also located in plasmids. In contrast to our findings, where protease, lecithinase and lipase production was higher in non plasmid strains; suggested that the presence of plasmids alone may not be a primary determinant of virulence factor expression in the studied bacterial population. However, further analysis including larger sample sizes, functional assays, molecular analyses and detailed plasmid map are needed to figure out the influence of plasmid on biofilm, hemolysin, protease, lecithinase and lipase capacities.

Conclusion and perspectives

Antibiotic resistance (AR) is one of the most pressing global health threats, severely compromising the treatment of bacterial infections. The emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and highly virulent strains has led to treatment failures, limited therapeutic options, and increased healthcare burdens. Beyond clinical settings, AR is now recognized across other reservoirs, such as animals, water, and soil, reinforcing the importance of a One Health approach.

This study was conducted over a three-year period (2021–2023) in the Sétif province of northeastern Algeria. It aimed to determine the epidemiological and bacteriological profiles of major clinical bacterial strains isolated from urine, pus, blood, vaginal swabs, and medical devices. Additionally, it assessed the evolution of AR over these years and explored the associated resistance mechanisms, virulence factors, and phylogenetic traits.

A wide range of bacterial strains was identified, with Gram-negative bacteria (GNB) being more prevalent than Gram-positive bacteria (GPB). *Escherichia coli* was the dominant species among Enterobacteriaceae, while *Staphylococcus aureus* was the most common GPB. Over the study period, an increasing trend of resistance was observed, especially in 2023, where resistance to cephalosporins, quinolones, phenicols, and monobactams rose significantly. XDR strains increased steadily across the years, indicating worsening resistance profiles and more complex treatment challenges.

Antibiotic susceptibility testing revealed a high level of resistance to first-line antibiotics. Fourth-generation cephalosporins, particularly cefepime, showed declining efficacy, while carbapenems lost substantial activity, notably against *E. coli*, *Proteus spp.*, and *Morganella morganii*. Tobramycin was less effective against *Pseudomonas aeruginosa*, *Enterobacter spp.*, and GPB. Although nitrofurantoin, colistin, and fosfomycin were considered effective options, significant resistance was also observed. Only amikacin retained strong activity across all tested strains.

Phenotypic analysis revealed a high prevalence of resistance profiles such as ESBL, AmpC, MRSA, CMLSB, IMLSB, and hypervirulent *Klebsiella pneumoniae* (hvKp). Biofilm formation was notably associated with resistance to polymyxins and amoxicillin, and was especially

strong in XDR strains. Hemolysin and protease were the most frequently produced virulence enzymes, with hemolysin strongly linked to XDR strains, while MDR strains were more likely to produce protease, lecithinase, and lipase.

Molecular analyses via multiplex PCR revealed widespread distribution of resistance genes, including *blaTEM*, *blaSHV*, *blaCTX-M* (groups I, II, IV), *blaOXA*, and AmpC genes such as *blaCMY II* and *blaDHA*. These genes were often plasmid-borne, facilitating rapid dissemination. The combined ESBL/AmpC genotype demonstrated the broadest resistance across antibiotic classes. Statistical correlations showed strong associations between certain *bla* genes and specific virulence factors, particularly hemolysin, protease, and lipase, suggesting a link between resistance and pathogenicity.

Phylogenetic analysis of imipenem-resistant *E. coli* from urinary tract infections revealed a predominance of the B2 group, followed by the E group. These phylogroups carried multiple resistance and virulence genes, with B2 harboring the highest load. Despite broad resistance, aminoglycosides remained among the most effective treatments for these strains.

Overall, between 2021 and 2023, this study documented a clear and concerning rise in AR, especially in 2023, across several antibiotic classes and resistance phenotypes. Our region, like the rest of the world, has witnessed a significant increase in resistant strains, which has led to the concerted efforts of international governmental and nongovernmental agencies to combating AR and to minimize the health and economic burdens on people. Further, recommended measures were needed to achieve these goals:

- Establishing infection prevention and control committees in healthcare settings.
- good microbiology practice, particularly good hand hygiene practices to reduce infection transmission.
- Proper diagnosis and successful treatment of bacterial infections.
- Encouraging the responsible use of antibiotics and fight against self-medication, probabilistic antibiotic therapy, overconsumption, and the anarchy in antibiotic prescription to prevent unnecessary resistance development.
- Continuous surveillance and monitoring of antibiotic use and AR.
- Good microbiological laboratory practices and raising awareness among all healthcare professionals: doctors, microbiologists, caregivers, hygienists.

Prevention is still the best strategy to reduce AR infections and their spread globally. urgent efforts must also be directed toward the development of novel, effective antimicrobial agents, alternatives to antibiotics, and innovative progress in diagnostics and vaccine development.

In the perspective of this work, it would be interesting to:

- To more focusing on genetic characterization of AR in all strains even GNB or GPB and identified the genetic support related with resistance to several antibiotic classes.
- Identified the genetic support in MRSA, IMLSB and CMLSB profiles.
- Characterize different virulence genes associated with the presence of phenotypic virulence profiles (biofilm, hemolysin, protease, lecithinase and lipase).
- Enzymatic characterization by zymography technic.
- Identified all the clones in all isolated strains (GNB and GPB) by MLST technic (Multilocus Sequence Typing) and determined genetic diversity and evolutionary relationships between isolates.

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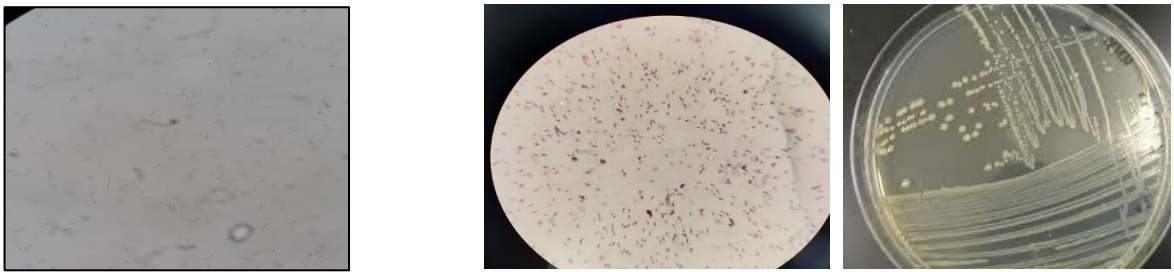
5 Annexes

Annex 1

Table 1. Culture media according to nature of sampling.

Nature of sampling	**Systematic media**
Urine	Chapman, nutrient agar
Pus	Chapman, chocolate blood agar, nutrient broth
Blood	Chapman, blood agar, chocolate blood agar
Vaginal swab	Chapman, chocolate blood agar, nutrient broth
Catheter	Blood agar, MacConkey agar



a b c

c d

Identification of strains (a: microscopic examination, b: Gram staining, c: bacterial culture, d: classic biochemical gallery, e: API 20E gallery).

Annex 2

Table2. Reading table of API 20E gallery for Enterobacteriaceae.

Caractere	**Active ingredient**	**Enzyme/reaction**	**Reagent**	**Results**	
				Positive	**Negative**
ONPG	ONPG	β-galactosidase	-	Yellow	Incolor
ADH	Arginine	Arginine dihydrolase	-	Red/orange	Yellow
LDC	Lysine	Lysine decarboxylase	-	Red/orange	Yellow
ODC	Ornithine	Ornithine decarboxylase	-	Red/orange	Yellow
CIT	Citrate	Citrate utilisation	-	Bleu	green
H2S	S_2O_3	Thiosulfate reductase	-	black	Incolor
URE	Urea	Urease	-	Red	Yellow
TDA	Tryptophane	Tryptophan deaminase	Ferric chloride	Brown	Yellow
IND	Tryptophane	Tryptophanase	Kovacs	Red	Incolor
VP	Pyruvate	Acetoin production	VP1 (KOH) + VP2 (α-naphthol)	Red	Incolor
GEL	Gelatine	Gelatinase	-	Black	Incolor
GLU	Glucose	Fer	-	Yellow	Bleu
MAN	Mannose	Fer	-		
INO	Inositol	Fer	-		
SOR	Sorbitol	Fer	-		
RHA	Rhamnose	Fer	-		
SAC	Sucrose	Fer	-		
MEL	Melibiose	Fer	-		
AMY	Amygdalin	Fer	-		
ARA	Arabinose	Fer	-		

ONPG: ortho-Nitrophenyl-β-galactoside, S_20_3: thiosulfate, Fer: fermentation.

Table 3. Reading table of API Staph gallery.

Caractere	Active ingredient	Enzyme/reaction	Reagent	Results	
				Positive	**Negative**
0	No substrate		Negative control	-	Red
GLU	Glucose	Aci	-	Yellow	Red*
FRU	Fructose	Aci	-		
MNE	Mannose	Aci	-		
MAL	Maltose	Aci	-		
LAC	Lactose	Aci	-		
TRE	Trehalose	Aci	-		
MAN	Mannitol	Aci	-		
XLT	Xylitol	Aci	-		
MEL	Melibiose	Aci	-		
NIT	Potassium nitrate	Reduction of nitrates to nitrites	NIT 1 + NIT 2	Red	Colorless-light pink
PAL	β-naphthyl phosphate	Alkaline Phosphatase	ZYM A + ZYM B	Violet	Yellow
VP	Pyruvate	Acetoin production	VP 1 + VP 2	Violet-pink	Colorless-light pink
RAF	Raffinose	Aci	-	Yellow	Red
XYL	Xylose	Aci	-		
SAC	Saccharose	Aci	-		
MDG	methyl-α-glucopyranoside	Aci	-		
NAG	N-acetyl-glucosamine	Aci	-		
ADH	Arginine	Arginine dihydrolase	-	Orange-red	Yellow
URE	Urea	Urease	-	Red-violet	Yellow

*: When MNE and XLT are preceded or followed by positive tests, then an orange test should be considered negative,

Aci: acidification.