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# Caractérisation moléculaire des Entérobactéries productrices de bêta-lactamases à spectre étendu et de carbapénèmases isolées des 3 compartiments (Humain, animaux et environnement) dans une approche “One Health”

Dina Daaboul

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Caractérisation moléculaire des Entérobactéries productrices de bêta-lactamases à spectre étendu et de carbapénèmases isolées des 3 compartiments (Humain, animaux et environnement) dans une approche ‘‘One Health’’

**Molecular characterization of Enterobacteriales producing extended-spectrum beta-lactamases and carbapenemases isolated from the 3 compartments (Humans, animals and environment) in a ‘‘One Health’’ approach.**

**Thèse de doctorat de l'université Paris-Saclay et de l'université Libanaise**

École doctorale n°569, Innovation thérapeutique : du fondamental à l'appliqué Spécialité de doctorat : Microbiologie

Graduate school : Santé et médicaments. Référent : Faculté de pharmacie

Thèse préparée dans l'unité de recherche Immunologie des maladies virales, auto-immunes, hématologiques et bactériennes (Université Paris-Saclay, Inserm, CEA), sous la direction du docteur Thierry NAAS et dans le laboratoire de microbiologie Santé et Environnement LMSE sous la direction de Professeur Fouad DABOUSSI et la co-direction du docteur Marwan OSMAN

**Thèse soutenue à Tripoli, Liban, le 22 Décembre 2023, par**

**Dina DAABOUL**

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## Résumé

Les bactéries résistantes aux antibiotiques et plus particulièrement les Entérobactéries productrices de carbapénèmases (EPC) augmentent à l'échelle mondiale, et constitue une menace pour la santé publique mondiale avec des infections bactériennes qui sont de plus en plus difficiles à traiter. Les EPCs, identifiées dans tous les compartiments, humains, animaux et environnementaux, nécessitent une approche globale dite 'One health' pour pouvoir les combattre efficacement. Le Liban, pays à faible revenus, souffrant d'une crise économique impactant sérieusement son système de santé, doit également prendre en charge de nombreux réfugiés venant des pays voisins. Les données épidémiologiques et moléculaires de la résistance aux antibiotiques au Liban chez l'homme, les animaux et dans le secteur alimentaire restent rares. L'objectif de cette thèse est de mieux comprendre l'épidémiologie des EPC, les voies de disséminations des bactéries et des gènes de résistance entre les différents compartiments au Liban. Une première étude, menée sur des isolats cliniques a révélé une dissémination principalement de *Escherichia coli* résistantes aux carbapénèmes, suggérant une dissémination communautaire importante. Les carbapénèmases identifiées appartenaient majoritairement à la classe B et D de Ambler, et une prévalence élevée des souches de *E. coli* productrices de *bla<sub>NDM-5</sub>* a été signalée. Une localisation plasmidique de 2 metallo  $\beta$ - lactamases chez une même souche de *Enterobacter* a été mise en évidence. Une deuxième étude menée au sein des camps de réfugiés syriens afin d'étudier la prévalence des gènes de résistance et la structure de la population des isolats résistants aux carbapénèmes a mis en évidence une situation alarmante avec la détection de souches multirésistantes chez les réfugiés, chez les animaux élevés dans les camps et dans l'environnement et tout particulièrement dans une rivière traversant ces camps. Enfin, nous avons mené une troisième étude sur des isolats vétérinaires pour explorer la présence et le portage de déterminants de la résistance aux antibiotiques. Nous avons ainsi pu décrire pour la première fois la présence du gène *bla<sub>NDM-5</sub>* chez des entérobactéries isolées d'animaux de compagnie. Nos résultats ont révélé une dissémination des EPC isolées à l'hôpital mais aussi chez les animaux, suggérant que ces derniers puissent être des réservoirs de bactéries multirésistantes. Ces résultats sont alarmants et soulignent la nécessité urgente de réévaluer la gestion efficace des antimicrobiens afin de limiter l'émergence de la résistance aux antibiotiques au Liban.

## Abstract

Antibiotic-resistant bacteria, and more particularly carbapenemase-producing Enterobacterales (CPE), continue to increase globally, posing a threat to global public health, with bacterial infections that are increasingly difficult to treat. CPE identified in all compartments, human, animal and environment, require a global approach called 'One health' to be able to combat them effectively. Lebanon, a low-income country suffering from an economic crisis seriously impacting its health system, must also take care of many refugees from neighboring countries. Epidemiological and molecular data on antibiotic resistance in Lebanon in humans, animals and in the food sector remain scarce. The objective of this thesis is to better understand the epidemiology of CPE and the pathways of dissemination of bacteria and resistance genes between the different compartments in Lebanon. A first study, carried out on clinical isolates, revealed a dissemination mainly of *Escherichia coli* resistant to carbapenems, suggesting significant community dissemination. The identified carbapenemases predominantly belonged to Ambler class B and D, and a high prevalence of *bla*<sub>NDM-5</sub>-producing *E. coli* strains was reported. Likewise, a plasmid localization of 2 metallo  $\beta$ -lactamases in the same strain of *Enterobacter* was demonstrated. A second study carried out in Syrian refugee camps to study the prevalence of resistance genes and the population structure of carbapenem-resistant isolates highlighted an alarming situation with the detection of multi-resistant strains among refugees, in animals raised in the camps and in the environment and particularly in a river crossing these camps. Finally, we conducted a third study on veterinary isolates to explore the presence and carriage of antibiotic resistance determinants. We were thus able to describe for the first time the presence of the *bla*<sub>NDM-5</sub> gene in Enterobacterales isolated from companion animals. Our results revealed a dissemination of carbapenem resistance genes in Enterobacterales isolated in hospitals but also in animals, suggesting that the latter could be reservoirs of multi-resistant bacteria. These results are alarming and highlight the urgent need to reassess effective antimicrobial stewardship to limit the emergence of antibiotic resistance in Lebanon.

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## **Abréviations**

ABC: ATP-binding cassette transporter

AMR: Antimicrobial resistance

BEL-1: Belgium extended-spectrum  $\beta$ -lactamase

BES-1: Brazilian extended-spectrum  $\beta$ -lactamase

BLSE :  $\beta$  -lactamase à spectre étendu

BMR : Bactéries multirésistantes

CA-SFM : Société Française de Microbiologie

CHDL: Carbapenem-hydrolyzing class D  $\beta$ -lactamases

CMI : Concentration minimale inhibitrice

C3G : Céphalosporines de 3ème génération

DAP: Acide diaminopimélique

DHP-1: Déhydropeptidase

EDTA: Acide éthylène diamine tétra-acétique

EPC: Entérobactéries productrices de carbapénèmase

ERC: Entérobactéries résistantes aux carbapénèmes

GES-1: Guiana Extended-Spectrum  $\beta$ -lactamase

IMI: Imipenem-hydrolyzing  $\beta$ -lactamase

IMP: Imipenemase

KPC: Klebsiella pneumoniae carbapenemase

LPS: Lipopolysaccharide

MATE: Multidrug and Toxic Compound Extrusion

MFS: Major Facilitator Superfamily

NAG: N-acétylglucosamine

NAM: Acide N-acétylmuramique

NAM-PP: Acide N-acétylmuramique-Pentapeptide

NDM: New-Delhi metallo  $\beta$ -lactamase

NMC-A: non-métallo carbapenemase

OMS: Organisation Mondiale de la santé

PER: Pseudomonas Extended Resistance

PLP: Protéines Liant les Pénicillines

PBP: Penicillin-Binding-Protein

RND: Resistance Nodulation Cell Division

SASM : *Staphylococcus aureus* sensible à la méticilline

SFO-1: *Serratia fonticola*

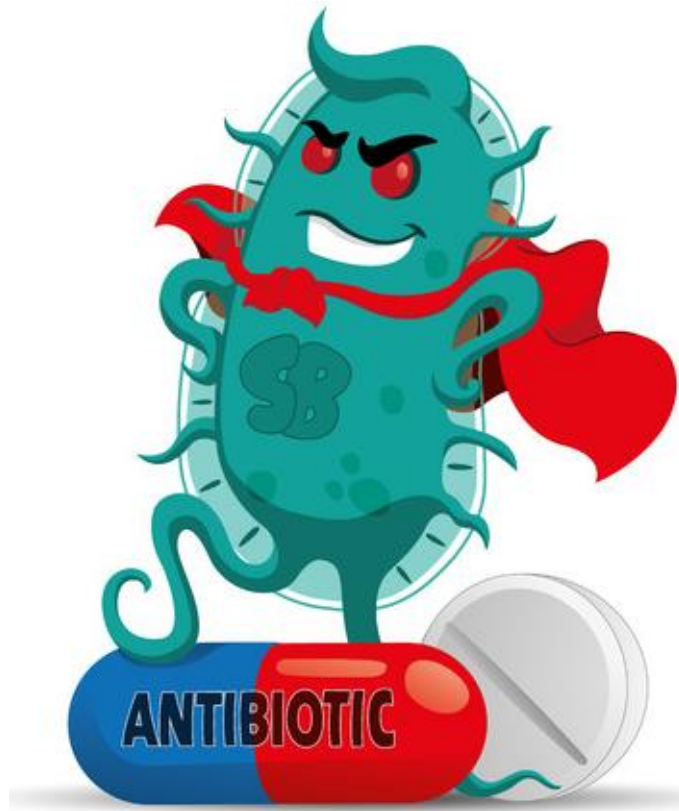
SME: *Serratia marcescens* enzyme

SMR: Small Multidrug Resistance

TLA-1: Tlahuicas, tribu indienne

VIM: Verona Integron-encoded Métallo-  $\beta$  -lactamase

VEB-1: Vietnamese Extended-spectrum  $\beta$ -lactamase



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## Chapitre 1 : Etude bibliographique

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Les bactéries multirésistantes (BMR) à Gram négatif, et particulièrement les Entérobactéries résistantes aux carbapénèmes (ERC), émergent à l'échelle mondiale. La résistance aux antibiotiques (AMR), constitue un problème majeur de santé publique, conduisant à des infections bactériennes de plus en plus difficile à traiter traitement des voire même à des impasses thérapeutiques. Selon l'organisation mondiale de la santé (OMS), les ERC représentent la plus grande menace avec un besoin critique de développer de nouvelles molécules antibactériennes (1).

## **I. Bacilles à Gram négatif (BGN)**

### **1. Les entérobactéries**

Au cours des deux dernières décennies, la famille des Entérobactéries, a subi des changements phylogénétiques importants. La famille des Entérobactéries, comprend des bacilles à Gram-négatif (BGN), capables de croître à la fois en aérobie et en anaérobie. La majorité de ses membres sont mobiles grâce à plusieurs flagelles disposés tout autour de la bactérie leur donnant une mobilité dite péritriche.

Ces bactéries sont retrouvées dans l'environnement (sol, eau et plantes) mais aussi font partie de la flore normale du tube digestif de l'homme et des animaux. En ce qui concerne leur pathogénicité, on distingue celles qui peuvent être accidentellement pathogènes dites pathogènes opportunistes (*Escherichia coli*), alors que d'autres telles que *Salmonella* et *Shigella*, sont strictement pathogènes pour l'homme (2). Les pathogènes opportunistes peuvent causer des pathologies chez des sujets immunodéprimés, mais leur pouvoir pathogène est insuffisant pour le développement spontané d'une pathologie chez un individu sain. Par exemple, *E. coli* constitue la première espèce responsable d'infections urinaires chez l'homme tout en étant un commensal de la flore intestinale. En ce qui concerne les pathogènes stricts, plusieurs facteurs de virulence sont impliqués dans la sévérité de la pathologie. Des souches de *E. coli* pathogènes strictes, produisant des toxines (*Shiga* toxines), sont responsables de formes sévères de pathologies digestives. A noter que les Entérobactéries peuvent être responsables de nombreuses infections nosocomiales du fait de leur capacité à coloniser le matériel médical. Les espèces les plus fréquemment associées à des infections chez l'homme sont : *Escherichia*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Proteus*, *Serratia*, *Shigella*, *Salmonella*.

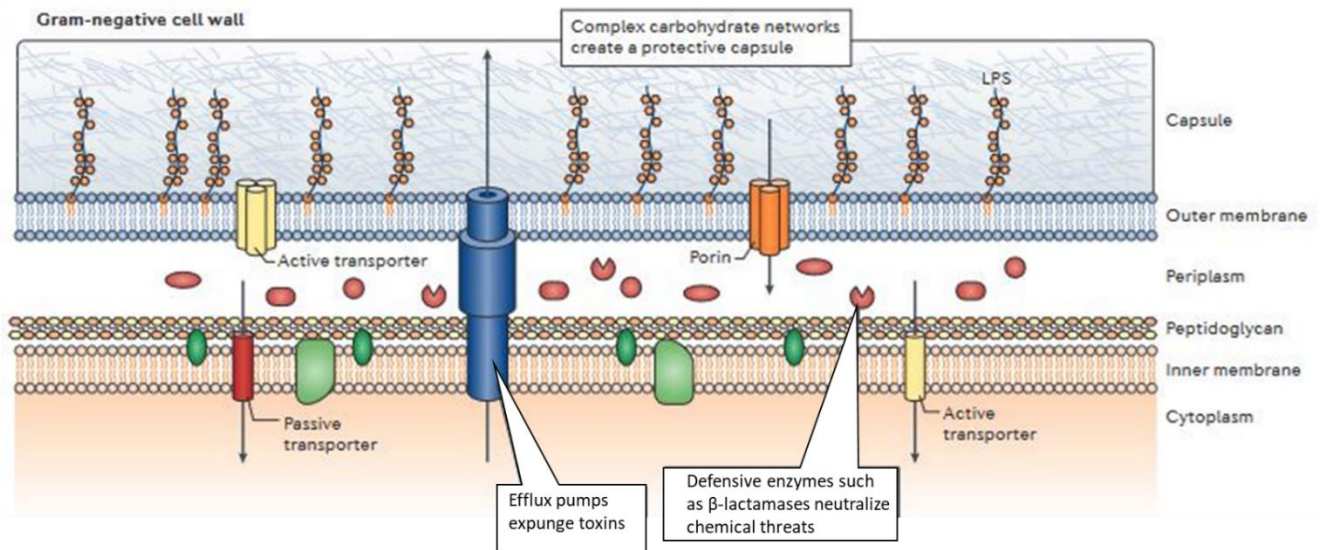


## **2. Les non-fermentants**

Les bacilles à Gram négatif non fermentants sont des bactéries de l'environnement responsables d'infections opportunistes chez les patients immunodéprimés. En plus de leur multirésistance naturelle aux antibiotiques, des mécanismes acquis peuvent être observés. Bien que les carbapénèmes soient les plus utilisés pour le traitement des infections potentiellement mortelles, ces bactéries acquièrent de plus en plus de résistance aux carbapénèmes. *Acinetobacter baumannii* et *Pseudomonas aeruginosa* résistants aux carbapénèmes sont classés comme agents pathogènes critiques par l'OMS (3).

## **3. Structure de la paroi des Bacilles à Gram Négatifs**

La paroi comprend 3 couches principales : La membrane externe, l'espace périplasmique contenant une couche de peptidoglycane et la membrane interne ou cytoplasmique (**Figure 1**). La présence de la membrane externe permet de distinguer la paroi des bactéries Gram négatives de celle des bactéries Gram positives. Le peptidoglycane qui est un polymère structurel qui confère la forme à la bactérie et de résister aux différences de pression osmotique entre le cytoplasme et l'extérieur de la bactérie. La paroi bactérienne est une structure complexe, essentielle au maintien de la forme et de l'intégrité structurelle des cellules (4).



**Figure 1: Représentation schématique de la paroi des bactéries à Gram-négatif. D'après Tommasi et al. (5).**

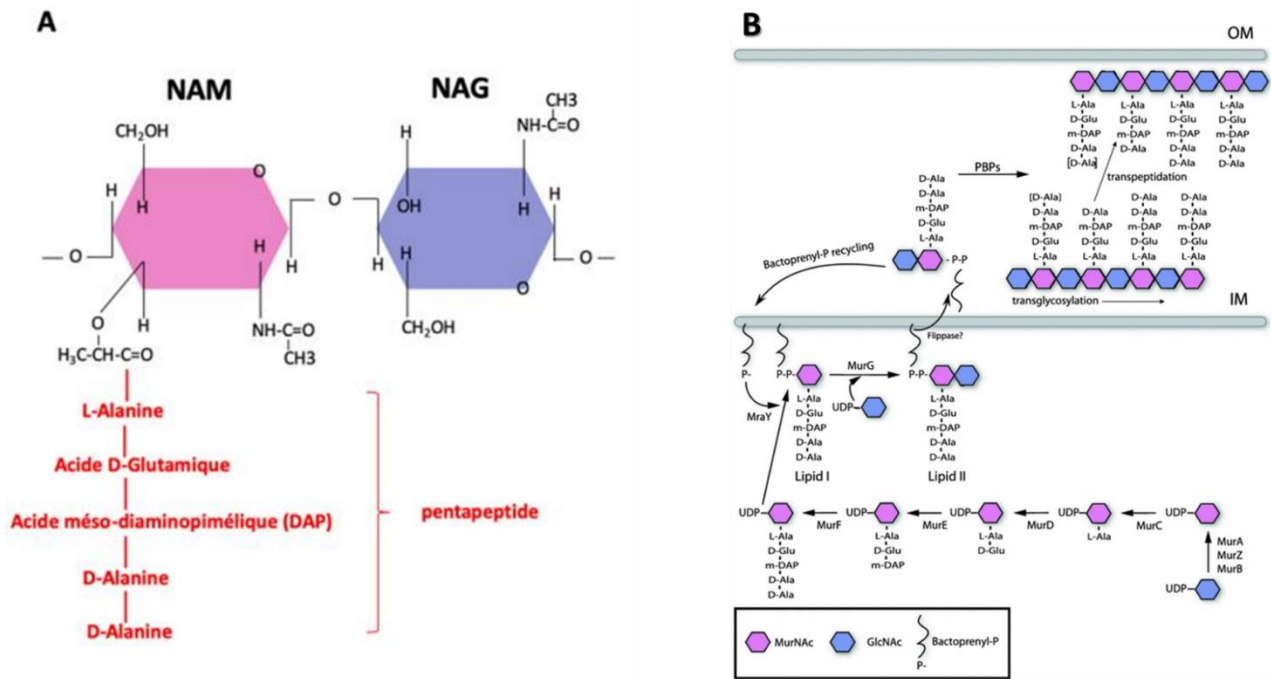
### a. Membrane externe

Cette membrane est caractéristique des bactéries Gram-négatif. Elle se présente sous forme d'une bicouche phospholipidique. La lipoprotéine de Braun, majoritairement présente dans sa partie interne, est attachée de façon covalente au peptidoglycane sous-jacent ainsi elle permet un ancrage de la membrane externe au peptidoglycane. Le lipopolysaccharide (LPS), qui est un glycopeptide, est présent dans sa partie externe (6) (**Figure 1**). Le LPS est responsable du choc endotoxique associé avec la septicémie causée par les microorganismes à gram négatif (6). Le système immunitaire humain est sensibilisé par cette molécule, qui constitue un indicateur d'infection. Le LPS constitue une véritable barrière pour les bactéries à Gram négatif puisqu'il les protège des substances hydrophobes (sels biliaries) et de masse moléculaire élevée (protéases, lipases). Les porines qui sont des protéines transmembranaires assure le transport de molécules hydrophiles de faible masse moléculaire comme les sucres, les acides aminés mais également les antibiotiques (tels que les  $\beta$ -lactamines hydrophiles), et en même temps empêchent l'entrée de grosses molécules grâce à leur structure formée de trois sous-unités. Donc, le transport passif de

ces molécules dépend de leurs masses moléculaires mais également de leurs formes et de leurs charges (7, 8).

### **b. Peptidoglycane**

Le peptidoglycane est un composant essentiel et spécifique de la paroi cellulaire bactérienne qui se trouve à l'extérieur de la membrane cytoplasmique de presque toutes les bactéries. Le peptidoglycane est une structure tridimensionnelle en réseau. Il est composé de chaînes polysaccharidiques formées de dimères de résidus alternés de N-acétylglucosamine (NAG) et d'acide N-acétylmuramique (NAM) qui sont reliés entre eux via des liaisons osidiques de type  $\beta$  (1-4) (**Figure 2.A**). La synthèse du peptidoglycane se fait en plusieurs étapes: dans un premier temps, dans le cytoplasme bactérien, chaque unité de NAM est liée à un pentapeptide (NAM-PP) composé en général du L-Ala-D-Glu-DAP-D-Ala-D-Ala, où DAP représente l'acide diaminopimélique. Le NAM-PP est ensuite expédié vers la membrane plasmidique, où le NAG y est fixé (NAG-NAM-PP). Ce complexe traverse alors la membrane cytoplasmique. Enfin, dans l'espace intermembranaire (ou périplasme), il y a polymérisation des précurseurs entre eux pour former les chaînes glucidiques, qui seront reliées entre elles par des ponts peptidiques (9). La réaction de pontage entre deux chaînes de glycanes est catalysée par des enzymes les DD-peptidases, aussi appelées protéines liant les pénicillines (PLPs) (ou « Penicillin Binding Proteins » (PBPs) en anglais) qui sont des transpeptidases (10) ou des carboxypeptidases et des transglycosylases (**Figure 2.B**).



**Figure 2 : A. Composition d'une sous-unité du peptidoglycane ; B. Représentation schématique de la synthèse du peptidoglycane (PG).** Elle est initiée par la synthèse du pentapeptide disaccharide (précurseurs) dans le cytosol (GlcNac-MurNac-L-Ala-D-Glu-DAP-D-Ala-D-Ala), qui est ensuite transféré au niveau de l'espace périplasmique via la formation d'un complexe lipidique avec le bactoprenyl. Les précurseurs monomères sont complexés entre eux via les PLPs permettant la transglycosylation et la transpeptidation. *D'après Cava et al. (11).*

### c. Protéines liant les Pénicillines PLP (Penicillin-Binding Protein PBP) ou les DD-peptidases

Les PLPs sont des enzymes très importantes car elles sont responsables de l'assemblage du peptidoglycane, ce dernier assurant la rigidité de la paroi et la protection de la bactérie contre le milieu extérieur. Le nombre et la nature des PLPs varient selon les espèces bactériennes, on distingue deux grands groupes en fonction de leur poids moléculaire : celles à haut poids moléculaire qui ont une activité transpeptidase et transglycosidase et celles de faible poids moléculaire pourvues d'une activité carboxypeptidase. Toutes les PLPs sont des enzymes à sérine active, à l'exception de la DD-peptidase de *Streptomyces albus* qui est une enzyme faisant intervenir des ions zinc ( $Zn^{2+}$ ) au niveau de son site actif (12). Les PLPs sont ancrées dans la membrane cytoplasmique avec leur site actif dirigé vers l'espace périplasmique. La réticulation

du peptidoglycane se fait via deux réactions: la transpeptidation et la carboxypeptidation. La transpeptidation conduit à la formation d'une liaison peptidique entre une D-Alanine d'un peptide et la fonction NH<sub>2</sub> libre de l'acide diaminopimélique (DAP) du peptide adjacent avec l'élimination d'un résidu D-Alanine (13). Cette réaction est catalysée par une transpeptidase qui se fixe à l'extrémité D-Alanyl-D-Alanine formant un acyl-enzyme instable, l'attaque nucléophile par la fonction NH<sub>2</sub> libre du DAP permet la libération de l'enzyme et la formation de la liaison peptidique. La réaction de carboxylation permet l'élimination d'un résidu D-Alanine terminal. La carboxypeptidase se fixant également à l'extrémité D-Alanyl-D-Alanine engendrant la formation d'un acyl-enzyme instable qui est hydrolysé en présence d'une molécule d'eau.

## II. Les Antibiotiques

### 1. Histoire

En 1928, Alexandre Fleming, a découvert le premier antibiotique : la pénicilline. Il a constaté qu'une culture de *Staphylococcus aureus* sur boîte de pétri, effectuée avant de partir en vacances, contaminé par un champignon du genre *Penicillium*, a été inhibée par ce dernier. Pour cela, il a suggéré que les champignons secrètent une substance capable d'inhiber la croissance bactérienne (14). C'est après 12 ans, en 1940, que Florey et Chain ont réussi à extraire la molécule de *Penicillium notatum* et à démontrer l'efficacité de son activité antibactérienne et l'absence de toxicité chez la souris (15). De même, en 1931, une autre substance, le Prontosil, a été découverte par Gerhard Domagk, capable de traiter les infections liées aux *Streptocoques* (16). Ces 2 molécules ont été largement utilisées durant la deuxième guerre mondiale pour traiter les infections bactériennes, telle que les septicémies.

En 1943, le microbiologiste Waksman, a défini les antibiotiques comme « des substances chimiques produites par des microorganismes capables d'inhiber le développement et de détruire les bactéries et d'autres microorganismes ».

Suite à la découverte de ces premiers antibiotiques, un grand nombre de molécules a été isolé à partir des bactéries du genre *Streptomyces*, qui sont des bactéries à Gram-positif et sont à l'origine de 80% des antibiotiques connus (17) (**Figure 3**).



Figure 3 : Chronologie de la découverte d'antibiotiques. D'après Procopio et al. (17).

## 2. Généralités

Les antibiotiques sont soit des molécules naturelles, semi-synthétiques ou synthétiques. Les molécules naturelles sont produites par des bactéries ou champignons. Elles peuvent être bactéricides c'est-à-dire capables de tuer les bactéries sensibles ou bactériostatiques, c'est à dire capables d'inhiber la croissance bactérienne. Les molécules semi-synthétiques sont des molécules naturelles modifiées par l'addition des groupements chimiques afin d'améliorer leur activité. Et enfin, les molécules synthétiques sont des analogues ou dérivés des molécules naturelles.

On peut regrouper les différentes familles d'antibiotiques selon leur mécanisme d'action ou leur cible (**Tableau 1**). Les principales familles d'antibiotiques peuvent agir sur: (i) la synthèse de la paroi bactérienne par inhibition de la synthèse du peptidoglycane, (ii) sur la membrane plasmidique en perturbant la perméabilité membranaire, (iii) la synthèse protéique (action sur les ribosomes), (iv) Inhibiteurs de la synthèse d'acides nucléiques, et enfin (v) sur les voies métaboliques essentielles.

**Tableau 1 : Classification des antibiotiques en fonction de leur mode d'action**

<b>MODE D'ACTION</b>	<b>ANTIBIOTIQUES</b>
<b>INHIBITION DE LA SYNTHÈSE DE LA PAROI</b>	β-lactamines Vancomycine Éthionamide et isoniazide fosfomycine
<b>ALTERATION DE LA FONCTION DE LA MEMBRANE PLASMIDIQUE</b>	Polymyxines Daptomycine
<b>INHIBITION DE LA SYNTHÈSE NUCLEIQUE</b>	Rifampicine Quinolones
<b>INHIBITION DE LA SYNTHÈSE PROTEIQUE</b>	Aminoglycosides ou aminosides Cyclines Phénicolés Macrolides Oxazolidinones
<b>INHIBITION DES VOIES METABOLIQUES</b>	Sulfamides Triméthoprim

### **3. L'utilisation des antibiotiques :**

Certaines classes d'antibiotiques sont réservées plus ou moins exclusivement aux humains, en particulier ceux utilisés pour traiter la tuberculose (par exemple, l'isoniazide) ou d'autres infections pour lesquelles les animaux ne sont généralement pas traités (par exemple, les bovins atteints de tuberculose bovine sont généralement euthanasiés plutôt que traités). Quelques autres sont limités à un usage vétérinaire (par exemple, les flavophospholipols, les ionophores), principalement en raison de leur toxicité pour l'homme. Cependant, la grande majorité des classes d'antimicrobiens sont utilisées à la fois chez les humains et les animaux, y compris les mammifères

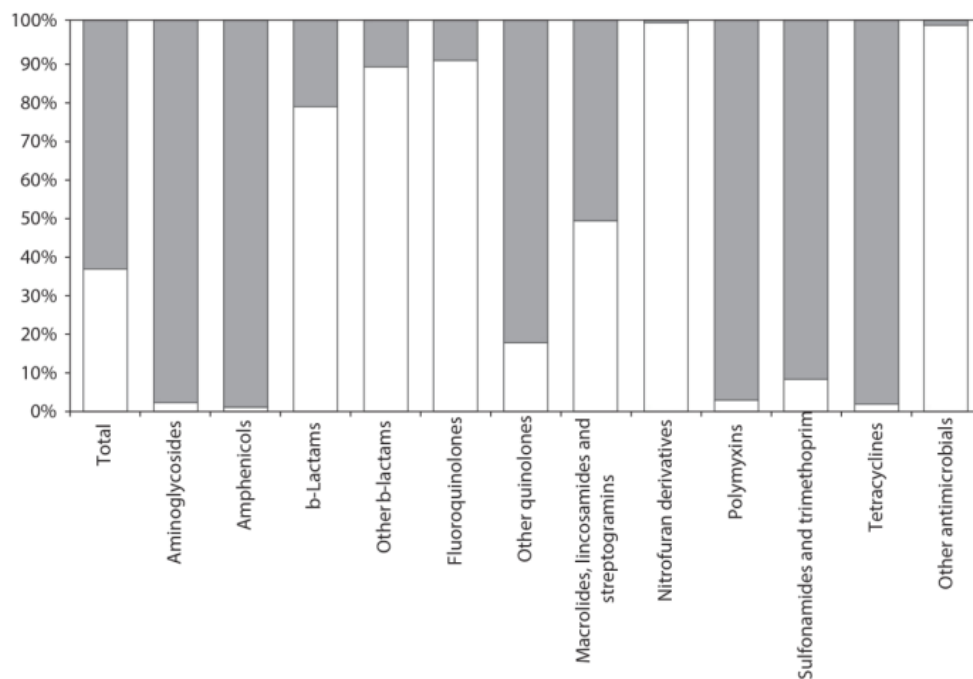
domestiques, les oiseaux, les poissons et crustacés d'élevage, les abeilles mellifères et autres (18, 19).

Ceux qui sont utilisés en vétérinaire et en agriculture sont souvent utilisés à des fins thérapeutiques (pour la thérapie), métaphylactiques (pour la prophylaxie et la thérapie), prophylactiques (pour la prévention des maladies) et sous-thérapeutiques (pour la promotion de la croissance et la prophylaxie des maladies) (18).

Dans les années 1950, l'utilisation d'antibiotiques chez le bétail, comme stimulateurs de croissance, a été largement répandue. et est devenue une pratique courante dans le monde entier (20). À des niveaux thérapeutiques, les antibiotiques affectent le système immunitaire des animaux (21). Les antibiotiques utilisés comme stimulateurs de croissance modifient également l'équilibre intestinal des espèces microbiennes et suppriment certaines bactéries sensibles (22). L'utilisation d'antibiotiques comme stimulateurs de croissance est interdite dans l'UE depuis 1999 (23).

Les antibiotiques utilisées dans le secteur clinique ou en agriculture sont pour la plupart identiques, mais ils sont utilisés dans des proportions différentes (24). Selon une étude comparative menée par Moulin *et al.* en 2008, les tétracyclines, les sulfamides, le triméthoprim, les  $\beta$ -lactamines et les aminoglycosides représentaient plus de 80 % des antimicrobiens vendus en médecine vétérinaire en France entre 1999 et 2005 (24). A l'inverse, durant la même période, les  $\beta$ -lactamines constituaient plus de 50 % de la consommation totale en médecine humaine (24). Ainsi, comme le montre la **figure 4**, certains antibiotiques ont été utilisés presque exclusivement soit chez les animaux comme l'apramycine (aminoglycosides), chloramphénicol, polymyxines et tétracyclines, ou chez l'homme comme dérivés du nitrofurane, carbapénèmes et autres antimicrobiens (24).





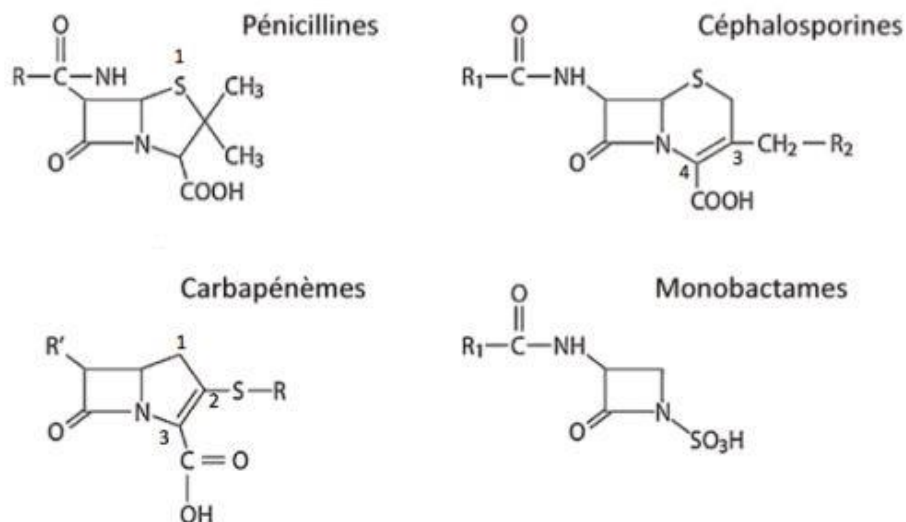
**Figure 4 : Une comparaison entre les ventes totales enregistrées en France en 2005 d'antimicrobiens approuvé pour un usage humain (en blanc) et animal (en gris) (D'après Moulin et al.)(24)**

#### 4. Les $\beta$ -Lactamines

Les  $\beta$ -lactamines représentent la famille d'antibiotiques la plus utilisée dans le monde en milieu hospitalier et communautaire. Leur importance réside dans le fait de leur faible toxicité, leur large spectre d'action, leur efficacité thérapeutique et leur faible cout.

##### a. Structure et Spectre d'activité

Les  $\beta$ -lactamines, se caractérisent par la présence d'une structure commune, le noyau  $\beta$ -lactame (support de l'activité bactérienne des  $\beta$ -lactamines) accolé, ou non, d'un hétérocycle de 5 à 6 atomes (**Figure 5**). Le succès clinique de la pénicilline G, la première  $\beta$ -lactamine, a suscité la recherche et le développement de nouveaux dérivés. Selon la nature de l'hétérocycle, on distingue quatre sous-familles: Les pénicillines, les céphalosporines, les carbapénèmes et les monobactames.



**Figure 5 : Structure des β-Lactamines**

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### **b. Les Pénicillines**

Les pénicillines sont des β-lactamines à noyau pénème, où le cycle β-lactame est associé à un cycle thiazolidine. La pénicilline G (Benzylpenicillin) était la première β-lactamine à être utilisée en clinique par voie injectable, pour traiter les infections à Streptocoques et Staphylocoques (25). La pénicilline V (Phenoxymethylpenicilline), une pénicilline administrée par voie orale, est encore utilisée jusqu'à présent, en thérapeutique et en prophylaxie pour les infections causées par *Streptococcus* spp, et aussi utilisé en pédiatrie (26). L'oxacilline et la cloxacilline, sont les β-lactamines de choix pour le traitement des infections cutanées, les infections de cathéter et les bactériémies causées par *S. aureus* sensible à la méticilline (SASM) (27). L'ampicilline et l'amoxicilline, introduit en 1970, présentent une activité améliorée sur les bactéries à gram-négatif. Dans les années 70-80, la production industrielle, de pénicillines semi-synthétiques (ticarcilline, pipéracilline, méticilline, cloxacilline et témocilline) a été amorcée. Ces molécules présentaient un spectre élargi aux staphylocoques producteurs de β-lactamases, aux entérobactéries et à *P. aeruginosa* (28).

### c. Les céphalosporines

Les céphalosporines sont des  $\beta$ -lactamines à noyau céphème, produit dans les années 70. Ce noyau est constitué du cycle  $\beta$ -lactame associé à un cycle dihydrothiazine. La céphalosporine C, isolée à partir de *Cephalosporium acremonium*, a une faible activité antibiotique mais présente une action contre les bactéries résistantes aux pénicillines. A partir de cette molécule, de nouvelles générations de céphalosporines ont été développées entre les années 70 et 90. Le spectre d'action est devenu plus large à chaque génération. Il en existe actuellement 4 générations, dont le spectre d'action est de plus en plus large avec une activité sur les Entérobactéries productrices de pénicillinases et de céphalosporinases (29).

- Les céphalosporines de 2<sup>ème</sup> génération: La famille des céphamycines se distingue par leur résistance à l'hydrolyse induite par  $\beta$ -lactamases à spectre étendu (BLSE).
- Les céphalosporines de 3<sup>ème</sup> génération : Fréquemment utilisés pour le traitement des infections probablistes par une Entérobactérie.
- Les céphalosporines de 4<sup>me</sup> génération : Elles ne sont pas inactivés par les céphalosporinases hyperproduites (29).

Les dernières céphalosporines synthétisées telles que la ceftaroline et le ceftobiprole se démarquent par leur activité dirigée contre certaines bactéries à Gram positif dont les *Staphylocoques* résistants à la méticilline (SARM) (30). De même, le ceftolozane démontre une très bonne activité anti-pyocyanique (31). Récemment, un nouvel antibiotique, le cefiderocol, a vu le jour. C'est est une  $\beta$ -lactamine de type céphalosporine couplée à un sidérophore Sa stratégie consiste à dissimuler un antibiotique auquel une bactérie résiste, à l'aide d'une molécule familière, et même indispensable, à cette bactérie. L'antibiotique masqué par cette molécule parvient ainsi à pénétrer la bactérie alors que seul il n'y parviendrait pas. Ce composé a démontré une activité *in vitro* puissante vis-à-vis des souches cliniques de *P. aeruginosa* (32), *A. baumannii*, *S. maltophilia* et des entérobactéries résistantes aux carbapénèmes (33-35)

### d. Les carbapénèmes

La famille des carbapénèmes est caractérisée par des molécules dont la structure de base dérive de la thiénamycine, antibiotique naturellement produit par *Streptomyces cattleya* (36) et

découvert dans les années 1970. Cette molécule naturelle étant instable, un dérivé plus stable a été développé: la N-formimidoyl thiénamycine ou imipénème.

La déhydropeptidase-1 (DHP-1), une enzyme humaine présente au niveau des tubules rénaux, est capable d'hydrolyser l'imipénème, d'où la nécessité de l'administrer avec un inhibiteur de cette enzyme: la Cilastatine. Les autres carbapénèmes (l'ertapénème, le meropénème et le doripénème) sont insensibles à l'activité de la DHP-1 (37).

Les carbapénèmes sont les  $\beta$ -lactamines possédant le spectre d'activité le plus large, comprenant des bactéries à Gram positif (tel que les *streptocoques*, *S. aureus* sensible à la méticilline) et les bactéries à Gram négatif aérobies et anaérobies (38). Du fait de leur fort pouvoir antimicrobien, ils sont devenus des outils thérapeutiques cruciaux pour le traitement empirique des infections nosocomiales sévères (39). Les carbapénèmes possèdent en particulier une bonne activité sur les Entérobactéries, y compris les souches productrices de BLSE. Tous les carbapénèmes ont des spectres d'action proches à l'exception de l'ertapénème, inactif sur *Enterococcus* spp., *P. aeruginosa*, *A. baumannii* et les autres bacilles à Gram négatif non fermentaires (37). L'imipénème et le meropénème sont actuellement utilisées dans le traitement des infections sévères et sont administrés plusieurs fois dans la journée, à cause de leur demi-vie courte. Par contre, l'ertapénème ayant une demi-vie plus longue (4h au lieu de 1h pour les autres molécules), n'est administré qu'une seule fois par jour.

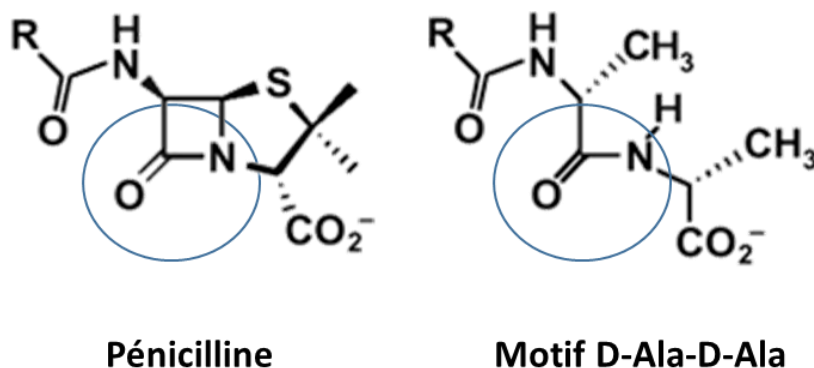
#### e. Les monobactames

Les monobactams sont des  $\beta$ -lactamines qui ne possèdent qu'un seul cycle  $\beta$ -lactame. L'aztréonam est le seul représentant de cette sous-famille, son spectre est comparable aux céphalosporines de 3<sup>ème</sup> génération. Leur spectre d'action couvre les Entérobactéries et *P. aeruginosa* mais pas les bactéries à Gram positif et les germes anaérobies.

### III. Le mécanisme d'action des $\beta$ -lactamines

Le cycle  $\beta$ -lactame est un substrat suicide des transpeptidases, enzyme impliquée dans la synthèse des peptidoglycanes. En fait le cycle  $\beta$ -lactame est analogue de D-Ala-D-Ala (substrat des transpeptidases) (**Figure 6**) (40). Les DD-peptidases ou les PLPs constituent la cible des  $\beta$ -lactamines qui jouent un rôle crucial dans la dernière étape de synthèse du peptidoglycane. Toute

altération du peptidoglycane aboutit plus ou moins rapidement à la lyse bactérienne. Pour accéder à leurs cibles (PLPs présents dans l'espace périplasmique), les  $\beta$ -lactamines doivent tout d'abord traverser la membrane externe, qui constitue une barrière de perméabilité. Un transport passif via des porines permet la pénétration des  $\beta$ -lactamines. Les molécules de petites tailles, hydrophiles et chargées positivement rendent leur transport plus facile. Seules les  $\beta$ -lactamines hydrophiles peuvent traverser la membrane externe. Les  $\beta$ -lactamines comme ayant une homologie structurale avec le peptide D-Alanine-D-Alanine, substrat naturel des PLPs, aboutit à une inhibition de ces PLPs et par conséquent à l'arrêt de la synthèse du peptidoglycane, aboutissant à un arrêt de la croissance bactérienne (effet bactériostatique). La lyse cellulaire intervient secondairement par la sécrétion d'auto lysines (effet bactéricide) (40, 41).



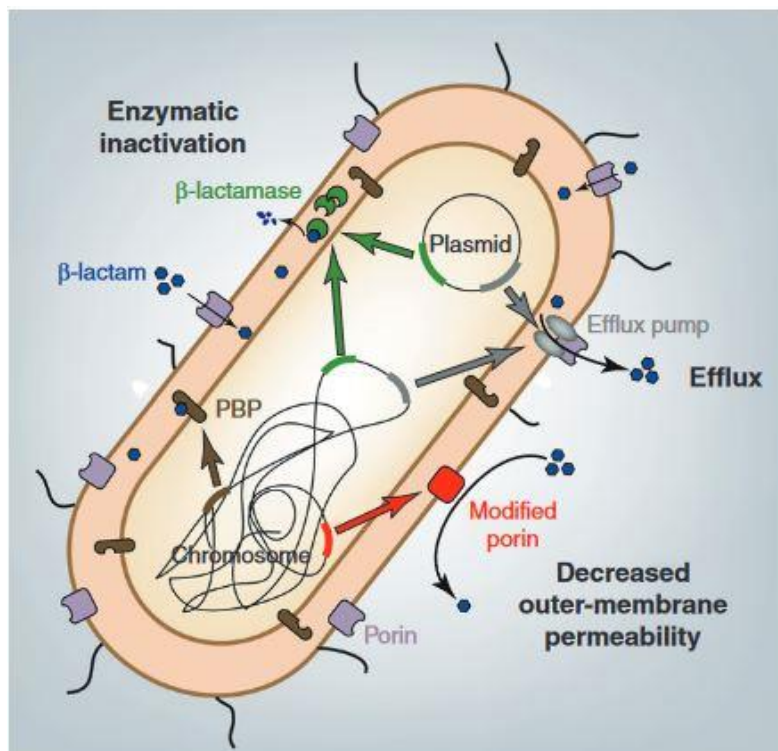
**Figure 6 : Homologie structurale entre le substrat naturel D-Ala-D-Ala des PLPs et la Pénicilline**

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#### **IV. Les mécanismes de résistance aux $\beta$ -lactamines**

La résistance aux antibiotiques constitue un problème majeur de santé publique qui menace la capacité à traiter, avec succès, les infections bactériennes (42, 43). L'organisation mondiale de la santé, a défini la notion de résistance aux antibiotiques, quand les bactéries résistent aux effets de ces médicaments. Les bactéries multirésistantes sont définies comme étant les bactéries résistantes à 3 classes ou plus d'agents antimicrobiens (44). Auparavant, les BMR étaient confinées aux milieux hospitaliers, par contre, aujourd'hui, ils sont largement répandues chez les animaux et dans l'environnement (45). En 1941, les premiers cas de résistance aux  $\beta$ -lactamines ont été

constatés, où moins de 1% des *S. aureus* étaient résistant à la pénicilline. La résistance aux  $\beta$ -lactamines peut être naturelle ou acquise. En général, 4 mécanismes de résistance aux  $\beta$ -lactamines sont connus: (i) diminution de la perméabilité membranaire (ii) modification de la cible (iii) Système d'efflux (iv) inactivation enzymatique des  $\beta$ -lactamines (**Figure 7**).



**Figure 7 : Mécanismes de résistance chez les Entérobactéries (46).**

## 1. La résistance intrinsèque chez les BGN

Certains organismes sont considérés résistant intrinsèquement à un ou plusieurs agents antimicrobiens. La plupart des composés antimicrobiens sont des molécules produites de façon naturelle par des bactéries co-résidentes qui ont élaboré des mécanismes pour surmonter leur action et survivre (47). Cette résistance intrinsèque ou encore dite naturelle complique la sélection des médicaments pour divers traitements (47).

## 2. L'imperméabilité

Les  $\beta$ -lactamines rentrent dans la cellule d'une façon passive à travers des porines (48). Une altération qualitative ou quantitative de ces porines peut être à l'origine de ce phénomène de résistance. Une altération qualitative se traduit par une mutation au niveau de l'un des gènes codant pour ces porines ou à une délétion de l'un d'entre eux. Alors que l'altération quantitative, survient avec une diminution de l'expression de ces porines. La réduction de la perméabilité empêche les  $\beta$ -lactamines d'atteindre leur cible (les PLPs) et donc de les inhiber (49, 50). Ce mécanisme aboutit généralement à une sensibilité diminuée aux antibiotiques et est souvent associé à une production de  $\beta$ -lactamases. Chez les Entérobactéries, ce mécanisme n'est pas spécifique des  $\beta$ -lactamines et peut affecter d'autres familles d'antibiotiques (49). La résistance acquise résultant d'une diminution de la perméabilité de la paroi bactérienne a été décrite chez *E.coli*, *Proteus* sp, *Shigella* sp, *Klebsiella* sp, *Enterobacter* sp, *Serratia* sp (49).

Chez *E. coli*, l'altération des porines OmpA, OmpC et OmpF est fréquemment décrite dans les isolats multi résistants, notamment en association avec la production d'une  $\beta$ -lactamase comme une céphalosporinase (51) ou une BLSE (52).

Chez *K. pneumoniae*, l'inactivation des porines OmpK35 et/ou OmpK36 peut être responsable de la résistance aux carbapénèmes en association avec une BLSE ou une céphalosporinase acquise (53, 54).

Chez *Enterobacter* spp. hyperproducteurs de céphalosporinase naturelle, il a été montré que les porines OmpF et OmpC sont moins exprimées dans les souches résistantes aux carbapénèmes en comparaison aux souches sensibles (54, 55)

## 3. Modification de la cible des $\beta$ -lactamines

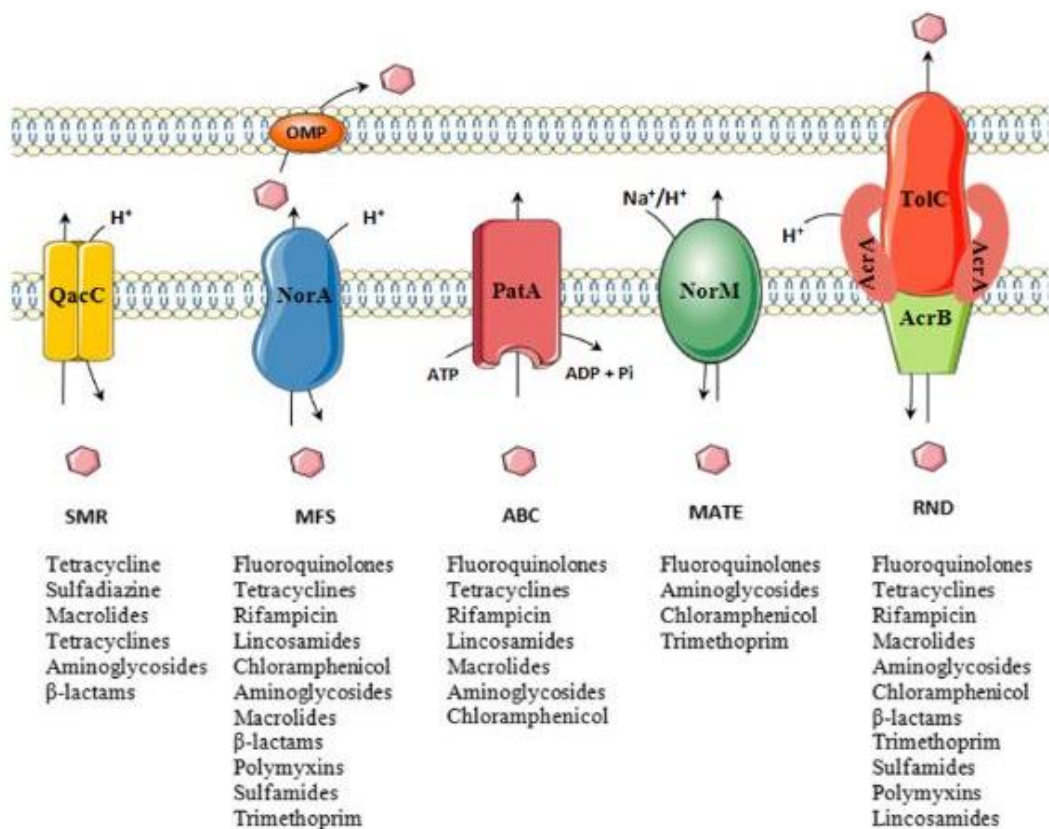
La modification de la cible se traduit par une diminution de l'affinité des PLPs, cible des  $\beta$ -lactamines. Cependant, les fonctions cellulaires des cibles (PLPs, ARN polymérase, ADN gyrase...), empêchent les microorganismes de s'échapper à l'action antimicrobienne, en inactivant les cibles complètement. Pour cela, des mutations au niveau des cibles peuvent avoir lieu, afin de réduire la sensibilité à l'inhibition, tout en conservant la fonction cellulaire. Ces mutations peuvent induire : une modification de la conformation des PLPs, une diminution de production d'une PLPs

de forte affinité ou encore une hyperproduction d'une PLP de faible affinité. Alors que ce mécanisme de résistance est prédominant chez les bactéries à Gram positif, il semble marginal chez les entérobactéries. Des souches de *Proteus mirabilis* résistantes à l'imipénème et au mécilinam ont ainsi été décrites par perte d'affinité de la PLP2 et diminution quantitative de la PLP1A. Plus récemment, des mutations dans les séquences protéiques des PLP2 ont également été identifiées chez 2 isolats cliniques de *E. coli* de sensibilité diminuée à l'imipénème uniquement (Concentration Minimale Inhibitrice (CMI) à 3 mg/L) en France. En plus, récemment, une altération dans la séquence d'acides aminés de la PLP3 chez *E. coli*, qui est une insertion YRIK ou YRIN après la position 333, a été décrite pour provoquer une diminution de la liaison de l'aztréonam à la cible. De même, les mutations de PLP3 sont à l'origine des CMI plus élevées de l'aztréonam/avibactam (56).

#### 4. Système d'efflux

Les pompes à efflux sont des transporteurs membranaires qui excrètent activement des molécules toxiques, dont les antibiotiques, en dehors du cytoplasme bactérien (**Figure 8**). Les pompes à efflux peuvent être classées selon la structure de la pompe, le type de la molécule véhiculée ou selon le phénotype de résistance attribué. Pour cela on distingue 5 familles de pompes à efflux associées à la résistance : (i) Famille ABC (ATP-binding cassette transporter), (ii) Famille SMR (Small Multidrug Resistance), (iii) Famille MATE (Multidrug and Toxic Compound Extrusion), (iv) Famille MFS (Major Facilitator Superfamily), (v) Famille RND (Resistance Nodulation Cell Division) (57). Des mutations au niveau des gènes qui régulent l'expression des systèmes d'efflux, peuvent conduire à une surexpression de ces systèmes et par la suite à une résistance croisée à différents familles d'antibiotiques.



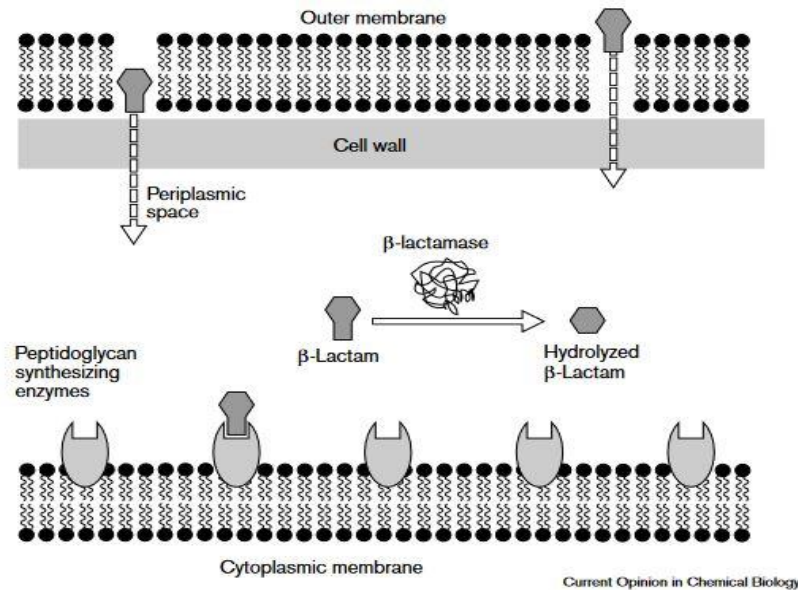


**Figure 8 : 5 Familles de pompes à efflux avec leur substrat antibiotiques connus.** Famille SMR « Small Multidrug Resistance » family, famille MFS « Major Facilitator Superfamily », Famille ABC « ATP-binding cassette », Famille MATE « Multidrug and Toxic Compound Extrusion », Famille RND « Resistance-Nodulation-Division. OMP (outer membrane protein) : protéine de membrane externe.

## 5. Production d'enzymes inactivant les β-lactamines: Les β-Lactamases

L'inactivation enzymatique des β-lactamines, constitue le mécanisme de résistance principal aux β-lactamines chez les Entérobactéries. Les β-lactamases inactivent les β-lactamines par ouverture du cycle β-lactame au niveau de la liaison amide, conduisant à une perte de leur activité (**Figure 9**). Chez les Entérobactéries, plusieurs espèces sensibles à toutes les β-lactamines (*E. coli*, *Salmonella* sp., *Shigella* sp., *P. mirabilis*). Par contre, de nombreuses espèces sont naturellement résistantes aux aminopénicillines et aux céphalosporines de première génération par production de β-lactamases naturelles, c'est à dire dont les gènes codant pour ces enzymes sont localisés sur le chromosome bactérien. Ce type de résistance peut également être acquis grâce à des transferts génétiques faisant intervenir des plasmides. La production de ces β-lactamases est

soit constitutive (ex : pénicillinase SHV chez *K. pneumoniae*), soit inducible par la présence d'un gène régulateur localisé en amont du gène codant pour la  $\beta$ -lactamase (ex : céphalosporinase chez *Serratia* spp., *Enterobacter* spp., *Morganella* spp., *Providencia* spp.).



**Figure 9 : Schéma du mode d'action des  $\beta$ -lactamines et des  $\beta$ -lactamases.** Les antibiotiques diffusent à travers la membrane externe et inactivent les PLPs, qui sont impliqués dans la biosynthèse du peptidoglycane. L'action des  $\beta$ -lactamases provoque l'ouverture du noyau  $\beta$ -lactame de l'antibiotique, le rendant inactif. Les substitutions à chacune des trois positions (indiquées par des lignes ondulées) donnent lieu à une famille de composés bioactifs, dont beaucoup sont clivés par les  $\beta$ -lactamases. *D'après Wang et al. (58)*

## V. Classification des $\beta$ -lactamases

Par définition, les  $\beta$ -lactamases sont des enzymes capables d'inactiver les  $\beta$ -lactamines en hydrolysant la liaison amide du cycle  $\beta$ -lactame à quatre chaînes, entraînant ainsi une perte complète de l'activité antibiotique. L'activité d'hydrolyse des  $\beta$ -lactamines, a été décrite pour la première fois en 1940 soit 12 ans après les travaux de *Fleming*, par *Abraham* et *Chain*, chez une souche de *Bacterium coli*. Cette enzyme capable d'hydrolyser les pénicillines, a été nommée Pénicillinase (59). De même, une souche de *S. aureus*, productrice d'une  $\beta$ -lactamase codée par un plasmide, a été décrite à la fin de cette même année.

L'hydrolyse des  $\beta$ -lactamines par des  $\beta$ -lactamases est le mécanisme de résistance le plus fréquent chez les bactéries Gram-négatives. De nombreuses  $\beta$ -lactamases disséminent par l'intermédiaire d'éléments génétiques mobiles à travers des agents pathogènes opportunistes tels que les Entérobactéries et les bactéries non fermentantes. Chez les bactéries à Gram négatif, les  $\beta$ -lactamases sont secrétées dans l'espace périplasmique, lieu d'action des  $\beta$ -lactamines sur les PLP. Ainsi, les  $\beta$ -lactamines seront dégradées par les  $\beta$ -lactamases avant même qu'elles ne puissent interagir avec les PLPs.

Actuellement, plus de 7997 séquences protéiques de  $\beta$ -lactamases sont disponibles dans les banques de données ([www.bldb.eu](http://www.bldb.eu)) (60). Les  $\beta$ -lactamases présentent une grande diversité de structure primaire et de propriétés catalytiques, ce qui a conduit à l'élaboration de plusieurs classifications, dont deux sont actuellement utilisées: La classification d'Amblar basée sur leur structure primaire (61) et la classification fonctionnelle de Bush et Jacoby (62).

### **1. Classification d'Amblar**

La classification moléculaire d'Amblar, proposée dans les années 1975, est basée sur la séquence d'acides aminés et divise les  $\beta$ -lactamases en enzymes de classe A, C et D qui utilisent la sérine pour l'hydrolyse des  $\beta$ -lactames et les métallo enzymes de classe B qui nécessitent des ions zinc divalents pour l'hydrolyse du substrat.

#### **a. Les $\beta$ -lactamases de classe A**

Ces enzymes sont considérées les plus importants. Ils sont capables d'hydrolyser les pénicillines (Amoxicilline, Ticarcilline, Piperacilline) et sont sensibles aux inhibiteurs de  $\beta$ -lactamases (acide clavulanique, tazobactam et sulbactam). Selon leur spectre d'hydrolyse, on distingue au sein de cette classe, trois grandes sous familles: les pénicillinases, les  $\beta$ -lactamases à spectre étendu (BLSEs) qui sont des pénicillinases ayant acquis une activité vis-à-vis de certaines céphalosporines à large spectre (ou céphalosporines de 3ème génération [C3G]) et les carbapénèmases de classe A qui, en plus d'hydrolyser les pénicillines et les céphalosporines, hydrolysent les carbapénèmes.

### **b. Les $\beta$ -lactamases de classe B**

Ce sont des métalloprotéines, dont l'activité nécessite la présence d'ions  $Zn^{2+}$  au niveau de leur site actif. Les métallo-  $\beta$ -lactamases sont des carbapénèmases capables d'hydrolyser toutes les  $\beta$ -lactamines, excepté l'aztréonam et sont insensibles aux inhibiteurs classiques, mais peuvent être inhibées par des agents chélateurs d'ions divalents, tel que l'EDTA (Acide éthylène diamine tétra-acétique) (63).

### **c. Les $\beta$ -lactamases de classe C ou céphalosporinases :**

Ces  $\beta$ -lactamases sont capables d'hydrolyser les aminopénicillines et les céphalosporines de première génération (et pour certaines de deuxième génération). En cas d'hyperproduction de ces enzymes (céphalosporinase de haut niveau), on observe une hydrolyse des carboxypénicillines (ticarcilline), des uréidopénicillines (pipéracilline), de l'aztréonam et des C3G. La majorité sont insensible aux inhibiteurs classiques mais sont inhibées par la cloxacilline.

### **d. Les $\beta$ -lactamases de classe D**

Du point de vue structure et spectre d'hydrolyse, c'est la classe la plus hétérogène (64). Initialement nommées oxacillinases du fait que ces enzymes hydrolysaient l'oxacilline plus rapidement que la pénicilline G. Au sein de cette classe, et selon leur spectre d'hydrolyse, on distingue : (i) Les oxacillinases à spectre étroit (hydrolyse des pénicillines et des céphalosporines de première génération), (ii) Les oxacillinases à spectre élargi aux céphalosporines (2ème, 3ème et 4ème génération) et des oxacillinases ayant une activité carbapénèmases (tel que type OXA-48 chez les entérobactéries). Ces enzymes ne sont pas affectées par les inhibiteurs classiques, mais pour certaines oxacillinases, une activité inhibitrice du chlorure de sodium a été identifiée *in vitro* (64).

## **2. Classification de Bush et Jacoby**

La classification fonctionnelle publiée par Bush et Jacoby en 1989 (65) et 1995 (66), a été mis à jour en 2010 (62). Cette classification prend en considération le profil d'hydrolyse ainsi que le profil d'inhibition de l'enzyme. Cette classification comprend 3 sous-groupes :

- Le groupe 1 : comprend les céphalosporinases de la classe C d'Ambler.

- Le groupe 2 qui regroupe les classes A et D d'Ambler (Pénicillinases, céphalosporinases et BLSE) qui sont divisées en sous-groupes allant de 2a à 2f.
- Le groupe 3 : correspond à la classe B d'Ambler. Deux sous-groupes (3a et 3b) peuvent être individualisés sur la base de données structurale, d'affinité pour l'ion  $Zn^{2+}$  et de caractéristiques hydrolytiques.

## VI. Les $\beta$ -lactamases à spectre étendu

### 1. Définitions

Le terme  $\beta$ -lactamases à spectre étendu (BLSE) a été initialement utilisé pour définir une  $\beta$ -lactamase, dont le spectre d'hydrolyse a été étendu suite à l'introduction d'une ou plusieurs substitution(s) dans sa séquence protéique (67). Deux types de BLSE sont à distinguer. Le premier type appartient au groupe 2be de la classification de Bush et Jacoby et à la classe A de Ambler, tandis que le deuxième type appartient à la classe D, correspondant aux oxacillinases dont le spectre est également étendu aux C3G et/ou C4G. La définition actuelle des  $\beta$ -lactamases à spectre étendu est restreint aux  $\beta$ -lactamases de classe A et appartenant au groupe fonctionnel 2be, capables d'hydrolyser au moins un représentant des oxyimino-céphalosporines (tels que le cefotaxime, la ceftazidime), l'oxyimino-monobactam (l'aztréonam) et inhibées par l'acide clavulanique (62).

### 2. Généralités

La première BLSE a été identifiée en 1983 chez deux souches cliniques de *K. pneumoniae* et *Serratia marcescens* (68). Il s'agissait de SHV-2, dérivant de la pénicilline à spectre étroit SHV-1. Depuis, plusieurs familles de BLSE ont été identifiées dans les bacilles Gram négatifs incluant trois familles majeures (type TEM, SHV et CTX-M) et des BLSE plus rarement identifiées dites mineures (62).

### 3. Les BLSE majeures: TEM, SHV et CTX-M

Les premières BLSE identifiées étaient des enzymes qui dérivait des pénicillinases de type TEM et SHV isolées classiquement chez *E. coli* et *K. pneumoniae* respectivement (69). Ces enzymes alors non-BLSE ont évolué au cours des années 1980 grâce à des mutations spécifiques qui ont contribué à élargir la poche catalytique des  $\beta$ -lactamases (68). Les BLSE type TEM et SHV

étaient portées par des plasmides transférables entre de nombreuses espèces d'Entérobactéries, mais étaient principalement présentes chez *K. pneumoniae* et *E. coli*.

Les enzymes de la famille des CTX-M (pour Cefotaximase-Munich) ont été décrites pour la première fois en 1989 en Allemagne, et ont supplanté les enzymes type TEM et SHV dès le début des années 2000 (69). Au sein des CTX-M, 5 principaux groupes, dont les séquences protéiques diffèrent de plus de 10%, sont actuellement reconnus : CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 et CTX-M-25. Cependant, ces groupes partagent des caractéristiques communes :

- Les gènes *bla* codant ces enzymes proviennent de différentes espèces du genre *Kluyvera*, Entérobactérie rarement impliquée en pathologie humaine.
- Les enzymes de type CTX-M hydrolysent préférentiellement le céfotaxime en comparaison à la ceftazidime, à l'inverse des enzymes de type TEM et SHV.

Grâce à leur présence sur un grand nombre de plasmides de différents groupes d'incompatibilité, les CTX-M sont devenues en quelques années les BLSE les plus répandues sur l'ensemble des régions du globe. L'enzyme la plus fréquemment rencontrée en pathologie humaine est CTX-M-15 (groupe CTX-M-1), suivie de CTX-M-14 (groupe CTX-M-9) (70).

#### 4. Les BLSE mineures

D'autres BLSE sont distribuées plus fréquemment chez *P. aeruginosa* et dans une bien moindre mesure chez *Acinetobacter* mais restent plus rarement identifiées chez les Entérobactéries. Il s'agit principalement des enzymes GES, PER et VEB.

- Les BLSE de type GES : le gène *bla*<sub>GES-1</sub> (pour Guiana Extended-Spectrum  $\beta$ -lactamase) a été identifié dans un isolat clinique de *K. pneumoniae* en France d'un patient originaire de Guyane française (71). Au sein de la famille des  $\beta$ -lactamases de type GES, les profils de substrat diffèrent. Certains variants tels que GES-1, GES-3 et GES-7 possèdent un profil de type BLSE de classe A, mais épargnent l'aztréonam. Certains variants ont un profil hydrolytique étendu aux carbapénèmes (GES-2 et GES-8) ou aux carbapénèmes et céphamycines (GES-4, -5, -6).
- Les BLSE de type PER (pour Pseudomonas Extended Resistance) ont été identifiées pour

la première fois dans un isolat de *P. aeruginosa* d'un patient turc (72). Douze variants ponctuels de PER ont été décrits incluant un groupe (PER-2 et PER-6) qui ne partage que 85% d'identité de séquence en acides aminés en comparaison avec PER-1.

- Les BLSE de type VEB-1 (pour Vietnamese Extended-spectrum  $\beta$ -lactamase) ont été caractérisées pour la première fois en 1999, dans une souche de *E. coli* isolée d'un patient du Vietnam et transféré à l'hôpital Antoine Bécère (Clamart, France) (73).
- les SFO-1 (*Serratia fonticola*) (74), BES-1 (Brazilian extended-spectrum  $\beta$ -lactamase) (75), BEL-1 (Belgium extended-spectrum  $\beta$ -lactamase) (76) et TLA-1 (Tlahuicas, tribu indienne) (77) sont rarement isolées à ce jour.

## VII. Les Carbapénèmases

### 1. Histoire et Généralités

Les carbapénèmases constituent une famille de  $\beta$ -lactamases capables d'hydrolyser les carbapénèmes (78). Les carbapénèmases appartiennent aux classes A, B et D de Ambler. Les enzymes de classe A possèdent une sérine active responsable de l'attaque nucléophile de la liaison amide du noyau  $\beta$ -lactam. Les enzymes de classe B nécessitent 1 ou 2 ions zinc pour coordonner une molécule d'eau responsable de l'attaque nucléophile (79). Les gènes codant pour les carbapénèmases peuvent être chromosomiques ou plasmidiques. Au début des années 90, des carbapénèmases naturelles, chromosomiques, de classe B ont été identifiées chez certaines espèces: *S. maltophilia*, *Aeromonas* sp., *B. cereus* et *Bacteroides fragilis* (80). Les premières carbapénèmases identifiées chez les Entérobactéries, étaient chromosomiques comme SME-1 *S. marcescens* à Londres en 1982 (81, 82), IMI-1 chez *Enterobacter* du complexe cloacae aux Etats-Unis en 1984 et NmcA chez *Enterobacter* du complexe cloacae en France en 1994 (81, 83). Après, les Entérobactéries résistantes aux carbapénèmes, ont commencées à être décrites partout dans le monde principalement en raison de l'acquisition de gènes codant pour des carbapénèmases (79). Les carbapénèmases plasmidiques de classe A comme KPC, de classe B comme NDM, VIM et IMP et de classe D surtout OXA-48, sont les plus répandues dans le monde et dans de nombreuses espèces d'Entérobactéries (84).

## 2. Les carbapénèmases de classe A

### a. Généralités

Les carbapénèmases de classe A, sont soit chromosomiques (SME, NmcA, SFC, BIC-1, PenA, FPH-1, SHV-38), soit plasmidiques (KPC, GES, FRI-1), soit les 2 (IMI).

*E. cloacae* et *S. marcescens* productrices de carbapénèmases de classe A, ont été rapportés dans les années 80. Ces bactéries isolées de l'environnement, ont été capables d'hydrolyser l'imipenème et l'ampicilline mais pas les C3G, et l'ajout d'acide clavulanique restaurait partiellement l'activité de ces molécules. NMC-A (non-métallo carbapenemase), SME (*Serratia marcescens* enzyme) et IMI (imipenem-hydrolyzing  $\beta$ -lactamase) étaient les enzymes responsables de cette résistance (84). Le spectre d'hydrolyse de ces carbapénèmases est large incluant les pénicillines, les céphalosporines uniquement de première génération, l'aztréonam et les carbapénèmes. Les gènes codant pour ces enzymes sont chromosomiques mais également plasmidiques tel que le gène *bla*<sub>IMI-2</sub> (85, 86).

Les quatre enzymes principales (SME, NMC-A, IMI et SFC-1) étaient codées par des gènes localisés au niveau du chromosome bactérien. Elles se caractérisent toutes par un spectre d'hydrolyse large, ces enzymes sont également inductibles par la Céfoxitine (céphalosporine de 2ème génération) et l'imipenème. NMC-A a été décrite en 1993 dans une souche de *Enterobacter asburiae* (NOR-1) (87) tout comme *bla*<sub>IMI-1</sub> en 1996 (83). SME a été décrite dans deux souches de *S. marcescens* isolées en 1982 (82). Enfin, SFC-1 n'a été rapportée, à ce jour, que dans une seule souche de *Serratia fonticola* décrite en 2003 au Portugal (88).

D'autre part, les enzymes plasmidiques, surtout KPC-2, répandus partout dans le monde, ont été isolées chez des espèces d'Entérobactéries à intérêt cliniques, *P. aeruginosa* et *A. baumannii* (89, 90).

Les carbapénèmases de classe A, sont capables d'hydrolyser plusieurs  $\beta$ -lactamines, incluant les pénicillines, les céphalosporines, les carbapénèmes et l'aztréonam. Elles sont plus ou moins inhibées par l'acide clavulanique et le tazobactam, les classifiant ainsi le sous-groupe fonctionnel 2f de la classification de Bush et Jacoby (62).



### **b. Les carbapénèmases de type KPC**

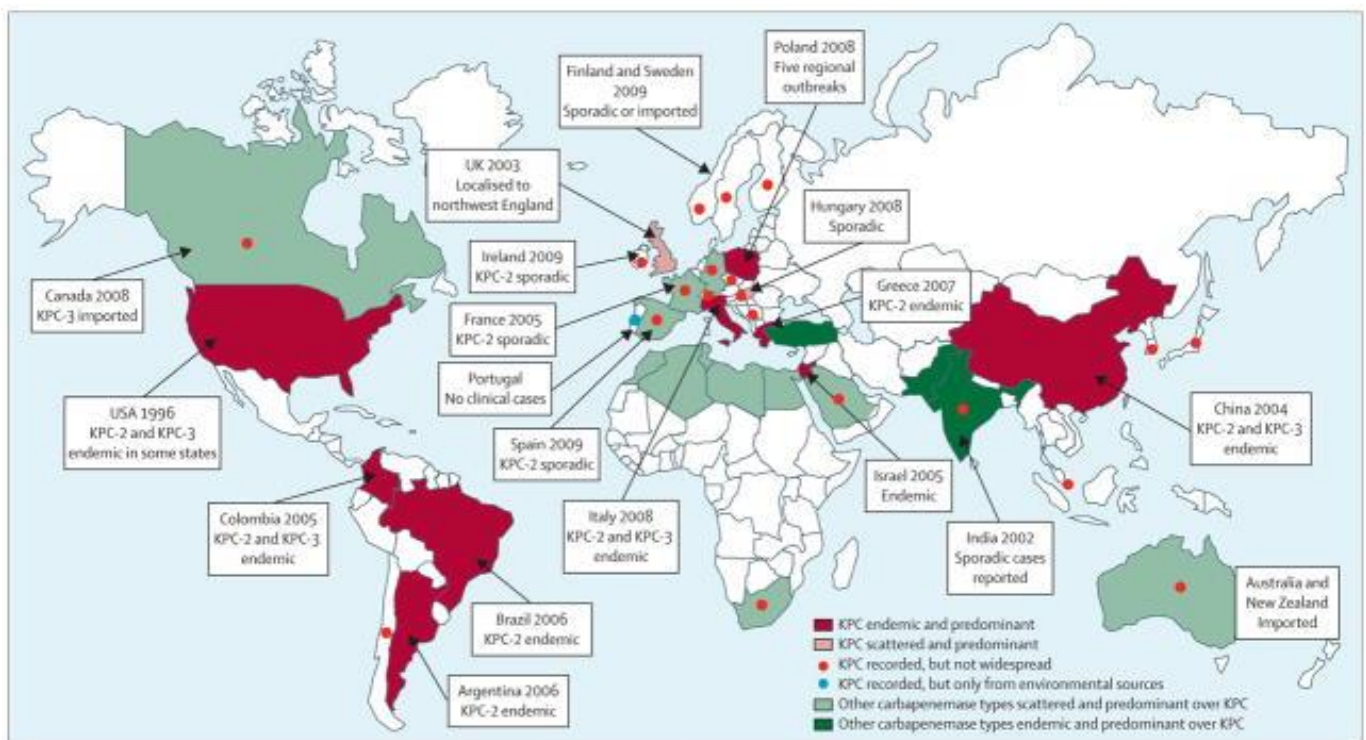
Cette famille de carbapénèmase de la classe A de Ambler a été décrite pour la première fois en 1996 aux Etats-Unis (91). Ces enzymes hydrolysent toutes les  $\beta$ -lactamines mais confèrent une résistance variable aux carbapénèmes. Les enzymes de type KPC sont peu inhibées par les inhibiteurs classiques de  $\beta$ -lactamases (acide clavulanique et tazobactam), le tazobactam étant le plus efficace (92). Les gènes *bla*<sub>KPC</sub> ont largement été caractérisés avec une localisation plasmidique (plasmides de différents types). En revanche, les gènes *bla*<sub>KPC</sub> sont largement associés au transposon Tn4401 (de type Tn3) et sont accompagnés par d'autres mécanismes de résistance aux antibiotiques notamment aux aminosides et/ou aux fluoroquinolones, et souvent à d'autres  $\beta$ -lactamases comme CTX-M-15 (89, 92, 93).

### **c. Epidémiologie**

Après la première identification de KPC-1 en 1996, un nouveau variant a été identifié, KPC-2 en 2003 également aux États-Unis (91), l'incidence des souches de *K. pneumoniae* productrices de cette carbapénèmase a augmenté régulièrement dans la région de New-York (94). La distribution géographique de ces enzymes chez les Entérobactéries, a été limitée jusqu'à l'année 2005, à l'Est des Etats Unis (94). Après quelques années, les souches productrices de KPC ont été décrites globalement dans les pays de l'Amérique du Nord et du Sud, en Grèce, Italie et Chine, considéré ainsi comme endémique (90). A ce jour, KPC est considéré comme endémique dans plusieurs pays dont l'Amérique du Nord, le Brésil, la Colombie, l'Argentine, l'Asie de Sud-Est, l'Italie, la Grèce, la Pologne et Porto Rico (95). Bien que la  $\beta$ -lactamase KPC soit fortement associée à *K. pneumoniae*, elle a été également identifiée dans de nombreuses espèces (*E. coli*, *Citrobacter* sp., *Enterobacter* sp., *S. marcescens*, *P. mirabilis*, *M. morgani*), ainsi que dans des souches de bacilles à Gram négatif non fermentant, comme *P. aeruginosa* en Amérique du Sud et *A. baumannii* à Porto Rico principalement (96).

#### d. Support génétique du gène *bla*<sub>KPC</sub>

La dissémination mondiale des gènes *bla*<sub>KPC</sub> est actuellement liée à la diffusion d'un seul clone de *K. pneumoniae* appartenant au séquence-type ST258 (95), mais dans une zone géographique donnée plusieurs clones de KPC de ST différent peuvent diffuser. Elles se distinguent de part les  $\beta$ -lactamases additionnelles, la taille, le nombre et la structure des plasmides. Le gène *bla*<sub>KPC</sub> est porté par des plasmides, appartenant à différents groupes d'incompatibilité: IncFII, FIA, I2, A/C, N, X, R, P, U, W, L/M et ColE (97-102). Ces plasmides portent de nombreux gènes de résistance aux  $\beta$ -lactamines et à d'autres familles d'antibiotiques comme les aminosides, les quinolones, les tétracyclines, les sulfamides (101).



**Figure 10 : Distribution géographique des bactéries productrices de KPC. D'après Munoz-price et al. (103).**

### 3. Les carbapénèmases de classe B

#### a. Généralités

Les carbapénèmases de classe B sont caractérisées par leur résistance aux inhibiteurs de  $\beta$ -lactamases classiques (acide clavulanique et tazobactam), et par une inhibition de leur activité par l'EDTA (acide éthylène diamine tétra-acétique), chélateur des ions  $Zn^{2+}$  (et autres cations divalents) nécessaires à l'interaction entre les  $\beta$ -lactamines et le site actif de l'enzyme. Elles possèdent également un spectre d'hydrolyse large puisque les pénicillines et toutes les céphalosporines sont hydrolysées à l'exception de l'aztréonam. Au sein de ce groupe, les carbapénèmases de type VIM, IMP et NDM sont les plus répandues.

#### b. Les carbapénèmases de type NDM

##### i. *Epidémiologie*

Le premier cas rapporté, d'une infection causée par une bactérie productrice de New-Delhi metallo-  $\beta$ -lactamases, a eu lieu en 2008, quand *bla*<sub>NDM</sub> a été détectée chez une souche de *K. pneumoniae* isolée chez un patient rentrant de l'Inde au Suède (104). Depuis, elle a fait l'objet d'une attention particulière du fait de sa dissémination rapide chez les entérobactéries mais également chez *Acinetobacter* sp., et *P. aeruginosa*. Une étude parue en 2010 a montré la forte prévalence de souches d'Entérobactéries productrices de la carbapénèmase NDM-1 en Grande-Bretagne, en Inde et au Pakistan (105). La majorité des patients infectés ou colonisés par *bla*<sub>NDM-1</sub>, ont déjà voyagé au sous-continent Indien ou ont des relations avec l'Inde, Pakistan ou Bangladesh (46). Il a donc pu être mis en évidence que le sous-continent indien était un réservoir du gène *bla*<sub>NDM-1</sub>. De plus, la présence de ce gène dans des espèces de BGN isolés à partir de prélèvements d'eau réalisés à New Delhi (106), et chez des germes responsables d'infections purement « communautaires » liées au péril fécal, comme *Salmonella* sp. (107) et *Vibrio cholerae* (108) suggère que cette carbapénèmase est fortement présente dans l'environnement.

Le gène *bla*<sub>NDM</sub> a été rapporté chez plusieurs espèces d'Entérobactéries surtout chez les clones épidémiques de *K. pneumoniae* ST147, ST11 et ST14, et chez *E. coli* 131 et 405. De même, il a été décrit chez *Vibrio cholerae*, *Pseudomonas* spp et *A. baumannii* (105, 108-110).

## **ii. Support génétique de *bla*<sub>NDM</sub>**

L'analyse de l'environnement génétique a révélée dans la plupart des cas, la présence de *ISAbal25* et le gène de résistance à la bléomycine, respectivement en amont et en aval du gène *bla*<sub>NDM</sub> (111). Il a été démontré que le transposon composite *Tn125*, délimité par 2 séquences *ISAbal25*, est responsable de la dissémination de *bla*<sub>NDM</sub> depuis *A. baumannii* vers les entérobactéries (112), qui possèdent toujours une version tronquée de *Tn125* (113). Les gènes codant pour NDM-1 et ses variants sont portés par différents plasmides (*IncFII*, *IncL/M*, *IncN*, *IncR*, *IncHIB-M/FIB-M*), *IncFI* étant le plus prévalent. De même, l'association de *bla*<sub>NDM-1</sub> avec l'intégron de classe 1 *ISCR1*, l'un des mécanismes de dissémination de la résistance entre les espèces, peut aussi expliquer la diffusion rapide de ce gène (90).

## **c. Les carbapénèmases de type IMP et VIM**

### **i. Epidémiologie**

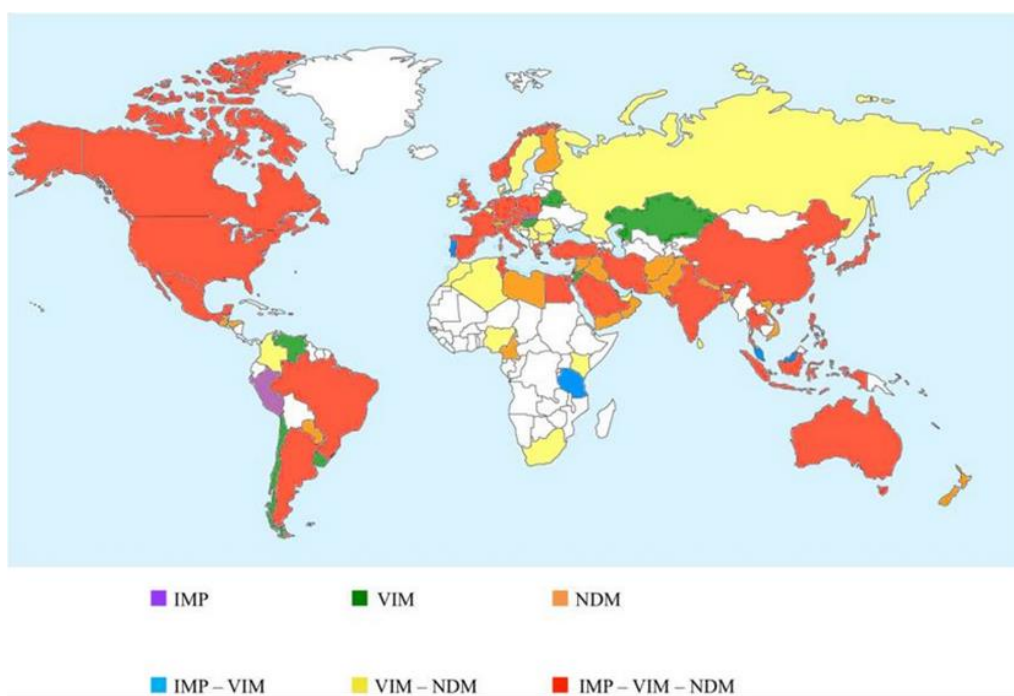
IMP-1 (pour « active on Imipenem ») fut la première enzyme décrite capable de conférer une résistance transférable aux carbapénèmes. La première bactérie exprimant cette carbapénémase a été décrite au début des années 90 au Japon ; il s'agissait d'une souche de *P. aeruginosa* (90). 85 variants d'IMP-1 ont été rapportés dans plus de 30 pays ([www.bldb.eu](http://www.bldb.eu)). Les carbapénèmases de type IMP sont endémiques au Japon et à Taiwan et ont disséminé en Chine (91) et en Corée (92). Des cas sporadiques ont été décrits en Europe (93-95), en Amérique du Sud (96), au Canada (97), aux Etats-Unis (98), en Australie (99), et en Afrique (100) (**Figure 11**).

VIM-1 (pour « Verona Integron-encoded Métallo- $\beta$ -lactamase) a été décrite pour la première fois en 1997 dans une souche de *P. aeruginosa* isolée à Vérone, Italie (114). Ensuite, le variant *bla*<sub>VIM-2</sub> a été identifiée chez une souche de *P. aeruginosa* isolée en France en 1996 (115). *Bla*<sub>VIM</sub> est prédominant en Europe, surtout dans les pays méditerranéens comme l'Italie et la Grèce, où les souches de *P. aeruginosa* (CC235 et CC111) sont impliquées dans les épidémies nosocomiales (63, 116). Les enzymes VIM-2-Like sont associés avec *P. aeruginosa* (117) alors que les enzymes VIM-1-Like surtout *bla*<sub>VIM-4</sub> sont fréquemment rapporté chez les Entérobactéries (79, 118, 119).

*ii. Support génétique de bla<sub>IMP</sub> et bla<sub>VIM</sub> :*

L'analyse de l'environnement génétique du gène *bla<sub>IMP</sub>* a montré qu'il était présent sous forme de gène cassette au sein d'intégrons de classe 1 ou 3 (120, 121), portant d'autres gènes de résistance comme les gènes de résistance aux aminoglycosides (*aac*, *aad*) ou de  $\beta$ -lactamases de classe D (*bla<sub>OXA-1,-2,-10</sub>*) (122). Ces intégrons sont localisés sur des transposons de type Tn21 et Tn5051, ce qui peut expliquer leur mobilité (123), et sont souvent portés par des plasmides à large spectre d'hôte et de groupe d'incompatibilité variable IncL/M et IncA/C (124, 125).

Comme *bla<sub>IMP</sub>*, *bla<sub>VIM</sub>* est un gène cassette associé à des intégrons de classe I et intégrés soit dans le chromosome soit dans des plasmides dans un transposon type Tn402 (ou Tn5090) (126, 127).



**Figure 11 : Distribution globale de *bla<sub>VIM</sub>*, *bla<sub>IMP</sub>* et *bla<sub>NDM</sub>* métallo-  $\beta$ -lactamases. D'après Mojica et al. 2016 (128).**

## 4. Les carbapénèmases de classe D

### a. Généralités

L'émergence de  $\beta$ -lactamases de classe D capables d'hydrolyser les carbapénèmes (Carbapenem-hydrolyzing class D  $\beta$ -lactamases CHDL) a commencé en 1995 avec l'identification d'OXA-23 (également connu sous le nom d'ARI-1) à partir d'un isolat de *A. baumannii* en Écosse en 1993 (129). Par la suite, l'expression d'OXA-23 a été rapportée dans le monde entier (130) et le portage plasmidique et chromosomique des gènes *bla*<sub>OXA-23</sub> a été décrit. Le groupe OXA-23 comprend OXA-27, trouvé dans un seul isolat de *A. baumannii* à Singapour (131). À l'exception de quelques isolats de *P. mirabilis* identifiés en France en 2002, ce groupe de  $\beta$ -lactamases a été exclusivement identifié au sein de *A. baumannii* (132). Les CHDL chez *A. baumannii* peuvent être regroupées en cinq sous-familles basées sur leurs séquences: OXA-23, OXA-24/40, OXA-143, OXA-58 et OXA-51. OXA-198 a été trouvé chez *P. aeruginosa* et quelques entérobactéries en France et en Belgique (133), tandis que OXA-48 a été trouvé exclusivement chez les Entérobactéries (64).

### b. Les carbapénèmases de type OXA-48

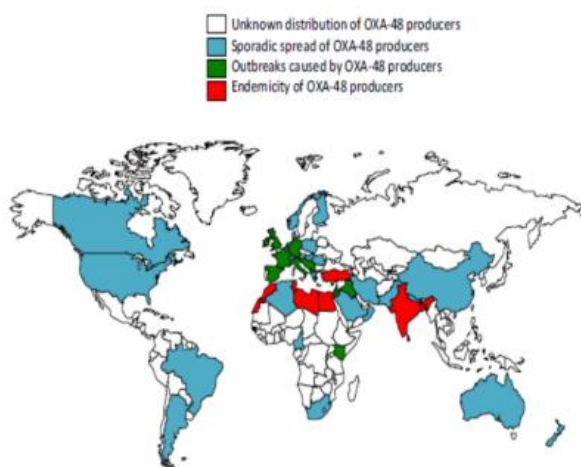
#### i. Histoire

Le gène *bla*<sub>OXA-48</sub> a été identifié pour la première fois dans une souche clinique de *K. pneumoniae* isolée en Turquie, en 2001 (134). Ensuite, ce gène a été identifié chez plusieurs Entérobactéries en Turquie (135, 136). La première souche de *K. pneumoniae* productrice de OXA-48, était résistante à toutes les  $\beta$ -lactamines comprenant les céphalosporines à large spectre, céphamycines, monobactams et les carbapénèmes. Le gène *bla*<sub>OXA-48</sub> était porté par un plasmide de type IncL et code pour une  $\beta$  CHDL faiblement reliée aux autres  $\beta$ -lactamases de classe D, partageant 46%, 36%, 32% et 21% avec OXA-10, OXA-23, OXA-40 et OXA-1, respectivement (134).

#### ii. Épidémiologie :

Durant plusieurs années, la majorité des bactéries productrices d'OXA-48 était isolée chez des patients hospitalisés en Turquie ou ayant un lien avec la Turquie (137). Rapidement, de rares cas sporadiques ont été décrits dans différents pays tels que la Belgique (138), la Tunisie (139), la

France et l'Égypte (140). Depuis, cette carbapénèmase est devenue endémique en Turquie et les bactéries productrices d'OXA-48 ont diffusé en Afrique du Nord et au Moyen-Orient. A l'heure actuelle, tous ces pays sont considérés comme étant d'importants réservoirs de bactéries productrices d'OXA-48 (**Figure 12**) (141). En Europe, plusieurs épidémies hospitalières ont été décrites comme en France, en Allemagne, en Espagne, aux Pays-Bas et en Angleterre (142). En Afrique sub-saharienne, des souches productrices d'OXA-48 ont été rapportées dans différents pays d'Afrique centrale et en Afrique du Sud. Enfin, plus récemment, des cas ont été décrits en Europe de l'Est (143), en Russie (144), au Canada et aux Etats-Unis (96).



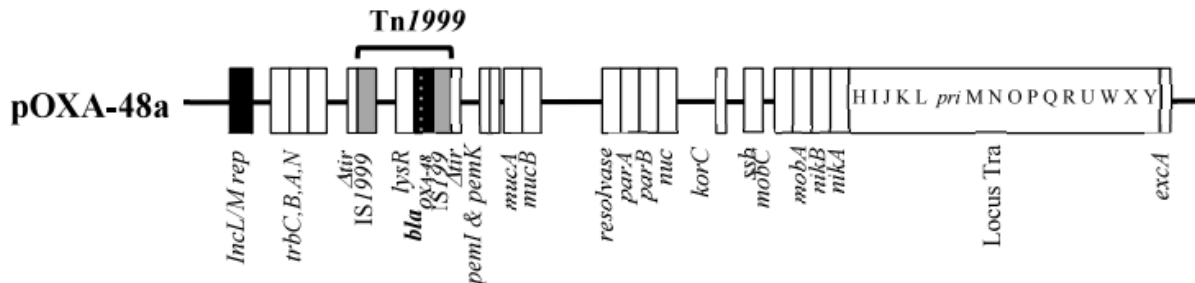
**Figure 12 : Distribution des Entérobactéries produisant une carbapénèmase de type OXA-48.**  
D'après Nordmann *et al.* (142).

### *iii. Spectre d'hydrolyse*

La  $\beta$ -lactamase OXA-48 a la particularité d'hydrolyser fortement toutes les pénicillines et les céphalosporines de 1<sup>ère</sup> génération. Cependant, les C3G sont peu, ou pas, hydrolysées. En effet, OXA-48 hydrolyse très faiblement le céfotaxime et elle n'a aucune activité vis-à-vis de la ceftazidime (134, 145). Enfin, elle présente une activité contre les carbapénèmes, qui reste néanmoins inférieure à celle des autres carbapénèmases telles que KPC et NDM. Les inhibiteurs des  $\beta$ -lactamases de classe A (acide clavulanique et tazobactam) sont inefficaces sur OXA-48.

#### iv. Localisation et Environnement génétique

Le gène *bla*<sub>OXA-48</sub> a été identifié sur un plasmide IncL de 62-Kb transférable ne possédant pas d'autres gènes impliqués dans une résistance associée (140), sur lequel le transposon Tn1999 avait été inséré (**Figure 13**). Les plasmides IncL/M sont des plasmides très répandus chez les entérobactéries. Ils sont responsables de l'acquisition de nombreux gènes de résistance aux antibiotiques. Le plasmide pOXA-48a a été identifié majoritairement dans les souches productrices d'OXA-48 isolées de différents pays (146).



**Figure 13 : Environnement génétique du gène *bla*<sub>OXA-48</sub>.** D'après Poirel et al.(146).

Initialement, le gène *bla*<sub>OXA-48</sub> a été identifié en association avec la séquence d'insertion IS1999 chez *K. pneumoniae* apportant ainsi une séquence promotrice au gène, permettant ainsi son expression (134). L'étude de l'environnement génétique du gène *bla*<sub>OXA-48</sub> a démontré qu'il est porté par un transposon composite, Tn1999 encadré par deux IS1999 (147). Après, le transposon Tn1999.2, identifiée chez une souche *K. pneumoniae*, diffère par une insertion de la séquence ISIR (140). Il a été montré que la séquence ISIR en amont du gène *bla*<sub>OXA-48</sub> augmente son expression (140). L'isoforme Tn1999.3 identifié chez *E. coli* en Italie possède 2 séquences ISIR(148) de part et d'autre du gène *bla*<sub>OXA-48</sub>. Le transposon Tn1999.4 identifié chez une souche de *E. coli* et de *E. cloacae* résulte de l'intégration au niveau du gène *lysR* du transposon Tn2015 qui véhicule le gène codant pour une BLSE CTX-M-15 (149). Enfin, dernièrement, un nouvel isoforme a été décrit, Tn1999.5. Il s'agit d'un variant du Tn1999.2 dans lequel est inséré l'élément ISKpn19 (150).



La présence du gène *bla*<sub>OXA-48</sub> a également été retrouvé dans le chromosome de deux souches de *E. coli* (151). L'acquisition d'une séquence d'insertion *ISIR* en amont du gène *bla*<sub>OXA-48</sub> (formant le Tn1999.2) a généré la formation d'un transposon composite, grâce à une autre copie d'*ISIR* située environ 21-kb en aval. Ce transposon appelé Tn6237 est capable de s'insérer à différents endroits du chromosome de *E. coli* (151, 152).

## 5. Les variants d'OXA-48

Depuis la première identification d'OXA-48, de nombreux variants ont été décrits. De nos jours, il existe une cinquantaine de variants chez les Entérobactéries. Tous ces variants diffèrent d'OXA-48 par des substitutions ou des délétions d'acides aminés, leurs pourcentages de similarité restent néanmoins supérieurs à 90 %. Les principales différences entre ces variants se situent dans la boucle qui relie le feuillet  $\beta 5$  au feuillet  $\beta 6$ . Ainsi, on distingue 4 spectres d'activités. Le premier est similaire à celui d'OXA-48, à savoir une hydrolyse des pénicillines et des carbapénèmes et une activité très faible, ou presque nulle, vis-à-vis des C3G et englobe les variants OXA-162, OXA-181.... Le deuxième possède des mutations ponctuelles dans la boucle  $\beta 5$ -  $\beta 6$ , notamment des changements R214 en S ou en G dans OXA-232 et OXA-244, respectivement, qui impacte fortement le niveau d'hydrolyse des carbapénèmes et de la témocilline (146). Le troisième, regroupant les variants OXA-163, 247 et 405 qui se caractérisent par une délétion de 4 AA dans la boucle  $\beta 5$  et  $\beta 6$  et par un spectre opposé : une hydrolyse des C3G et une perte quasi totale de l'hydrolyse des carbapénèmes. Enfin, le 4ème spectre regroupant les enzymes de type OXA-517, 438, 793 présentent une délétion de 2 AA seulement dans la boucle  $\beta 5$ - $\beta 6$  qui leur permet d'hydrolyser les C3G et les carbapénèmes (147).

Parmi les différents variants d'OXA-48, OXA-181 (qui diffère par quatre substitutions en AA) est la deuxième plus répandue. La première souche décrite fut isolée chez un patient transféré d'Inde en 2011 (148). Ce variant a été identifié dans plusieurs pays mais un lien avec l'Inde est systématiquement retrouvé (64, 148, 149). En l'espace de peu de temps, la carbapénémase OXA-48 et de ses variants sont isolées mondialement (150).

OXA-244 (OXA-48 R214G) ou OXA-232 (OXA181 R214S) ou OXA-484 (OXA-181 R214G) sont particulièrement intéressants car ils confèrent des CMI aux carbapénèmes et de témocilline plus faibles par rapport aux producteurs d'OXA-48 ou OXA-181 (146).

## VIII. Voies de transmission de la résistance

La résistance acquise peut être causée par des mutations chromosomiques ou des gènes acquis par des éléments génétiques mobiles (MGE). La résistance médiée par des mutations chromosomiques est généralement stable et peut être transférée de la cellule mère à la descendance de manière verticale (Transfert vertical). Au contraire, les MGE peuvent favoriser le transfert horizontal de gènes (HGT), qui peut impliquer une transmission intra ou inter-espèces. La propagation de la résistance aux antibiotiques parmi les bactéries est médiée par (i) les plasmides, (ii) des transposons ou (iii) des intégrons porteurs de gènes de résistance par transduction, conjugaison ou transformation (**Tableau2**).

**Tableau 2 : Caractéristiques des différents éléments impliqués dans la dissémination des gènes de résistance**

Elément	Caractéristiques	Rôle dans la diffusion des gènes de résistance
Plasmide autotransmissible	Circulaire, réplication autonome, porte les gènes nécessaires au transfert conjugatif d'ADN	Transfert de gènes de résistance ; mobilisation d'autres éléments qui portent des gènes de résistance
Plasmides mobilisables	Possède l'origine de transfert, mais requiert les gènes de transfert à partir d'un autre plasmide	Transfert de gènes de résistance ; mobilisation d'autres éléments qui portent des gènes de résistance
Transposon	Peut se déplacer d'un segment d'ADN à un autre au sein d'une même cellule	Peut transporter des gènes de résistance de chromosome en plasmide ou vice versa
Gène cassette	Segments d'ADN circulaires et non répliatifs contenant uniquement des cadres de lecture ouverts ; s'intègre dans les intégrons	Porter des gènes de résistance
Intégron	Segment d'ADN intégré qui contient un intégrase, un promoteur et un site d'intégration pour cassettes de gènes	Forme des groupes de gènes de résistance, le tout sous le contrôle du promoteur de l'intégron

### 1. Les plasmides

La transmission de gènes de résistance médiée par les plasmides est la voie la plus fréquente pour l'acquisition de matériel génétique exogène (153, 154). Comme décrit dans

(**Tableau 2**), les plasmides sont molécules d'ADN double brin, circulaires extra-chromosomiques capables de répllication autonome et peuvent médier la résistance à plusieurs classes d'antimicrobiens, notamment les  $\beta$ -lactamines, les tétracyclines, aminoglycosides, sulfamides, chloramphénicol, triméthoprime, colistine, macrolides et quinolones (124, 155). Les plasmides mobilisent les gènes de résistance et favorisent le transfert horizontal de déterminants de la résistance parmi les bactéries de différentes espèces, genres et règnes, en fonction de leur gamme d'hôtes étroite ou large, leurs propriétés conjugatives et l'efficacité de la conjugaison (156). Une trentaine de groupes d'incompatibilité ont été identifiés chez les Entérobactéries (157). Certains sont particulièrement associés à la résistance aux antibiotiques, notamment les plasmides de type IncF, IncA/C, IncL/M, IncI1, IncH et IncN (158). Actuellement, la base de données PlasmidFinder permet d'identifier plus de 120 réplicons à partir des données de séquençage (159).

Une autre caractéristique des plasmides est leur capacité à être transférés par conjugaison ou non. Les plasmides conjugatifs, généralement de grande taille, peuvent se transmettre horizontalement entre bactéries. Chez les bacilles à Gram négatif, la conjugaison est sous la dépendance de l'opéron *tra* qui commande la synthèse de pili « sexuels » formant un pont cytoplasmique entre la bactérie donneuse et la bactérie réceptrice (160).

## **2. Les éléments transposables**

Les éléments transposables sont des séquences d'ADN capables de promouvoir leur translocation d'un site à un autre, qu'il soit localisé sur une même molécule d'ADN ou non. Ils doivent s'intégrer dans un réplicon pour être dupliqués avec lui. À la différence de la recombinaison classique, la transposition s'effectue en l'absence d'homologie génétique entre l'élément transposable et la molécule d'ADN cible. Elle conduit à l'insertion d'un élément génétique transposable dans l'ADN cible; il y a donc addition et non échange de matériel génétique. Les éléments transposables bactériens impliqués dans la résistance aux antibiotiques comprennent essentiellement les séquences d'insertion et les transposons

### **a. Les séquences d'insertion**

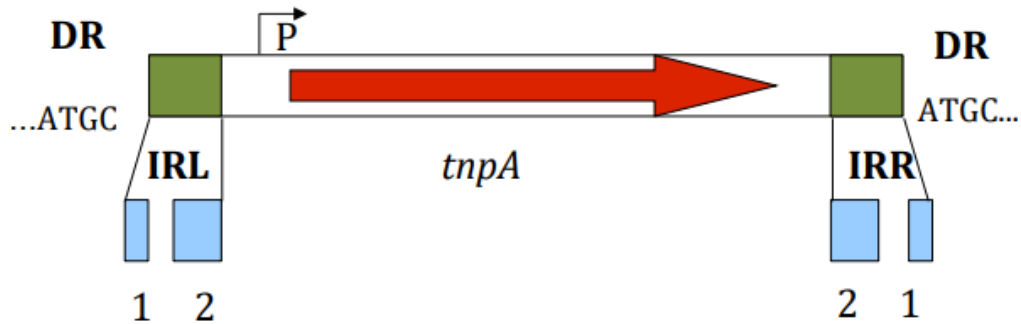
Les séquences d'insertion (IS) sont des fragments d'ADN de taille variable mais généralement inférieure à 2,5 kb. Elles sont capables de s'exciser et de s'insérer à différents

endroits dans une séquence d'ADN cible. Leur séquence code pour une transposase qui contribue à la mobilité de l'élément ainsi qu'à son intégration au sein de la séquence cible (161). Il existe actuellement plusieurs milliers de séquences déposées sur la base de données ISFinder (<http://www-is.biotoul.fr>) (162). Les IS identifiées chez les procaryotes sont regroupées en une vingtaine de familles. Elles sont classées en fonction des similarités dans leur organisation génétique, des homologies de séquence de leur transposase, de leurs séquences répétées inversées, des duplications de site générées et de leur fréquence de transposition (161). Les IS peuvent être présentes en une ou plusieurs copies sur le chromosome bactérien mais aussi sur des plasmides.

Les IS ne codent généralement pour aucune autre fonction que celles qui permettent leur mobilisation.

- La transposase est la protéine indispensable pour la mobilisation de la séquence d'insertion, par une ou deux phases ouvertes de lecture occupant la quasi-totalité de la séquence de l'IS. Elle possède deux domaines fonctionnels. Le premier est impliqué dans l'activité catalytique et se situe dans le domaine C terminal de la protéine, alors que l'extrémité N terminale assure la reconnaissance de certains domaines de l'ADN. Le domaine catalytique possède une triade catalytique DDE très conservée parmi les IS et qui joue un rôle important dans les réactions de transposition (161).
- Les séquences inversées répétées (IR) plus ou moins parfaites de 5 à 40 pb appelées « Inverted Repeat Left (IRL) et Inverted Repeat Right (IRR) », délimitent les IS. Ces séquences sont indispensables pour la transposition et sont constituées de deux domaines fonctionnels : le premier correspond aux 2-3 dernières paires de bases et est reconnu lors de l'étape de clivage médiée par la transposase alors que le second est impliqué dans la fixation de la transposase se trouvant à l'intérieur des séquences inversées répétées (**Figure14**) (161, 163).
- Séquences en répétition directe également nommées Direct Repeat (DR) ou Target Site Duplication (TSD), sont générées par les IS lors de leur insertion. Ces DR représentent une duplication du site d'insertion formant, aux extrémités de l'IS, deux séquences en répétition directe. Les DR sont de taille variable (entre 2 et 15 paires de bases). La duplication de site d'insertion est une signature d'un événement de transposition (**Figure 14**). Cependant, en

fonction du type de transposase, certaines IS ne font pas de DR lors d'un événement de transposition. Cela dépend du mécanisme de clivage de l'ADN.



**Figure 14 : Organisation générale d'une séquence d'insertion.** La flèche rouge représente le gène *tnpA* codant la transposase. Les boîtes vertes représentent les séquences répétées inversées gauche (IRL) et droite (IRR) (inverted repeat right and left). Les séquences nucléotidiques ATGC correspondent à des exemples de séquences répétées direct (DR). Les boîtes 1 représentent les dernières bases impliquées dans le clivage et transfert des brins. Les boîtes 2 représentent les bases impliquées dans la reconnaissance de la séquence d'insertion par la transposase. La flèche P indique le promoteur et le site de fixation du ribosome.

## b. Les transposons

Les transposons (Tns) sont un groupe d'éléments génétiques mobiles qui peuvent se déplacer à différents endroits du génome. L'existence de l'enzyme nommée Transposase, dans les Tns, provoquent leur transposition de la molécule d'ADN vers d'autres endroits sur le même ADN ou sur d'autres molécules d'ADN (164). Les Tns, qui contiennent des gènes de résistance aux antibiotiques provoquent la transmission de la résistance entre les bactéries. Les transposons les plus fréquemment impliqués dans la mobilisation de gènes de résistance sont les transposons composites et les transposons de type Tn3.

- Les transposons composites, ou transposons de classe I, sont constitués d'un fragment d'ADN encadré par deux copies de la même IS. Lors de la transposition d'une IS, la transposase reconnaît ses deux IR. Il peut arriver que cette IS reconnaisse son IRL et une IRR (ou vice versa) appartenant à une autre IS située dans son environnement proche. Il y a alors transposition des deux IS simultanément. Le fragment d'ADN encadré par ces deux IS, qui peut contenir des gènes de résistance, de virulence ou de métabolisme, est ainsi mobilisé.

- Les Transposons non-composites de type Tn3 ou transposons de classe II, se caractérisent par l'absence des IS de part leurs extrémités et la présence d'ADN exogène à l'intérieur de la séquence délimitée par les IR. Une transposase permet la mobilisation des transposons de type Tn3. Ces transposons transposent de façon très efficace en entraînant une duplication du site d'insertion de 5-pb. Ne s'excisant pas du réplicon donneur, deux copies du transposon initial sont donc obtenues après le mécanisme de transposition.

### c. Les intégrons

Comme le montre le **Tableau 2**, les intégrons sont des segments d'ADN intégrés contenant une intégrase (*intI*), un promoteur et un site d'intégration pour les cassettes génétiques (CG). L'intégrase est capable d'exciser et d'intégrer les gènes cassettes au niveau d'un site de recombinaison spécifique adjacent au gène *intI* et nommé *attI*

Ce sont des éléments génétiques bactériens capables de favoriser l'expression et l'acquisition de gènes intégrés dans les CG (48). Les CG sont des segments d'ADN circulaires et non répliatifs contenant uniquement des cadres de lecture ouverts et qui peuvent s'intégrer dans les intégrons (165). Les études décrivent deux grands groupes d'intégrons : les intégrons chromosomiques (IC) et les intégrons mobiles (IM).

Les IC sont situés sur le chromosome des espèces bactériennes, tandis que les IM ne sont pas des éléments auto-transposables mais sont situés sur des éléments génétiques mobiles tels que les transposons et plasmides. Les IM contiennent un nombre limité de CG codant pour des déterminants de la résistance aux antibiotiques (166).

Cinq classes d'IM ont été décrites, parmi lesquelles les classes 1 et 2 sont les plus couramment étudiés (167). Les IM de classe 1 présentent une large répartition parmi les BGN et sont les plus signalés chez les animaux et les humains. Ils sont principalement associés avec des transposons fonctionnels et non fonctionnels dérivés du Tn402 (168). Les IM de classe 2 sont principalement composés du CG *sat2* (impliqué dans la résistance à la streptothricine), *dfrAI* (impliqué dans la résistance au triméthoprim) et *aadAI* (impliqué dans la résistance à la streptomycine et à la spectinomycine) (169).

## **IX. Méthodes de détection des Entérobactéries productrices de carbapénèmases**

Le niveau de résistance aux carbapénèmes varie d'une souche à l'autre, en fonction de la carbapénémase exprimée mais également en fonction des autres  $\beta$ -lactamases associées (BLSE, céphalosporinases...) et de l'association de mécanismes non enzymatiques (impermeabilité, efflux...). La détection précoce des EPC a pour but (i) d'adapter au plus vite l'antibiothérapie en cas d'infection par une bactérie multi-résistante et (ii) de pouvoir mettre rapidement en place des mesures de contrôle visant à limiter la dissémination de ces germes multi-résistants d'un patient à l'autre (isolement des patients, personnel dédié ...).

### **1. Les méthodes phénotypiques**

#### **a. Antibiogramme et concentration minimale inhibitrice (CMI)**

Quand les souches d'Entérobactéries présentent une diminution de sensibilité à au moins une des carbapénèmes, la production de carbapénémase doit être suspectée.

Depuis 2015, le Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM), a établi des recommandations nationales pour aider les laboratoires à détecter les EPC. Comme l'ertapénème est le carbapénème permettant d'obtenir une sensibilité maximale dans le dépistage des souches d'EPC, l'algorithme commence par la détermination de la sensibilité à l'ertapénème (170). Ainsi toute souche ayant une sensibilité diminuée à l'ertapénème (CMI > 0.5 mg/L ou un diamètre d'inhibition < 25 mm), doit être suspectée comme EPC et faire l'objet de tests supplémentaires. La notion de cut-offs épidémiologiques ou screening cut-offs ou encore seuil de dépistage, a été introduite par l'EUCAST (171). Ces cut-offs présentent des valeurs basses permettant ainsi l'amélioration de la détection des EPC, surtout celles qui ne présentent phénotypiquement qu'une faible diminution de sensibilité aux carbapénèmes. Cependant, la spécificité est faible car les CMIs des souches associant des BLSE et/ou des céphalosporinases et/ou impermeabilité dépassent souvent ces cut-offs.

### **b. Disques combinés avec inhibiteur**

La caractérisation de la carbapénémase produite peut se faire à l'aide de l'utilisation d'inhibiteurs spécifiques aux différentes classes. Ainsi, les MBLs, par exemple, sont inhibées par l'acide dipicolinique ou EDTA alors que les carbapénémases de type KPC sont inhibées, partiellement, par l'acide boronique. L'acide boronique inhibe également les céphalosporinases (Classe C de Ambler). L'avibactam est, quant à lui, un nouvel inhibiteur capable d'inhiber les  $\beta$ -lactamases de classe A (BLSE et carbapénémases), les carbapénémases de type OXA-48 ainsi que les céphalosporinases. La détection de ces carbapénémases de type OXA-48 fait également intervenir l'utilisation d'un disque contenant de la témocilline. En effet, 90% des EPC exprimant OXA-48 sont très résistantes à cet antibiotique (diamètre d'inhibition inférieur à 12 mm pour des disques chargés à 30  $\mu$ g, soit une CMI > 256 mg/L) (172).

### **c. Test de Hodge modifié**

Ce test permet la mise en évidence d'une synergie d'activité enzymatique entre souches productrices de carbapénémases (souche à tester) et une souche sauvage de référence sensible aux carbapénèmes. Pour cela, un disque contenant un carbapénème est déposé sur une gélose de Mueller-Hintonensemencée avec une souche sauvage *E. coli* ATCC25922 sensible aux carbapénèmes. Quelques colonies de la souche à tester sont prélevées et déposées sur la gélose de manière rectiligne depuis le disque contenant le carbapénème vers la périphérie de la boîte. Un contrôle positif est également réalisé de la même façon. La boîte est ensuite incubée de 16 à 24 heures à 37°C. La production d'une carbapénémase est détectée si la souche à tester permet la croissance de la souche sauvage dans le diamètre d'inhibition (en direction du disque). On parle alors d'un profil « clover leaf-like ». En effet, la production d'une carbapénémase par la souche à tester dégradera l'antibiotique ce qui permettra à la souche de *E. coli* sauvage de croître au plus près du disque contenant le carbapénème. Ce test présente de nombreux faux positifs avec notamment des *E. cloacae* hyperproduisant l'AMPC et des faux négatifs avec notamment des metallo- $\beta$ -lactamases.



#### **d. Carbapenem-Inactivation Method (CIM):**

Le *Carbapenem-Inactivation Method* (CIM) test peut être considéré comme un dérivé du test de Hodge, reposant sur l'hydrolyse du méropénème contenu dans un disque chargé à 10 µg pendant 2h par la souche à tester, puis l'étude de la sensibilité d'une souche de *E. coli* sensible ATCC 29522 à ce disque (173). Si la souche à tester produit une carbapénémase, le méropénème est hydrolysé pendant l'incubation, et la souche de *E. coli* ATCC 29522 poussera au contact du disque. A l'inverse, si la souche à tester ne produit pas de carbapénémase, le méropénème restera actif et une zone d'inhibition sera visible.

Le modified-CIM (mCIM) est une version légèrement modifiée du CIM qui permet d'augmenter la sensibilité du test pour la détection des carbapénémases de type OXA-48, notamment en prolongeant l'incubation du disque de méropénème avec la souche à tester à 4h. Le mCIM a fait l'objet d'une évaluation multicentrique (174), et a été intégré aux recommandations américaines du CLSI (Clinical and Laboratory Standards) pour la détection des EPC avec spécification de diamètres limites.

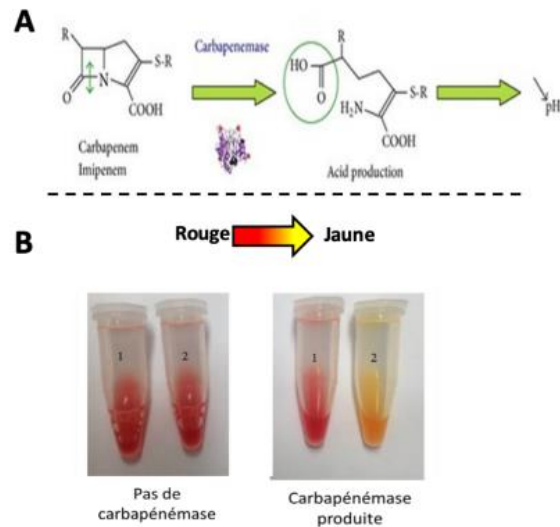
Enfin, un dérivé de cette méthode, le rapid CIM (rCIM), permet la détection des EPC sans passer par une croissance sur un milieu gélosé permettant ainsi un gain de temps considérable. En effet, le délai de réponse passe d'une durée de 16h/24h à 2h30. Ce test repose sur l'hydrolyse de méropénème contenu dans deux disques (10 µg). Après incubation, le surnageant est récupéré puis mis en présence d'une suspension à 1 MacFarland de la souche *E. coli* sensible ATCC 25922. On suit la culture de la souche de *E. coli* au néphélomètre pendant au maximum deux heures. Si la bactérie testée produit une carbapénémase, le méropénème a été hydrolysé et on observera une croissance normale de la souche ATCC en présence du disque de méropénème ayant été incubé avec la souche à tester ( $\Delta DO$  en 2h > 1 MacFarland). A l'inverse, si la bactérie testée ne produit pas de carbapénémase, le méropénème présent dans le surnageant reste actif et inhibe la croissance de la souche ATCC ( $\Delta DO$  en 2h < 0,5 McF).

## 2. Les tests d'hydrolyse des carbapénèmes

### a. Tests colorimétriques

Le CarbaNP test est une technique biochimique colorimétrique de diagnostic rapide visant à détecter une activité carbapénémase (175). Son principe se base sur la détection de la production d'enzyme, par la mise en évidence de l'acidification du milieu lors de l'hydrolyse de l'imipénème par une carbapénémase. Le milieu contient un indicateur de pH, le rouge de phénol. L'hydrolyse de l'imipénème par une carbapénémase, aboutit à une acidification du milieu, et par la suite un virage de l'indicateur coloré du rouge au jaune. Ce test est capable de détecter l'ensemble des enzymes possédant une activité carbapénémase sans restriction de classe. Il possède une excellente spécificité. Sa sensibilité 73 varie de 90% à 100% selon les études ; un défaut de sensibilité a été rapporté pour certains variants de type OXA-48 et certains variants GES pour lesquels l'activité carbapénémase est faible (176, 177).

Le  $\beta$ -CARBA test est également un test colorimétrique (commercialisé par la société BioRad) de détection rapide de carbapénémase, basé sur l'hydrolyse d'un substrat, une  $\beta$ -lactamine chromogénique dont l'identité reste inconnue. Les avantages de ce test sont sa grande facilité d'utilisation et d'interprétation, tout comme le CarbaNP test. En revanche, on note l'absence de détection de certaines enzymes de classe A non-KPC, comme IMI, SME et FRI, très minoritaires en France, et de certains variants d'OXA-48 (176). 231 Ce kit présente une sensibilité de 84,9% et une spécificité de 95,6% (176).



**Figure 15 : A. Principe des tests colorimétriques ; B. Résultats du Carba NP test D'après Nordmann et al. (175).**

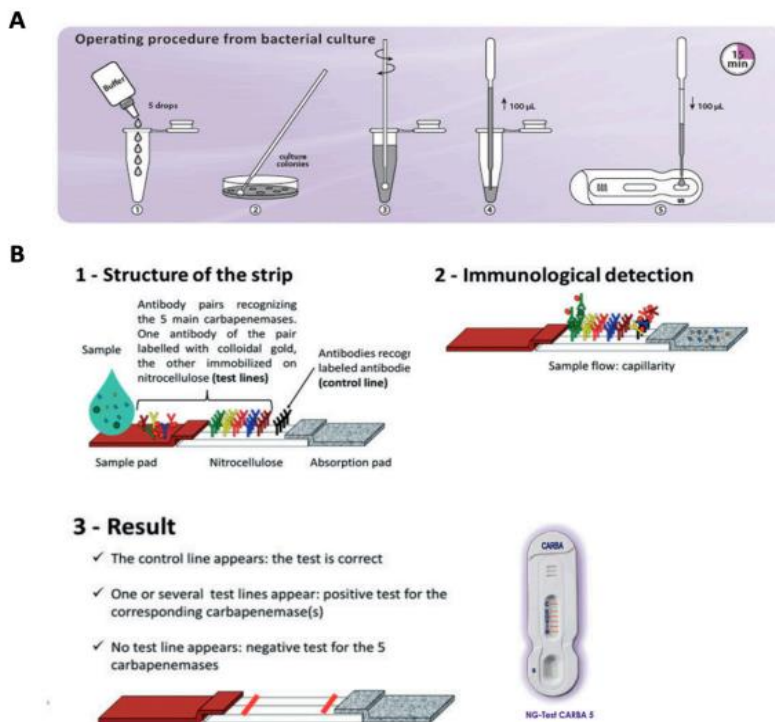
### b. Test spectrométrique (MALDI-TOF)

Cette technique repose sur la détection d'un carbapénème et de son éventuel produit de dégradation par spectrométrie de masse de type MALDI-TOF. Plusieurs techniques « maison » ont été développées, mais une seule est actuellement commercialisée : MBT STAR®-Carba IVD Kit (Bruker Daltonics) (179-181). Ces techniques présentent de très bonnes sensibilités et spécificités mais nécessitent une mise au point fine, du personnel particulièrement formé et un spectromètre de masse avec système ouvert (182). La spectrométrie de masse a également été utilisée pour détecter les souches produisant spécifiquement la carbapénémase KPC (183). En effet, sur les spectres utilisés pour l'identification de la bactérie, un pic situé à 11,109 Da est présent uniquement chez les souches produisant cette carbapénémase (183, 184). Ce pic correspond à une protéine hypothétique appelée p019, dont le gène est localisé sur le plasmide pKpQIL, en aval du site d'insertion du Tn4401a. Il est cependant nécessaire d'indiquer que ce gène est absent des plasmides portant les autres isoformes du Tn4401 ou les structures de type NTEKPC. p019 est présent dans 97,8% des génomes possédant Tn4401a (184). Par son manque de sensibilité vis à vis de l'ensemble des structures génétiques portant *bla*<sub>KPC</sub>, cette technique ne peut donc pas être utilisée pour le diagnostic des EPC dans des zones à faible prévalence en KPC mais son intérêt

réside principalement dans le suivi d'épidémies après caractérisation génétique de la souche incriminée (185).

### **3. Tests immunochromatographiques**

Plusieurs tests immunochromatographiques qui détectent directement la production de l'enzyme responsable de la résistance ont été commercialisés pour la détection des EPC. De plus, contrairement aux tests phénotypiques et biochimiques, ils ne sont pas influencés par l'activité enzymatique même faible de l'enzyme. Des résultats faux négatifs peuvent avoir lieu, à cause de l'émergence de variants conduisant à une modification de la structure tertiaire empêchant ainsi la reconnaissance de la carbapénèmase par les anticorps. Le principe de ce test est simple, il suffit de mettre les bactéries en contact avec un tampon de lyse, puis déposer l'extrait sur la cassette. Les résultats sont généralement obtenus dans un délai de 15 minutes. Les premiers tests étaient unitaires et permettaient la détection des carbapénémases de type OXA-48 ou KPC (186, 187). Rapidement, des tests multiplexés ont été commercialisés pour la détection de 3 types de carbapénèmase (RESIST-3 O.K.N (CORIS Bioconcept) pour la détection d'OXA-48-like/KPC/NDM, puis de 4 carbapénémases avec RESIST-4 O.K.N.V (CORIS Bioconcept) permettant la détection de VIM en plus (188). Enfin, le kit NG-Test CARBA 5 (NG Biotech) est capable de détecter les enzymes de type OXA-48-like/KPC/NDM/VIM et IMP (189, 190).



**Figure 16 :** NG-Test CARBA 5 : **A.** Protocole ; **B.** Principe et résultat. *D'après Boutal et al.(189) et <https://ngbiotech.com/carbapenemases/>*

#### 4. Techniques moléculaires

Ces méthodes permettent de révéler la présence des gènes codant pour les carbapénèmases. Pour cela, les gènes d'intérêt sont amplifiés par PCR. L'analyse de la PCR en point final se fait par visualisation du ou des produits de l'amplification s'il s'agit du mono-test ou d'un multi-tests respectivement (191). Il faut compter en moyenne 4h pour avoir les résultats. Il existe également une autre méthode : la PCR en temps réel, ici, l'amplification du gène recherché est mise en évidence par une émission de fluorescence qui est mesurée au cours de la réaction. De nombreux kits sont commercialisés, et s'utilisent à partir de colonies ou directement à partir d'échantillons cliniques tels que des écouvillons ou encore des hémocultures positives. La plupart de ces kits, sont basés sur l'analyse syndromique, reposant sur la réalisation de PCR multiplex permettant ainsi l'identification de plusieurs gènes sur un même échantillon. La sensibilité ainsi que la spécificité de ces outils de diagnostic sont très bonnes (192-195). L'inconvénient majeur de ces techniques

moléculaires réside dans le fait qu'ils ne détectent que les gènes recherchés et donnent un résultat positif même si ces gènes ne sont pas exprimés. De plus, une mutation survenant au niveau du site d'hybridation des amorces peut conduire à de faux négatifs (196).

De même, la technique d'amplification isotherme par boucle, ou *Loop-mediated isothermal amplification* (LAMP), est une méthode de biologie moléculaire qui permet d'amplifier rapidement et spécifiquement l'ADN à une température constante. Contrairement à la PCR qui nécessite des cycles thermiques, le LAMP se déroule à une seule température, simplifiant ainsi le processus. Cette technique utilise des amorces spécifiques en forme de boucle, qui se lient à l'ADN cible, et une ADN polymérase à activité élevée pour produire rapidement des copies multiples de la séquence cible. Le LAMP est largement utilisé pour la détection rapide de pathogènes, la génétique moléculaire et d'autres applications diagnostiques, offrant une alternative efficace et pratique aux méthodes conventionnelles d'amplification de l'ADN (197).

## **X. Situation de la résistance aux antibiotiques au Liban**

La production de carbapénémase est la principale cause de résistance aux carbapénèmes chez les Enterobacterales (90). Malgré la mise en œuvre de stratégies et des mesures cohérentes pour prévenir et contrôler l'émergence et la propagation de la résistance dans les pays en cours de développement, ce problème continue de prendre de l'ampleur dans un grand nombre de ces pays (198). Cependant, le problème de la résistance est plus compliqué dans les pays à faible ou moyen revenus, comme le Liban, car de nombreux facteurs, conduisant à l'émergence et à la propagation de souches multi-résistante, restent incontrôlables.

La disponibilité croissante des antibiotiques au Liban est généralement une bonne chose, mais l'utilisation abusive de ces médicaments a créé et favorisé la transmission des bactéries résistantes aux antibiotiques, en particulier dans les communautés les plus pauvres telles que les réfugiés syriens et palestiniens et la population libanaise vulnérable (199). Outre la disponibilité d'antibiotiques sans ordonnance dans les pharmacies, divers facteurs contribuent à la résistance aux antibiotiques au Liban, notamment (i) une mauvaise sensibilisation aux antibiotiques des prescripteurs et du grand public, (ii) une utilisation intensive des antibiotiques chez les humains, les animaux et dans l'agriculture, (iii) l'utilisation excessive d'antibiotiques comme facteurs

favorisant la croissance du bétail, (iv) le non-respect de la durée de l'antibiothérapie, (v) la promotion de l'automédication aux antibiotiques, (vi) une vente incontrôlée des antibiotiques et (vii) l'absence de mesure visant à contrôler la diffusion de ces bactéries en milieu hospitalier.

Malgré les progrès remarquables réalisés dans l'élaboration d'un plan d'action national pour lutter contre la propagation de la résistance au Liban, les taux de résistance se situent à l'extrémité supérieure des niveaux signalés à l'échelle mondiale (200). De même, de grandes quantités d'antibiotique sont utilisées dans les domaines vétérinaire, de l'élevage et de l'agriculture sans surveillance, ni contrôle, provoquant une augmentation rapide de la prévalence de la résistance au Liban. D'autre part, une étude transversale nationale menée en 2017, en synchronisation avec la première : la campagne libanaise de sensibilisation aux antibiotiques a révélé des connaissances très insuffisantes, de mauvaises attitudes et des pratiques inadéquates à l'égard des antibiotiques parmi les populations résidentes au Liban (201). Malheureusement, les bactéries multirésistantes et les gènes de résistance ont désormais largement disséminés parmi les humains, les animaux et l'environnement, et leur présence dans ces secteurs a également été régulièrement rapportée au Liban (202-207).

### **1. La résistance aux carbapénèmes en milieu clinique:**

Les Entérobactéries résistantes aux carbapénèmes constituent une menace alarmante pour la communauté libanaise. Des isolats cliniques isolés à partir de différents sites et de différents hôpitaux partout au Liban sont de plus en plus signalés. Une première étude concernant la sensibilité bactérienne aux antibiotiques a été réalisée au Centre médical de l'Université américaine de Beyrouth (AUBMC). Au total, 3 773 bactéries à Gram négatif ont été examinées entre mars 1992 et le 30 juin 1993. Toutes les Entérobactéries, y compris *Citrobacter* spp., *Enterobacter* spp., *E. coli*, *Proteus* spp. et *Serratia* spp., se sont révélées sensibles à 100 % à l'imipénème (208).

Après cette étude, les données étaient rares jusqu'aux années 2007-2008, où plusieurs études sur les Entérobactéries résistantes aux carbapénèmes, ont été publiées. En 2007, la première souche *K. pneumoniae* productrice d'une M $\beta$ L, a été isolée à partir d'une lésion cutanée chez un patient de 58 ans qui avait subi plusieurs interventions chirurgicales. *Bla*<sub>IMP</sub> a été mise en évidence par des méthodes phénotypiques et ensuite identifiée par d'autres tests : *bla*<sub>IMP-1</sub> (209).

En 2008, Matar *et al.*, ont rapporté la première souche de *K. pneumoniae* productrice d'OXA-48 chez une fille de 7 ans ayant des antécédents d'infection récurrente des voies urinaires (IVU) (210).

En 2010, une souche de *K. pneumoniae* et une souche de *E. coli* isolées chez 2 patients souffrant d'infection urinaire acquise dans la communauté, ont été identifiées résistantes aux carbapénems. Les souches ont été productrices de *bla*<sub>OXA-48</sub> (211). Ces résultats ont évoqué une situation alarmante, du fait que les Entérobactérie productrices d'OXA-48 sont devenues répandue même en tant qu'agent communautaire. Dans cette même année, 3 patients Irakiens, ont été identifiés avec des souches des Entérobactéries productrices de carbapénemase. Les 3 souches ont été isolées à partir d'hémoculture : Une souche de *E. coli* productrice du gène *bla*<sub>OXA-48</sub>, une souche de *K. pneumoniae* coproductrice de *bla*<sub>OXA-48</sub> et *bla*<sub>NDM-1</sub> et une souche de *K. pneumoniae* productrice de *bla*<sub>NDM-1</sub>. Les trois isolats abritaient également OXA-1, TEM-1 et CTXM-15 avec des mutations de porine de la membrane externe OmpF et OmpC (212).

En 2011, une étude menée par Baroud *et al.* a montré que 1.07% des *E. coli* et 2.45% des *K. pneumoniae* productrices de BLSE, étaient résistantes à l'ertapénème (213).

Selon une étude réalisée au nord du Liban par Beyrouthy *et al.* sur la propagation du gène *bla*<sub>OXA-48</sub> chez les Entérobactéries, le taux d'Entérobactéries présentant une diminution de la sensibilité à l'ertapénème est passé de 0,4% en 2008-2010 à 1,6% en 2012 pour des isolats cliniques prélevés des patients hospitalisés. De plus, cette étude a montré que parmi les EPC isolées, 88% produisent des OXA-48, indiquant une forte prévalence de ce type d'enzyme au Liban (202).

Une autre étude rétrospective, menée par Moghnieh *et al.*, a montré que 8.8% des souches de *E. coli* isolées chez des patients traités pour un cancer, sont résistantes à l'imipénème (214).

Durant les années 2011-2012, Hammoudi *et al.*, dans le cadre de surveillance des bactéries gram négatif résistantes aux carbapénèmes à l'hôpital Hôtel Dieu de France (HDF), a montré que les souches de *K. pneumoniae* résistantes à l'imipénème et productrices d'OXA-48 a augmenté de 0.8% en 2011 à 0.9% en 2012 (215).

Chamoun *et al.*, a mené une étude rétrospective sur des isolats bactéries provenant des laboratoires de bactériologie 16 hôpitaux au Liban. Les résultats ont montré qu'au cours des 3



années, *E. coli* présente le taux le plus faible de résistance à l'imipénème avec un taux stable de 0,7%, alors que 2% des *K. pneumoniae* étaient résistantes à l'imipénème (200).

Entre 2015 et 2016, une étude rétrospective est menée dans 13 hôpitaux Libanais, pour examiner les derniers changements concernant la résistance aux antibiotiques, y compris les Entérobactéries résistantes aux carbapénèmes. Les données ont été comparées au rapport de surveillance réalisé par Chamoun *et al.* au cours des années 2011-2013. Les données montrent désormais une nette augmentation des EPC, où 3 % des *E. coli* étaient résistants à l'imipénème, contre 0,7 % dans le rapport de surveillance 2011-2013 et 4 % des *K. pneumoniae* étaient résistants au carbapénème, contre 2 % dans le rapport 2011-2013.(216).

Une étude rétrospective menée à l'hôpital Saint Georges à Beirut entre 2010 et 2018 a montré que le pourcentage d'Entérobactéries productrices de carbapénèmases (EPC) parmi les souches résistantes aux carbapénèmes était 3% en 2010-2011 et a augmenté à 8% en 2017. Ensuite cette prévalence a quadruplé à 32% en 2018 (217). Cette étude a montré un changement épidémiologique parmi les organismes résistants aux carbapénèmes, passant de la prédominance des fermenteurs sans lactose à la prédominance des fermenteurs du lactose.

Durant cette même période, 2010 et 2018, Moussally *et al.* a étudié la prévalence des organismes résistants à l'hôpital AUBMC et a montré que des souches de *E. coli* et *K. pneumoniae* résistantes aux carbapénèmes ont augmenté de 0% en 2010 jusqu'à 3,3% et 7% en 2018, respectivement (218).

Enfin, AL-Bayssari *et al.* a montré dans une étude concernant 23 EPC collectées à partir de l'hôpital de Zgharta (Zgharta, Liban Nord), que la majorité des souches de *K. pneumoniae* étaient productrices d'OXA-48 alors que les souches de *E. coli* étaient productrices de *bla*<sub>NDM-4</sub>, de *Enterobacter cloacae* productrices de *bla*<sub>NDM-1</sub> et quelques souches de *K. pneumoniae* coproductrices de *bla*<sub>OXA-48</sub> et *bla*<sub>NDM-6</sub> (219). Cette étude a identifié pour la première fois le gene *bla*<sub>NDM-1</sub> chez *Enterobacter cloacae* et a suggéré l'augmentation de la prévalence du gene *bla*<sub>NDM</sub> chez les EPC.

**Tableau 3 : Les carbapénèmases identifiées au Liban.** Un résumé de tous les types de carbapénèmases détectés au Liban depuis la première carbapénèmase signalée en 2007. IMP : imipenemase métallob- $\beta$ -lactamase ; NDM : métallob- $\beta$ -lactamase de New Delhi ; OXA : oxacilline carbapénèmase/oxacillinase. *D'après et Fadlallah et al. (220).*

Years	Studies	Types of detected carbapenemase
2007	Daoud <i>et al.</i>	IMP
2008	Matar <i>et al.</i>	OXA-48
2010	El-Herte <i>et al.</i>	OXA-48 and NDM-1
2011	Baroud <i>et al.</i>	OXA-48 and NDM-1
2011-2012	Hammoudi <i>et al.</i>	OXA-48
2012	Hammoudi <i>et al.</i>	OXA-48
2012	Beyrouthi <i>et al.</i>	OXA-48
2008-2014	Kissoyan <i>et al.</i>	OXA-48 and NDM-1
2011-2015	Hajj <i>et al.</i>	OXA-48 and OXA-244
2012-2016	Dagher <i>et al.</i>	OXA-48 and OXA-181
2018-2019	Bayssari <i>et al.</i>	OXA-48, NDM-1 and NDM-4

Dans l'ensemble, les carbapénèmases les plus fréquemment signalées parmi les Entérobactéries en milieu hospitalier étaient OXA-48, OXA-232 chez *E. coli*, NDM-1, OXA-48, OXA-162, OXA-232 chez *K. pneumoniae* (221, 222). De plus, le gène *bla*<sub>OXA-181</sub> a été identifié récemment sur le plasmide IncX3 chez un *E. coli* ST940 résistant aux carbapénèmes, isolé d'un patient en soins intensifs (223). Dans deux études, le gène *bla*<sub>OXA-48</sub> était localisé sur le même plasmide IncL (chez *E. coli* et *K. pneumoniae*), suggérant la propagation de ce déterminant de résistance entre espèces (221, 224). Cependant, Moussa *et al.* a montré que *bla*<sub>OXA-48</sub> peut également être porté sur un plasmide IncFII dans Tn6237 (225).

## 2. La résistance aux carbapénèmes en milieu extra clinique:

Al Bayssari *et al.* a été le premier qui a rapporté des souches de *P. aeruginosa* et *A. baumannii* productrices des gènes *bla*<sub>VIM 2</sub> et *bla*<sub>OXA-23</sub>, respectivement, isolées de bétail et volaille (226). De plus, ils ont également détecté la co-occurrence des gènes *bla*<sub>OXA-23</sub> et *bla*<sub>OXA-58</sub> dans la même souche de *A. baumannii* isolée de volaille. De même, Rafei *et al.* a identifié une souche d'*A. baumannii* hébergeant *bla*<sub>OXA-143</sub> et un *Acinetobacter pittii* hébergeant *bla*<sub>OXA-24</sub>, tous deux chez

des chevaux (45). De plus, deux souches de *A. calcoaceticus* portant le *bla*<sub>OXA-72</sub> ont également été isolé à partir des légumes (227). Par ailleurs, une souche de *E. coli* ST38 productrice d'OXA-48 a été décrite chez une volaille libanaise. Cette souche correspond à un clone humain émergent au Liban et le pourtour du bassin méditerranéen et qui produit la carbapénèmase OXA-244, également isolées dans les eaux des estuaires du Liban (206, 228, 229). En outre, une souche de *K. pneumoniae* ST530 productrice d'OXA-48 a été détectée dans des laits de vache (205). De plus, les souches de *E. coli* et de *K. pneumoniae* hébergeant *bla*<sub>OXA-48</sub> ont également été signalées dans les eaux de l'estuaire, confirmant la diversité des espèces bactériennes produisant OXA-48 et leur probable statut endémique au Liban (205, 227, 228, 230). De même, une étude récente a signalé pour la première fois une souche de *E. cloacae* productrice de *bla*<sub>NDM-1</sub> au Liban (231). En outre, les BGN produisant des BLSE et des carbapénèmases sont co-résistantes à d'autres antibiotiques vétérinaires et cliniquement autorisés, aggravant la situation de la résistance aux antibiotiques.

## **XI. Résistance aux antibiotiques et réfugiés syriens au Liban**

La population libanaise a récemment augmenté de manière importante, en partie attribuable à l'afflux de réfugiés syriens suite à la guerre civile en Syrie en 2012 (232). De nombreux réfugiés syriens actuellement au Liban vivent dans des mauvaises conditions d'extrême précarité, avec un accès limité aux soins de santé, à l'eau potable et à une nourriture inadéquate (233, 234). Il n'existe aucune base de données fiable sur la santé pour promouvoir de meilleurs soins pour cette population mal desservie. Plus important encore, il existe peu de données disponibles concernant l'épidémiologie de la résistance aux antimicrobiens dans la population de réfugiés syriens. Le tractus gastro-intestinal est connu pour être un réservoir d'Entérobactéries à Gram négatif et il a été démontré que la colonisation par les Entérobactéries multi-résistante multiplie par au moins deux, le risque d'infection ultérieure (235).

Les facteurs qui favorisent l'émergence de la résistance aux antibiotiques, existaient déjà en Syrie, même avant le conflit (236, 237). Bien qu'il existe des lois en Syrie (numéro 2/T) qui interdisent la vente d'antimicrobiens en vente libre et qui remontent à 1988, la Syrie connaît encore des réglementations et des lois faiblement appliquées associées à l'utilisation prudente des antimicrobiens (237). En effet, des rapports existants sur la résistance aux antimicrobiens chez les populations syriennes déplacées, ont décrit une augmentation de l'émergence d'Entérobactéries

multi résistantes, notamment de SARM et de *Mycobacterium tuberculosis* multirésistantes aux antituberculeux (233, 238-245). Dans l'ensemble, on peut affirmer qu'il existe une menace imminente pour les populations syriennes, en particulier les réfugiés qui vivent dans des camps fortement exposés à la pollution et à un accès limité aux programmes d'eau, d'assainissement et d'hygiène et à des soins médicaux adéquats. De plus, l'augmentation de la résistance aux antimicrobiens dans les populations syriennes a de graves implications régionales et mondiales, car elle peut se répercuter sur les pays qui accueillent un nombre important de réfugiés. En 2022, le Liban a hébergé presque 1.5 million de réfugiés syriens (246).

Plusieurs rapports ont mis en évidence une forte prévalence d'agents étiologiques résistants aux antibiotiques, parmi les populations syriennes déplacées et les réfugiés et ont appelé à des actions préventives pour s'attaquer au problème. La première description d'un cas de tuberculose ultrarésistante (XDR) au Liban concernait des réfugiés ou des demandeurs d'asile de Syrie, du Soudan et d'Ukraine (239).

## **XII. L'approche "One Health" appliquée à la résistance aux antibiotiques**

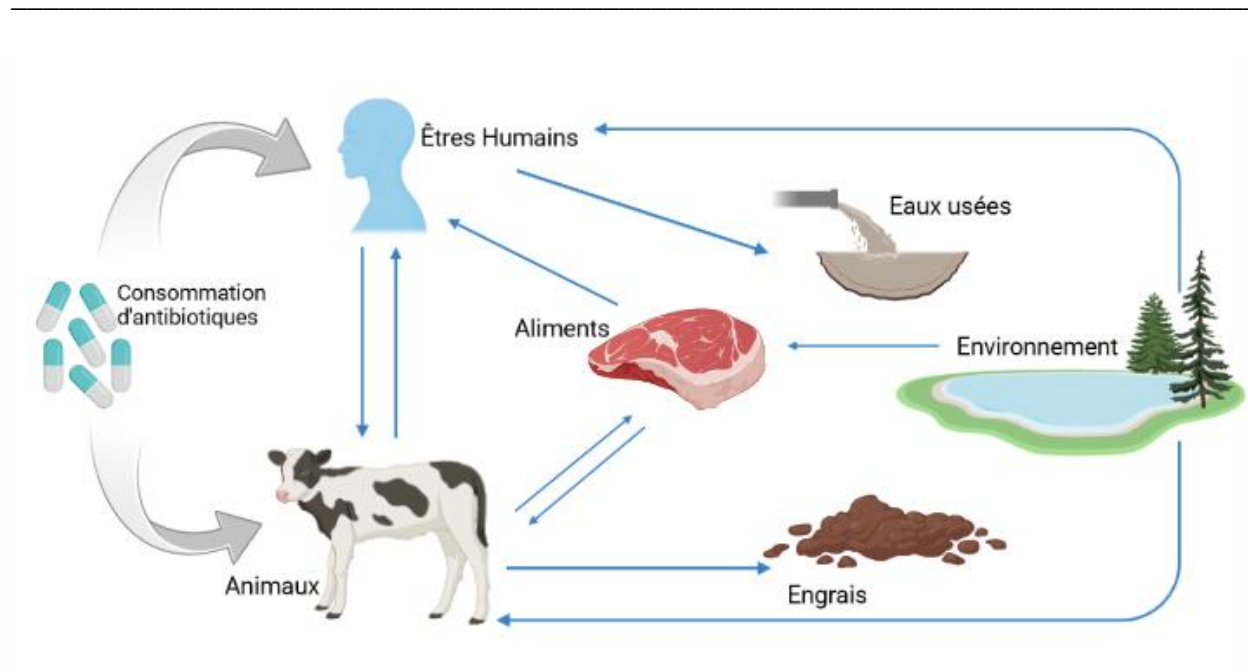
La résistance aux carbapénèmes peut être due soit à la production de carbapénèmases soit à une combinaison de mutations structurelles et la production d'autres  $\beta$ -lactamases, telles que la  $\beta$ -lactamase à spectre étendu (BLSE) et les céphalosporinases AmpC (247). Les carbapénèmases, sont capables d'hydrolyser les carbapénèmes, ainsi que d'autres  $\beta$ -lactamines, notamment les pénicillines, les céphalosporines et les monobactames (248).

La dissémination globale des Entérobactéries productrices de carbapénèmases, constitue un défi de plus en plus grand de santé publique (249). L'utilisation contrôlée des agents antimicrobiens et la mise en place des stratégies efficaces de contrôle des infections sont des moyens pour empêcher et limiter la propagation des Entérobactéries résistantes aux carbapénèmes (249).

Le concept One Health est défini comme « une approche collaborative, multisectorielle et transdisciplinaire, travaillant aux niveaux local, régional, national et mondial, pour obtenir une optimisation en terme de santé et de bien-être, en reconnaissant les interconnexions entre les personnes, les animaux, les plantes et leur environnement commun » ([https://www.onehealthcommission.org/en/why\\_one\\_health/what\\_is\\_one\\_health/](https://www.onehealthcommission.org/en/why_one_health/what_is_one_health/)).

L'organisation mondiale de la santé (OMS) définit l'approche "One Health", comme étant une approche intégrée et unificatrice pour équilibrer et optimiser la santé des personnes, des animaux et de l'environnement. Cette approche mobilise plusieurs secteurs, disciplines et communauté à différents niveaux de la société pour travailler ensemble. Plusieurs secteurs sont impliqués dans l'approche One Health, surtout les secteurs de santé publique et de l'environnement. Cette approche est pertinente pour la sécurité de l'eau et des aliments, le contrôle des zoonoses et la lutte contre la résistance aux antibiotiques.

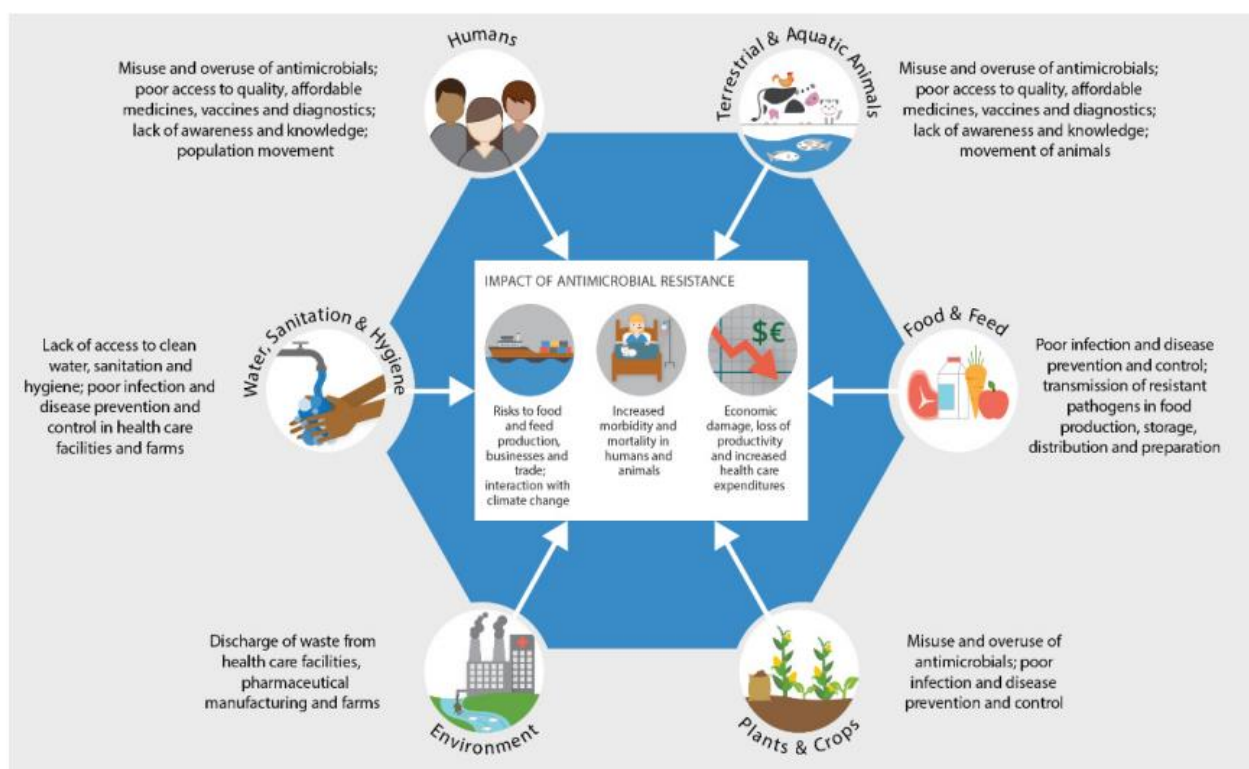
De même, l'approche One Health est «l'effort de collaboration de plusieurs professions des sciences de la santé, ainsi que de leurs disciplines et institutions connexes - travaillant aux niveaux local, national et mondial - pour atteindre une santé optimale pour les personnes, les animaux domestiques, la faune, les plantes et notre environnement" (250).



**Figure 17 :** Représentation schématique des voies de transmission de la résistance aux antibiotiques entre les animaux d'élevage, l'environnement au sens large et les humains.

Tous les secteurs de l'écosystème sont connectés et les antibiotiques pénètrent dans l'environnement dans un cercle complexe (251, 252) (**Figure 18**). L'usage abusif et inapproprié

des antibiotiques dans de multiples secteurs est l'un des principaux facteurs de résistance aux antibiotiques, conduisant à la création d'un réservoir de bactéries résistantes et de gènes de résistance qui retournent dans l'environnement par contamination fécale, les stations d'épuration des eaux usées étant des points chauds réputés (253). Ainsi, un traitement inadéquat des déchets résidentiels, industriels et agricoles accroît encore la résistance dans l'environnement. La présence de pathogènes bactériens humains dans l'environnement augmente le risque d'émergence de nouveaux pathogènes consécutifs au transfert de nouveaux gènes de résistance provenant des bactéries environnementales.



**Figure 18 : Une réponse One Health pour lutter contre les facteurs déterminants et l'impact de la résistance aux antimicrobiens** (adapté du résumé final de l'IACG 2019 ([https://www.who.int/antimicrobial-resistance/interagency-coordination-group/IACG\\_final\\_summary\\_EN.pdf?ua=1](https://www.who.int/antimicrobial-resistance/interagency-coordination-group/IACG_final_summary_EN.pdf?ua=1)))

### XIII. Apport du NGS dans l'étude de la résistance aux antibiotiques

Les techniques de séquençage haut débit (*High Throughput Sequencing*) ou aussi appelée NGS (*Next -Generation Sequencing*), ont remplacé la technique de séquençage de Sanger qui a

permis le séquençage des premiers génomes bactériens (*Haemophilus influenzae* et *Bacillus subtilis*) et du génome humain. Le NGS regroupe des techniques et des technologies développées dans le but de générer et lire en parallèle à un haut débit des millions de séquences d'ADN indépendantes. Les résultats sont générés sous forme brute appelée *Reads* qui seront ensuite réorganisés par traitement informatique pour reconstituer la séquence du brin d'ADN cible.

Dans cette section, la technique de séquençage Illumina et le système nanopore seront abordés. L'accès au NGS a notamment permis le développement des études de méta génomique, le typage bactérien associé à l'étude d'épidémies hospitalières, la découverte de nouveaux mécanismes de résistance.

### 1. Séquençage par Illumina :

En première étape, le génome d'intérêt est fragmenté en millions de petits fragments d'ADN, qui seront liés à des adaptateurs pour permettre leur amplification et leur séquençage (254). Cette collection de fragments amplifiés est appelée « librairie » (anglicisme pour Library, banque de fragments d'ADN). Les fragments d'ADN « étiquetés » d'une petite séquence nucléotidique spécifique d'un échantillon (ou code-barre), se fixent sur un support « flowcell » par hybridation de l'adaptateur à des séquences complémentaires. À cette étape, une amplification est réalisée pour multiplier ces fragments d'ADN immobilisés, puis des nucléotides couplés à des fluorochromes sont chargés sur la « flowcell » (**Figure 19**). Ces nucléotides possèdent une propriété de terminaison réversible, permettant à la réaction de séquençage de se produire en présence des quatre nucléotides simultanément. On parle de séquençage par synthèse (254).

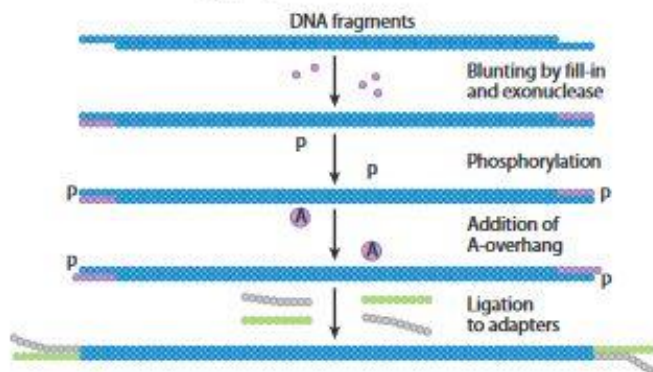
La société Illumina propose plusieurs types de séquenceurs dont (a) le séquenceur à très haut débit de type HiSeq 2000, pouvant fournir un téraoctet de données et pouvant rivaliser avec le seuil des 1000\$ pour un génome humain (255), (b) le séquenceur de « pailleasse » de type MiSeq, recourant à la même technologie mais avec un débit beaucoup plus faible, et (c) le séquenceur de type NextSeq 500 qui offre une solution intermédiaire rapide et simple pour la caractérisation de n'importe quel génome (256).

Le séquençage Illumina® permet d'obtenir une grande profondeur de séquençage, correspondant au nombre de lectures (ou reads) obtenues indépendamment pour chaque base

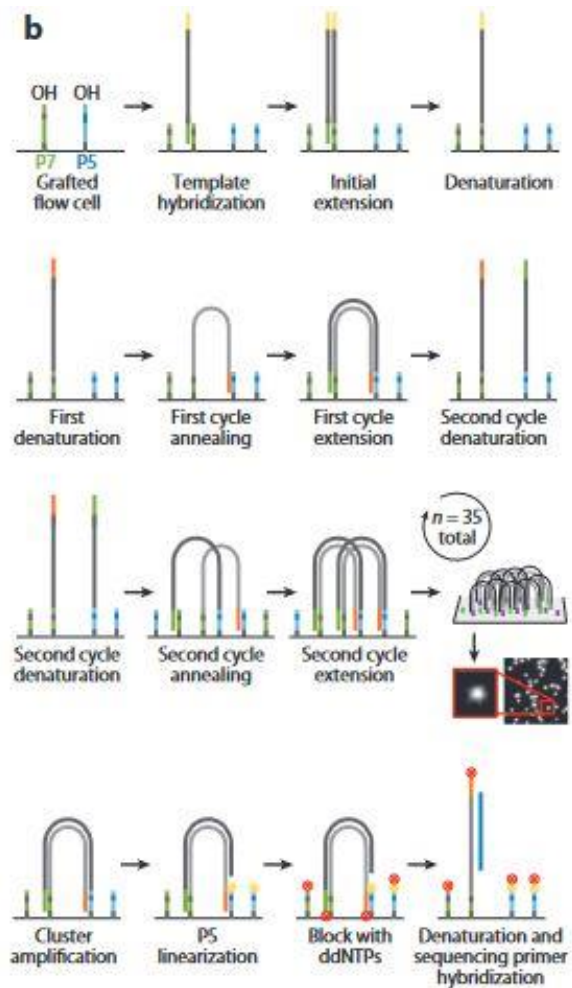
ciblée : elle s'exprime en nombre de fois (X). Ainsi, une « profondeur de 100X » signifie que la base, ou la région considérée, a été séquencée 100 fois de manière indépendante. Très souvent, la profondeur de séquençage est une valeur moyenne sur l'ensemble des régions ciblées.



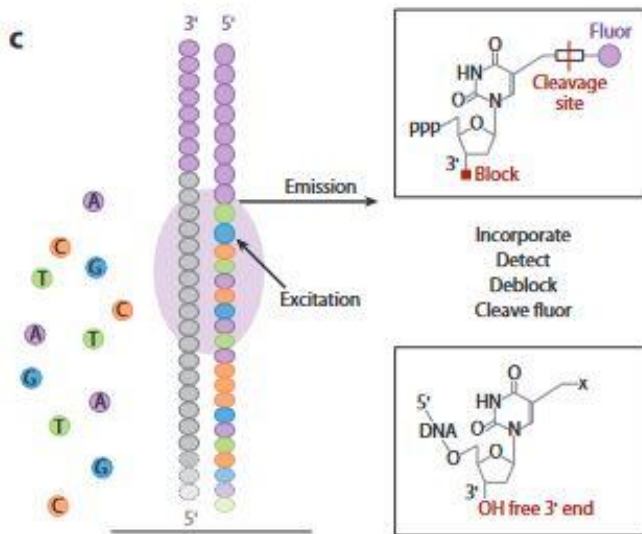
**a** Illumina's library-preparation work flow



**b**



**c**



**Figure 19 : Séquençage de type Illumina.** a) La préparation de la librairie permet la fixation d'adaptateurs aux extrémités 5' et 3' des fragments d'ADN à séquencer. Cette librairie est ensuite chargée sur la flowcell. (b) Les fragments d'ADN s'y fixent par hybridation aux séquences oligonucléotidiques P5 et P7 complémentaires des adaptateurs. L'action de la polymérase permet la synthèse du 1er brin d'ADN complémentaire qui sera lié de façon covalente à la flowcell. Le fragment d'ADN initial ayant servi de matrice est éliminé au lavage. La synthèse du 2e brin d'ADN se fait après formation d'un pont grâce à la présence de l'adaptateur 3' complémentaire de l'un des deux oligonucléotides fixés sur la flowcell. Un fragment d'ADN est amplifié plusieurs fois ( $n=35$ ) jusqu'à l'obtention de copies identiques multiples formant un cluster. Les brins formant le cluster sont ensuite linéarisés. Les extrémités 3'-OH sont bloquées pour éviter les réactions d'hybridations et permettre la réaction de séquençage. (c) Des dNTPs marqués par 4 fluorochromes différenciant les 4 bases sont ajoutés. À chaque nucléotide incorporé, un signal fluorescent dont la longueur d'onde dépend du fluorochrome est émis. Une caméra est capable de détecter le signal fluorescent et son intensité pour l'ensemble des clusters séquencés. *D'après Mardis (257).*

## **2. Le système Nanopore:**

Le système Nanopore, commercialisé par Oxford Nanopore technologies sous le nom minION®, est un système de séquençage en temps réel à partir d'une molécule unique, sans amplification préalable (258, 259). Le principe repose sur le passage d'une molécule d'ADN simple brin à travers un pore protéique extrêmement fin permettant de calculer la taille de la base franchissant ce pore et donc d'en déduire l'identité exacte de la base. Selon Oxford Nanopore technologies, des séquences pouvant dépasser les 200 kb peuvent être générées, mais dans les faits, sont comprises, en moyenne, entre 5 et 30 kb. La longueur des reads est favorisée par rapport au nombre de reads, limitant la profondeur de séquençage obtenue. Ce type de séquenceur est particulièrement indiqué pour du séquençage de novo, y compris pour des génomes possédant de nombreuses séquences répétées (cas des eucaryotes supérieurs).

## **XIV. Review 1: Carbapenemase Dissemination in the Middle East and North Africa: Alarming Situation or Delusion?**

### **Abstract**

Pathogenic resistant bacteria keep on disseminating worldwide leaving doctors with very few therapeutic options and expanding treatment failure rates in the light of menacing nosocomial infections. In Gram-negatives, this threatening issue is highly associated with the spread of  $\beta$ -lactamases with broad spectrum of activity, especially carbapenemases that are capable of hydrolyzing carbapenems, the last resort antibiotic to treat gram negative infections. Carbapenemases may be rapidly transferred between the different compartments: humans, animals and the environment. The prevalence of carbapenemases and of their variants is specific to each country with its own epidemiological pattern. A prerequisite to propose intervention strategies to limit their spread is to know which enzyme is present, and where are their reservoirs. In this review, we were interested in the spread of carbapenemases, especially OXA-48-types which seem to increase in the Middle Eastern area (MENA). We focused on the different variants of OXA-48 in each country of the MENA region to provide an assessment of the current situation.

## Introduction

The discovery of antibiotics made a huge change in the therapeutic field, by significantly increasing lifespan through the cure of otherwise deadly infections. With their clinical use antimicrobial resistance (AMR) appeared more or less rapidly and resulting in drastic efficacy problems. Decades have passed, and AMR has continuously increased turning bacterial infections again in life-threatening situations with high burdens on patient's health and world economy. Several factors have been incriminated: (i) the overuse and abuse of these drugs, is among the most dangerous causes, since patient's non-compliance to prescribed treatment, not only induces recovery failure, but also a selection of resistant mutants allowing them to spread rapidly, (ii) poor diagnostic tools increases the problem as well, where an inaccurate prescribed treatment results in the same outcome, and (iii) the over-the counter use of antibiotics, (vi) their extensive use in agricultural and veterinary fields, as prophylactic agents or growth factors, and (v) the absence of the development of new drugs that could replace ineffective conventional antibiotics (1) (**Figure 1**).



**Figure 1:** Various causes behind the exacerbation of antimicrobial resistance (AMR)

Several resistance mechanisms have been described to contribute to the failure of antibiotic treatment including (i) outer membrane permeability problems caused by bacterial porin defects, (ii) overexpression of efflux pumps, (iii) alteration of the target of the antibiotics, and (vi) enzymatic inactivation of the antibiotic (2). In Gram-negatives, enzymatic inactivation of  $\beta$ -lactams by the production of  $\beta$ -lactamases is the most important mechanism, while the other mechanisms contribute to reduced susceptibilities. In particular carbapenems, last resort antibiotics to treat gram-negative infections are increasingly inactivated by specific  $\beta$ -lactamases: carbapenemases (3, 4). Carbapenemase producing Enterobacterales (CPE) are highly disseminating especially in the Middle Eastern area (5, 6).

While some carbapenemases are chromosomally encoded, others are carried by highly mobile genetic elements (Transposons, plasmids), which contribute to their rapid spread (7, 8). They have a versatile hydrolytic capacities, with the ability to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems (9). They belong to molecular classes A, B and D of Ambler, which is based on amino acid homology (10). Classes A and D carbapenemases contain a serine active residue responsible of the nucleophilic attack of the  $\beta$ -lactam ring, while class B carbapenemases are metallo- $\beta$ -lactamases (MBLs) with a divalent Zinc cation in their active site that coordinates a water molecule responsible of the nucleophilic attack (11) (**Table 1**). Among class C enzymes, even though a few have been described to hydrolyze weakly carbapenems, they may confer resistance to the latter only if associated with impaired outer membrane permeability (12).

Class A carbapenemases are capable of hydrolyzing a broad variety of  $\beta$ -lactams, including carbapenems, cephalosporins, penicillins and aztreonam, and remain susceptible to clavulanate and tazobactam. This group of enzymes includes members of the *S. marcescens* enzyme (SME), imipenemase (IMI), non-metallo carbapenemase (NMC), Guiana extended-spectrum (GES), and *K. pneumoniae* carbapenemase (KPC) families (5, 9, 13-17). These carbapenemases, may be found in Enterobacterales as well as in non-fermenting Gram-negative bacilli like *Acinetobacter* spp, *Pseudomonas aeruginosa* and *Pseudomonas putida* (18). They may be encoded on chromosomes (SME, NmcA, PenA ...), on plasmids (KPC, GES) or on both (IMI).

Class B carbapenemases, also known as metallo- $\beta$ -lactamases and represented mainly by IMPenmase (IMP), Verona integron-borne metallo- $\beta$ -lactamase (VIM), Sao Paulo metallo- $\beta$ -lactamase (SPM), German imipenemase (GIM), and Seoul imipenemase (SIM) and New Delhi Metallo- $\beta$ -lactamase (NDM) families (9). This class of carbapenemases is able to hydrolyze all  $\beta$ -lactams except aztreonam, resist to the action of commercially available  $\beta$ -lactamase inhibitors, but are inhibited *in vitro* by metal ion chelators, such as EDTA. Even though frequently described in *P. aeruginosa*, they are increasingly identified in Enterobacterales and *Acinetobacter baumannii*, especially for NDM enzymes (9).

The class D carbapenemases also known as oxacillinases, are frequently detected in Enterobacterales and *A. baumannii* (9). They differ from class A enzymes, by their ability to hydrolyze oxacillin in addition to penicillin. Class D oxacillinases are usually not inhibited by clavulanic acid, tazobactam, and sulbactam, whereas their activities may be inhibited *in vitro* by sodium chloride (NaCl) (19). These enzymes are able to hydrolyze penicillins, narrow-spectrum cephalosporins and weakly hydrolyze carbapenems and have limited activities against the broad spectrum cephalosporins (20). Many OXA variants were described including OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like, OXA-48-like family members and OXA-198 were found in the Enterobacterales (21-25).

**Table 1** : Characteristics of carbapenemases - Pl: Plasmidic, Ch: Chromosomal, CLA: Clavulanic acid, ATM: Aztreonam, R: Resistant, S: Susceptible.

Molecular Class	Enzyme	Gene Location	Organisms	Penicillins	Aztreonam	Cephalosporins 1st and 2nd generation	Cephalosporins 3rd and 4th generation	Carbapenems	Inhibitors		
									CLA	EDTA	ATM
Class A	KPC-2 to KPC-13	Pl	<i>Enterobacteriaceae</i> <i>Pseudomonas aeruginosa</i> <i>Acinetobacter baumannii</i>	→					+/-	-	R
	Sme-1 to Sme-3 IMI-1 to IMI-3	Ch	<i>Serratia marcescens</i> , <i>Enterobacter cloacae</i>	→					+/-	-	R
Class B: Metallo-enzymes	GES-1 to GES-20	Pl	<i>Enterobacteriaceae</i> , <i>P. aeruginosa</i> , <i>A. baumannii</i>	→					+	-	S/R
	IMP-1 to IMP-33 VIM-1 to VIM-33 NDM-1 to NDM-6, SPM-1, SIM, GIM	Pl/Ch	<i>Enterobacteriaceae</i> , <i>P. aeruginosa</i> , and other GNNFB	→					-	+	S
Class D	OXA-23 group (OXA-23, OXA-27, OXA-49)	Pl/Ch	<i>A.baumannii</i> , <i>P. aeruginosa</i> , <i>Enterobacteriaceae</i>	→					+/-	-	R
	OXA-48 group (OXA-48, OXA-244, OXA-181, OXA-232...)			→					+/-	-	R
	OXA-24 group			→					+/-	-	R
	OXA-40 group			→					+/-	-	R
	OXA-58 group			→					+/-	-	R

## Oxacillinases

Since they are among the earliest detected carbapenemases, we focus in this review on oxacillinases. Interestingly, many new OXA variants emerged with various hydrolytic profiles observed which touches scientists' curiosity to investigate more in this domain (26). At first, those enzymes were rare and exclusively plasmid mediated, soon after, they spread rapidly and chromosome encoded oxacillinases were described (27, 28). Currently, over 1000 oxacillinases variant have been identified according to the  $\beta$ -lactamase database "BLDB", and some of them seem to be specie specific. In 1985, OXA-23 was originally found in an *Acinetobacter baumannii* isolate in United Kingdom, to confer resistance to imipenem (29). OXA-23, OXA-24, and OXA-58 were then described as the most prevalent enzymes responsible for carbapenem resistant *A. baumannii* (CRAB) (30). OXA variants, could be classified in different subgroups according to their hydrolytic patterns (**Tables 2 and 3**) (20).

**Table 2:** Classification of OXA-48 Like enzymes and their hydrolytic profile.

OXA group	Variants	Hydrolytic pattern
<b>Subgroup 1</b>	OXA-48, OXA-162, OXA-181, OXA-204	- Poor carbapenem hydrolysis - Poor cefotaxime hydrolysis - Strong penicillin hydrolysis - No ceftazidime hydrolysis - Temocillin strongly resistant
<b>Subgroup 2</b>	OXA-163, OXA-252, OXA-405	- Loss of carbapenemase activity - Gain of cephalosporinase activity
<b>Subgroup 3</b>	OXA-244, OXA-232	- Reduced carbapenemase activity

**Table 3:** Classification of non-OXA-48 like enzymes – Present mainly in *A. baumannii*

Subgroup	Subgroup name	Carbapenemase variants
<b>Ia</b>	OXA-23-like $\beta$ -lactamases	OXA-23, -27, -49, -73, -102, -103, -133, -146, -165, -166, -167, -168, -169, -170, -171, -225, -239, -366, -398, -422, -423, -435, -440, -481, -482, -483, and -565
<b>Ib</b>	OXA-24/40-like $\beta$ -lactamases	OXA-24, -25, -26, -72, -139, -160, -207, and -437
<b>Ic</b>	OXA-51-like $\beta$ -lactamases	OXA-64, -65, -66, -67, -68, -69, -70, -71, -75, -76, -77, -78, -79, -80, -82, -83, -84
<b>Id</b>	OXA-58-like $\beta$ -lactamases	OXA-58, -96, -97, -164, -397, -420, and -512

OXA-48  $\beta$ -lactamases are by far the most important enzymes. Initially described in *K. pneumoniae* isolates in Turkey in 2004, *bla*<sub>OXA-48</sub>-like genes have spread in several countries, especially of the Mediterranean rim and subsequently all over the world (31) (32) (33). The rapid rise of OXA-48-like producing Enterobacterales (OPE), is the most recent and worrying issue. OXA-48 producing Enterobacterales have also been reported from several countries in Middle East, North Africa and Europe (34). These enzymes are weakly related to other class D  $\beta$ -lactamases with less than 50% amino acid identity with other OXA members. The OXA-48 family members share 87% of amino



acid identity and include now more than 48 OXA-48 LIKE variants (see BLDB)(20). OXA-48, OXA-162, OXA-181 OXA-204, OXA-232, OXA-244, OXA-245, OXA-247, OXA-436, OXA-484 and OXA-519 are the most common acquired OXA-48 like carbapenemases causing carbapenem resistance among Enterobacterales (20), (35).

While some OXA-48-variants with single amino acid substitutions have similar hydrolytic profiles as OXA-48, others especially those with changes in the  $\beta$ 5- $\beta$ 6 loop located close to the substrate binding site, which represents 22/45 variants described have significant changes in the hydrolytic spectrum of  $\beta$ -lactams. This is the case for variants with a change at position R214, such as OXA-244 (R214G) and OXA-232 (R214S) that have decreased carbapenemase activity (36, 37), but also for variants with a four-amino-acid deletion inside the loop such as OXA-163 or OXA-405, which results in the loss of carbapenemase activity and a gain of the ability to hydrolyze expanded-spectrum cephalosporins (5, 7-9). Finally, other variants such as OXA 438, OXA517, OXA-793 with two AA deletions in the  $\beta$ 5- $\beta$ 6 loop, have been described with the ability to hydrolyze carbapenems and oxyimino-cephalosporins equally well (Pablo Power, Dabos AAC, Dabos poster ECCMID)) (38, 39).

### **What are the factors sustaining the rapid dissemination of carbapenemase producing Gram-negatives in the MENA region?**

Different surveillance studies conducted in the MENA and North Africa region revealed epidemiological changes occurring continuously and likely related to several factors. The lack of hygiene has been pointed out as a major cause of resistant bacteria dissemination allowing the spread of bacteria in hospital and community settings (40). This is emphasized by the refugees' camps present in a large number of Middle Eastern countries such as Lebanon and Jordan, and by the civil wars present in others. For instance, Syrian civil war affected the whole region through the scarcity of suitable shelters for refugees, and the sharing of housing units resulting in overcrowded refugee camps as well as the lack of hygiene infrastructure, and the inaccessibility to healthcare services (41). In addition, the lack of efficient diagnostic tools prevents identification of infected/colonized patients, thus allowing for further spread and improper empirical treatment (42). Poverty in some rural areas, lack of sanitation, and over the counter purchase of antibiotics are additional factors contributing to this situation (ref).

## What are the carbapenemases identified in the Middle East?

The predominance of *bla*<sub>OXA-48</sub> genes among Enterobacterales, was reported in several regional studies. Prevalence varied from 49% in the Arabian Gulf, 53.3% in the United Arab Emirates (UAE), 88% in Lebanon, 49.2% in Egypt to 86% in Turkey (43). With non-fermenters of the middle east regions, high prevalence of MDR have been described in hospitalized patients in the intensive care units, with a high prevalence of infections caused by bacteria resistant to cephalosporins, carbapenems, aminoglycosides...(44).

Here, the majority of works published about carbapenemases in the Middle East Region, among Gram-negative bacteria have been reviewed. A comprehensive literature search of PubMed was conducted using relevant keywords including “Carbapenemase” or “OXA” and (“Bahrain” or “Egypt” or “Iran” or “Iraq” or “Jordan” or “Kuwait” or “Lebanon” or “Oman” or “Palestine” or “Qatar” or “Saudi Arabia or “Syria” or “Turkey” or “United Arab Emirates” or “Yemen” or “Libia” or “Tunisia” or “Morocco” or “Gulf cooperation Council” or “Middle east” or “Arabian Peninsula”). Articles and reviews published only in English, reporting any data on carbapenemase-producing bacteria from Middle East countries were included.

### 1- IRAN

According to the latest studies, health care associated infections in Iran, requires special attention in detection, reporting and in setting efficient control measures (45-48). The main concern in this country is carbapenem resistance due to carbapenemase production, particularly among *Acinetobacter* and *Pseudomonas* spp. (5), where MBLs are the most prevalent among *P. aeruginosa* (49, 50), with *bla*<sub>NDM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, being the most widespread genes (49, 51), especially, NDM-1 (52). A recent study, showed that from 236 carbapenem-resistant *P. aeruginosa* (CRPA), 116 isolates harbored MBL genes and 29 isolates were found positive for *bla*<sub>NDM-1</sub> (50), which ensures the high prevalence of class B MBLs among *P. aeruginosa* in Iran. According to the same study, CRPA, harbored *bla*<sub>IMP-1</sub>, *bla*<sub>VIM-2</sub> and *bla*<sub>OXA-10</sub> in 27.5%, 21.1% and 32.2% of isolates respectively, and interestingly, a co-production of *bla*<sub>NDM-1</sub>, *bla*<sub>IMP-1</sub>, *bla*<sub>OXA-10</sub>, *bla*<sub>VIM-2</sub>, were determined in 11 (4.6%), 8 (3.4%) and 27 (11.4%) of isolates respectively which is alarming (50). In this study infections caused with imipenem resistant *P. aeruginosa* isolates

that harbor *bla*<sub>OXA-23</sub> and *bla*<sub>OXA24/40</sub> genes, normally encountered in *A. baumannii*, have also reported (53). Finally, the alterations of the porin D2 (*oprD*) and/or the overexpression of the efflux systems (MexAB system) in *P. aeruginosa* are responsible for a vast majority of carbapenem resistance described in that species (54-56).

Carbapenem-resistant *A. baumannii* (CRAB) have also been described, as it is considered an important pathogen causing hospital acquired infections (47, 48, 57). In particular, *A. baumannii* shows high genetic diversity of acquired OXA genes in Iran, *bla*<sub>OXA-23-like</sub>, *bla*<sub>OXA-58-like</sub>, *bla*<sub>OXA-24/40-like</sub> and *bla*<sub>OXA-143-like</sub> (52, 58-64). Beigverdi *et al.* found prevalence of OXA-23, OXA-24/40, and OXA-58 with values of 73.7%, 21.9%, and 6.2%, respectively among CRAB in Iran (47). Similarly, *bla*<sub>23-like</sub> genes was found to be dominant in carbapenem-resistance in *A. baumannii* from Hamadan hospitals (60). In addition to oxacillinases, MBLs were detected in *A. baumannii*, notably, VIM-1 and IMP-1 reported in clinical samples isolated from southwest Iran (65). MDR *A. baumannii* harboring *bla*<sub>OXA-317</sub>, *bla*<sub>OXA-64</sub>, and *bla*<sub>OXA-69</sub>, were detected in Iran, from nasal and hand samples in healthcare workers and burn patients who are at high risk of acquiring *A. baumannii* from endogenous sources (66). Imipenem resistant *A. baumannii* harbored also other oxacillinases. For instance, clinical *A. baumannii* isolates carrying *bla*<sub>OXA-68</sub>, *bla*<sub>OXA-66</sub>, *bla*<sub>OXA-71</sub>, *bla*<sub>OXA-90</sub> belonging to ST10, ST2, ST3, and ST513 respectively were found in Tehran (67).

The occurrence of carbapenemase production among Enterobacterales is also significant (68). Fifteen out of 307 Enterobacterales originating from five medical centers in Azerbaijan, Iran, (4.9%) were found to produce carbapenemases including MBL, KPC and OXA-48 (69). Thoroughly, these 15 isolates were all *K. pneumoniae*. As for the repartition of produced carbapenemases, MBLs were found in 9 out of 57 *K. pneumoniae* (15.8%), KPC were found in 4 out of 57 isolate (7%), and OXA-48 in 2 out of 57 (3.5%) (69), suggesting that the most common CRE is *K. pneumoniae*. Many reports that focused on carbapenem-resistant *K. pneumoniae* in Iran, revealed that OXA-48-like and NDM enzymes were highly prevalent. For instance, Babazadeh *et al.* showed that out of 100 *K. pneumoniae* isolates collected from October 2018 until the end of April 2020, *bla*<sub>OXA-48</sub> (24%) and *bla*<sub>IMP</sub> (13%) genes had the highest prevalence, while *bla*<sub>KPC</sub> and *bla*<sub>GIM</sub> genes were not detected among Iranian *K. pneumoniae* isolates (70). In another study, 3 OXA-48 (10%) and 15 NDM-1 (45%) were found among 33 carbapenem resistant *K. pneumoniae*

in 2020 (71). Similar results were observed by Ghamari *et al.* that found 28.5% of OXA-48 and 22 % NDM out of 60 carbapenem resistant *K. pneumoniae* clinical isolates (72). Carbapenem-resistance is often mediated by NDM-1, and OXA-48 associated with *K. pneumoniae* ST15 (73), ST11 (74, 75), ST893 (75) followed by KPC (KPC-2), VIM (VIM-1 and VIM-4) and IMP (69, 76-89). *K. pneumoniae* ST13 and ST392, were the most disseminated among NDM-1 producers in south Iran (90). Moreover, the first detection of another variant, NDM-6 in *K. pneumoniae* ST147 in Iran, was reported in 2019 (91). Hospital circulation of a hypervirulent isolate of *K. pneumoniae* producing both OXA-48 and NDM-1 was reported in a tertiary care center of Iran highlighting the importance of reinforcing the surveillance system to avoid such epidemic spread (92).

Co-expression of CTX-M and TEM has frequently been described (93, 94).

A study conducted by Armin *et al.*, on Enterobacterales (58 *E. coli*, 95 *K. pneumoniae* and 60 *Enterobacter*) Out of 58 *E. coli*, 9 harbored OXA-48, 5 harbored NDM-1, one isolate had KPC and another one had IMP. Regarding *K. pneumoniae* 46 isolates out of 95 expressed OXA-48, 29 had NDM-1 and only one isolate had IMP. As for *Enterobacter* spp. most isolates showed NDM-1 (19 out of 60), while the few had OXA-48 (7 out of 60) (95). Talking about NDM-1, this variant was also detected in two *E. coli* isolated from leukemia patients in Iran, which is considered a major life threatening danger for them (96). Other studies aiming to characterize the mechanisms behind carbapenem resistance, also showed that the highest prevalence was for OXA-48 like enzymes followed by NDM. Among these studies, Alizadeh *et al.* found a 72.8%, 50.8%, 18.6%, 11.8% and 6.7% prevalence for OXA-48 like, NDM, IMP, VIM and KPC respectively in 60 carbapenem resistant Enterobacterales (97). To summarize, OXA-48 and NDM seem to be predominant in Iran in Enterobacterales. These findings ensures that OXA-48 and NDM-1 are widely spread among Enterobacterales in Iran.

Besides IMPs, and NDM-1, other MBLs such as VIM-2 were also found in *E. coli* isolates, with the ability to conjugate and transmit imipenem resistance (98). These MBLs were also detected in cancer patients (99) which increases their risk of nosocomial infections, and put their life in danger since their immune system is altered. Finally, in another study investigating reduced susceptibility to carbapenems of 91 MDR *E. coli* isolates from patients having urinary tract infections revealed

the presence of 2.1% of *bla*<sub>KPC</sub>, while *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, and *bla*<sub>OXA-48</sub> genes were also found in 8.7%, 9.8%, and 15.3% of respectively (100). Another study showed fecal carriage of ESBLs and carbapenemases in rectal swabs specimen from clinical and community settings, where among 120 rectal swabs, 72 were carriers for ESBL- *E. coli* with *bla*<sub>CTX-M-15</sub>, *bla*<sub>NDM-1</sub>, *bla*<sub>OXA-48</sub> and *bla*<sub>IMP</sub> being detected in 90.2%, 4.1%, 4.1%, and 1.3% of the isolates (101). Co-production of two carbapenemases among *E. coli* was reported, for example, OXA-181 and NDM-5 were co-produced in the same isolate (102). Regardless of our major focus in this review on carbapenemases, we can't deny that other mechanisms such as AmpC and ESBL production are also responsible for alarming resistance percentages (103). These mechanisms are well spread among *K. pneumoniae* and *E. coli* in Iran hospitals (70, 104-112).

## 2- LEBANON

In Lebanon, several studies have described an alarming epidemiology of AMR among Lebanese population. Very early, the study conducted by Beyrouthy *et al.* showed that the prevalence of ertapenem resistant clinical Enterobacterales, recovered from hospitalized patients, increased from 0.4% in 2008-10 to 1.6% in 2012, of which 88% were OXA-48 carbapenemase producer (113). Between 2012 and 2016, the emergence of OXA-48 and OXA-181-producing, clinical *E. coli* isolates was also reported, where an in-depth WGS analysis was performed revealing 48.1% of OXA-48 prevalence, along with 7.4% of OXA-181 production (114). Co-production of NDM-4 along with *mcr-1* gene in *E. coli* isolates in northern Lebanon, was the first to be described in the world (115). In addition, *K. pneumoniae*, co-producing NDM-6 and OXA-48 was identified in the same study by Al-Bayssari *et al.* in 2021 (115). Resistance to colistin affects global health especially when accompanied with carbapenem resistance, rendering those two antibiotics ineffective to treat GNB infections. This was the case for the description of a colistin resistant *K. pneumoniae* isolate producing NDM-5 (116) The northern area of Lebanon was largely investigated through several studies: Rima *et al.* revealed that carbapenem-resistance among Enterobacterales in Tripoli area between 2015 and 2019 was 2.7% (311 out of 11210 isolates) and that the main mechanism of carbapenem resistance among gram-negative bacilli, with 74% of studied carbapenem-resistant isolates showing enzymatic resistance to carbapenems by the production of OXA-48, OXA-181, OXA-244 and OXA-162 (117). In 2016, the first OXA-181-

producing *E. coli* was reported in Lebanon from a patient in the intensive care unit of the American University Hospital in Beirut (118). In Lebanon, the predominant mechanism remains OXA-48 production (119). Hammoudi *et al.* published a study performed on a large number of isolates consisting of 8717 Enterobacterales, among which 1.2% showed reduced susceptibility to carbapenems. Out of the 44 studied isolates, 70% have OXA-48 (31/44) (15 *K. pneumoniae*, 8 *E. coli*, 4 *S. marcescens*, 3 *E. cloacae*, 1 *M. morgani*) (119). Other studies were investigating the prevalence of intestinal carriage of carbapenem-resistant bacteria in cancer patients under chemotherapy in 2016 and reported a carriage rate of 24.4% among Gram negative Bacilli (120). In addition to their presence in clinical isolates, OXA-48 and OXA-244 producing Enterobacterales were also detected in water sources in Lebanon (Estuaries, rural wells, spring water) (121). Hammoudi *et al.* reported the presence of imipenem-resistant *A. baumannii* strains isolated from nine Lebanese hospitals located in diverse geographic area, co-harboring OXA-23 and GES-11, and other strains having *bla*<sub>OXA-23</sub>, *bla*<sub>OXA-24</sub>, *bla*<sub>GES-11</sub> or *bla*<sub>OXA-24</sub> with *bla*<sub>GES-11</sub>. Carbapenemases in Lebanon are not restricted to oxacillinases: The emergence of VIM-2 and IMP-15 carbapenemases in carbapenem-resistant *P. aeruginosa* was reported in Lebanon by Al Bayssari *et al.* (122). Actually, *P. aeruginosa* is predominantly resistant through the production of VIM-2 in the region (123). Add to this, the detection of the first *Acinetobacter pittii* clinical isolate carrying either the *bla*<sub>NDM-1</sub> or the *bla*<sub>OXA-72</sub> in 2015 (124). Dual-carbapenemase secretion of GES-6 and VIM-2 or IMP-15, was reported in carbapenem-resistant *Pseudomonas aeruginosa* isolated from patients suffering from nosocomial urinary tract infections in a Lebanese hospital (125).

Water contamination was studied further by Diab *et al.* through a study published in 2018, where ESBLs and carbapenemases were shown to pollute it and the identified isolates were CTX-M-15-producing *E. coli* belonging to ST38, ST10, ST131 which are well known to be associated with animal and human reservoirs (121). In 2015, OXA-143 and OXA-23 producing *A. baumannii* were reported by Rafei *et al.* and Al Bayssari *et al.* from horse's mouth (126), and pigs and cattle respectively (127). OXA-48-producing *K. pneumoniae* ST530 was detected in raw milk in Lebanon, where milk is mostly consumed raw, and may be a source of human exposure to carbapenemases (128).

The numerous refugee camps have worsened the AMR situation in Lebanon as they are overcrowded and lack of proper hygiene measures. Several studies have addressed the carriage of carbapenem-resistant bacteria, in Syrian refugees in Lebanon. For instance, *K. pneumoniae* isolated from refugees harbored plasmid-encoded *bla*OXA-48 gene (129). A study conducted by Hajjar *et al.*, showed that among one hundred isolates collected from various clinical specimens from Lebanese patients admitted to Saint Georges Hospital, University Medical Center between June 2013 and June 2014. *Bla*OXA-23-like in 79% and *bla*OXA-40-like in 3%. Two isolates harbored both *bla*-OXA-23-like and *bla*-OXA-40-like (130). At last, as economic crisis that the country is going through, affects the surveillance systems and the national action plans, the situation is likely to get worse with even faster dissemination of resistant bacteria.

### **3- Syria**

Studies on carbapenem resistance situation in Syria remain scarce especially after the war, which impacted the healthcare system and affected resistance patterns in the region. Resistance profiles following Syrian displacement were studied rather than the real situation in the country, since the studies are limited to resistance profiles without characterization of the resistance mechanism (131).

Following refugee crisis, it was claimed that *A. baumannii* ST85 harboring NDM-1 was imported by Syrian refugees to Turkey and neighboring countries (132). Nevertheless, this same variant harbored by *A. baumannii* ST85 was isolated in 2012 in the Tripoli Government Hospital, Lebanon, from civilians wounded during the Syrian war, in addition to OXA-94 (133, 134).

### **4- EGYPT**

Regarding the situation in Egypt, the gathered information below reveal the dissemination of OXA-48 Like variants and MBLs. El Badawy *et al.* found a carbapenem resistance prevalence of 19.79% (19/96) among *K. pneumoniae* isolates recovered as a part of Egyptian routine microbiological laboratory. Selected clinical carbapenem resistant *K pneumoniae* isolates were found to carry *bla*NDM, *bla*OXA-48, *bla*OXA-181, *bla*OXA-51, and *bla*OXA-23 genes with prevalences of 26.32% (5/19), 73.68% (14/19), 21.05% (4/19), 10.53% (2/19), and 5.26% (1/19) respectively (135). VIM-2-producers have also been reported in Egypt, along with NDM-1-producing

*Providencia stuartii* in university hospital in Egypt (136). Furthermore, another study conducted in 2019, aimed to investigate the prevalence of carbapenemase producers among extensive drug resistant clinical gram negative bacteria: *bla*<sub>KPC</sub> (63.5%) followed by *bla*<sub>OXA-48</sub> (55.7%) and *bla*<sub>VIM</sub> (28.8%) were the most prevalent resistance genes (137). Enterobacterales strains isolated from cultured rectal swabs from children in the intensive care unit in Cairo, harbored *bla*<sub>OXA-48</sub> and *bla*<sub>NDM-1</sub> genes (138). Out of 150 *P. aeruginosa* isolates isolated from various infections as part of routine hospital laboratories in Minia governate, Egypt, 21% were resistant to carbapenems (139). Detected enzymes were *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>GIM</sub>, *bla*<sub>SPM</sub> with percentages of 42.8%, 52.3%, 52.3%, and 38% respectively. (140). In 2015, carbapenem resistance among *A. baumannii* isolates during a 4-month outbreak in 2015 at Tanta university hospitals (TUH), was correlated with the presence of OXA-23, NDM-1 and -2, VIM-1 and -2 genes (141). In the context of an epidemiological regional survey investigating hidden community acquired infections in Egypt, MDR *A. baumannii* were isolated from non-hospitalized symptomatic patients from private medical and microbiological analysis laboratories across Egypt. The study revealed that more than 60% were at least resistant to one carbapenem, and that the most prevalent resistance genes were *bla*<sub>OXA-24/40</sub> (65.2%), followed by *bla*<sub>OXA-23</sub> (30.4%) and *bla*<sub>OXA-58</sub> (17.4%). *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, and *bla*<sub>NDM</sub> genes were also detected occasionally (142). In this same period of time, another study conducted on carbapenem resistant *A. baumannii* from cancer patients revealed a high prevalence of *bla*<sub>NDM</sub>, *bla*<sub>OXA-23-like</sub>, and *bla*<sub>KPC</sub> genes (143). Similarly, in 2019 a large variety of acquired carbapenem-hydrolyzing  $\beta$ -lactamases have been described, including *bla*<sub>KPC</sub>, *bla*<sub>GES</sub>, *bla*<sub>NDM</sub>, *bla*<sub>SIM</sub>, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub> and *bla*<sub>OXA-23-like</sub> (144).

In 2013, Metwally *et al.* reported at a tertiary care center, the first KPC-producing *K. pneumoniae* (145). Clinical *K. pneumoniae* isolates recovered from four hospitals from July 2015 to April 2016, co-harbored both *bla*<sub>NDM-1</sub> and *bla*<sub>OXA-48</sub> genes (146). Between 2018 and 2019, among carbapenemase-producing *K. pneumoniae*, *bla*<sub>KPC-2</sub> and/or *bla*<sub>NDM-1</sub> were reported. Inc FIIK and FII plasmids were found to be predominant among isolates by molecular typing based on replicon typing (147). A study published in 2020, reported a novel ST4497 as well as the emergence of *K. pneumoniae* ST231 producing OXA-232 for the first time in Egypt among clinical carbapenem resistant *K. pneumoniae* (148). Multiple carbapenemase producing *K. pneumoniae* isolate co-harboring *bla*<sub>OXA-48</sub>, *bla*<sub>NDM-1</sub>, and *bla*<sub>IMP-1</sub> genes, was reported for the first time in Egypt by Osama



*et al.* in a study performed in Children's Cancer Hospital for pediatric cancer in Cairo (149). *bla*<sub>NDM-1</sub>, *bla*<sub>NDM-5</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>OXA-181</sub> and *bla*<sub>KPC2</sub> were also detected among geographically diverse randomly selected MDR *K. pneumoniae* isolates from nine Egyptian hospitals (150). In 2018, a study that *bla*<sub>OXA-48</sub> gene was the most identified carbapenemase followed by *bla*<sub>NDM</sub>, *bla*<sub>VIM</sub> and *bla*<sub>KPC</sub> in febrile neutropenic pediatric cancer patients in Egypt (151). As the prevalence is very high in Hospitals, carbapenemases have now been described in several compartments. *Blavim-1* and *blavim-2* genes have been reported in two *E. cloacae* complex strains isolated from kofta and beef burger and one *P. aeruginosa*, isolated from minced meat (152, 153). Another study, reported for the first time *P. aeruginosa* clinical isolates harboring *bla*<sub>VEB</sub> together with *bla*<sub>GES</sub> in Egypt (154). *Blavim* and *bla*<sub>IMP</sub> were also detected among *P. aeruginosa* clinical isolates (155). OXA-48 and IMP are the most prevalent carbapenemase genes among *Escherichia coli* clinical isolates (156). Many outbreaks of *S. marcescens* infection have been reported in neonates, and also in Egypt where *S. marcescens* harboring the *bla*<sub>IMP-4</sub> and *blavim-2* genes was reported for the first time in 2015 in the neonatal intensive care unit of Cairo University Hospital (157). *bla*<sub>OXA-51</sub>, *bla*<sub>OXA-23</sub> and *blavim* were detected among all CRAB isolates from Egypt. *bla*<sub>OXA-58</sub> and *bla*<sub>NDM-1</sub> were present in 1.4% and 12.1% of the isolates, respectively (158). Furthermore, OXA-23, NDM and GES were detected in carbapenem resistant *A. baumannii* isolated from the surgical intensive care unit. While genes coding for VIM, GES, NDM and IMP were detected CR *P. aeruginosa* (159).

Far from clinical strains, *bla*<sub>KPC</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>NDM</sub> were described in Enterobacterales strains isolated from fish farms in Egypt (160). Among carbapenem resistant, shiga toxin-producing *E. coli*, isolated from aw food samples and diarrheic samples (cattle and human), the *blavim* gene was detected in all isolates, and one human isolate carried the *bla*<sub>NDM-1</sub> gene (161).

## 5- JORDAN

The emergence of OXA-48-like and NDM carbapenemases was found to be associated with multi-drug resistant (MDR) *K. pneumoniae* isolates in Jordan (162). A study conducted by Aqel *et al.* showed a prevalence of 1% of carbapenem resistance (in 28 out of 2759 isolates originating from five hospitals in Amman, Jordan). It was shown that 23 of the resistant isolates (82.1%) were *K. pneumoniae* (OXA-48-like, n = 7; NDM, n = 14; OXA-48-like and NDM, n = 2), four (14.2%)

were *Enterobacter cloacae* complex (NDM, n = 3 and VIM, n = 1), and one (3.5%) was *E. coli* (NDM)(163). Another study published in 2018, in which 2,759 Enterobacterales isolates were collected from five hospitals in Amman revealed that only 28 (1%) were carbapenem resistant (163). Out of the 28 CR isolates, 23 (82.1%) were *K. pneumoniae* (seven isolates harbored OXA-48-like, 14 NDM, and OXA-48-like and NDM), four (14.2%) were *E. cloacae* complex (three had NDM, and one has VIM), and one (3.5%) was *E. coli* (NDM) (163). A recent study performed on patients admitted to King Abdullah University Hospital (KAUH), 23 (12.8%) of 179 *K. pneumoniae* isolates were resistant to carbapenems with 12/26 (46 %) of them harboring KPC enzyme genes, 6/26 (23 %) IMP genes, 5/26 (19 %) OXA-48 genes, 3/26 (11.5 %) NDM-1 genes and no VIM gene

Regarding *A. baumannii*, a study evaluating the situation between 2010 and 2020, showed that 76.8% of 622 tested *A. baumannii* isolates collected from three tertiary hospitals in Jordan were MDR with 99.2% of them being carbapenem-resistant (164). OXA-23 gene was predominant (98.5%), then 26.6% of CRAB harbored VIM gene, and 0.8% had IMP genes and no KPC were detected (164). Similarly, *A. baumannii* isolates, collected from major hospitals in Jordan in 2018, showed that *bla*<sub>OXA-23-Like</sub> (88.3%) and *bla*<sub>NDM-1</sub> (10.4%) were the most prevalent enzymes (165). Additionally, investigations of drug-resistant *E. coli* isolates from February to June 2015 in samples from all drinking water sources reveal the lack of safe water in Amman, where carbapenemase production rate was 2.8%, with *bla*<sub>VIM</sub> (n= 75/109; 68.8%) followed by *bla*<sub>NDM</sub> (n= 12/109; 11%), and *bla*<sub>KPC</sub> (n= 5/109; 4.6%) (166).

## **6- TURKEY**

Interestingly, back to 2001, OXA-48 was reported was reported in Turkey. This region was then considered as endemic for OXA-48 Like enzymes, where it's the most frequent enzyme (167-170). In 2009, OXA-162, a single amino acid variant of OXA-48, with increased carbapenem hydrolysis was described in Turkey in a *K. pneumoniae* isolate (171). The same year, the first *K. pneumoniae* strain co-producing OXA-48 and KPC was described (172). Coproduction of OXA-48 and NDM in *K. pneumoniae* was also found in two out of 44 CRKP (carbapenem resistant *K. pneumoniae*) isolates (173). Talking about co-production, OXA-48 was also found to be co-produced with *bla*<sub>VIM</sub> gene in 6.3% out of 83 *K. pneumoniae* isolates, while 74.1% harbored OXA-48 alone among

carbapenem-resistant *K. pneumoniae* isolated from various clinical samples in Ankara University Faculty of Medicine, Ibni Sina Hospital, Central Microbiology Laboratory between June 2010 and May 2014 (174). Moreover, out of 100 *K. pneumoniae*, 45 expressed OXA-48 Like variants including OXA-48, OXA-245, OXA-181 and OXA-244 (175). In 2019, 28 hospitals from 26 different regions, were included in a study that aimed to evaluate the results of the pilot study for the establishment of molecular-based carbapenem surveillance system in *E. coli* and *K. pneumoniae* isolates and to investigate the carbapenemase epidemiology in Turkey. OXA-48, KPC, NDM-1, OXA-48 with NDM-1, KPC with NDM-1, VIM and OXA-48 with VIM were the carbapenemases identified among the carbapenemase producing strains (176). Since, several studies confirmed the co-production of carbapenemases (172, 177).

Carbapenem resistance in *A. baumannii* associated to outbreak situations has been described several times (178-180). In 2016, molecular characteristics of clinical *A. baumannii* strains from 3 Turkish university hospitals were investigated. Results showed that OXA-23-like and OXA-58-like were the most common carbapenemase genes (181). A study conducted over a 6 months period in 2012, reported OXA-23 and/or OXA-58 producing *A. baumannii* in Turkey among clinical and environmental isolates where *bla*<sub>OXA-23</sub> gene has become the most prevalent carbapenemase in Turkey (182). Generally, *bla*<sub>OXA-23</sub>, *bla*<sub>OXA-24</sub>, *bla*<sub>OXA-51</sub>, *bla*<sub>OXA-58</sub>, *bla*<sub>IMP</sub>, *bla*<sub>NDM</sub>, *bla*<sub>KPC</sub>, *bla*<sub>OXA-48</sub> are spread among *A. baumannii* in Turkey (182).

Between 2017 and 2018, a study showed the co-existence of OXA-48 and NDM-1 genes in colistin-resistant *P. aeruginosa* (183). Over and above that, the coexistence of *bla*<sub>NDM-1</sub> and *bla*<sub>VIM-48</sub> metallo- $\beta$ -lactamase (MBL) encoding genes was noted, conferring resistance to carbapenems (184). Lately, a study conducted over a 14 month period, revealed the expression of VIM-5 and IMP-7 in ST308 and ST357 respectively, two high risk clones of *P. aeruginosa* (185)

Moving to non-fermenters, Çekin *et al.* reported high-risk clones of *P. aeruginosa* carrying VIM-5- and IMP-7-type for the first time in Turkey in 2020 (185). In addition, MDR *A. baumannii* are well spread in Turkey. For instance, a study published in 2019 by Boral *et al.* showed that out of 172 isolates, 166 (96.5%) carried *bla*<sub>OXA-23</sub>, 5 (2.9%) *bla*<sub>OXA-58</sub> and one isolate (0.6%) was positive for both genes. *Bla*<sub>IMP</sub>, *bla*<sub>KPC</sub>, *bla*<sub>NDM</sub> and *bla*<sub>OXA-48</sub> genes were not detected (182).

## 7- Algeria

The first report of CPE in Algeria, dates back to 2008, and mainly described resistance in *K. pneumoniae* and *E. coli* (186). Since then, MDR Enterobacterales are largely found in Algeria with carbapenem-resistance mostly described in *K. pneumoniae* and *E. coli* (187). OXA-48 is one of the most widespread enzymes there. For instance, a nosocomial outbreak in Batna hospital unveiled the dissemination of OXA-48 after the death of five hematology unit patients with OXA-48 harboring *K. pneumoniae* (188).

Among a collection of 168 isolates from clinical specimens in Annaba University Hospital (Algeria) between April 2016 and December 2018, 17.3% (29/168) were CRE, predominantly, *K. pneumoniae* (n=23), *E. coli* (n=5) and *E. cloacae* (n=1) with *bla*<sub>OXA-48</sub> (n= 26/29; 90%) and *bla*<sub>NDM-1</sub> (n= 3/29; 10%) being the most prevalent genes (189). In addition to NDM-1 metallo-beta-lactamase to be discovered in Algeria, NDM-5 was also discovered lately (190). Another MBL, *bla*<sub>VIM</sub> was also detected, especially the variant VIM-19 in *E. coli* and *K. pneumoniae* isolated from clinical rectal swab samples (191, 192).

Interestingly, various carbapenemases were detected in *P. mirabilis*, notably, TEM-1, TEM-2, PER-1, SHV-11 and OXA-24 which was detected for the first time in *P. mirabilis* strain in Algeria in 2019 (193). More importantly, *P. mirabilis* harboring OXA-24 was also found to harbor *armA* 16S rRNA methylase and *aac(6)-Ib-cr* genes.

Wild animals are also an important source of carbapenemase producing isolates. Especially OXA-48 found in wild boars and Barbary macaque (194), and in Columba Livia pigeons (195). Following its first description in a clinical *K. pneumoniae*, NDM-5 was then found in long-distance migratory bird species *Ciconia Ciconia* (196). Unfortunately, the occurrence of carbapenem resistant bacteria is rising in vegetables also, such as bacteria encoding *bla*<sub>OXA-48</sub> and *bla*<sub>VIM-4</sub> genes (197).

## 8- Morocco

As for the majority of countries, serious precautions should be taken in Morocco, to contain the spread of carbapenemase. Non fermenters seem to follow the trend, *A. baumannii* usually carry *bla*<sub>OXA-51</sub> and *bla*<sub>OXA-23</sub> gene (198-200). MDR *A. baumannii* harboring OXA-23 were also found

among newborns hospitalized in Moroccan neonatal intensive care unit (201). A recent study reported the first isolation of *A. baumannii* harboring the MBL Spm-1 (202). Out of 70 CRAB, OXA-51 gene, OXA-23 and OXA-58 were detected in 100%, 82.85% and 10% of isolates respectively (202).

The coexistence of *bla*<sub>OXA-23</sub> and *bla*<sub>NDM</sub> genes was also detected in Morocco. *P. aeruginosa* is well known to carry MBLs especially *bla*<sub>VIM-2</sub> (200).

In a study published in 2022, out of 484 clinical isolates, from Mohammed VI University Hospital of Marrakech, Morocco, 388 (80.16%) were Carbapenemase-positive. Out of the latter, 170 produce NDM, and 162 produce OXA-48-like (203). Both found enzymes are very widespread in the region (204). Another study was conducted on strains isolated from the same hospital, it has been shown that 85.5% of the isolates were resistant to carbapenem through the production of OXA-48, NDM, OXA-48/NDM and VIM). NDM-1 and VIM-1 were the only found MBL variants found (205).

Unfortunately, the widespread OXA-48 was also found in neonatal intensive care unit in *K. pneumoniae* isolates according to a study conducted by Taoufik *et al.* in 2019 (206). This alarming issue highlights the importance of isolation of patients with infections caused by MDR, carbapenemase producing isolates.

## 9- Libya

Recent studies in Libya are scarce, but they have similar findings, where the majority of *A. baumannii* isolates were found to carry OXA-23, moreover, NDM-1 MBL was also found in one of the isolates tested by Slimene *et al.* (207). This was found by other studies as well where *A. baumannii* isolates were identified to have *bla*<sub>OXA-23-like</sub>, *bla*<sub>OXA-24-like</sub>, and *bla*<sub>OXA-48-like</sub> genes (208).

VIM-2 was detected in *P. aeruginosa* in several studies conducted in Libya (209). OXA-48 is widespread in Libya as it disseminates horizontally through IncL/M plasmid (210). MBLs exist also in Libya, where an *Achromobacter xylosoxidans* isolate originating from Tripoli central hospital produced a unique metallo- $\beta$ -lactamase, designated TMB-1 (211).

## 10- TUNISIA

In Tunisia, the spread of carbapenemases among Gram negative bacteria constitutes a real concern (212). Usually, VIM, NDM-1, and OXA-48 enzymes are well reported with the predominance of OXA-48 among Enterobacterales (212, 213), with OXA-181 identified for the first time in *Serratia* in 2021 (214). KPC and IMP carbapenemases were uncommonly detected in Tunisia and OXA-23, OXA-51, and OXA-58 carbapenemases constitute the main carbapenem resistance mechanism among *Acinetobacter baumannii* (212).

The first report of carbapenemase producing *K. pneumoniae* in Tunisia, dates back to 2006 (215). Since then, several types of carbapenemases were detected in different species isolated from clinical and environmental specimens (212). KPC is recently reported in Tunisia among *Klebsiella pneumoniae* and *Escherichia coli* strains but is still uncommon (216, 217). Metallo- $\beta$ -lactamases are described in Tunisia, for instance, IMP metallo-beta lactamase, was described only in *Klebsiella pneumoniae* environmental isolate (218, 219). VIM enzyme was also frequently reported among *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and scarcely among *Escherichia coli* and *Enterobacter cloacae* strains in Tunisia (220, 221). In spite of being lately reported, NDM-1 is found to be dramatically disseminated in Tunisia comparing with other metallo- $\beta$ -lactamases (220, 221). Concerning class D carbapenemases, OXA-48-like, OXA-23-like, OXA-51-like (OXA-66, OXA-82, OXA-94), and OXA-58-like (OXA-58 and OXA-97) were the four major subfamilies found in Tunisia (212). OXA-48, OXA-204 and OXA-232 are the OXA-48-like variant commonly found among Enterobacterales (222). OXA-48- was reported for the first time in 2010 by Ktari *et al.* (223). Moreover, the co-occurrence of  $\beta$ -lactamases is also well reported in Tunisia, for instance, in a study conducted lately, *K. pneumoniae* isolates co-harbored  $bla_{NDM-1}$  and  $bla_{OXA-48}$ , while others carried  $bla_{NDM-1}$  with  $bla_{VIM-1}$  (n = 1) (224).

Among *A. baumannii* strains, carbapenem resistance is usually associated with the production of OXA-23, OXA-58 and OXA-51 carbapenemases where OXA-51 is intrinsic to *A. baumannii* isolates and can only lead to carbapenem resistance when adjacent to the insertion sequence ISAbal which promotes its expression (21, 225). Carbapenem resistant bacterial strains were not limited to clinical sources in Tunisia, but also it was observed in natural sources. These strains were found in a polluted river in the country (219). OXA-23 producing *A. baumannii* ST2 was

detected in seafood collected from a region contaminated with hospital effluents (226). The co-expression of OXA-58 with OXA-23 was described in 2018, by Mathlouthi *et al.* (227), similarly, *A. baumannii*, co-producing VIM-2 with OXA-23, was described in intensive care units (228).

In *P. aeruginosa*, the first detection of GES-5 and GES-9 as well as the co-occurrence of the *bla*<sub>GES-5</sub> and *bla*<sub>VIM-11</sub> carbapenemase genes occurred in 2022 (229).

## **11- Gulf countries - Bahrain, Iraq, Kuwait, Oman, Qatar, Saudi Arabia, and the United Arab Emirates.**

The burden of carbapenem resistance is not well studied in Gulf region, however, we know that carbapenem resistant gram-negative organisms are spreading in Gulf Cooperation Council (GCC) countries. Studies conducted in Saudi Arabia, Bahrain, Kuwait Oman, Qatar, and the United Arab Emirates point out the domination of NDM-type and OXA-48-type enzymes produced by multiple clonal lineages of *E. coli* and *K. pneumoniae* (230-232). A recent study, aimed to determine the prevalence of carbapenem resistant genes among gram negative organisms from patients in GCC. Results showed that 26.1% of the specimens carry carbapenem resistance genes among which, OXA-48 was the most commonly detected (233). The resistome of *K. pneumoniae* isolates from the Arabian Peninsula, were studied to understand the reasons of spread of carbapenem-resistant *Klebsiella pneumoniae* ST14. NDM-1, OXA-48, OXA-162, OXA-232, KPC-2, or co-production of NDM-1 and an OXA-48-like carbapenemase, were described (234). Note that, the first identification of a KPC-2-producing CRE in the Gulf region dates back to 2019 (235), in Saudi Arabia in a *K. quasipneumoniae* isolate.

Among the available data, VIM type enzyme was found to be the most prevalent in carbapenem-resistant *P. aeruginosa* (CRPA) collected from hospitals in the countries of the gulf (236).

Until 2017, four *E. coli* isolates in Kuwait, Oman, and the UAE, respectively, were identified to be NDM-7 producers (237). Between 2009 and 2014, *bla*<sub>NDM-1</sub>, *bla*<sub>NDM-4</sub>, *bla*<sub>NDM-5</sub>, *bla*<sub>NDM-7</sub>, *bla*<sub>OXA-181</sub> or *bla*<sub>KPC-2</sub> carbapenemase genes were identified among carbapenem resistant Enterobacterales from UAE (238). *K. pneumoniae* isolates harbored double carbapenemase genes were identified in UAE. Different isolates were reported: NDM-1 and OXA-232, NDM-1 and OXA-162, NDM-1 and OXA-48 carriers on 2 different plasmids for each isolate (239). A study

showed that ISEcp1-driven *bla*<sub>OXA-181</sub> insertion, in a *Klebsiella pneumoniae* isolate, was the cause of resistance to carbapenems (240).

In Saudi Arabia, Abd El Ghani *et al.* showed the presence of *bla*<sub>NDM-5</sub>, *bla*<sub>OXA-1</sub> and *bla*<sub>OXA-181</sub> (241).

In particular, among carbapenem-resistant *Escherichia coli* clinical isolates, from Iraqi patients with urinary tract infection, *bla*<sub>OXA-48</sub> was the most frequently identified carbapenemase gene, followed by *bla*<sub>PER</sub>, *bla*<sub>KPC</sub>, *bla*<sub>VEB</sub> and *bla*<sub>VIM</sub> (242). As in other countries, reports show a spread of OXA-23 in *A. baumannii*: A study published in 2016, reported that *A. baumannii* isolates harbored *bla*<sub>OXA-51-like</sub> genes and *A. calcoaceticus* complex isolates additionally carried the *bla*<sub>OXA-23-like</sub> gene *bla*<sub>OXA-24-like</sub> (243). Regarding metallo-β-lactamases, a low prevalence of IMP gene, was detected among *K. pneumoniae* strains isolated from patients suffering of urinary tract infections and burns in Al-Kufa hospital in Al-Najaf province, Iraq (244). *Bla*<sub>NDM-1</sub> was as well reported for the first time among clinical *K. pneumoniae* isolates in Iraq by Hussein *et al.* (245). For most studies, conventional PCR technique is used to detect resistance genes e.g. IMP, VIM, and SPM-1 were detected by conventional PCR technique among carbapenem resistant *P. aeruginosa* isolated from public and private hospitals in Baghdad, Iraq (246). A similar study carried in Najaf City, Iraq, showed that *bla*<sub>VIM</sub> and *bla*<sub>IMP</sub> gene were detected among *P. aeruginosa* isolated from diabetic patients (247). Ismail *et al.* also aimed to detect the prevalence of *bla*<sub>NDM</sub> variants metallo-beta lactamase among clinical *P. aeruginosa*. *P. aeruginosa* isolates carrying *bla*<sub>NDM-1</sub> and *bla*<sub>NDM-2</sub> were reported for the first time in Iraq (248).

In Kuwait, several articles reported the presence of *bla*<sub>NDM-1</sub> along with *bla*<sub>OXA-48</sub> (249). The enzymes *bla*<sub>OXA-23</sub>, *bla*<sub>IMP</sub>, *bla*<sub>OXA-24/40</sub>, *bla*<sub>GES</sub>, *bla*<sub>OXA-48</sub>, and *bla*<sub>OXA-58</sub>, are also found in Kuwait, but a lack of *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub>, *bla*<sub>SIM</sub>, and *bla*<sub>NDM</sub> was noted (250). Moghnia *et al.* showed the presence of KPC-19, KPC-35, OXA-10, OXA-59, in addition to NDM-2, and NDM-32 in CRE (251). Genes of *bla*<sub>OXA-181</sub> and *bla*<sub>NDM-5</sub> carrying plasmids were also detected in some strains (252).

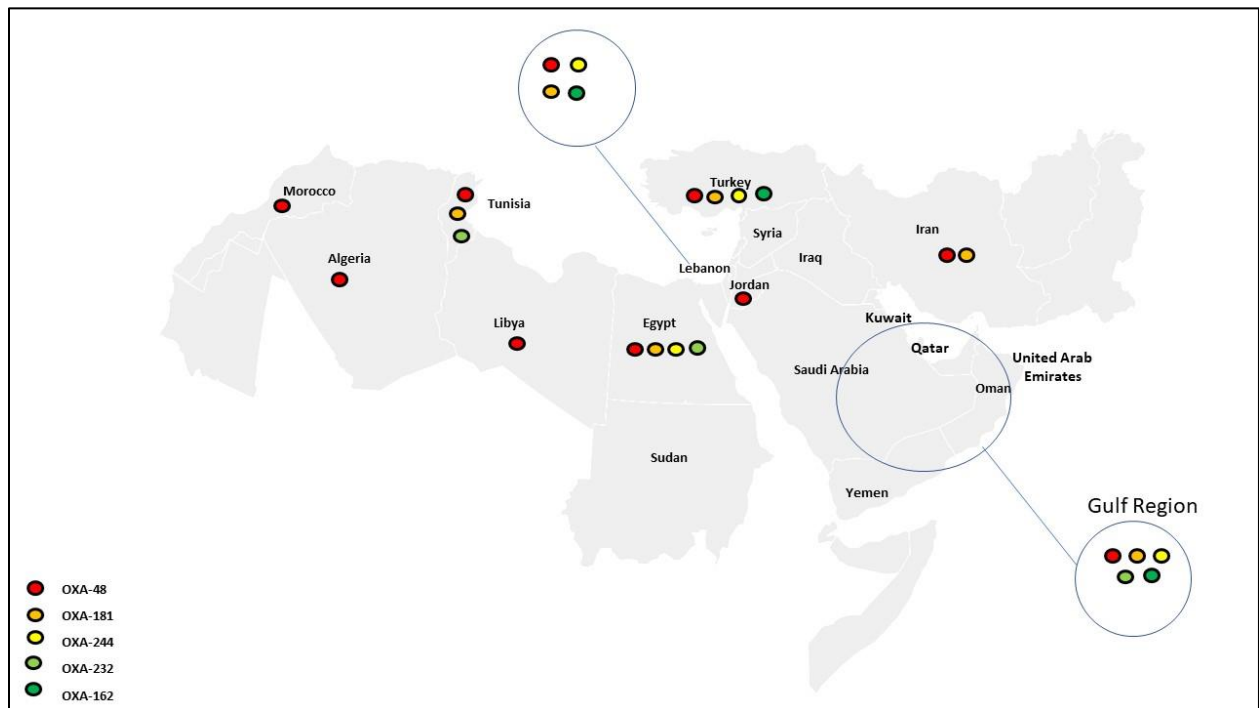
In Oman, Double carbapenemase genes were identified among *K. pneumoniae* isolates in Oman, where *bla*<sub>NDM-1</sub> carried on IncHI1b plasmid was identified with a chromosomally encoded *bla*<sub>OXA-181</sub> (239).



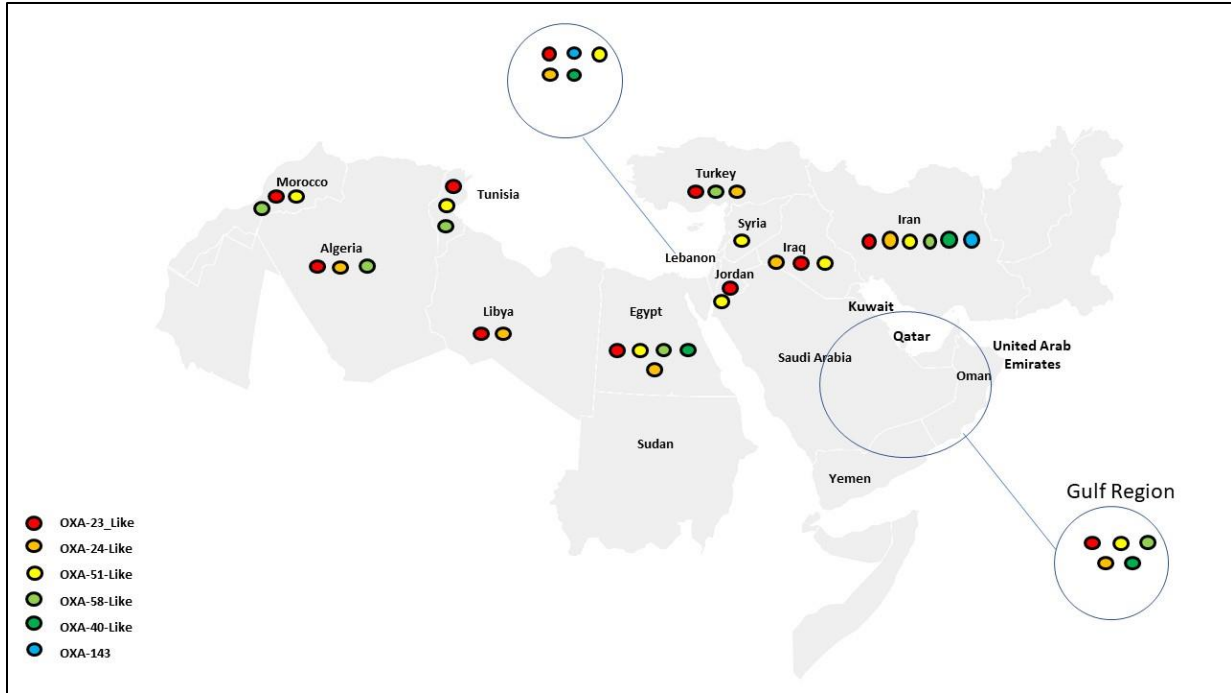
In Bahrain, the first study to report the presence of the VIM family gene and NDM-1 genes in imipenem-resistant *P. aeruginosa* was in 2019 (253).

Studies conducted in Qatar, show that among clinical carbapenem-resistant Enterobacterales isolates, *E. coli* and *K. pneumoniae* were the most common species, with OXA-48-like, and NDM-1 enzymes were the most frequent (231). OXA-244, was also detected in patients with no travel history, in Qatari health care facilities (254).

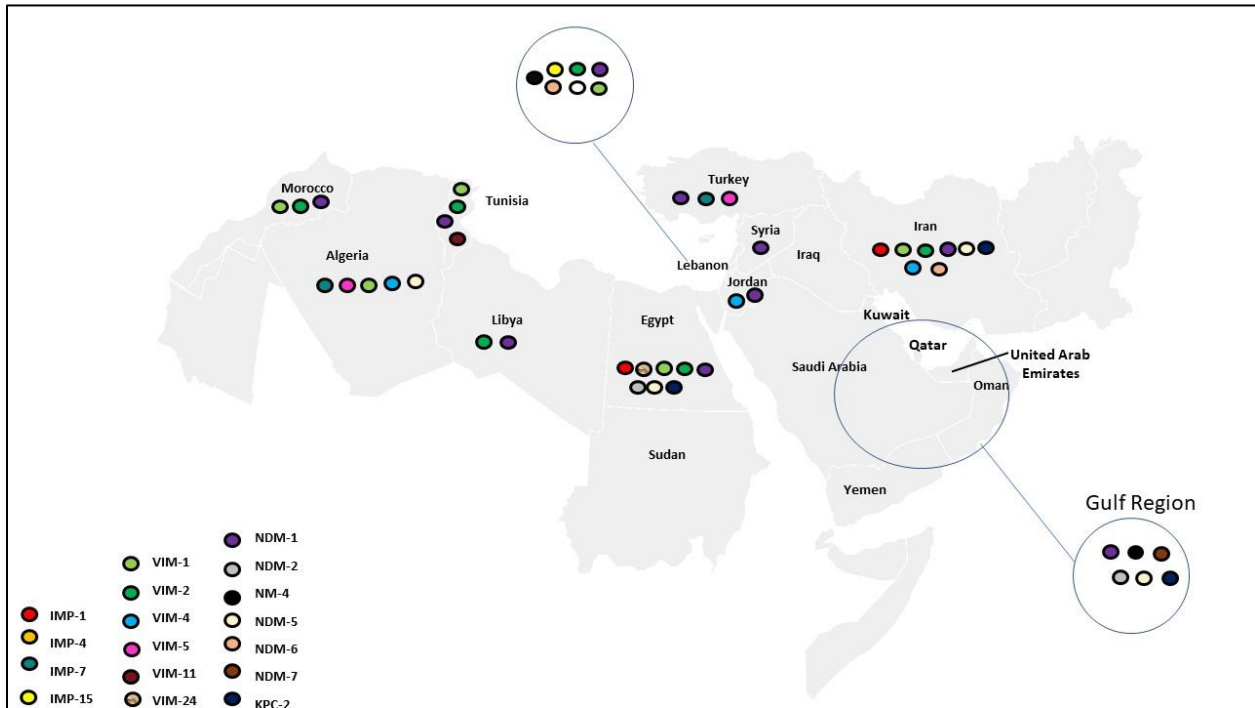
The following maps sums up the carbapenemase variants found in each country.



**Figure 1:** Repartition of OXA-48 Like variants in the Middle East



**Figure 2:** Repartition of various oxacillinases in the Middle East



**Figure 3:** Repartition of some carbapenemase variants in the Middle East

**Conclusion**

Even though some countries don't have enough published data regarding carbapenemase situation, all the regions of the Middle East seem to follow a similar trend with a predominance of OXA-48 Like enzymes in Enterobacterales, OXA-23 Like enzymes in *A. baumannii* and MBLs in *P. aeruginosa*. In the light of all the stated findings, following few steps remain crucial to limit the spread of  $\beta$ -lactamases. Ensuring a vigorous national plan aiming to survey antibiotic resistant infections, along with the implementation of an efficient prevention and control protocol will significantly help. Quality medicines should be handled with care as well.

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## **XV. Review 2: A silent conquest: A snapshot on mobile colistin resistance genes in the MENA region**

La colistine est un antibiotique de dernier recours, largement utilisé en médecine vétérinaire pour plus de 50 ans, mais négligé chez l'homme en raison de sa neurotoxicité (260). Par conséquent, les quantités de colistine utilisées chez les animaux destinés à l'alimentation dépassent largement celles utilisées chez l'homme, et les isolats résistants sont apparus pour la première fois dans le domaine vétérinaire (261). Liu *et al.* a identifié pour la première fois, un gène de résistance à la colistine portée par un plasmide, isolé chez les poules en 2015 (155). Cette découverte fut suivie par un nombre important d'études à travers le monde qui ont révélé que le gène *mcr-1* était répandu dans les isolats provenant de la volaille, du porc et de sources environnementales. La localisation plasmidique du gène *mcr-1*, principalement sur IncX4 et IncI2 hautement transférables, a également suscité des inquiétudes mondiales quant à la perspective de transfert horizontal de ce gène de l'animal à l'homme, dans lequel la colistine est une molécule importante de dernier recours (262). De nombreux pays de la région MENA manquent de compréhension, de cartographie et de caractérisation complètes des gènes *mcr* ce qui constitue une menace importante, agissant comme un catalyseur silencieux pour la propagation de ce marqueur génétique dans le monde entier. Ici, nous avons pour objectif de décrire leur distribution des gènes *mcr* dans la région MENA, souligner la nécessité de mettre en œuvre de solides programmes de gestion des antimicrobiens et d'interdire l'utilisation de la colistine dans les pratiques agricoles, et insister sur l'importance de la mise en œuvre de plans d'action nationaux utilisant l'approche One Health pour lutter contre la propagation de la résistance aux antibiotiques.

# **A silent conquest: A snapshot on mobile colistin resistance genes in the MENA region**

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**Abstract:** Colistin has recently been reintroduced into human clinical use as a consequence of the rapid emergence of multidrug-resistant bacteria. This old antibiotic is nowadays used to treat serious drug-resistant infections, particularly those caused by carbapenem-resistant strains. However, its inappropriate use in animal production and clinical settings enhanced the dissemination of the plasmid-borne mobile colistin resistance (*mcr*) genes. Despite the global concern, the healthcare systems in the Middle East and North Africa (MENA) region are still behind in understanding how critical is the antimicrobial resistance (AMR) issue. In addition to globalization, many MENA countries face calamitous multi-sectoral challenges and depend on food imports, making them an ideal hotspot for AMR. The lack of a comprehensive understanding, mapping, and characterization of *mcr* genes in the region pose a substantial threat, acting as a silent catalyst for the propagation of this genetic marker worldwide. Here, we aimed to discuss existing evidence on *mcr* genes in the MENA region to (i) describe their distribution, (ii) emphasize the need for implementing robust antimicrobial stewardship programs and banning the use of colistin in agricultural practices, and (iii) urge for the implementation of national action plans using a One Health approach to combat the spread of AMR.

**Keywords:** colistin; *mcr* genes; antimicrobial resistance, multidrug resistance; MENA region

## 1. Introduction

The discovery of antimicrobials brought a paradigm shift in the field of medicine, improving the health and well-being of humans and animals. Nowadays, these wonder drugs are regrettably losing their effectiveness, and the antimicrobial resistance (AMR) issue emerges as one of the top threats to global health, food security, and sustainability [1]. Difficult-to-treat infections and high carriage of multidrug-resistant (MDR) bacteria among humans and animals are increasingly reported across the globe, resulting in a dramatic impact on morbidity and mortality, a lengthened treatment duration, an increased cost of hospitalization, and challenges to find a suitable treatment [2]. According to estimations, the AMR issue is expected to cause a global annual death of 10 million people by the year 2050, total loss of 100 trillion US dollars, and more than 28 million people will be pushed below the poverty line, out of which 26 are from low and middle income countries (LMIC) [1,3]. Although novel antimicrobial agents are urgently required, the development of new drugs is declining [4]. The lack of new effective antimicrobials along with the worrying upsurge in life-threatening infections has urged the health community to reconsider old therapeutic agents, such as colistin. Even though this cationic polymyxin was disregarded in the 1970s due to its nephrotoxicity and neurotoxicity, it was revitalized in the early 2000s, as Gram-negative bacterial strains became more resistant and challenging to treat. Consequently, colistin was recognized by the World Health Organization (WHO) among the highest priority critically important antimicrobials used to treat complicated MDR infections in human medicine [5].

The advent of complicated drug-resistant infections is an amalgam of several factors such as the lack of awareness and the inappropriate use of antimicrobials in clinical settings and agricultural and veterinary practices [6]. It is well-established that countries with high antimicrobial resistance burden heavily depend on clinically important broad spectrum antimicrobials for veterinary practices to treat and prevent diseases as well as growth promotion purposes in food-producing (e.g., livestock, poultry) [7]. The overreliance on colistin in clinical settings and its non-judicious use in animals have substantially compromised the effectiveness of this antimicrobial agent and facilitated the emergence of resistant strains and plasmid-borne mobile colistin resistance (mcr) genes [8]. Resistance to colistin is mainly due to the altered physicochemical features of the

bacterial lipopolysaccharides (LPS). Chromosomal mutations are the major cause of the acquisition of resistance to colistin, especially through activation of LPS modifying operons such as *arnBCADTEF* and *pmrCAB* that result in the addition of positively charged 4-amino-4-deoxy-L-arabinose. In 2016, the expression of a plasmid-encoded phosphoethanolamine transferase, named *MCR-1* has been firstly described to be involved in colistin resistance in Enterobacterales [9]. The phosphoethanolamine (pEtN) transferase enzymes catalyze the addition of a positively charged phosphoethanolamine to the lipid A moiety (i.e., changing the negative net charge of the membrane) thus limiting the capacity of colistin to bind to it [10]. Interestingly, *mcr* genes can laterally disseminate between bacterial hosts, progeny, and niches through horizontal gene transfer (HGT); further increasing the chance to disseminate resistance to colistin [11]. So far, *mcr* genes (*mcr-1* to *mcr-10*) have been promptly reported worldwide in various clinical, agricultural, and environmental matrices, transcending geographic and ecological borders [12]. Although the global spread of the *mcr* genes has been driven by multiple plasmid types (*IncI2*, *IncX4*, *IncX3-X4*, *IncHI1*, *IncHI2*, *IncP*, *IncY*, *IncK2*, *IncFI*, *IncFI*, *IncFII*, and *IncFIB-I2*), the major plasmid harboring colistin resistance correspond to *IncX4*, *IncHI2*, and *IncI2* scaffolds [13]. The high transfer capacity of these plasmids can probably explain their broad geo-graphical distribution and their occurrence in a wide variety of human and animal hosts.

Despite the global concern over losing the effectiveness of critically important antimicrobials, LMIC, especially the Middle East and North Africa (MENA) region, are still struggling to comprehensively understand and address the breadth of the problem on their premises. As the MENA region faces calamitous challenges with debilitated infra-structures, overcrowding, weakened antimicrobial stewardship programs, and political unrest, the countries remain among the least studied and surveilled concerning AMR, including colistin resistance [14-17]. Even though current AMR data remain insufficient in the MENA region, available reports suggest a high burden of colistin resistance and associated *mcr* genes in humans, animals, and the environment. The lack of comprehensive national data and the inefficiency of national action plans on AMR from the region hinders any efforts to collectively decrease the spread of colistin-resistant isolates. Here, we compiled and mapped the latest findings on *mcr* genes in different matrices in the MENA region to understand interactions at the human-animal interface and preserve the effectiveness of colistin in these presumed high-risk communities.

## 2. Colistin resistance and *mcr* genes in humans

The detection of *mcr* genes is less common in isolates from humans as compared to food producing animals; however, most countries in the MENA region reported the emergence and dissemination of these genetic markers in clinical settings. Although many *mcr* variants have been reported in the region, the *mcr-1* gene is predominant and widely distributed among colistin-resistant isolates, consistently with global surveillance data [12]. As described in most reports from the MENA region, colistin-resistant isolates showed high clonal diversity, demonstrating that horizontal transfer and plasmids play the main role in the dissemination of *mcr* genes rather than clonal spread of single clones. On the other hand, the co-existence of ESBL and/or carbapenemase and *mcr* genes, in addition to biofilm and toxin-encoding genes, creates more virulent clinical strains and enhances their ability to persist and cause severe life-threatening infections.

In Algeria, several reports revealed the emergence of *mcr-1* (n= 5) and *mcr-8* (n=1) in hospitalized patients [18-23]. The first reported *mcr-1* gene was identified in a *bla*<sub>CTX-M-15</sub> extended-spectrum beta-lactamase (ESBL)-producing ST405 *Escherichia coli* isolate recovered from the semen of a 29-year-old patient. The gene was located on a high-frequency transferable IncFIB plasmid, highlighting the potential risk of its rapid dissemination in the Algerian community [18]. Moreover, a *mcr-1*- and *bla*<sub>CTX-M-15</sub>-positive ST405 *E. coli* isolate has also been described in an 18-year-old polytrauma Algerian man with no history of traveling abroad, which suggests a likely local acquisition of the genes [19]. A *mcr-1* gene carried on an IncHI2 plasmid was found in ST19 *E. coli* isolated from the urine of a 75-year-old patient [20]. Other autochthonous cases of the *mcr-1* gene were also described [21]. *Mcr* genes were also described among carbapenem-resistant clinical isolates, such as *mcr-1* in *Pseudomonas aeruginosa* [22] and *mcr-8* in ST336 *Klebsiella pneumoniae* (*bla*<sub>OXA-48</sub> and *bla*<sub>CTX-M-15</sub> producing) [23]. Interestingly, the *mcr-8* gene was located on an IncFII plasmid and showed a high similarity to the previously reported *mcr-8*-positive *K. pneumoniae* from a pig sample in China [24]. The identification of both colistin and carbapenem resistance in the same isolate is very problematic since colistin is used as a salvage therapy to treat complicated carbapenem-resistant infections. *Mcr-8*-positive carried by a non-typable self-conjugative plasmid ST967 *K. pneumoniae* was also reported in France from a repatriated patient

from Morocco. The isolate has a genetic environment identical to that previously described in Algeria [23] and possesses an ESBL genotype (*bla<sub>SHV-27</sub>*) associated with a decreased phenotypic susceptibility to carbapenems [25]. *Mcr-1* was also observed among *E. coli* and *K. pneumoniae* isolated from Algerian and Moroccan pilgrims upon their return from the multi-national Hajj event in Saudi Arabia [26]. The *E. coli* isolates belonged to different STs (ST10, ST93, ST155, ST453, ST602, ST648, ST656, ST1300) [26]. Traveling, especially to overcrowded or endemic areas, represents an effective driver for the silent dissemination of plasmids harboring Antimicrobial Resistance Genes (ARGs) globally.

In 2015, the plasmid-borne *mcr-1* gene was first described in Egypt in a *bla<sub>CTX-M-15</sub>* producing *E. coli* (ST1011) isolated from a hospitalized patient with no history of travel abroad [27]. Moreover, the presence of the *mcr-1.1* gene was identified in three clinical ESBL-producing *E. coli* isolates. The *mcr-1*-bearing plasmid corresponded to IncHI2 type with a notable similarity to other *E. coli* plasmids previously observed in Egypt [28]. Alarming, *mcr-1* was reported in an NDM-1-producing *E. coli* isolated from a cancer patient [29]. Cancer patients are prone to bacterial infections due to their weakened immune systems; thus, MDR isolates (i.e., resistant to extended-spectrum cephalosporin/carbapenem and colistin) may lead to complicated and sometimes life-threatening outcomes. The *mcr-2* gene has emerged in both colistin-resistant *E. coli* and *K. pneumoniae* isolates from fecal samples of healthy farmers [30]. Recently, a *mcr-9* and *bla<sub>VIM-4</sub>*-carrying MDR *Enterobacter hormaechei* (ST133) clinical isolate has been described in Egypt. Notably, the isolate did not express a phenotypic resistance to colistin (i.e., reported as susceptible to colistin), indicating the possible silent transmission of *mcr-9*-positive isolates in clinical settings. The authors suggested that the carrying plasmid (IncHI2) and the two detected insertion sequences (IS1, and IS903) might serve as vehicles for the *mcr-9* dissemination. In Sudan, only one study assessed the prevalence of colistin resistance among Gram-negative bacteria in clinical samples, showing the emergence of *mcr-1*-positive *K. pneumoniae* and *P. aeruginosa* [31]. Moreover, the only data from Tunisia showed a high prevalence (10%) of clinical *E. coli* isolates harboring the *mcr-1* gene, located on either IncY or IncFIB plasmid [32].

In Lebanon, it is well-documented that colistin is highly used in the clinical settings in recent years, whereby imports of this polymyxin increased five folds between 2010-2017 [7]. Despite its clinical

importance, only a few studies were conducted to assess the spread of *mcr* variants in humans. To date, *mcr-1*, *mcr-8*, and *mcr-9* have been described in Lebanese healthcare settings. The *mcr-1* variant carried on IncX4 plasmid was described among *E. coli* isolates resistant to all classes of beta-lactams, except carbapenems [33]. IncX4 plasmids, highly prevalent in Lebanon, are self-transferable at high frequency and have been responsible for the dissemination of *mcr-1* across the globe. Notably, the *mcr-1*-positive isolates belonged to various genetic backgrounds (ST2705, ST1011, and ST1431), indicating the non-clonal spread of the gene in Lebanese clinical settings. Furthermore, *E. coli* ST405 isolates co-harboring *mcr-1* and *bla*<sub>NDM-4</sub> genes have been isolated from hospitalized patients [34]. Taken together, this data highlights an alarming dissemination of highly resistant *E. coli* strains in Lebanese hospitals, especially in a period of extreme shortage in antibiotics and medical supplies [35,36]. Worryingly, *mcr-1* was also documented to be circulating in the community. *Mcr-1* was detected in *E. coli* isolates re-covered from fecal samples of food workers [37] and healthy toddlers [38]. As previously found in clinical settings, the isolates from food workers belonged to different genetic backgrounds and were carried by the IncX4 (83%) or IncHI2 (17%) [37]. Moreover, they were different than those identified among patients (ST1303, ST69, ST10, and ST8140) [37]. The colonization of food workers with *mcr* poses a significant public health and food safety concerns since cross-contamination of food products can easily occur through the fecal-oral route due to improper hygienic practices. Regarding healthy toddlers, the *mcr-1* variant was located on diverse globally disseminated plasmid types including IncI2, In-cII $\alpha$ , IncX1, IncX4, IncF2, and IncFIB [38]. The *mcr-1*-positive isolates were genetically diverse (ST57, ST69, and ST1011) and previously associated with *mcr-1*-positive *E. coli* from clinical and the community. Additionally, a single *mcr-1*-positive *Proteus mirabilis* was detected in the diapers of a 2-year-old toddler [39]. As this opportunistic pathogen is naturally colistin-resistant, these findings confirm the ability of intrinsically resistant isolates to silently spread *mcr* to other bacterial species. *Mcr-8* [40] and *mcr-9* [41] variants were re-reported in *K. pneumoniae* and *E. hormaechei* strains, respectively. Both isolates were MDR and have been isolated from urine tract infections. As found in the Egyptian study, the *mcr-9* variant was associated with a colistin-susceptible *E. hormaechei* (ST78), confirming the ability of this variant to silently disseminate in the ecosystem. It is worth mentioning that the *mcr-9* was located on the bacterial chromosome. A recent study

conducted in Jordan documented for the first time the emergence of an *mcr-1*-positive *K. pneumoniae* clinical isolate out of 89 tested colistin-resistant strains [42].

In Saudi Arabia, approximately 40% of Gram-negative bacteria are colistin-resistant [43]. *Mcr-1*, *mcr-2*, *mcr-5*, and *mcr-8* have been reported in healthcare settings. The first case of *mcr-1*-producing *E. coli* was recovered from a patient's blood sample. The isolate was MDR co-harboring a *bla<sub>NDM-1</sub>* carbapenemase gene and belonged to ST68 [44]. A second *mcr-1*-positive *E. coli* (ST131) isolate has been isolated from a 2-year-old patient with urinary tract infection [45]. The *mcr* gene in both cases was located on an IncHI2 plasmid, which is one of the major carriers of *mcr* genes in the MENA region. As part of Saudi Arabian efforts to monitor MDR clinical pathogens, a clinical ST3513 *K. pneumoniae* isolate co-expressing MCR-1 and MCR-8 was lately detected in the country [43]. *Mcr-5* was isolated from *E. coli* with no further details about the molecular characteristics and properties of the isolate [46]. Recently, an *mcr-2*-positive *K. pneumoniae* clinical isolate co-harboring *bla<sub>NDM-1</sub>* and *bla<sub>CTX-M-1</sub>* genes has been described, in addition to 18 other *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* clinical isolates harboring the *mcr-1* gene [47].

In Qatar, an *mcr-1*-positive MDR *E. coli* strain was isolated from the respiratory secretions of a Nepali patient with a subarachnoid hemorrhage [48]. The isolate belonged to the ST95, which represents a globally disseminated ST frequently associated with invasive infections (e.g., bloodstream, meningitis). The *mcr-1* gene was shown to be located on an IncHI2 plasmid, which co-harbored *bla<sub>CTX-M-1</sub>* and other ARGs. Additionally, two *mcr-1*-positive MDR *E. coli* strains recovered from the rectal swabs of pediatric patients were found [49]. The *mcr-1* gene was carried by an IncI2 plasmid in both isolates, which also co-harbored beta-lactamase genes. The first isolate belonging to the ST115 (commonly reported in animals) co-harbored *bla<sub>NDM-1</sub>*, *bla<sub>CTX-M-15</sub>*, and *bla<sub>OXA-9</sub>*, and the second belonging to the ST540 (commonly reported in food and environmental sources) co-harbored *bla<sub>CTX-M-15</sub>* and *bla<sub>TEM-141</sub>* [49]. Moreover, a colistin-resistant *bla<sub>CTX-M-55</sub>*-producing *E. coli* (ST156) isolate was described, carrying the *mcr-1.1* variant on the IncI2 plasmid [50]. *Mcr-8* and *mcr-9* have been observed in Qatar. The *mcr-8.1* gene, located on an IncFII plasmid, has been found in two ESBL-producing *K. pneumoniae* isolates belonging to ST716 and ST383 [50]. The first co-harbored *bla<sub>SHV-26</sub>* gene and the second co-harbored *bla<sub>CTX-M-15</sub>* and

*bla*<sub>SHV-27</sub> genes. The *mcr-9* gene, located on an IncHI2 plasmid, has been recently described in *bla*<sub>SHV-12</sub>-producing *Klebsiella oxytoca* isolate recovered from the rectal swab of a child in the intensive care unit [51]. The strain was colistin-susceptible, consistent with previous reports from the MENA region and beyond. Indeed, resistance to colistin in the presence of *mcr-9* can only occur when *qseC* and *qseB* regulatory genes are expressed [52].

In Iraq, colistin has been excessively used in hospitals in recent years because of the spread of carbapenem-resistant *Acinetobacter baumannii* (CRAB), particularly among wounded patients [53]. As predicted, colistin resistance and *mcr* variants emerged promptly. Alarming, *mcr-1*, *mcr-2*, and *mcr-3* were reported in 73.5%, 64.5%, and 67.8% of the tested *A. baumannii* isolates, respectively [54]. The *mcr-1* gene was also detected in 11% of CRAB isolates in hospital settings in Baghdad during 2014–2018 [55]. The *mcr-1* gene was not limited to *A. baumannii*, but also found among three pan-drug-resistant *K. pneumoniae* isolates belonging to different STs including two high risk clones (ST11 and ST147) [56].

In the United Arab Emirates (UAE) and Bahrain, *mcr-1*-positive ESBL-producing *E. coli* were reported. Although the *mcr-1* genes were all carried by an IncI2 plasmid, the iso-late from the UAE belonged to ST131 (co-harbored *bla*<sub>CTX-M-15</sub> gene) and the two isolates from Bahrain belonged to ST224 (co-harbored *bla*<sub>CTX-M-15</sub> gene) and ST648 (co-harbored *bla*<sub>CTX-M-64</sub> gene) [44]. However, in Sultanate Oman, the *mcr-1* gene was detected in a non-ESBL *E. coli* isolated from the blood of a hospitalized patient with multiple comorbidities [57]. The gene is also carried by an IncI2, and the strain belonged to the ST10, which has been previously postulated to have been locally acquired by Hajj pilgrims [26]. Also, the ST10 *mcr-1.1 E. coli* occurred in the Lebanese community [37]. Notably, this clonal group has been already shown to disseminate ESBL and other ARGs across the globe [58]. In Iran, *mcr-1* and *mcr-2* have been reported among *E. coli* and *Klebsiella* spp. in clinical settings [59,60]. Both genes were detected among *bla*<sub>IMP</sub>-producing *K. oxytoca* isolates from hospitalized patients with antibiotic-associated hemorrhagic colitis [60].

Although other studies might show colistin resistance in other MENA countries [61], such as Kuwait, Palestine, Syria, Yemen, and Libya, *mcr* genes have not been yet reported. Undoubtedly, colistin resistance and *mcr* genes are highly prevalent in the MENA region; however, the lack of comprehensive surveillance data and the limited scope of screening efforts of AMR in clinical



settings hinder the understanding of the impact of this burden on patients and the community. Additionally, emergent data from Lebanon emphasized that intrinsically colistin-resistant isolates should not be overlooked during screening efforts since they have an essential role in the silent dissemination of *mcr* genes.

### **3. Colistin resistance and *mcr* genes in poultry, livestock, birds, fish, and wild animals**

#### **3.1. Poultry**

The poultry sector is a significant contributor to the global AMR burden. In recent years, colistin has been heavily used in agricultural practices and veterinary medicine in the MENA region, particularly for prevention and treatment of infections and as growth promoter. The inappropriate use of this antimicrobial in food-producing animals is recognized as the reason for the selection of colistin-resistant bacteria and spread of *mcr* genes, thus, the dissemination of these plasmid-borne genes is different between countries. Consequently, *mcr* genes can be transmitted by direct (e.g., lick) or indirect animal-to-human contact (e.g., food consumption).

In Algeria, the first report on the emergence of *mcr-1*-positive *E. coli* in chicken was a warning sign about the possible spread of this genetic marker in the poultry industry [62]. Other investigations subsequently confirmed the presence of *mcr-1* gene in chicken in Algeria [63,64], as well as in other countries such as Morocco [65], Tunisia [66,67], Egypt [68,69], Lebanon [70-75], Qatar [76], and the UAE [77]. Replicon typing and hybridization experiments demonstrated that *bla*<sub>CTX-M-1</sub> and *mcr-1* co-localized in Tunisian *E. coli* (ST4) isolates on IncHI2 plasmid [66]. This close genetic relationship can be explained by the fact that the tested chicken were either imported from France or derived from French imported chickens [66]. Interestingly, *mcr-1* gene carried in a conjugative IncP plasmid has been detected in an avian pathogenic *E. coli* ST2197 isolate from a Tunisian poultry farm [78]. Recently, a study identified the presence *mcr-1* gene among *E. coli* isolates with high clonal diversity (ST4187, ST3882, ST5693, and ST8932) in diseased chickens from Tunisia [79]. Moreover, sporadic isolates of the *mcr-1* gene, belonging to different subtypes (ST10, ST57, ST69, ST349, and ST1011) and co-harboring the *bla*<sub>CTX-M-1</sub> gene, carried by an IncHI2 plasmid has been described among healthy chicken in Tunisia [67]. However,

a recent study from the UAE showed that the *mcr-1* gene was mostly carried by IncI2, followed by IncHI2 and IncX4 [77].

In Lebanon, the data on *mcr* genes in animals is insufficient due to the lack of active surveillance and nationwide monitoring systems [80]. However, the available findings from the poultry industry are worrisome. Poultry is the sector where the *mcr-1* gene was reported for the first time in Lebanon [72]. Several studies on poultry farms have demonstrated that colistin-resistant *E. coli* are widespread in chicken and their environment, with massive dissemination of the *mcr-1* gene [70-75]. The high prevalence of the *mcr-1* gene can be explained by the unregulated use of this antimicrobial in the animal sector, without the implementation of antimicrobial stewardship programs. Twelve different brands of antimicrobials containing colistin are legally accessible to farmers in agriculture markets without the need for a veterinarian's prescription [7]. Several beta-lactamase genes were identified, including *bla*<sub>CTX-M-3</sub>, *bla*<sub>CTX-M-14</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>CTX-M-55</sub>, *bla*<sub>CTX-M-64</sub>, *bla*<sub>CTX-M-65</sub>, *bla*<sub>TEM-57</sub>, *bla*<sub>TEM-122</sub>, *bla*<sub>SHV-12</sub>, and *bla*<sub>CMY-2</sub> genes [71,74,75]. In Contrast to other MENA countries, *mcr-1* genes in Lebanon were mostly detected on IncX4, followed by In-cI2 and IncHI2 [71,74,75]. As found in humans, the *E. coli* population structure is very diverse, confirming that the evolution of resistance to colistin appears to be multi-clonal and related to the diffusion of plasmids carrying *mcr-1* genes. Taken together, wide dissemination of *mcr-1 E. coli* strains will most probably occur in the Lebanese community and the environmental sectors.

Recently, *mcr-1*-positive *K. pneumoniae* has also been reported in broilers in Lebanon [73] and the UAE [77]. In addition, *mcr-1*-positive-*Escherichia albertii* and *Salmonella minnesota* have been identified in the UAE [77].

The limited number of studies conducted in this geographical area cannot provide a comprehensive understanding of the dissemination and impact of *mcr* variants in the poultry industry and its surrounding environment. Current data showed a wide dissemination of the *mcr-1* gene in poultry farms, which represents a potential public health concern since the poultry industry is considered an important reservoir of transmission of colistin-resistant bacteria and *mcr-1* genes to consumers through food production, international trade, and handling and eating of raw or undercooked meat. Indeed, poultry farms can easily contaminate nearby natural resources such as water, which is

mainly used for the irrigation of crops and recreational activities. Moreover, animal wastes are used as biofertilizers in sustainable agricultural practices. These results should urge local authorities in this region and international stakeholders to implement efficient guidelines for the use of antimicrobials in the poultry sector. Interestingly, no other *mcr* variants (*mcr-2* to *mcr-10*) were detected in poultry in the MENA region, while other still unknown non-*mcr* genes most likely also contribute to colistin resistance.

### **3.2. Livestock and aquaculture**

Although livestock and aquaculture are considered reservoirs of drug-resistant bacteria and AMR determinants, little information is available on colistin resistance in live-stock in the MENA region. In Tunisia, three CTX-M-1-producing and eight non-ESBL-producing *E. coli* isolates harboring *mcr-1* were recovered from veal samples [81]. As previously reported in the poultry sector in this country [66,67], the *mcr-1* gene was carried on IncHI2 plasmids [81]. Similarly, the colocalization of the *bla*<sub>CTX-M-1</sub> and *mcr-1* genes on an IncHI2 plasmid was also reported in a *E. coli* isolate from a meat-producing camel in Tunisia [82]. This plasmid type, co-harboring *bla*<sub>CTX-M-1</sub> and *mcr-1* genes, has been frequently reported in animals across the globe [58]. It is worth mentioning that the isolates showed high clonal diversity (ST88, ST162, ST260, ST533, and ST744), confirming the non-clonal spread of this gene in animals in Tunisia [81,82]. In Egypt, an *mcr-1*-positive *E. coli* (ST10) was isolated from a cow with subclinical mastitis [83]. The ST10 has commonly been reported among colistin-resistant isolates in humans and poultry from the MENA region. In Lebanon, high levels of AMR were present in live-stock-associated bacteria [80]. Dandachi *et al.* [84] reported for the first time the detection of *mcr-1 E. coli* in pork in this country. Even though many isolates harbored the *mcr-1* gene, only two were ESBL-producing [84]. Recently, an *mcr-1*-positive ESBL-producing *E. coli* strain has been reported in a cow in Iran [85].

### **3.3. Birds, fish, and wild animals**

Wild animals play a critical role in the harboring and environmental spread of resistant bacteria and genetic factors associated with AMR, posing a potential hazard to human and animal health. Migratory and non-migratory birds are now recognized as reservoirs of *mcr* genes in the MENA

region and beyond. Flying around, they can potentially spread *mcr* genes directly or indirectly to different matrices through uncontrolled animal defecation, contaminating humans, animals, and vital resources such as water. In Egypt, *mcr-1*- and *mcr-2*-positive *E. coli*, *K. pneumoniae*, and *P. aeruginosa* have been found among resident and migratory birds [30]. In Lebanon, *mcr-1.26* located on an IncX4 plas-mid has been reported in an MDR *E. coli* (ST2207) isolate from the fecal droppings of a domesticated pigeon (*Columba livia domestica*) [86]. Recently, five MDR *mcr-1.1*-positive *E. coli* (two ST48 and three ST101) isolates were detected in rainbow trout in Lebanese aqua-culture [87]. Both STs were previously associated with humans and fish isolates, posing a significant public health concern because pathogens and resistance determinants can be potentially transferred through the aquaculture supply chain [88]. As observed in the poultry sector, the *mcr-1.1* gene was shown to be carried on IncX4 plasmids, but none of the isolates were ESBL-producing. Moreover, one *mcr-1*-positive *E. coli* isolate was detected in Barbary macaques (*Macaca sylvanus*) in Algeria [89]. This isolate belonged to a CTX-M-15-producing ST405 that had been previously reported in two different Algerian patients in clinical settings [18,19]. Another *mcr-1*-positive *E. coli* isolate was isolated from a fennec fox (*Vulpes zerda*) imported from Sudan to China, emphasizing on the implication of fennec fox as a vector for the global dissemination of *mcr* genes and other AMR determinants [90].

Taken together, further large-scale genomic studies and One Health interventions, including several domesticated and wild animal species, are urgently required to evaluate and understand the dynamics of AMR spread, persistence, and evolution.

#### **4. Colistin resistance and *mcr* genes in retail food products**

The spread of MDR bacteria through food is a global issue. Moreover, as the consumption of raw food (e.g., meat, milk, and cheese) is common in the MENA region, there is a high risk of dissemination of drug-resistant pathogens and AMR determinants through ready-to-eat products. There is growing evidence for the dissemination of *mcr*-positive Enterobacteriales in retail food products in the MENA region, particularly in chicken and beef meat and dairy products. Indeed, contaminated food with drug-resistant opportunistic bacteria is considered a potential source of complicated infections for humans and ani-mals.

In Algeria, 11 *mcr-1*-positive non-ESBL-producing *E. coli* isolates were detected in poultry meat. The isolates belonged to a wide variety of STs (ST10, ST155, ST224, ST260, ST648, ST744, ST1196, ST1241, ST4246, ST4654, and ST5161) suggesting that the diffusion of colistin resistance had multiple origins. The *mcr-1* gene was associated with IncFV and IncFIIK plasmids [91]. The *mcr-1.1*-positive *E. coli* (ST3270) was isolated from poultry meat in a local market in Saudi Arabia. The isolate was ESBL-producing (*bla*<sub>CTX-M-15</sub>, *bla*<sub>TEM-141</sub>, *bla*<sub>TEM-206</sub>, and *bla*<sub>TEM-214</sub>) and carried numerous virulence factors, such as the EAST-1 heat-stable enterotoxin 1 (*astA*) and uropathogenic-specific protein (*usp*) genes [92]. In Egypt, *mcr-1* was detected in retail meat, milk, and cheese products. A *bla*<sub>CTX-M-9</sub>-producing *E. coli* carrying a transferable plasmid with the *mcr-1* gene was recovered in hard cheese made from raw milk [93]. An *mcr-1*/IncHI2-carrying non-ESBL-producing uropathogenic *E. coli* ST69 lineage strain was isolated from raw milk cheese [94]. An *mcr-1*/IncI2-carrying non-ESBL-producing *E. coli* ST101 isolate has been recovered from beef sausage [95]. Similarly, eight *mcr-1* positive *E. coli* strains with high genetic diversity (ST10, ST58, ST109, ST155, ST1172, ST1431, and ST1720) were recovered from raw beef samples. Of them, three were ESBL-producers (two harbored *bla*<sub>CTX-M-28</sub> and one *bla*<sub>TEM-116</sub> genes) [96]. These findings are consistent with a larger study on retail chicken meat in Egypt, showing that the *mcr-1* gene is mainly driven by IncI2, followed by IncX4 and IncHI2 [97]. Overall, 19 *mcr-1*-positive *E. coli* (ST101, ST156, ST371, ST373, ST398, ST986, ST1011, ST1125, ST1196, ST5687) and one *mcr-1*-positive *C. freundii* were recovered, including nine ESBL-producing strains (*bla*<sub>CTX-M-9</sub>, *bla*<sub>CTX-M-14</sub>, *bla*<sub>CTX-M-15</sub>, and *bla*<sub>SHV-12</sub>) [97]. The presence of *mcr-1*-carrying *E. coli* was reported for the first time in Turkey in chicken meat purchased from butchers and retail markets [98]. The isolates belonged to different genetic backgrounds (ST1049 and ST3941) and *mcr-1* genes were carried by the IncX4 (100%); one of the isolates was also positive for *bla*<sub>CTX-M-15</sub>-gene [37]. A similar study reported the presence of an *mcr-1* positive *bla*<sub>CTX-M-8</sub>-producing *E. coli* strain from retail raw chicken meat in Turkey. The isolate belonged to ST6094 and was carried by the IncI2 plasmid [99].

## 5. Colistin resistance and *mcr* genes in the environment

The global dissemination of colistin resistance is not limited to clinical or agricultural settings. Indeed, environmental ecosystems are a substantial reservoir of resistant micro-organisms, mobile

genetic elements, and ARGs [100]. The inappropriate use of colistin in humans and animals drastically increased the emergence of *mcr* genes in the environment since 60% of colistin taken are excreted unchanged by the host, causing the efflux of high levels of antimicrobial residues into natural resources and consequently selecting for resistant isolates [101]. Although it is well-established that several countries in the MENA region struggle to properly preserve natural resources due to deteriorated infrastructure, improper wastewater management, governmental oversight, economic collapse, and political unrest, only a handful of reports studied colistin resistance in the environment.

In Algeria, the *mcr* genes were detected among *E. coli* isolates in agriculture soil, irrigation water, and animal manure. Six and two isolates carrying the *mcr-1* and *mcr-3* gene, respectively. The isolates belonged to four different STs (ST10, ST155, ST345, and ST405) and was carried by a mobilizable plasmid [102]. These data argued that the transfer of manure from animals to water and soil might be disseminating a combination of AMR determinants, posing a serious threat to human health. Additionally, *mcr-1*-positive non-ESBL-producing *E. coli* (ST23 and ST115) were reported from contaminated seawater of the Algiers Coast [103]. The first (ST21) harbored an *mcr-1.1* gene localized on IncHI2A plasmid, while the second (ST708) carried an *mcr-1.5* gene localized on an IncI2-type plasmid [104]. Furthermore, a recent study reported the emergence of *mcr-5*-positive *Cupriavidus gilardii* isolates from well water of a maternity hospital in Algeria [105]. In addition to the ability of these isolates to silently spread the *mcr-5* gene to other bacterial species in their ecosystem, human *C. gilardii* infections may occur since this untreated water is used in many applications in the hospital (e.g., cooking, bathing of newborns, cleaning, hand washing). This species has earned increasing attention as an emerging pathogen over the last few years [106]. The *mcr-5* gene is on a Tn3-family transposon present in this species; thus, it has been previously suggested that this gene might have been transmitted from environmental *C. gilardii* to *Salmonella enterica* [107].

In Tunisia, only one report is available describing an *mcr-1* and *bla*<sub>CTX-M-15</sub>-producing *E. coli* (ST8900) isolate in raw sewage water [108]. To our knowledge, this is the only report describing this ST to date. In Egypt, as in humans and animals, the *mcr-1* and *mcr-2* genes have been reported in both colistin-resistant *E. coli* and *K. pneumoniae* isolates from surface water sources [30]. In

Lebanon, the *mcr-1* gene has emerged among *E. coli* isolates with various genetic backgrounds in irrigation water; the third of the isolates also harbored at least one *bla*<sub>CTX-M</sub> gene [109]. Worryingly, two isolates also harbored *bla*<sub>NDM-1</sub> and *bla*<sub>OXA-48</sub>, respectively [109]. Another study on water samples collected from a major agricultural area confirmed initial results, describing a contamination with two *mcr-1*-positive *E. coli* (ST10 and ST162); the *E. coli* ST10 also carries the *bla*<sub>CTX-M-3</sub> gene [110]. The contamination of the Lebanese seawater with *mcr* genes has also been documented. *Mcr-1.1*-positive *E. coli* isolates with high clonal diversity (ST93, ST602, and three unknown STs) were detected in ten different locations in the Mediterranean Sea along the coastline of Lebanon [111]. It is worth mentioning that both STs have been similarly described among Algerian and Moroccan pilgrims upon their return from Saudi Arabia [26] and have been associated with *mcr* and extraintestinal pathogenic and diarrhoeagenic strains in humans, animals, and food products across the globe. Notably, *mcr-1*-positive *E. coli* were found from drinking-, well-, and sewer-water in Syrian refugee camps in Lebanon [112]. The study indicated a potential cross-contamination between the different domestic and sewer water sources reflecting the unsanitary conditions in the makeshift camps. Indeed, the affected sewer effluent enhances the risks for amplifying the spread of AMR determinants beyond the camps via a debilitated sewage system. As in irrigation water, *bla*<sub>OXA-48</sub> gene, was detected in two *mcr-1*-positive *E. coli* isolates [112]. Moreover, other findings documented the presence of *mcr-1* in *P. mirabilis* in domestic and sewer waters of Syrian refugee camps in Lebanon [113]. The contamination of the camps' environment with this species combined with the carriage of the *mcr-1* gene presage a serious threat to the refugees in the camps and host communities through the sewer effluent and cross-contamination. Altogether, the prevalence of *mcr-1* in environmental samples in Lebanon is among the highest re-reported in the literature; therefore, robust surveillance data is needed in different environmental to accurately identify the dissemination of this gene in Lebanon.

## 6. Conclusion

The global dissemination of colistin resistance and *mcr* genes has recently received significant attention. To promote the optimal use of colistin, the antimicrobial stewardship programs in many countries (e.g., United States, European Union, China, India) strengthened national AMR surveillance systems, banned the use of this drug in agricultural practices, and promoted the

rational colistin use in human and animal medicine. Unfortunately, similar interventions remain insufficient in the MENA region, whereby there is an underestimation of the colistin resistance issue by local public health authorities and stakeholders.

Although the data is limited in the MENA region, available evidence showed that the prevalence of *mcr* genes is among the highest worldwide. We highlighted the wide distribution of these genes in different compartments, including humans, animals, food products, and the environment. In addition to the essential role of inappropriate colistin use in clinical settings, the food chain and environment are involved in *mcr* transmission. Additionally, international trade has been considered a potential route for the dissemination of *mcr* within countries and beyond. Indeed, the MENA region represents nowadays an important reservoir of transmission of colistin-resistant bacteria and *mcr* variants, including *mcr-1*, *mcr-2*, *mcr-3*, *mcr-5*, *mcr-8*, and *mcr-9*. Despite that the circulating *mcr*-positive strains belonged to various genetic backgrounds, similar STs have been described in different compartments locally and regionally. The most frequent types of plasmids associated with human, animal, and environmental isolates carrying *mcr* genes were IncX4, IncHI2, and IncI2. Interestingly, most findings confirmed the ability of these plasmids to transfer the *mcr* genes to other bacterial species. Alarmingly, most of the *mcr*-positive isolates were MDR and co-harbored other ARGs, particularly ESBL and/or carbapenemase genes. Furthermore, silent dissemination of *mcr* genes has been identified through the existence of naturally colistin-resistant *mcr-1*-positive *P. mirabilis* and colistin-sensitive *mcr*-positive isolates, respectively.

The widespread occurrence of *mcr* can be explained by the unregulated use of colistin in the human and animal sector, associated with strained infrastructure and insufficient resources in MENA countries, particularly those facing calamitous challenges (e.g., Lebanon). To combat the spread of resistance to colistin and other critically important antimicrobials, there is a crucial need to implement effective One Health antimicrobial stewardship. Sustained genomic surveillance, water sanitation and hygiene (WASH) and infection prevention and control (IPC) programs, and wastewater management in different sectors are the cornerstones to mitigate the dissemination of ARGs at the human-animal interface.



Supplementary Materials: The following supporting information can be downloaded at: [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Figure S1: title; Table S1: title; Video S1: title.

### **Data Availability Statement**

Not applicable.

### **Conflicts of Interest**

The authors declare no conflict of interest.

### **Institutional Review Board Statement**

Not applicable.

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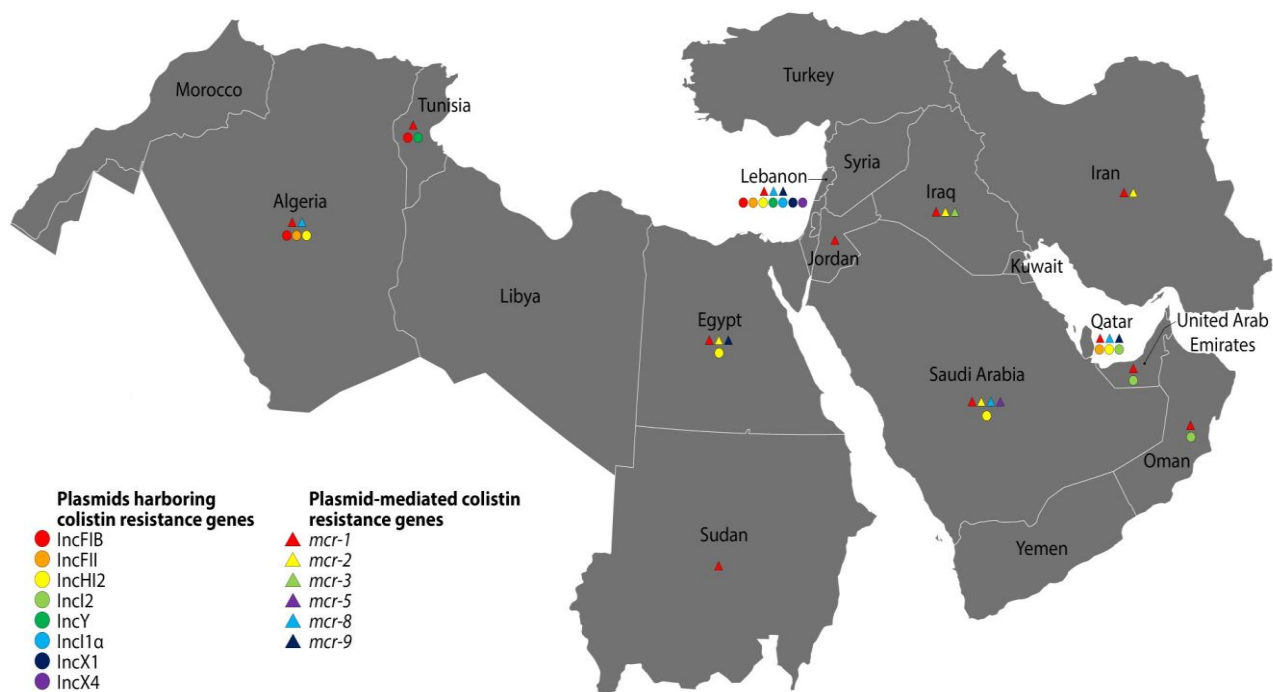
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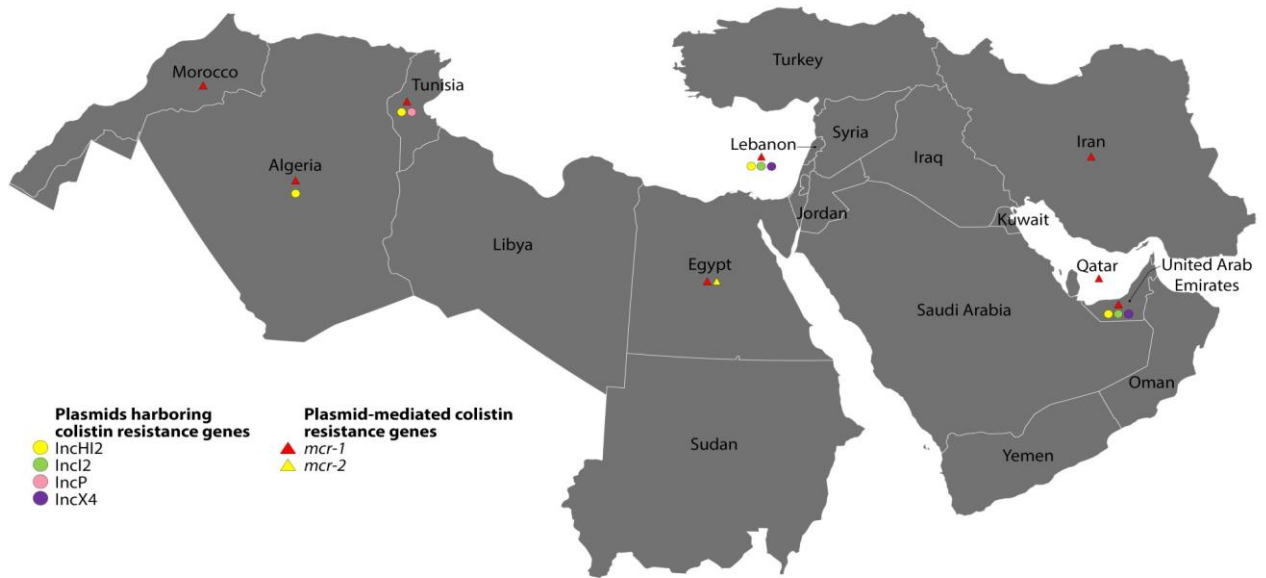
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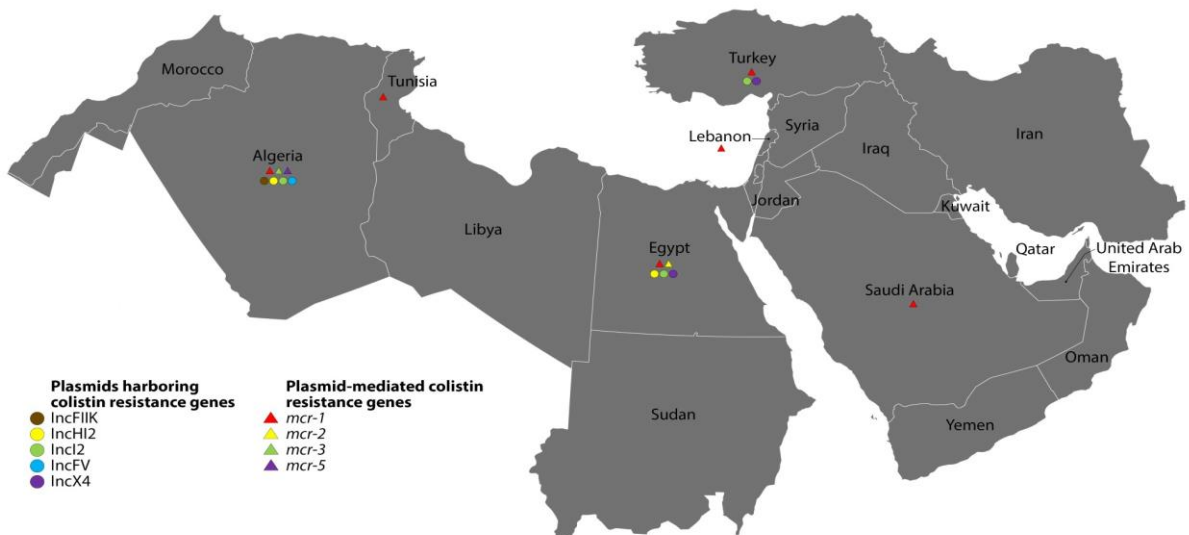


**Figure 1.** Geographic distribution of *mcr* genes and associated plasmids among humans in the MENA region.





**Figure 2.** Geographic distribution of *mcr* genes and associated plasmids in animals (poultry, livestock, birds, fish, and wild animals) in the MENA region.



**Figure 3.** Geographic distribution of *mcr* genes and associated plasmids in food products and the environment in the MENA region.

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## **Chapitre 2: Objectifs Scientifiques**

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La résistance aux antibiotiques est un problème complexe qui menace la santé publique mondiale et provoque une perte économique mondiale. La crise de la résistance aux antibiotiques ne se limite plus au contexte clinique mais a diffusé dans la communauté et dans tous les secteurs, de sorte qu'il doit désormais être abordé dans le cadre d'une approche One Health. Au Liban, les études menées sur l'épidémiologie de la résistance aux antibiotiques sont peu nombreuses, et l'augmentation de la résistance aux antibiotiques constitue un problème majeur de santé Publique surtout avec la consommation abusive et non-contrôlée des antibiotiques. Malheureusement, la lutte contre le problème de la résistance aux antibiotiques reste encore compliquée au Liban, en raison du manque d'informations sur la structure de la population ainsi que les plasmides porteurs des gènes de résistance.

Les bêta-lactamases à spectre étendu (BLSE) et les carbapénèmases constituent les principaux mécanismes de résistance aux  $\beta$ -lactamines. Leur production est souvent associée à une multi résistance, voire toto résistance aux antibiotiques. Plusieurs études ont décrit la diffusion des Entérobactéries productrices de CTX-M-15 et d'OXA-48, dans le milieu clinique et non-clinique.

Afin de répondre à ce problème de santé publique, il convient de mieux comprendre la dissémination de ces bactéries dans les trois compartiments (humain, animal, et environnemental) pour détecter ces bactéries rapidement dans un contexte 'One health'. Notre objectif de recherche était de déterminer la prévalence des Entérobactéries productrices de BLSE et de carbapénèmases isolées de l'environnement, en communauté et en milieu hospitalier, d'identifier les gènes ainsi que leurs supports génétiques et de déterminer le caractère clonal de ces bactéries.

Enfin, ce travail de thèse nous permettra d'accéder à une large collection d'isolats cliniques, et environnementaux, ce qui devrait permettre d'identifier les carbapénèmases répandues au Liban et leurs réservoirs afin de développer des mesures globales et intégratives et des stratégies efficaces de prévention et de contrôle pour lutter contre le fardeau de la résistance aux antibiotiques dans le pays.

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## **Chapitre 3: Etudes Expérimentales**

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## I. Résistance aux carbapénèmes en milieu Clinique au Liban

Dans cette partie, nous avons étudié l'épidémiologie et les caractéristiques moléculaires des bactéries productrices de carbapénèmases en milieu clinique au Liban. Nous nous sommes concentrés sur la recherche et la caractérisation des Entérobactéries résistantes aux carbapénèmes, isolées des hôpitaux au Nord du Liban. Cette étude montre que nos résultats sont concordants avec des études précédentes et révèlent une situation préoccupante de la résistance au Liban. Les carbapénèmases mises en évidence dans notre étude appartenaient aux classes B et D de la classification d'Ambler. Et un changement dans le gène le plus fréquemment rapporté a été remarqué. *Bla*<sub>OXA-48</sub> était l'enzyme la plus répandue parmi les Entérobactéries, alors que notre étude a montré une augmentation de la prévalence du gène *bla*<sub>NDM-5</sub> par rapport à celle de *bla*<sub>OXA-48</sub>. Cette étude met en évidence l'émergence et la large diffusion des gènes *bla*<sub>NDM-5</sub> et *bla*<sub>OXA-244</sub> dans les milieux cliniques libanais.

En plus, une souche de *Enterobacter* co-productrice de 2 metallo  $\beta$ -lactamases a été identifiée. La localisation plasmidique de ces 2 gènes, qui augmente la capacité de propagation horizontale de ces gènes, soulève d'importantes inquiétudes quant à l'émergence de clones multirésistants dans les systèmes de santé.

La faible sensibilisation au problème de la résistance au sein de la communauté libanaise et les défis économiques et sanitaires actuels ont stimulé les pratiques d'automédication. Dans l'ensemble, nos résultats signalent une situation alarmante et mettent en évidence un besoin urgent d'approches transformatrices pour lutter contre la résistance aux antimicrobiens en milieu communautaire et hospitalier.

# 1. Article 1: Prevalence and Molecular Mechanisms of Carbapenem Resistance among Gram-Negative Bacilli in Three Hospitals of Northern Lebanon



antibiotics



Article

## Prevalence and Molecular Mechanisms of Carbapenem Resistance among Gram-Negative Bacilli in Three Hospitals of Northern Lebanon

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**Abstract:** Carbapenem resistance (CR) is an emerging health issue. Epidemiological surveys on carbapenem-resistant Gram-negative bacilli (CR-GNB) in Lebanon remain scarce. In this study, we determined the prevalence of CR-GNB isolated between 2015 to 2019 in three hospitals in northern Lebanon: 311 CR-*Enterobacterales* (out of 11210; 2.8%), 155 CR-*Pseudomonas* (out of 1034; 15%) and 106 CR-*Acinetobacter* (out of 184; 57.6%) were identified. CR mechanisms were determined for 146 randomly chosen isolates: the Carba NP test revealed an enzymatic resistance to carbapenems in 109 isolates (out of 146, 74.7%). Produced carbapenemases were evaluated by the NG-Test Carba5, NG-Test OXA-23 immunochromatographic assays and PCR. Carbapenemase-producing (CP) *Enterobacterales* expressed *bla*<sub>OXA-48</sub>-like, *bla*<sub>NDM</sub>-like and *bla*<sub>VIM</sub>-like genes and CP-*Pseudomonas* expressed *bla*<sub>VIM</sub>-like and *bla*<sub>VIM</sub>-like genes, whereas CP-*Acinetobacter* expressed *bla*<sub>OXA-23</sub>-like genes. The NG-Test Carba5 results were confirmed by PCR sequencing and revealed several variants, such as NDM-19, VIM-62 and OXA-162, never described so far in Lebanon. Isolates with discordant results were sequenced by WGS and highlighted novel variants of the natural oxacillinases of *Pseudomonas aeruginosa*: *bla*<sub>OXA-50</sub>-like genes. Their role in carbapenem resistance should be further studied. Overall, our findings highlight an alarming situation and encourage health care centers to establish performant registration systems that could help in limiting resistance spread.

**Keywords:** antimicrobial resistance; carbapenem; carbapenemase; Gram-negative bacilli

### 1. Introduction

Antibiotic-resistant bacteria emergence is increasing drastically worldwide, thus limiting the efficacy of antibiotics. Their overuse or misuse, and the low rate of new drug development in the pharmaceutical industry, make the problem worse. Gram-negative multidrug-resistant bacterial infections remain one of the most dangerous public health threats, and carbapenems constitute the last-resort class of antibiotics for such infections

because of their stability against  $\beta$ -lactamases and their broad spectrum of action. For these purposes, resistance to carbapenem should never be underestimated. Mechanisms of this resistance are emerging by several ways, including: (i) impermeability, (ii) efflux, (iii) alteration of the target site and (iv) enzymatic inactivation. The production of enzymes remains one of the leading causes for carbapenem and other  $\beta$ -lactam inactivation, hence the importance of studying the various emerging  $\beta$ -lactamases [1]. Over-production of efflux pumps and the production of AmpC-type  $\beta$ -lactamase accompanied by the reduction of porin expression were also reported to play a major role in carbapenem-resistant strains [2]. According to Ambler,  $\beta$ -lactamases are classified in four groups based on their amino acid sequence differences. Classes A, C and D are serine-based enzymes with a serine residue in their active site, while in class B, we find metallo- $\beta$ -lactamases (MBL) that require a divalent cation, usually  $Zn^{2+}$ , for their activity [3]. Carbapenemases belong to class A serine  $\beta$ -lactamases, class B MBLs and class D oxacillinases of Ambler's classification, with KPC (*Klebsiella pneumoniae* carbapenemase), NDM (New Delhi metallo- $\beta$ -lactamase), VIM (Verona Integron-encoded MBL), IMP and OXA-48-like (Oxacillinases) being the major carbapenemases worldwide, and classes B and D being dominant in Lebanon [4].

Despite the lack of official national data on antimicrobial resistance (AMR) in Lebanon, each hospital generates its own annual data of antibiotic sensitivity [5]. Data generated by hospitals each year and several publications describing the epidemiology in north Lebanon show that this region follows the same global trend of increasing AMR [5]. The rate of isolates exhibiting reduced susceptibility or resistance to ertapenem increased from 0.4% in 2008–2010 to 0.9% in 2011 and 1.6% in 2012, according to a study conducted by Beyrouthi et al. [6]. This rise was associated with the emergence of the carbapenemase OXA-48. Other nationwide studies underscored the evolution of the prevalence of carbapenem resistance in Lebanon. According to Chamoun et al., *Enterobacteriales* had resistance values ranging from 0.7 to 2% over 2011, 2012 and 2013 [7]. High levels of resistance were also noted in *Acinetobacter* spp. and *Pseudomonas* spp. resistance, which exceeded 20% [7]. Other data collected in 2015 and 2016 from three hospitals revealed carbapenem resistance in 3% of *Enterobacteriales*, 30% of *Pseudomonas* spp. and 88% of *Acinetobacter* spp. [5]. Carbapenemases encountered in *Enterobacteriales* in Lebanon are mostly OXA-48-like enzymes [6]. Carbapenem resistance in *Pseudomonas aeruginosa* is mostly related to non-enzymatic mechanisms, especially those involving mutations in the OprD, the porin responsible for carbapenem uptake, but VIM-2, IMP-1, IMP-2 and IMP-15 carbapenemases have also been described [8]. The prevalence of OXA-23 of *Acinetobacter baumannii* is considerably high in Lebanon [9]. For instance, 91.3% of carbapenem-resistant isolates collected in a study conducted by Dahdouh et al. harbored OXA-23 [10]. Carbapenemases circulate in both environmental and clinical bacterial strains. Carbapenemases present in environmental strains isolated from north Lebanon had OXA-48, OXA-244 and NDM-1 noted mainly in *Enterobacteriaceae*, VIM-2 in *Pseudomonas* and OXA-23, OXA-24, OXA-58, OXA-72 and OXA-143 in *Acinetobacter* [11]. In the following study, we aimed at depicting the type of resistance that circulates in northern Lebanon and pinpointing the genes responsible for enzymatic resistance to carbapenems. The prevalence data of carbapenem resistance collected from three hospitals in northern Lebanon—Nini hospital, El Youssef Hospital Center, and Tripoli Governmental Hospital—from clinical samples between 2015 and 2019, were elaborated. Molecular characterization was carried out on 146 randomly selected Gram-negative bacterial isolates with carbapenem resistance. These include 72 *Enterobacteriales*, 29 *P. aeruginosa* and 45 *A. baumannii* isolates.

## 2. Results

### 2.1. Carbapenem Resistance Levels among Gram-Negative Bacteria

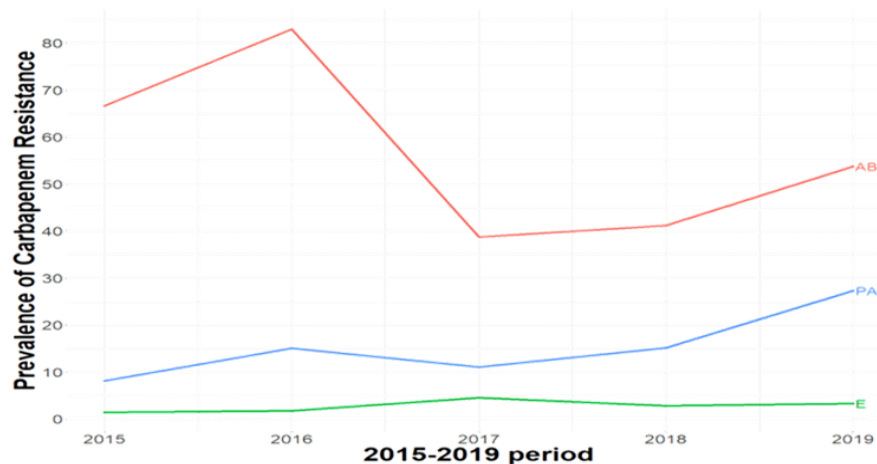
In 2015, the prevalence of carbapenem-resistant *Enterobacterales* was 1.4%; this prevalence increased to reach 3.3% in 2019, with a peak noted in 2017 (4.5%). The total prevalence among the 5 years is 2.7%. In *Pseudomonas* sp. Isolates, an increasing trend from 8.1% in 2015 to 27.3% in 2019 was noted. In *Acinetobacter* sp. isolates, the annual prevalence varied by year, with the highest percentage reached in 2016 (83%) and the lowest noted in 2017 (38.7%) (Table 1).

**Table 1.** Prevalence of carbapenem resistance in *Enterobacterales*, *P. aeruginosa* and *A. baumannii*, from 2015 to 2019, isolated in northern Lebanon, selected based on their resistance to ertapenem according to EUCAST guidelines [10].

Year	<i>Enterobacterales</i>		<i>P. aeruginosa</i>		<i>A. baumannii</i>	
	N	Resistant isolates (%) <sup>a</sup>	N	Resistant isolates (%) <sup>a</sup>	N	Resistant isolates (%) <sup>a</sup>
2015	2081	31 (1.4%)	244	20 (8.1%)	18	12 (66.7%)
2016	2157	37 (1.7%)	193	29 (15%)	47	39 (83%)
2017	1696	78 (4.5%)	172	19 (11%)	31	12 (38.7%)
2018	2125	60 (2.8%)	238	36 (15.1%)	34	14 (41.2%)
2019	3151	105 (3.3%)	187	51 (27.3%)	54	29 (53.7%)
Total	11210	311 (2.7%)	1034	155 (15%)	184	106 (58%)

<sup>a</sup> Carbapenem-resistant isolates.

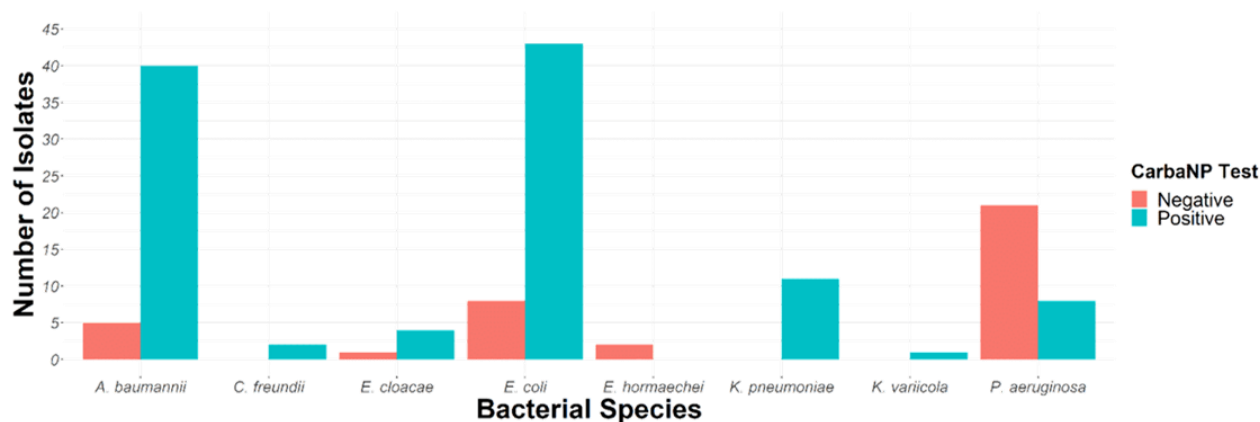
The evolution of carbapenem resistance is presented in Figure 1. No significant temporal trends of carbapenem resistance were detected by the Mann–Kendall test and Sen’s slope among *Enterobacterales* ( $Z = 1.22$ , Sen’s  $s = 0.005$ ,  $p$ -value = 0.221), *P. aeruginosa* ( $Z = 1.71$ , Sen’s  $s = 0.041$ ,  $p$ -value = 0.086) and *A. baumannii* ( $Z = -0.24$ , Sen’s  $s = -0.059$ ,  $p$ -value = 0.807) over the study period (2015–2019). In total, 146 isolates were randomly picked for molecular characterization, yet were representative of the global prevalence in each species. These included 51 *Escherichia coli*, 11 *Klebsiella pneumoniae*, one *Klebsiella variicola*, seven *Enterobacter* spp., two *Citrobacter freundii*, 29 *P. aeruginosa* and 45 *A. baumannii* isolates.



**Figure 1.** The evolution of carbapenem resistance among *Enterobacterales* (E), *P. aeruginosa* (PA) and *A. baumannii* (AB) isolates in northern Lebanon from 2015 to 2019.



All *K. pneumoniae* and *C. freundii* isolates and the majority of *E. coli* (84.3%) and *E. cloacae* (80%) isolates were positive with the Carba NP test, thus suggesting the presence of a carbapenemase. However, all *Enterobacter hormaechei* isolates were negative for the Carba NP test, suggesting a non-enzymatic resistance mechanism to carbapenems (Figure 2). In total, 84.7% of *Enterobacterales* displayed carbapenem-hydrolyzing activity. Similarly, the majority of *A. baumannii* (88.9%) were positive using the CarbAcineto NP test, suggesting the presence of a carbapenem-hydrolyzing enzyme. In contrast, only 27.6% (8/29) of the *P. aeruginosa* isolates had a positive Carba NP test (Figure 2). Altogether, carbapenem resistance in *Enterobacterales* and *A. baumannii* are linked to enzymatic hydrolysis, while in *Pseudomonas*, non-enzymatic mechanisms are most likely the origin.



**Figure 2.** Carba NP test (*Enterobacterales* and *P. aeruginosa*) and CarbAcineto (*A. baumannii*) test results.

## 2.2. Carbapenemase Characterization

The Carba NP test revealed isolates with imipenem hydrolytic activity. In order to identify the type of carbapenemase likely at the origin of carbapenem hydrolysis, lateral flow immunoassay (LFIA) and NG-Test Carba5 (NG-Biotech, Guipry, France) were used for *Enterobacterales* and *P. aeruginosa* and the NG-Test OXA-23 (NG-Biotech) for *A. baumannii*.

*K. pneumoniae* and *C. freundii* isolates expressed OXA-48-like carbapenemases, while *E. cloacae* expressed OXA-48, VIM and/or NDM. *E. coli* isolates were positive for two enzymes: OXA-48 and NDM. In *Pseudomonas*, only class B enzymes were detected, and in *A. baumannii*, the NG-Test Carba5 was negative, and the OXA-23-specific LFIA and the NG-test OXA-23 were positive for 39 out of 45 *A. baumannii* isolates. These results were confirmed by PCR sequencing. All Altogether, NG-Test Carba 5 allowed the detection of carbapenemases in strains with enzymatic resistance, while NG-test OXA-23 and PCR allowed the detection of OXA-23 in *Acinetobacter*. The other isolates, positive for Carba NP, but negative using LFIA or PCR, consist of three *K. pneumoniae*: one *K. variicola*, three *P. aeruginosa* and two *A. baumannii*. They were subjected to whole-genome sequencing.

Sanger sequencing of the entire carbapenemase gene revealed the presence of genes coding for OXA-48, OXA181 and NDM-5 in *K. pneumoniae*; OXA-48, VIM-1, VIM-4 and NDM-1 in *E. cloacae*; OXA-48 in *C. freundii*; OXA-48, OXA-162, NDM-5 and NDM-19 in *E. coli*; IMP-15, VIM-2 and VIM-62 in *P. aeruginosa*; and OXA-23 in *A. baumannii* (Table 2). NDM-19, VIM-62 and OXA-162 have never been described so far in Lebanon.

**Table 2.** Carbapenemases identified by Sanger sequencing in different multidrug-resistant GNB and the origin of each isolate.

Bacteria	Number of Isolates	CarbaNP-Positive Isolates	Carbapenemases (Sanger Sequencing)
<i>K. pneumoniae</i>	n = 11	11 (100%)	OXA-48 (6) OXA-181 (1) NDM-5 (1) (3) <sup>a</sup>
<i>K. variicola</i>	n = 1	1 (100%)	(1) <sup>a</sup>
<i>E. cloacae</i>	n = 5	4 (80%)	OXA-48 (1) VIM-4 (1) VIM-1 (1) NDM-1 (1)
<i>E. hormaechei</i>	n = 2	0	-
<i>C. freundii</i>	n = 2	2 (100%)	OXA-48 (2)
<i>E. coli</i>	n = 51	43 (84%)	OXA-48 (11) OXA-162 (1) OXA-181 (12) OXA-244 (6) NDM-5 (4) NDM-19 (9)
<i>P. aeruginosa</i>	n = 29	8 (27%)	VIM-62 (2) VIM-2 (2) IMP-15 (1) (3) <sup>a</sup>
<i>A. baumannii</i>	n = 45	33 (73%)	OXA-23-LIKE (31) (2) <sup>a</sup>

<sup>a</sup> WGS performed.

### 2.3. Whole-Genome Sequencing of Discrepant Results

Nine Isolates (three *K. pneumoniae*, one *K. variicola*, three *P. aeruginosa* and two *A. baumannii*) with positive CarbaNP test results, but negative LFIA results, were further characterized using Illumina WGS and subsequent analysis using software available at the center of genomic epidemiology. In two out of three sequenced *K. pneumoniae*, no carbapenemase was detected: the first one had *bla*<sub>CTX-M-15</sub>, *bla*<sub>SHV-1</sub> and *bla*<sub>OXA-1</sub> genes and the other one had *bla*<sub>CTX-M-15</sub>, *bla*<sub>SHV-81-like</sub> (99.8% nucleotide sequence identity) and *bla*<sub>TEM-1B</sub> genes, respectively. The *bla*<sub>NDM-1</sub> gene was identified in the third sequenced *K. pneumoniae*, as well as in *K. variicola*. Three *P. aeruginosa* isolates underwent WGS. Novel variants of the chromosomally encoded genes, *bla*<sub>OXA-50-like</sub> and *bla*<sub>AmpC-PAO-like</sub>, were found; for *P. aeruginosa* 1, *bla*<sub>OXA-50-like</sub> mutant (T16A, K112E) and *bla*<sub>PDC-11</sub> were found, whereas *bla*<sub>OXA-50-like</sub> (R49C, A133G, A181T) and *bla*<sub>PDC-45</sub> were noted for the second one, as well as *bla*<sub>OXA-50-like</sub> (R167H, D109E) and *bla*<sub>PDC-1-like</sub> for *P. aeruginosa* 3 (Table 3). The WGS of the *A. baumannii* isolate revealed the presence of the natural and chromosome-encoded *bla*<sub>OXA-64</sub> and *bla*<sub>ADC-26</sub> genes, together with a *bla*<sub>OXA-72</sub> gene encoding an OXA-40-like acquired carbapenem-hydrolyzing oxacillinase, and, for the last strain of *A. baumannii*, OXA-23, OXA-66 and ADC-73 were found. Furthermore, MLST results revealed that the three *K. pneumoniae* isolates belonged to ST-15, ST-37 and ST-48, the *K. variicola* belonged to ST-3195, the two *P. aeruginosa* isolates belonged to ST-357, ST-893 and ST-277 and the *A. baumannii* belonged to ST-229 and ST-1841 (Table 3).

**Table 3.** WGS analysis of selected isolates using Resfinder and MLST.

Species	MICs (µg/mL)		Phenotypic Tests		β-Lactamases Genes	Resfinder Other Resistant Genes	MLST	Accession Numbers
	IMP	MER	Carba NP	NG-Test Carba5				
<i>K. pneumoniae</i> 1	0.19	0.032	+	-	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>SHV-1</sub> , <i>bla</i> <sub>OXa-1</sub>	<i>aac</i> (6′)- <i>Ib-cr</i> , <i>aac</i> (3)- <i>Iia</i> , <i>OqxA</i> , <i>OqxB</i> , <i>fosA</i> , <i>tet</i> (A)	ST-48	JALHBC000000000
<i>K. pneumoniae</i> 2	0.38	1.5	+	-	<i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>SHV-81</sub> , <i>bla</i> <sub>TEM-1-B</sub>	<i>sul1</i> , <i>sul2</i> , <i>oqxA</i> , <i>dfrA17</i> , <i>OqxA</i> , <i>OqxB</i> , <i>qacE</i> , <i>aadA5</i> , <i>tet</i> (A), <i>fosA</i>	ST-37	JALHBA000000000
<i>K. pneumoniae</i> 3	6	6	+	Faint NDM band	<i>bla</i> <sub>NDM-1</sub> , <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>SHV-106</sub>	<i>aac</i> (6′)- <i>Ib-cr</i> , <i>msr</i> (E), <i>sul1</i> , <i>OqxA</i> , <i>dfrA12</i> , <i>OqxB</i> , <i>fosA</i> , <i>aadA2</i> , <i>mph</i> (E)	ST-15	JALHBB000000000
<i>K. variicola</i>	1.5	2	+	Faint NDM band	<i>bla</i> <sub>NDM-1</sub> , <i>bla</i> <sub>SHV-12</sub> , <i>bla</i> <sub>LEN-9</sub> , <i>bla</i> <sub>OXa-10</sub> , <i>bla</i> <sub>OXa-1</sub>	<i>aac</i> (6′)- <i>Ib-cr</i> , <i>rmtC</i> , <i>rmtH</i> , <i>erm</i> (B), <i>aph</i> (3′)- <i>Ia</i> , <i>sul1</i> , <i>sul2</i> , <i>OqxB</i> , <i>OqxA</i> , <i>dfrA14</i> , <i>floR</i> , <i>qacE</i> , <i>fosA</i> , <i>qacE</i> , <i>tet</i> (B), <i>aadA1</i> , <i>ARR-2</i> , <i>catB3</i> , <i>cmlA1</i> , <i>catA2</i>	ST-3195	JALHAY000000000
<i>P. aeruginosa</i> 1	>32	>32	+	-	<i>bla</i> <sub>OXa-50-like</sub> (T16A, K112E), <i>bla</i> <sub>PDC-11</sub>	<i>sul1</i> , <i>crpP</i> , <i>fosA</i> , <i>catB7</i> , <i>aph</i> (6)- <i>Id</i> , <i>aph</i> (3′′)- <i>Ib</i>	ST-357	JALHAX000000000
<i>P. aeruginosa</i> 2	>32	>32	+	-	<i>bla</i> <sub>OXa-50-like</sub> (R49C, A133G, A181T), <i>bla</i> <sub>PDC-45</sub>	<i>aph</i> (3′)- <i>IIb</i> , <i>fosA</i> , <i>catB7</i>	ST-893	JALHAW000000000
<i>P. aeruginosa</i> 3	>32	>32	+	-	<i>bla</i> <sub>OXa-50-like</sub> (R167H, D109E), <i>bla</i> <sub>PDC-1-like</sub>	<i>Sul1</i> , <i>fosA</i> , <i>aph</i> (3′)- <i>IIb</i> , <i>crpP</i> , <i>catB7</i>	ST-277	Pending
<i>A. baumannii</i> 1	>32	>32	+	-	<i>bla</i> <sub>OXa-72</sub> , <i>bla</i> <sub>OXa-64</sub> , <i>bla</i> <sub>ADC-26</sub>	<i>aac</i> (6′)- <i>Ian</i> , <i>aph</i> (6)- <i>Id</i> , <i>aph</i> (3′)- <i>Ib</i> , <i>aac</i> (3)- <i>Iia</i> , <i>sul2</i> , <i>aac</i> (3)- <i>Iia</i> , <i>tet</i> (B)	ST-229 *	JALHAZ000000000
<i>A. baumannii</i> 2	>32	>32	+	-	<i>bla</i> <sub>OXa-23</sub> , <i>bla</i> <sub>OXa-66</sub> , <i>bla</i> <sub>TEM-1D</sub> , <i>bla</i> <sub>ADC-73</sub>	<i>armA</i> , <i>msr</i> (E), <i>aph</i> (3′)- <i>Ia</i> , <i>sul2</i> , <i>tet</i> (B), <i>aph</i> (6)- <i>Id</i>	ST-1841-like *	Pending

\* using the Pasteur MLST scheme.

### 3. Discussion

Despite the efforts put into basic research concerning antimicrobial resistance over the last years in Lebanon, easily accessible epidemiological data remains somehow scarce due to a lack of or low-performing informatic systems in hospitals and healthcare systems. In the present study, we showed high, yet rising, rates of carbapenem resistance in Gram-negatives isolated over a 5-year period in three hospitals in the northern part of Lebanon.

Several previously published studies describe the antimicrobial susceptibility patterns based on records retrieved from different hospitals all over Lebanon. For example, between 2011 and 2013, resistance to imipenem was 0.7% in *E. coli* and 2% in *Klebsiella* [7]. According to Moghnieh et al., the mean resistance of *Enterobacterales* to imipenem slightly increased in 2016 and accounted for 2% [5]. The carbapenem resistance in *Enterobacterales* even reached alarming rates of 6.6% in 2017 in northern Lebanon [12]. Our results are in line with these previous studies.

As previously reported, carbapenem resistance in *Pseudomonas* spp. was as high as 27% in 2011–2013 and 30% in 2016 [5,7]; our results confirm these data, with an average level of 27.2% in 2019. In *Acinetobacter* spp., high antimicrobial resistance rates have been described in the period from 2011 to 2013 in 16 different Lebanese hospitals, with a imipenem resistance rate varying from 15 to 49% over this period [7]. In our study, 2.8% of *Enterobacterales*, 15% of *P. aeruginosa* and 57.6% of *A. baumannii* are resistant to carbapenem. Those percentages were lower in previous years, except for *Acinetobacter* spp., which showed continuously high resistance rates to most antimicrobials [7]. Careful analysis of our data did not reveal a statistically significant increasing trend of carbapenem resistance in the city of Tripoli, northern Lebanon. The issue of AMR is complicated in low and middle-income (LMIC) countries, such as Lebanon, where many of the factors leading to the emergence and spread of MDR isolates, especially carbapenemase-producing isolates, remain difficult to control.

In the present study, 146 carbapenem-resistant isolates were randomly chosen among the most prevalent species encountered in the clinical settings and presenting reduced susceptibility to ertapenem and/or imipenem. The mechanism sustaining the carbapenem resistance was investigated for 72 *Enterobacterales*, 29 *P. aeruginosa* and 45 *A. baumannii* collected between the years 2015 and 2019.

Initial biochemical screening using the Carba NP test or CarbaAcineto NP test revealed 109/146 isolates (74.7%) had carbapenem hydrolytic activity. The remaining 37 isolates (25.4 %) had most likely non-enzymatic resistance mechanisms to carbapenems, as revealed by contact growth to a moxalactam antibiotic disc-on-disc diffusion routine antibiogram. Indeed, moxalactam, a  $\beta$ -lactam belonging to the cephamycins, is rarely hydrolyzed by  $\beta$ -lactamases, and thus constitute a good marker for impermeability [13]. The most prevalent resistance mechanism in *Enterobacterales* and *A. baumannii* was the production of carbapenemases, unlike *P. aeruginosa*, for which non-enzymatic resistance mechanisms, such as loss of porin D2 and/or the production of a  $\beta$ -lactamase lacking significant imipenem-hydrolyzing properties, associated with permeability problems (loss of porin or hyper expression of the pumps efflux), represented 75%. Isolates with a positive Carba NP test were further studied using LFIA detecting the five main carbapenemases (KPC, VIM, NDM, IMP and OXA; NG-Test Carba5) and OXA-23-like enzymes (NG-Test OXA-23) from a bacterial colony [14]. These results were subsequently confirmed using the PCR/sequencing approach.

Our results were in line with previous data that showed that OXA-48-like-producing *Klebsiella* spp. isolates are present in Lebanon [15], but we also evidenced NDM-1 and NDM-5-producing *K. pneumoniae*, described for the first time in Lebanon in 2012 and 2018, respectively [16,17]. In *Enterobacter* spp., OXA-48, VIM-1, VIM-4 and NDM-1 were described, which have already been described in Lebanon [17–19]. As in our study, the *E. coli*-producing OXA-48, OXA-162, OXA-181, OXA-244 and NDM-5, have already been described [17,19], but our study reports OXA-162 and NDM-19 for the first time. The OXA-

162 carbapenemase was initially reported in Greece in 2015, which is geographically close to Lebanon [20].

In *Pseudomonas*, a novel VIM-2-variant, VIM-62, has never been described to date in Lebanon. As NG-Test Carba5 does not detect OXA-23, which is very common among *Acinetobacter* spp, a companion assay, the NG-Test OXA-23 was performed, and revealed the presence of OXA-23 in all but one *A. baumannii* isolate. In a previous study, OXA-23 was identified as the main carbapenemase in *A. baumannii* in Lebanon, as well as the Mediterranean region [4], which is still the case in our study [21]. For isolates presenting discrepant results (four *Klebsiella* spp., three *P. aeruginosa* and two *A. baumannii*) with positive Carba NP test with negative LFIA results, WGS was carried out. For two *K. pneumoniae* isolates, no carbapenemase gene was found, but the presence of CTX-M-15 ESBLs with disrupted porin-encoding genes are likely at the origin of reduced carbapenem susceptibility, but do not explain the positive Carba NP test, suggesting a false positive test. In one *K. pneumoniae* and one *K. variicola* isolate, WGS revealed the presence of the carbapenemase *bla*<sub>NDM-1</sub> gene, which was suggested by a faint NG-Test Carba5 signal for NDM and is compatible with the positive Carba NP test. Re-testing of these isolates with the NG-Test Carba5 revealed reproducibly a slight band for NDM, suggesting low expression of NDM1 in these isolates.

For the *P. aeruginosa* isolates with positive Carba NP tests, negative NG-Test Carba5 and negative for SPM, GES and GIM PCRs, the WGS results revealed the presence of single-point mutant derivatives of the naturally and chromosomally encoded OXA-50 and PDC. These mutations are new, and it is difficult to speculate whether these mutations may contribute to an increased carbapenem hydrolysis, as shown for ACT-28 [22]. Further characterization is necessary to determine the precise role of these variants in the carbapenem-resistant profile. OXA-50 has been initially described as a weak carbapenem-hydrolyzing enzyme [23], and hyperproduction of AMPC may also lead to decreased carbapenem susceptibility.

The WGS results for the *A. baumannii* isolate with a positive Carba NP test, negative NG-Test OXA-23 and OXA-23-specific PCR revealed the presence of the natural and chromosome-encoded OXA-64, without an *ISAba1* inserted upstream, suggesting a low-level-expressed enzyme and an acquired carbapenemase OXA-72 belonging to the OXA-40 carbapenem-hydrolyzing oxacillinases of *A. baumannii* [24], and thus not detected using the NG-Test OXA-23 and OXA-23-specific PCR. In addition, WGS results revealed different MLSTs for the nine WGS isolates, suggesting sporadic cases rather than outbreak-related isolates.

Carbapenemases observed in our study belonged to classes B and D of Ambler's classification; however, for Carba NP-negative isolates, the most likely carbapenem resistance mechanism is the production of an ESBL and/or AMPC with low carbapenem-hydrolyzing activity and impaired outer membrane, such as loss of porin or overexpression of efflux pumps [16].

Our results confirm previous findings and reveal a worrying AMR situation in Lebanon, notably during this critical period [25–28]. This country, which hosts ~1.5 million refugees, is currently experiencing severe economic and political collapse, resulting in a shortage of medicine, appropriate diagnostic tools, food and other essential necessities [29–32]. Taken together, there is a paramount need to tackle the AMR challenge at national and global levels. Promoting infection control measures, active screening of AMR carriers and antimicrobial stewardship programs are required in healthcare facilities. Finally, as colistin-resistant GNB are increasingly reported over recent years, colistin susceptibility needs to be determined precisely in routine clinical laboratories in Lebanon [26,33–37].

#### 4. Materials and Methods

##### 4.1. The Bacterial Isolates and Prevalence

This study is based on records of antimicrobial susceptibility tests performed on 12,428 clinical Gram-negative isolates collected in three hospitals in northern Lebanon—Nini hospital, Al Youssef hospital and Tripoli governmental hospital—between 2015 and 2019, following EUCAST guidelines [38]. The origin of collected isolates is shown in Figure 3. Species were identified by testing through Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (entero-TOF MS). Prevalence percentages were calculated based on antibiotic susceptibility tests made on all the clinical isolates using routine disk diffusion antibiograms. All isolates were from clinical samples, including pus, bronchoalveolar lavages, blood, urine, catheters and sputum.

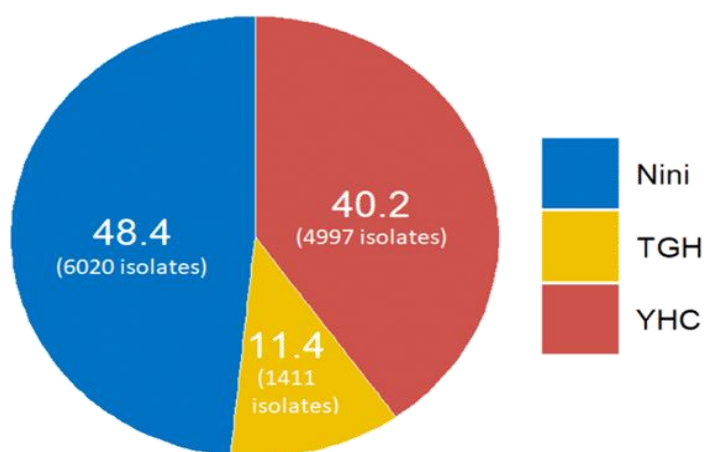


Figure 3. Origin of the isolates (%).

A total of 146 carbapenem-resistant Gram-negative isolates were picked randomly to study the underlying carbapenem resistance mechanisms. Selected isolates' number, species, origin of isolation and type of infection are shown in Table 4.

Table 4. Origin and percentage of tested clinical isolates.

Species	N	Hospital <sup>a</sup>	Type of Infection	Year
<i>Klebsiella pneumoniae</i>	11	Nini (7) YHC (4)	- Vaginal secretions (1)	2015–2018
			- Sputum (2)	
			- Urine (7)	
			- Nasal secretions (1)	
<i>Klebsiella variicola</i>	1	YHC (1)	- Urine (1)	2018
<i>Enterobacter cloacae</i>	5	Nini (3) TGH (2)	- Catheter (2)	2017–2018
			- Ear swab (2)	
			- Wound (1)	
<i>Enterobacter hormaechei</i>	2	Nini (1) TGH (1)	- Pus (1) - Deep abdominal wound (1)	2017
<i>Citrobacter freundii</i>	2	YHC (2)	- Pus (2)	2017
<i>Escherichia coli</i>	51	Nini (22) YHC (25) TGH (4)	- Urine (34)	2016–2018
			- Pus (7)	
			- Blood (2)	
			- Nasal secretions (1)	

			- Gastric liquid (4)	
			- Sputum (1)	
			- Wound (1)	
			- Rectal swab (1)	
<i>Pseudomonas aeruginosa</i>	29	YHC (25) TGH (4)	- Urine (18) - Blood (3) - Pus (5) - Wound (2) - Catheter (1)	2016–2018
<i>Acinetobacter baumannii</i>	45	Nini (8) YHC (22) TGH (15)	- Sputum (19) - Catheter (5) - Tracheal aspiration (3) - Cavum (1) - Wound (5) - Urine (1) - Blood (2) - Pleural liquid (1) - Bronchoalveolar lavage (4) - Pus (4)	2016–2019

<sup>a</sup>Nini: Nini hospital; TGH: Tripoli governmental hospital; YHC: Youssef Hospital Center.

Samples were stored in the biobank of the “Laboratoire de Microbiologie, Santé et Environnement (LMSE)” of the Lebanese University. A total of 41 isolates were picked from Nini Hospital, 27 from Tripoli government hospital and 78 from El Youssef Hospital Center. These included 72 *Enterobacterales*, 29 *P. aeruginosa* and 45 *A. baumannii* isolates. Susceptibility to imipenem and meropenem was further studied by the E-test method and analyzed according to EUCAST clinical breakpoints [38].

#### 4.2. Biochemical and Immunoenzymatic Assays for Carbapenemase Detection

The homemade Carba NP test was carried out as previously described [39]. Any change in the color of the solution from red to yellow after two hours incubation at 37 °C was considered positive for imipenem hydrolysis and thus for the presence of a carbapenem-hydrolyzing enzyme. For *A. baumannii* isolates, the modified Carba NP test known as the CarbaAcineto NP was used [40]. Similarly, a red to yellow color change indicates the presence of a carbapenemase-producing isolate. A control tube with no imipenem was performed for each test.

#### 4.3. The Lateral Flow Immunoassays (LFIA)

The NG-Test Carba 5 and the NG-Test OXA-23 (NG Biotech, Guipry, France) were carried out on Carba NP-positive isolates according to the manufacturer’s instructions [14], where one colony grown on Mueller–Hinton agar plates was resuspended in five drops of extraction buffer. 100 µL of this suspension were transferred either to the NG-Test Carba5 cassette or NG-Test OXA-23 (NG Biotech). The results were eye read after 15 min of migration at room temperature.

#### 4.4. Molecular Detection of Carbapenemase Genes and Sanger Sequencing

A few colonies of each bacterium to be analyzed were taken from Mueller–Hinton agar plates and resuspended in 100 µL of sterile distilled water. DNA was extracted using the boiling extraction procedure by incubating the tube for 10 min at 95 °C, then for 10 min at –80 °C and 5 min at 95 °C (lysis by thermal shock). Supernatant containing the DNA was collected after centrifugation for 10 min at 10,000 rpm and used for PCR

analysis. Carbapenemase genes were sought by PCR using primers specific for *bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub>, *bla*<sub>NDM</sub>, *bla*<sub>VIM</sub> and *bla*<sub>IMP</sub>, as previously described [41].

PCR products were analyzed by agarose gel electrophoresis containing ethidium bromide [41]. DNA migrated under a voltage of 120 V, at an amperage of 400 mA, for 30 min. Reading took place under a UV lamp (Ultra-Violet) with an imager, integrating the Vision Capt computer software. Each PCR product positive for the gene tested was purified using GeneJet PCR purification Kit® (ThermoFisher, Les Ulis, France) according to the manufacturer's instructions. Each purified PCR product was subsequently sequenced using the Big Dye™ Terminator V1.1 and Applied Biosystem 3130 genetic analyzer and run on an Applied Biosystem 3130 automated sequencer (Applied Biosystems, Les Ulis, France), as previously described [41].

#### 4.5. Whole-Genome Sequencing

Genomic DNA was extracted using the PureLink™ genomic DNA Mini Kit (ThermoFischer) following the manufacturer's instructions. Dual-indexed sequencing libraries were constructed using the NEBNext® library preparation kit and the Multiplex Oligos for Illumina® (NEB, Boston, MA, USA). Libraries were pooled and 100 pM were sequenced on the Illumina Next 500 (2 × 150 bp). Genome assembly was performed using CLC Genomics Workbench v12 (Qiagen, Les Ulis, France). Resistant genes were identified using ResFinder 4.1 [42], and multi locus sequence type (MLST) was determined using MLST V2.0.42 [43].

#### 4.6. Nucleotide Sequence Accession Number

The whole genome sequences generated in the study have been submitted to the Genbank nucleotide sequence database, Bioproject PRJNA820555, under the accession numbers of JALHBC000000000, JALHBA000000000, JALHBB000000000, JALHAY000000000, JALHAX000000000, JALHAW000000000 and JALHAZ000000000 (Table 3).

#### 4.7. Statistical Analysis

Data were analyzed using R software (R Core team, version 4.1.0; R Studio, version 2022.07.1+554). We used non-parametric statistical tests, the Mann–Kendall test and Sen's slope to detect monotonous temporal trends of carbapenem resistance among *Enterobacteriales*, *P. aeruginosa* and *A. baumannii* over the study period (2015–2019). Figures were illustrated using the ggplot2 R package.

### 5. Conclusions

Our study revealed high rates of carbapenem resistance at the level of three north Lebanese hospitals in *Enterobacteriales*, *P. aeruginosa* and *A. baumannii*. Several variants, including NDM-19 and OXA-162 (in *E. coli*), and VIM-62 (in *P. aeruginosa*), have never been described so far in Lebanon, which suggests an ongoing spread of variants of these enzymes. Novel OXA-50-like variants have also been identified, but further characterization is required to determine their precise contribution to the overall carbapenem resistance. In the present study, imipenem-resistant isolates were studied, but as carbapenemase producers may have MICs below the breakpoints, it might well be that our numbers are underestimated. Future work is required, using the EUCAST epidemiological screening cut off for meropenem or ertapenem to increase the potential isolates expressing a carbapenemase. To confirm that the spread of these variants is sporadic, the number of strains analyzed must be increased, in addition, the complete genome of a significant number of strains must be determined.

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**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** WGS sequences have been submitted to Genbank database

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## 2. Article 2: The emergence of carbapenemase-producing *Enterobacterales* in hospitals: A major challenge for a debilitated healthcare system in Lebanon

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# The emergence of carbapenemase-producing *Enterobacterales* in hospitals: a major challenge for a debilitated healthcare system in Lebanon

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**Background:** Carbapenem- and extended-spectrum cephalosporin-resistant *Enterobacterales* (CR-E and ESCR-E, respectively) are increasingly isolated worldwide. Information about these bacteria is sporadic in Lebanon and generally relies on conventional diagnostic methods, which is detrimental for a country that is struggling with an unprecedented economic crisis and a collapsing public health system. Here, CR-E isolates from different Lebanese hospitals were characterized.

**Materials and methods:** Non-duplicate clinical ESCR-E or CR-E isolates ( $N = 188$ ) were collected from three hospitals from June 2019 to December 2020. Isolates were identified by MALDI-TOF, and their antibiotic susceptibility by Kirby-Bauer disk diffusion assay. CR-E isolates ( $n = 33/188$ ) were further analyzed using Illumina-based WGS to identify resistome, MLST, and plasmid types. Additionally, the genetic relatedness of the CR-E isolates was evaluated using an Infrared Biotyper system and compared to WGS.

**Results:** Using the Kirby-Bauer disk diffusion assay, only 90 isolates out of the 188 isolates that were collected based on their initial routine susceptibility profile by the three participating hospitals could be confirmed as ESCR-E or CR-E isolates and were included in this study. This collection comprised *E. coli* ( $n = 70$ ; 77.8%), *K. pneumoniae* ( $n = 13$ ; 14.4%), *Enterobacter* spp. ( $n = 6$ ; 6.7%), and *Proteus mirabilis* ( $n = 1$ ; 1.1%). While 57 were only ESBL producers the remaining 33 isolates (i.e., 26 *E. coli*, five *K. pneumoniae*, one *E. cloacae*, and one *Enterobacter hormaechei*) were resistant to at least one carbapenem, of which 20 were also ESBL-producers. Among the 33 CR-E, five different carbapenemase determinants were identified: *bla*<sub>NDM-5</sub> (14/33), *bla*<sub>OXA-244</sub> (10/33), *bla*<sub>OXA-48</sub> (5/33), *bla*<sub>NDM-1</sub> (3/33), and *bla*<sub>OXA-181</sub> (1/33) genes. Notably, 20 CR-E isolates were also ESBL-producers. The analysis of the genetic relatedness revealed a substantial genetic diversity among CR-E isolates, suggesting evolution and transmission from various sources.

**Conclusion:** This study highlighted the emergence and broad dissemination of *bla*<sub>NDM-5</sub> and *bla*<sub>OXA-244</sub> genes in Lebanese clinical settings. The weak AMR awareness in the Lebanese community and the ongoing economic and healthcare challenges have spurred self-medication practices. Our findings highlight an urgent need for transformative approaches to combat antimicrobial resistance in both community and hospital settings.

#### KEYWORDS

Enterobacterales, antimicrobial resistance, one health, ESBL, carbapenemases

## 1 Introduction

The order Enterobacterales includes the most common human bacterial pathogens responsible for community- and healthcare-associated infections. These species have the ability to rapidly evolve through horizontal gene transfer (e.g., mobile genetic elements) (1). This includes the ability to develop resistance to multiple antibiotics, which complicates the treatment of infections and increases, potentially mortality and morbidity in patients. Of particular concern is the emergence of carbapenem-resistant Enterobacterales (CR-E), which poses a major concern in clinical as well as community settings all across the globe (2). Carbapenems referred to as last-resort antibiotics, possess a broad spectrum of activity against most clinically-relevant Gram-negative bacteria (3, 4). Consequently, it is crucial to continuously monitor and assess the spread of carbapenem resistance, especially in low- and middle-income countries (LMICs) that face established challenges in antimicrobial stewardship and public health systems.

Available observations suggest that antimicrobial resistance (AMR) has been precipitously increasing in Lebanon, a country with a plethora of issues resulting from an unprecedented economic collapse (5–7). The latter has amplified critical issues such as access to medical care, sanitation, and nutritious and safe food, as well as promoted lax medical practices, including self-medication and the reliance on widely and easily available antibiotics as cheaper alternatives across the country. This is important because excessive and inappropriate use of antimicrobials in human and veterinary medicine and agriculture has been well-documented in Lebanon, even before the economic collapse (8–10). Taken together, these challenging conditions have been predicted to enhance the emergence of AMR and the cycle of complicated infections, especially in the most vulnerable populations in Lebanon (5–7, 10, 11). Nevertheless, studies on AMR in Lebanon are generally scant and, when available, can be limited, in scope or (e.g., low sample number) and/or descriptive (e.g., phenotypic AMR evaluation and absence of in-depth genomic analysis). Despite this, available studies have reported rates of extended-spectrum cephalosporin-resistant Enterobacterales (ESCR-E; ~50% of tested isolates) and CR-E (~3%) among clinical isolates in Lebanon between 2015 and 2019 (12, 13). The enzymatic nature of the carbapenem-resistance has also been evidenced, by the detection of several carbapenemase genes, including *bla*<sub>OXA-48</sub>, *bla*<sub>NDM-5</sub>, and *bla*<sub>NDM-19</sub>, in *Escherichia coli*, *bla*<sub>OXA-48</sub>, *bla*<sub>OXA-181</sub>, and *bla*<sub>NDM-5</sub> in *Klebsiella pneumoniae*, *bla*<sub>OXA-48</sub>, *bla*<sub>VIM-1</sub>, *bla*<sub>VIM-4</sub>, and *bla*<sub>NDM-1</sub> in *Enterobacter cloacae*, and *bla*<sub>OXA-48</sub> in *Citrobacter freundii* isolated in Lebanese hospitals (12). In addition, high rates of multidrug-resistant

(MDR) Enterobacterales (60.7%) and the dissemination of extended-spectrum  $\beta$ -lactamase (ESBL) producing-, carbapenemase-producing (CP)-, and colistin-resistant *E. coli* isolates among healthy people in the Lebanese community (14), animals (15), and the environment (16) has been documented. Hence, in-depth studies to evaluate the emergence and spread of AMR in Lebanon, focusing on the determinants that contribute to the dissemination of ESBL-E and/or CP-E is now mandatory. Specifically, details of the sequence types of these bacteria, their resistome, and the plasmids carrying these genes may be crucial to identify the transmission routes and propose intervention strategies to limit their spread. To fill these gaps, we evaluated the occurrence of ESBL- and/or CP-E in three different hospitals in Lebanon and determined the underlying mechanisms of resistance, the population structure of the isolates, and the associated plasmid types using whole genome sequencing analysis (WGS), a powerful technique for investigating AMR, but still not commonly available in LMICs, such as Lebanon, due to its relative expensiveness and requirement of specialized equipment and skills (17).

This study focused only on hospital isolates, as (1) anecdotal evidence suggests that hospitals play an important role in the transmission of ESBL-E- and CP-E, and (2) hospitalized patients are more susceptible to infections/colonization with these species.

## 2 Materials and methods

### 2.1 Ethical approval

This study was approved by the Azm Center/Lebanese University ethical committee and the Lebanese Ministry of Public Health (CE-EDST-1-2020). All the specimens were analyzed anonymously, without any patient identifiers, and the patients were not physically involved in this study.

### 2.2 Isolation and identification of bacteria from clinical samples

A total of 188 clinical Enterobacterales isolates being either resistant to Expanded Spectrum Cephalosporin (ESCR) or Carbapenem Resistant were collected between 2019 and 2020 by the bacteriology laboratories of three hospitals, including El Youssef Hospital Center (50 isolates), the Nini Hospital (137 isolates), and the Tripoli Governmental Hospital (1 isolate), which are located in the Akkar and North governorates of Lebanon, respectively. These isolates

were collected based on their susceptibility profile established in the hospitals as part of routine clinical testing. These clinical isolates were recovered from different sample types, including urine, pus, wound, rectal, axillary, pleural fluid, gastric fluid, and bronchial fluid. They were identified at the hospitals using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) with VITEK MS protocol (bioMérieux, Version 3.0, Marcy L'Etoile, France). The isolates were subsequently stored at the Lebanese University bacterial bank (CMUL).

### 2.3 Antimicrobial susceptibility testing

The isolates were screened for ESBL and CR phenotypes using the Kirby-Bauer disk diffusion assay (including Ticarcillin, Ticarcillin/clavulanic acid, ceftaxime, ceftazidime, cefepime, temocillin, and imipenem). As for the CR-E isolates, a total of 15  $\beta$ -lactams (amoxicillin, amoxicillin/clavulanic acid, ticarcillin, ticarcillin/clavulanic acid, piperacillin, piperacillin/tazobactam, ceftaxime, cefepime, ceftazidime, aztreonam, ertapenem, imipenem, meropenem, and temocillin) antibiotics of human interest were tested. The minimum inhibitory concentrations (MICs) were also determined by E-test (bioMérieux) for ertapenem, imipenem, and meropenem, while temocillin and colistin MICs were assessed using the broth microdilution method.

Additionally, 13 CP-E. coli isolates were selected according to their resistance phenotype, genotype, and MLST type for further determination of MIC values using broth microdilution (BMD) test (Sensititre, ThermoFisher, Grenoble, France) for a complementary list of novel beta-lactam and non-beta-lactam antibiotics of clinical and veterinary interest (i.e., ceftazidime/avibactam, ceftiofur, ceftaroline, ceftobiprole, aztreonam-avibactam, mecillinam, imipenem/relebactam, meropenem/vaborbactam, cefiderocol, eravacyclin, apramycin, gentamicin, neomycin, streptomycin, sulfonamides, and nitrofurantoin). Susceptibility patterns were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST)<sup>1</sup> guidelines when available (18). For aztreonam/avibactam, interpretation was done using aztreonam breakpoints alone (18). For ceftiofur, apramycin, neomycin and streptomycin veterinary breakpoints were used.<sup>2</sup>

### 2.4 Evaluation of the ESCR- and CR-E isolates using enzymatic assays

The  $\beta$  LACTA™ test (Bio-Rad, Marne-la-Coquette, France) was used to further evaluate the ESCR-E isolates. Briefly, this test is based on the hydrolysis of a chromogenic cephalosporin that turns red upon hydrolysis. Notably, the chromogenic cephalosporin is not hydrolyzed by acquired penicillinases (e.g., SHV-1, TEM-1) but by ESBL, carbapenemase, and acquired AmpC (19). Furthermore, the NG-Test® CTX-M MULTI (NG Biotech, Guipry, France)

immunochromatographic assay (ICA) was performed on the  $\beta$  LACTA™ positive isolates to infer the presence of CTX-M-type ESBLs (20). The CR-E isolates were also evaluated using the Carba NP hydrolysis test, which detects carbapenemase activity based on *in vitro* hydrolysis of imipenem (21). The NG-Test® CARBA-5 ICA (NG Biotech) was used to detect members of the five main families of carbapenemases (i.e., KPC-, NDM-, VIM-, IMP-, and OXA-48-like enzymes) produced by the CR-E isolates as described in the manufacturers' instructions (22).

### 2.5 Molecular characterization of the ESCR- and CR-E isolates

The ESCR-E isolates (positive using the  $\beta$ -LACTA™ and NG-Test® CTX-M MULTI tests) were screened by PCR and subsequent Sanger sequencing to identify the *bla*<sub>CTX-M</sub> allele as described previously (23, 24). For the ESCR-E isolates (positive using the  $\beta$ -LACTA™ but negative with the NG-Test® CTX-M MULTI) and the CR-E isolates, the total DNA was extracted using the PureLink™ Genomic DNA Mini-Kit (ThermoFisher Scientific, Waltham, MA, United States) following the manufacturer's instructions and stored at -20°C. Genomic DNA was used for library preparation using the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (NEB, France) according to the manufacturer's instructions. Whole genome sequencing was performed on an Illumina NextSeq 500 instrument (Illumina). After sequencing, raw data were assembled *de novo* using the CLC genomics 10.2 program (Qiagen, Les Ulis, France), and the genomes were analyzed online using software available at the Center for Genomic Epidemiology-CGE.<sup>3</sup> The latter included multilocus sequence typing (MLST) with the CGE MLST 2.0 software to determine sequence types (ST), and acquired resistance gene determinations using ResFinder 4.1 (25–27). Similarly, plasmid replicon types and virulence genes were identified using PlasmidFinder 2.0 (26, 28) and VirulenceFinder 2.0., respectively (26, 29, 30). Reference plasmids sequence were retrieved from the NCBI database, using a local BLAST algorithm. Reads and/or Contigs carrying carbapenemase genes were mapped to reference plasmids, using the CLC genomics 10.2 program (Qiagen).

### 2.6 Plasmids from CR-E isolates

Plasmids were extracted from the carbapenem-resistant isolates by the Kieser method as previously described (31). Transfer of plasmid-borne resistance markers was assessed by electroporation of the plasmids into electro-competent *E. coli* TOP10 (Invitrogen, Saint-Aubin, France). Transformants were selected on Trypticase soy agar (TSA) supplemented with ticarcillin (100  $\mu$ g/mL). Transformants were PCR screened for the carbapenemase genes likely transferred to the recipient *E. coli*. Plasmids were visualized using electrophoresis on 0.7% agarose. *E. coli* NCTC 50192, which harbors four plasmids (7, 48, 66, and 154 kb) was used as size markers during electrophoresis (31).

1 [https://www.eucast.org/clinical\\_breakpoints](https://www.eucast.org/clinical_breakpoints)

2 [https://www.sfm-microbiologie.org/wp-content/uploads/2021/12/CASFM\\_VET2021.pdf](https://www.sfm-microbiologie.org/wp-content/uploads/2021/12/CASFM_VET2021.pdf)

3 <https://cge.food.dtu.dk/services/ResFinder/>

## 2.7 Typing of the CR-E isolates using infrared spectrometry

The CR-E isolates were typed using the Bruker IR Biotyper spectrometer (IRBT, Bruker, Hamburg, Germany). An amount of 1  $\mu$ L of bacterial colonies selected from the confluent part of the culture was re-suspended in 50  $\mu$ L of 70% ethanol solution in an IR Biotyper suspension vial. After vortexing, 50  $\mu$ L of deionized water was added, and the solution was mixed by pipetting. The bacterial suspensions (15  $\mu$ L) were spotted in three technical replicates onto the 96-spot silicon IR Biotyper target and let dry for 15–20 min at 35°C  $\pm$  2°C. In each run, prior to sample spectra acquisition, quality control was performed with the Infrared Test Standards (IRTS 1 and 2) of the IR Biotyper kit. IRTS 1 and IRTS 2 were re-suspended in 90  $\mu$ L deionized water and 90  $\mu$ L of absolute ethanol was added and mixed. Subsequently, 12  $\mu$ L of the suspension was spotted onto the IR Biotyper target and let dry as previously described. The relationships between the isolates were analyzed using the Bruker IR Biotyper Software (version 2.1.0.195, Bruker) (32). An online tool<sup>4</sup> was used to assess the quantitative data of discriminatory power and concordance of the typing methods. Simpson's index of diversity (SID) was used to evaluate the discriminatory power of the typing method, calculating the probability that two unrelated isolates from the test strain set will be clustered into different typing groups. Adjusted Rand index (ARI) with 95% confidence intervals was used to evaluate the concordance of IRBT typing results (33).

## 3 Results

### 3.1 Bacterial isolates

Based on the susceptibility profile (Resistance to ESC and/or carbapenems) derived from the microbiology laboratories of the three participating hospitals, 188 isolates, *E. coli* ( $n=151$ ; 80.3%), *K. pneumoniae* ( $n=20$ ; 10.6%), *Enterobacter* spp. ( $n=7$ ; 3.7%), *Proteus mirabilis* ( $n=6$ ; 3.2%), *Salmonella* spp. ( $n=3$ ; 1.6%), and *Serratia marcescens* ( $n=1$ ; 0.5%) were collected. Using the Kirby-Bauer disk diffusion assay, only 90 isolates could be confirmed and were included in this study. This collection comprised *E. coli* ( $n=70$ ; 77.8%), *K. pneumoniae* ( $n=13$ ; 14.4%), *Enterobacter* spp. ( $n=6$ ; 6.7%), and *Proteus mirabilis* ( $n=1$ ; 1.1%). Routine antimicrobial susceptibility testing revealed that out of these 90 isolates, 57 were only ESBL producers (e.g., fully susceptible to carbapenems), while the remaining 33 (i.e., 26 *E. coli*, five *K. pneumoniae*, one *E. cloacae*, and one *Enterobacter hormaechei*) were resistant to at least one carbapenem, of which 20 were also ESBL-producers, as revealed by synergy images between ESCs and clavulanic acid containing disks.

### 3.2 Susceptibility testing and ESBL gene characterization among the ESBL-producers

The 57 ESBL-producing isolates (Table S1) were composed of *E. coli* ( $n=44$ ; 77.2%), *K. pneumoniae* ( $n=8$ ; 14%), *Enterobacter* spp.

( $n=4$ ; 7%), and *Proteus mirabilis* ( $n=1$ ; 1.8%). These isolates were MDR and remained consistently susceptible only to carbapenems.

The NG-Test® CTX-M MULTI showed that all but two isolates were CTX-M positive (Table S1). PCR amplification of the entire *bla*<sub>CTX-M</sub>-gene and subsequent sequencing revealed that *bla*<sub>CTX-M-15</sub> was predominant (91.2%, 52/55), followed by *bla*<sub>CTX-M-55</sub> (3.5%, 2/55) and *bla*<sub>CTX-M-3</sub> gene (1.8%, 1/55). The whole genome sequencing analysis showed that the two non-CTX-M producing ESBL isolates: one *E. coli* isolate harboring a *bla*<sub>SHV-12</sub> ESBL gene and one *K. pneumoniae* isolate harboring a chromosomally encoded *bla*<sub>SHV-187</sub> gene.

### 3.3 Carbapenemase detection and susceptibility testing of CR-E isolates

The Carba NP hydrolysis test and the NG-Test® CARBA-5, are displayed in Table 1 and Table S2. Among the 33 CR-E isolates, 23 were positive using the Carba NP test, while the remaining isolates were repeatedly negative. Using the NG-Test® CARBA-5 ICA, all 33 isolates were CPs: 17 isolates (51.5%) were positive for NDM and 16 (48.5%) for OXA-48-like enzymes. Out of the 26 *E. coli* isolates different AMR phenotypes were observed (Figure S1). NDM ( $n=14$ ) and OXA-48-like ( $n=12$ ) were the only carbapenemases detected in these isolates. Three *K. pneumoniae* isolates were positive for OXA-48-like, and two for NDM. The two *E. cloacae* complex isolates produced either an OXA-48-like or an NDM.

Using disk diffusion antibiograms, all CR-E isolates were found to be resistant to nearly all antibiotics tested routinely in Lebanon, including ertapenem ( $n=33$ ; 100%). However, 31% (8/26) of these *E. coli* isolates were found susceptible to ertapenem (i.e., MIC  $\leq$  0.5 mg/L) using the broth microdilution method; of them, six isolates were negative with the Carba NP test, while all were positive by ICA for OXA-48. While NDM- and some OXA-48-like-producing *E. coli* displayed high MIC levels for temocillin, surprisingly most isolates (75%, 9/12) carrying *bla*<sub>OXA-48-like</sub> had relatively low MICs ( $\leq$  64  $\mu$ g/mL) for this antibiotic (as compared to 14.3%; 2/14) of the NDM-producing *E. coli* isolates.

Colistin MIC results revealed that almost all CR-E isolates (97%, 32/33) remained consistently susceptible to this antibiotic (MIC  $\leq$  2 mg/L). Furthermore, the activity of different last-resort beta-lactam and non-beta-lactam antibiotics was assessed against a subpopulation of CR-E *E. coli* isolates (6 *bla*<sub>NDM</sub> and 7 *bla*<sub>OXA-48-like</sub>-producers). All the tested isolates were resistant to ceftaroline and ceftibiprole but susceptible to eravacyclin, nitrofurantoin, apramycin, and tigecycline. Resistance to cefiderocol, ceftazidime/avibactam, imipenem/relebactam, and meropenem/vaborbactam was observed among the *bla*<sub>NDM</sub>-producing isolates (Table 2). Additionally, increased MICs to aztreonam/avibactam (4, 8, and 16 mg/L) were noticed among *bla*<sub>NDM</sub>-producing *E. coli* isolates.

The whole genome sequencing of the 33 CR-E isolates was performed. After read assembly, the contigs were submitted to ResFinder 4.0. In total, 47 different resistance genes were identified (Table S3), which code for resistance determinants to clinically-important classes of antibiotics, including  $\beta$ -lactams, aminoglycosides, tetracycline, quinolones, trimethoprim-sulfamethoxazole, fosfomicin, and sulfonamides. Among *E. coli* isolates, the most frequently identified carbapenemase was NDM-5 (53.8%; 14/26), followed by OXA-244 (38.5%; 10/26), OXA-181 (3.8%; 1/26), and OXA-48 (3.8%,

<sup>4</sup> [www.comparingpartitions.info](http://www.comparingpartitions.info)

TABLE 1 Antibiotic susceptibility patterns using Kirby-Bauer disk diffusion of carbapenemase-producing Enterobacteriales (except colistin).

Isolate code	Sample	Hospital	CarbaNP	NG-Test Carba5	$\beta$ LACTA	NG-Test CTX-M MULTI	FOX	CAZ	CTX	ATM	FEP	IMP	MEM	FDC	MEC	FSF	TGC	CIP	LVX	CHL	SXT	FUR	TMN	AMK	GMN	CST	
<b>Escherichia coli</b>																											
O84C6	Urine	YHC	+	OXA-like	+	-	R	R	R	R	R	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	2
O84C10	Gastric fluid	YHC	-	OXA-like	+	+	S	R	R	R	R	S	S	S	R	S	S	S	S	S	R	R	S	S	S	S	4
O84D2	Urine	YHC	+	OXA-like	-	ND	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	1
O84D6	Urine	YHC	-	OXA-like	+	+	S	S	S	S	S	S	S	S	R	S	S	S	S	S	R	R	S	S	S	S	1
O84E5	Urine	YHC	-	OXA-like	-	ND	R	R	R	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	1
O84E7	Urine	YHC	+	NDM	-	ND	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	S	R	R	R	2
O84E8	Urine	YHC	-	OXA-like	-	ND	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	1
O84E9	Urine	NH	+	NDM	+	+	R	R	R	R	R	R	R	S	R	R	S	R	R	R	R	S	R	R	R	R	2
O84F2	Urine	YHC	-	OXA-like	+	+	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	1
O84F5	Urine	YHC	+	NDM	+	-	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	S	S	S	S	S	0.5
O86D2	Urine	YHC	+	NDM	+	+	R	R	R	R	R	R	R	S	R	S	S	R	R	R	R	S	R	S	R	R	1
O84F9	Urine	YHC	+	NDM	-	ND	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	S	S	S	S	S	1
O84G8	Urine	YHC	-	OXA-like	-	ND	R	R	R	S	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	1
O85A1	Urine	YHC	-	OXA-like	+	+	S	R	R	R	R	S	S	S	S	S	S	R	R	S	R	S	R	S	R	R	1
O85A3	Urine	NH	-	OXA-like	-	ND	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	1
O85C3	Urine	YHC	+	NDM	+	+	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	2
O85C6	Urine	YHC	+	NDM	+	+	R	R	R	R	R	R	R	R	S	S	S	R	R	S	R	S	S	S	S	S	2
O85C10	Urine	YHC	+	NDM	+	+	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	R	R	R	S	S	0.5
O85D6	Pleural fluid	NH	+	NDM	-	ND	R	R	R	S	R	R	R	R	R	S	S	R	R	S	R	S	S	S	S	S	1
O85D8	Urine	YHC	-	OXA-like	-	ND	S	R	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	0.5
O85E2	Axillary	NH	+	NDM	-	ND	R	R	R	S	R	R	R	R	R	S	S	R	R	S	R	S	S	S	S	S	2
O85F3	Pus	NH	+	NDM	-	ND	R	R	R	S	R	R	R	R	R	S	S	R	R	S	R	S	S	S	S	S	1
O85G1	Wound	NH	+	NDM	-	ND	R	R	R	S	R	R	R	S	R	S	S	R	R	S	R	S	S	S	S	S	2
O86A2	Urine	NH	-	OXA-like	-	ND	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	2
O85C4	Urine	NH	+	NDM	+	+	R	R	R	R	R	R	R	R	S	S	S	R	R	S	R	S	R	S	S	S	1
O86A6	Rectal	NH	+	NDM	+	+	R	R	R	R	R	R	R	R	R	S	S	R	R	S	R	S	R	S	S	S	1

(Continued)



TABLE 1 (Continued)

Isolate code	Sample	Hospital	CarbaNP	NG-Test Carba5	β LACTA	NG-Test CTX-M MULTI	FOX	CAZ	CTX	ATM	FEP	IMP	MEM	FDC	MEC	FSF	TGC	CIP	LVX	CHL	SXT	FUR	TWN	AMK	GMN	CST		
<b>Klebsiella pneumoniae</b>																												
O84C9	Blood culture	TGH	+	NDM	+	+	R	R	R	S	R	S	R	R	R	S	S	S	S	S	R	S	R	R	R	R	1	
O84D5	Pus	YHC	+	OXA-like	+	-	R	R	R	R	R	S	R	S	R	S	S	S	S	R	S	R	R	R	R	R	1	
O84E1	Gastric fluid	YHC	+	OXA-like	+	+	S	R	R	R	R	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	1	
O85D10	Urine	NH	+	NDM	+	+	R	R	R	R	R	S	S	S	S	R	S	R	S	R	R	S	R	R	R	R	2	
O85E3	Axillary	NH	+	OXA-like	+	+	R	R	R	R	R	S	R	S	S	R	S	S	S	R	S	S	S	S	S	S	2	
<b>Enterobacter spp.</b>																												
O84D8 <sup>II</sup>	Urine	NH	+	NDM	+	-	R	R	R	S	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R	1
O84F3 <sup>II</sup>	Urine	YHC	+	OXA-like	+	-	R	R	R	R	R	S	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	1

\*Susceptibility results were interpreted according to the EUCAST guidelines. All isolates are resistant to amoxicillin, amoxicillin/Clavulanate, Ticarcillin, Ticarcillin/Clavulanate, piperacillin, piperacillin/tazobactam, temocillin, and ertapenem; (18). Gray: Resistant; No color: Susceptible; Abbreviations: FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; FEP, cefepime; MEM, meropenem; FDC, Cefiderocol; MEC, Mecillinam; FSF, Fosfomicin; TGC, Tigecycline; CIP, ciprofloxacin; LVX, levofloxacin; CHL, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; FUR, nitrofurantoin; TMN, tobramycin; AMK, Amikacin; GMN, gentamicin; CST, colistin; YHC, El Youssef Hospital Center; TGH, Tripoli Governmental Hospital; NH, Nimi Hospital. Colistin MIC was assessed using the broth microdilution method according to the EUCAST guidelines (18). The NG-Test CTX-M MULTI was only performed on positive β LACTA isolates. <sup>II</sup>O84D8, Enterobacter hormaechei; O84F3, Enterobacter cloacae (as identified by MALDI-TOF MS).

1/26). OXA-48 has also been identified in three *K. pneumoniae* and one *E. cloacae* isolates, while NDM-1 ( $n = 3$ , 9.1%) was detected in two *K. pneumoniae* and one *E. hormaechei* isolates (Table 2). Notably, two plasmid-encoded cephalosporinases, *bla*<sub>CMY-145</sub> and *bla*<sub>DHA-1</sub> genes that confer resistance to ESCs were detected in three *E. coli* isolates. ESBL genes such as *bla*<sub>CTX-M-15</sub> ( $n = 15$ ), *bla*<sub>CTX-M-27</sub> ( $n = 1$ ), *bla*<sub>TEM-35</sub> ( $n = 1$ ), *bla*<sub>SHV-33</sub> ( $n = 1$ ), *bla*<sub>SHV-12</sub> ( $n = 2$ ), *bla*<sub>SHV-26</sub> ( $n = 1$ ), were also identified. Specifically, the *bla*<sub>CTX-M-15</sub> gene was the most frequently detected in *E. coli* ( $n = 11$ ); including one isolate co-harboring two CTX-M alleles: *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-27</sub>) and *K. pneumoniae* ( $n = 4$ ) isolates. 16S RNA methylase genes were detected among 6 isolates including 5 *bla*<sub>NDM-5</sub>-producing *E. coli* and one *bla*<sub>NDM-1</sub>-producing *E. hormaechei* isolate.

MLST analysis using the whole genome sequence showed that the CR-*E. coli* belonged to 11 different STs; including ST69 ( $n = 6$  isolates), followed by ST648 ( $n = 4$ ), ST167 ( $n = 3$ ), ST361 ( $n = 3$ ), ST405 ( $n = 3$ ), ST10 ( $n = 2$ ), ST90 ( $n = 1$ ), ST940 ( $n = 1$ ), ST38 ( $n = 1$ ), ST46 ( $n = 1$ ), and ST8881 ( $n = 1$ ). Additionally, the CR-*K. pneumoniae* isolates belonged to four different STs, namely ST35, ST37, ST45, and ST1770. *E. cloacae* and *E. hormaechei* belonged to ST1006 and ST182, respectively (Table S3).

Furthermore, alignment of the *ftsI* gene sequences with that of a wild-type gene (*E. coli* NCTC 9022, accession number LRI34237) revealed a four amino-acid insertion in PBP3 after residue 333 in 13 of 14 NDM-5-producing *E. coli* isolates. Two types of insertions were detected: YRIN ( $n = 7$ ) and YRIK ( $n = 6$ ). YRIN insertion was found among isolates with ST167 and ST361, and YRIK insertion was present in isolates with ST648 and ST405. ST90 isolate contained neither YRIN nor YRIK insertions. These 4 AA insertions could be correlated with increased MICs to aztreonam/avibactam (4, 8, and 16 mg/L) and cefiderocol among *bla*<sub>NDM-5</sub>-producing *E. coli* isolates, as compared to similar isolates lacking a 4 AA insertion (0.06 mg/mL).

### 3.4 Virulence determinants and plasmids in the CR-*Escherichia coli* isolates

Virulence factors (VFs) in the CR-*E. coli* isolates were identified using the CGE VirulenceFinder 2.0. TraT protein, previously shown to mediate resistance to bacterial killing by serum, was detected in 14 *E. coli* isolates, including a *bla*<sub>OXA-48</sub><sup>+</sup> positive isolate belonging to ST69. Genes encoding adhesins (*ipfA*, *fimH*, *afaA*, *afaC*, *afaD*, *afaE*) were found in five *E. coli* that were positive for *bla*<sub>OXA-244</sub> (Table 3). Additionally, *iss* encoding an outer membrane lipoprotein that enhances serum resistance, was detected only in one *bla*<sub>OXA-244</sub><sup>+</sup> positive *E. coli* isolate. The capsular genes, *kpsE* and *kpsM*, were both detected in eight isolates, of which three were *bla*<sub>NDM-5</sub><sup>+</sup> positive (ST648), and 5 were *bla*<sub>OXA-244</sub><sup>+</sup> positive (ST69). Most VFs were detected in two isolates belonging to ST69.

Using PlasmidFinder 2.1, 9 plasmid replicon types were identified. Specifically, the following plasmid types were detected in the *E. coli* isolates; Col ( $n = 9$  isolates), IncFII ( $n = 18$ ), IncX3/X4 ( $n = 4$ ), IncI1-I ( $n = 3$ ), and IncFIA ( $n = 11$ ). Additionally, in the *K. pneumoniae* isolates, IncL ( $n = 3$ ), IncM2 ( $n = 1$ ), and IncFIB ( $n = 4$ ) were detected. The *E. cloacae* and *E. hormaechei* isolates carried IncFIB/II and IncL/X3, respectively (Table 4). Transformants were obtained after Kieser plasmid extraction and electro-transformation for 25/33 isolates. For 8 *bla*<sub>OXA-244</sub><sup>+</sup> producing isolates, even with repeated attempts no plasmids were observed on Kieser gel, and no transformants were



TABLE 4 Carbapenemase gene location was determined by plasmid extraction using Kieser technique and by applying a BLASTN algorithm for carbapenemase-genes-carrying contigs and then these contigs were mapped to reference plasmids using CLC genomics.

Isolate code	MLST type	Carbapenemase gene	Location (chromosomal or plasmid-borne)	IncX3	IncX4	IncFIA	IncFIB	IncFII	Col	IncI1-I	IncL	IncM
<i>Escherichia coli</i>												
O84D6**	ST10	<i>bla<sub>OXA-244</sub></i>	P									
O85D8**	ST10	<i>bla<sub>OXA-244</sub></i>	C									
O84F2**	ST38	<i>bla<sub>OXA-244</sub></i>	C									
O84C10**	ST46	<i>bla<sub>OXA-244</sub></i>	C									
O84D2**	ST69	<i>bla<sub>OXA-48</sub></i>	P									
O84E5**	ST69	<i>bla<sub>OXA-244</sub></i>	C									
O84E8**	ST69	<i>bla<sub>OXA-244</sub></i>	C									
O84G8**	ST69	<i>bla<sub>OXA-244</sub></i>	C									
O85A3*	ST69	<i>bla<sub>OXA-244</sub></i>	C									
O86A2*	ST69	<i>bla<sub>OXA-244</sub></i>	C									
O86D2**	ST90	<i>bla<sub>NDM-5</sub></i>	P									
O85C6**	ST167	<i>bla<sub>NDM-5</sub></i>	P									
O85C4*	ST167	<i>bla<sub>NDM-5</sub></i>	P									
O85C10**	ST167	<i>bla<sub>NDM-5</sub></i>	P									
O84F5**	ST361	<i>bla<sub>NDM-5</sub></i>	P									
O84F9**	ST361	<i>bla<sub>NDM-5</sub></i>	P									
O86A6*	ST361	<i>bla<sub>NDM-5</sub></i>	P									
O84E7*	ST405	<i>bla<sub>NDM-5</sub></i>	P									
O84E9*	ST405	<i>bla<sub>NDM-5</sub></i>	P									
O85C3**	ST405	<i>bla<sub>NDM-5</sub></i>	P									
O85E2*	ST648	<i>bla<sub>NDM-5</sub></i>	P									
O85F3*	ST648	<i>bla<sub>NDM-5</sub></i>	P									
O85G1*	ST648	<i>bla<sub>NDM-5</sub></i>	P									
O85D6*	ST648	<i>bla<sub>NDM-5</sub></i>	P									
O85A1**	ST8881	<i>bla<sub>OXA-244</sub></i>	C									
O84C6**	ST940	<i>bla<sub>OXA-181</sub></i>	P									

(Continued)

TABLE 4 (Continued)

Isolate code	MLST type	Carbapenemase gene	Location (chromosomal or plasmid-borne)	IncX3	IncX4	IncFIA	IncFIB	IncFII	Col	IncI1-I	IncL	IncM
<i>Klebsiella pneumoniae</i>												
O84E1**	ST35	<i>bla<sub>OXA-48</sub></i>	P									
O84D5**	ST35	<i>bla<sub>OXA-48</sub></i>	P									
O85E3*	ST37	<i>bla<sub>OXA-48</sub></i>	P									
O84C9***	ST45	<i>bla<sub>NDM-1</sub></i>	P									
O85D10*	ST1770	<i>bla<sub>NDM-1</sub></i>	P									
<i>Enterobacter</i> spp.												
O84D8*	ST182	<i>bla<sub>NDM-5</sub></i>	P									
O84F3**	ST1006	<i>bla<sub>OXA-48</sub></i>	P									

Plasmid incompatibility groups present in the 33 clinical carbapenemase-producing Enterobacterales isolates, were identified using PlasmidFinder 2.1 online tool available at the Center for Genomic Epidemiology -CGE (<http://www.genomicepidemiology.org/>) using 99% identity cut-off (26, 28–30). \*\*El-Youssef Hospital; \*\*\*Tripoli Governmental Hospital; Different colors represent different sequence types (STs).

TABLE 5 Comparison of different typing methods for carbapenemase-producing *Escherichia coli* clinical isolates.

Method	No. of types	Simpson's ID (95% CI)
MLST <sup>a</sup>	7	0.875 (0.813–0.937)
IRBT <sup>b</sup>	11	0.933 (0.857–1.000)
WGS <sup>c</sup>	12	0.967 (0.935–0.998)

Simpson's index of diversity used to determine the discriminatory power and concordance of the three typing methods was determined using the online tool 690 ([www.comparingpartitions.info](http://www.comparingpartitions.info)). <sup>a</sup>MLST: Multi-locus sequence typing; <sup>b</sup>IRBT: IR Biotyper; <sup>c</sup>WGS: Whole-genome sequencing.

obtained suggesting a chromosomal location. For *K. pneumoniae* and *E. hormaechi*, the *bla<sub>OXA-48</sub>* gene was carried on an IncL plasmid.

### 3.5 Genetic relatedness as revealed by IR Biotyper in comparison to WGS

Sixteen CR-*E. coli* and the five CR-*K. pneumoniae* isolates positive with the Carba NP test were assessed with the IR Biotyper for strain typing and the results were compared with those obtained by WGS analysis (Figures S2, S3). Overall IRBT results corroborated WGS for the typing of the five CR *K. pneumoniae*, which were classified into four IR types (Figure S2). However, IRBT differentiated 16 *E. coli* isolates that belonged to 7 STs into 11 IR types, with 8 IR types comprising only a single isolate (Table 5). The Simpson Index of Diversity (SID) was used to determine the discriminatory power of the typing methods. WGS had the highest discriminatory power (0.967) followed by IRBT (0.933), while the lowest SID was for MLST (0.875). Main discrepancies were observed between IRBT and WGS with *E. coli* ST-648 isolates (O85D6, O85E2, O85G1, and O85F3), which clustered into IR type 7 (Figure S3), but differed from each other by 53, 400, and 1,360 SNIPs.

## 4 Discussion

The increase in ESCR- and CR-E isolates has been observed in hospitals worldwide. In Lebanon, two nationwide hospital-based retrospective studies documented a high prevalence of MDR pathogens, including ESBL-producing Enterobacterales (34% in 2016) (5). Recently, a community-based study revealed that approximately half of the population carries ESCR-*E. coli* (14), with an important dynamic of acquisition and loss of MDR strains and limited plasmid spread. The occurrence of Enterobacterales with decreased susceptibility to carbapenems, raised from 0.4% in 2008–2010 to 1.6% in 2012 and 3.3% in 2019 in hospitalized patients (12, 35). Despite the concerning increase and reports, there is a lack of molecular data on the epidemiology of ESBL- and CR-E in Lebanon.

Our results highlighted the predominance of the *bla<sub>CTX-M-15</sub>* gene (52/57, 91.2%) among ESBL-producing isolates regardless of the co-existence of other β-lactam resistance determinants, as observed globally (36). CTX-M-15-producing Enterobacterales clones have been widely reported in Lebanon among human, animal, and environmental sources (14, 15, 37–39). The finding that one single *E. coli* isolate may carry two CTX-M (–15 and –27) variants capable

of strongly hydrolyzing ceftazidime and belonging to two different groups of CTX-Ms (Group 1 and 9) is another example of concentration of restriction mechanisms.

Our findings further support the shift from *bla*<sub>OXA-48-like</sub> variants toward *bla*<sub>NDM-5</sub> among *E. coli* in hospital settings in Lebanon (12, 35). OXA-48-like enzymes were the most prevalent carbapenemases among Enterobacterales in Lebanon over the last decade while NDM-5 was uncommon in hospital and community settings (12, 40). In our study, the *bla*<sub>NDM-5</sub> gene has become the predominant carbapenemase gene (14 of 26 *E. coli* isolates). The *bla*<sub>OXA-244</sub> gene (10/26) came in second place, while *bla*<sub>OXA-48</sub> (1/33), and *bla*<sub>OXA-181</sub> (1/33) genes are rare. OXA-244 producing *E. coli* isolates are increasingly described worldwide (41), and represents a threat to public health because of the difficulties in their detection using classical screening media based on carbapenems and temocillin (42). Indeed, our results confirmed that OXA-244-producing isolates have lower MICs to temocillin and carbapenems as compared to other CP-E, which results in the absence of growth on screening media and thus an underestimation and silent spread. Furthermore, these isolates were negative for the Carba NP test, and only the use of an ICA (here the NG-Test Carba5) revealed a positive signal for OXA-48-like carbapenemase, suggesting that both tests should be used together to maximize the chances of detecting all OXA-48-like carbapenemases. Additionally, the CR *K. pneumoniae* isolates harbored *bla*<sub>OXA-48</sub> (3/5) and *bla*<sub>NDM-1</sub> (2/5), while the *E. cloacae* and *E. hormaechei* isolates harbored *bla*<sub>OXA-48</sub> and *bla*<sub>NDM-1</sub>, respectively.

MLST results demonstrated several distinct genetic backgrounds for the CR-E isolates, suggesting horizontal gene transfer of the carbapenemase gene carrying plasmids, particularly those carrying the *bla*<sub>NDM-5</sub> gene, rather than a clonal spread of a single clone. Interestingly, we found that the carbapenemase genes were plasmid-borne (except in eight *bla*<sub>OXA-244</sub>-producing *E. coli*) and on different plasmid types (Table 4). Of the NDM-5-producing *E. coli* isolates, six and seven had the insertion of YRIN or YRIK in BBP3, respectively, which resulted in a significant increase of MICs to aztreonam/avibactam and cefiderocol as well as to other BBP3-targeting  $\beta$ -lactams (34). Surprisingly, the *bla*<sub>OXA-244</sub> gene was chromosomally encoded in 8 isolates, while it was plasmid-mediated in only two isolates belonging to ST69 and ST10. The chromosomal location of the *bla*<sub>OXA-244</sub> gene was previously reported in ST38 and ST69 in France (43). Taken together, the occurrence of different resistance markers in diverse genetic backgrounds as well as plasmid types constitute a risk to patients, potentially highlighting a more severe problem and highlighting an urgent need to monitor and control the spread of resistance in hospitals in Lebanon.

The study identified various MLST types, including ST648, ST167, ST361, and ST405, in *bla*<sub>NDM-5</sub>-producing *E. coli*. These STs are recognized as high-risk global clones that contribute to the widespread dissemination of drug resistance determinants among Enterobacterales (44). For instance, ST648 is recognized as a major global ESBL-producing *E. coli* clone (45, 46), particularly associated with *bla*<sub>CTX-M-15</sub>, in humans, birds, and companion animals (47). Additionally, ST648 was identified in Lebanon among clinical CRE (48), including *bla*<sub>NDM-5</sub>-producing *E. coli* (49). Besides, ST167 has been linked to the global spread of *bla*<sub>NDM</sub> in humans, animals, and food (50). ST405 has been detected in several countries, including the United States (51), Japan (52), and Lebanon (48), allowing the

transmission of *bla*<sub>CTX-M-15</sub> and *aac(6′)-Ib-cr* genes (53). Notably, both ST361 and ST648 have been reported among Syrian refugees in Lebanon (54), and ST90 was found in the effluent of Al-Qaa refugee camp (55). Additionally, we identified ST38 among *bla*<sub>OXA-244</sub>-producing *E. coli*, previously described in estuary water in Lebanon (56). Several European countries have reported increased dissemination of the *bla*<sub>OXA-244</sub> gene (57, 58), with ST38 being the most common sequence type among *bla*<sub>OXA-244</sub>-producing *E. coli* isolates (59). Moreover, ST38 *bla*<sub>OXA-48</sub>-producing *E. coli* has also been found in fowls in Lebanon (60). As observed in our study, ST940 *E. coli* carrying the *bla*<sub>OXA-181</sub> gene was previously reported at the American University of Beirut Medical Center (61). Among the *K. pneumoniae* isolates, there were five isolates belonging to four different sequence types. One of them, *K. quasipneumoniae* subsp. *similipneumoniae* ST1770, has been previously reported in hospital wastewater effluents in Japan (62), but had not been reported in Lebanon. Additionally, clinical *K. pneumoniae* isolates ST35 and ST45 have been documented in Lebanon (63, 64). ST37 has also been closely associated with ESBLs (65). Furthermore, the ST182 *bla*<sub>NDM-1</sub> *E. hormaechei* isolate, previously reported in Lebanon (66), has been frequently isolated from clinical specimens in China, Mexico, the Czech Republic, and the United States (67).

Although there is a paramount need to monitor the spread of critical AMR strains in hospitals in Lebanon, these efforts are complicated by the unavailability of resources. Molecular typing methods such as whole genome sequencing are relatively time-consuming and expensive (32). Therefore, we evaluated the Bruker IR Biotyper for reliable detection of the relatedness and discrimination between strains. Our findings showed that Fourier Transform Infrared (FT-IR) technology is a powerful tool for strain typing, showing slightly better results to MLST and comparable results to WGS among CR-*E. coli* and *K. pneumoniae* isolates. Its advantages are summed up in the simple preparation of samples, ease of use, and low running costs. Together with its relatively high discriminatory power, the FT-IR seems to be a good tool for outbreaks real-time surveillance and infection control in clinical settings.

In conclusion, our study showed that in-depth studies are crucial to better understand the emergence and dissemination of drug-resistant determinants within and across healthcare institutions. Specifically, we highlighted an unprecedented diversity of ESBL- and CR-E determinants compared to other studies in Lebanon. Furthermore, the MLST and the associated plasmid types suggested that these determinants were circulating in diverse strains, complicating the control efforts and suggesting the need for evidence-based antimicrobial stewardship programs. In the distressing situation of Lebanon, the accessibility to novel antibiotic molecules remains a major concern, impacting public health. The economic crisis had a profound impact leading to significant challenges in antibiotics procurement and even more so to new molecules, which are unavailable and not yet routinely tested. Furthermore, our data support that the fight against MDR bacteria in LMICs, such as Lebanon, requires a comprehensive One Health approach because of the diffuse sources and factors that affect the spread of resistance in hospitals and the community. The latter corroborates our previous research that showed a wide reliance on and sometimes indiscriminate use of critically important antibiotics in healthcare settings, agriculture, and the community in Lebanon (8, 9). This approach and science-based interventions are urgently needed to control the spread

of AMR in Lebanon, which represents an essential threat nationally and globally as AMR is known to spill across international borders.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at <http://www.ncbi.nlm.nih.gov/bioproject> and PRJNA973232.

## Ethics statement

The studies involving humans were approved by Azm Center/Lebanese University ethical committee and the Lebanese Ministry of Public Health (CE-EDST-1-2020). The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from bacterial isolates collected based on susceptibility profiles from three hospitals. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

## Author contributions

DD: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. SO: Supervision, Validation, Writing – review & editing. MR: Investigation, Writing – review & editing. IK: Investigation, Resources, Writing – review & editing. HM: Investigation, Writing – review & editing. AB: Formal analysis, Validation, Writing – review & editing. DG: Formal analysis, Investigation, Writing – review & editing. MH: Methodology, Writing – review & editing. FD: Formal analysis, Investigation, Writing – review & editing. MO: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. TN: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing, Project administration, Validation.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpubh.2023.1290912/full#supplementary-material>

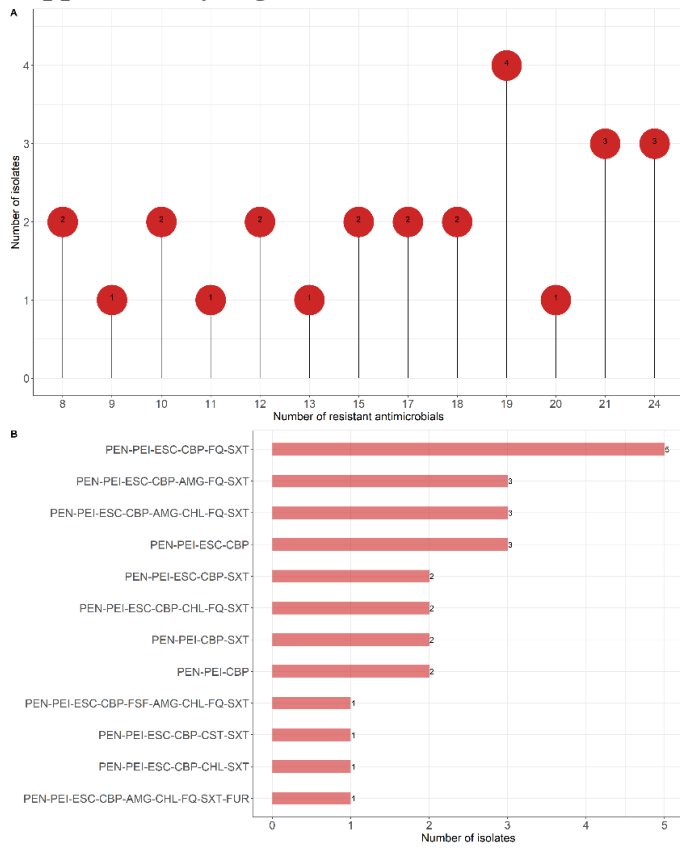
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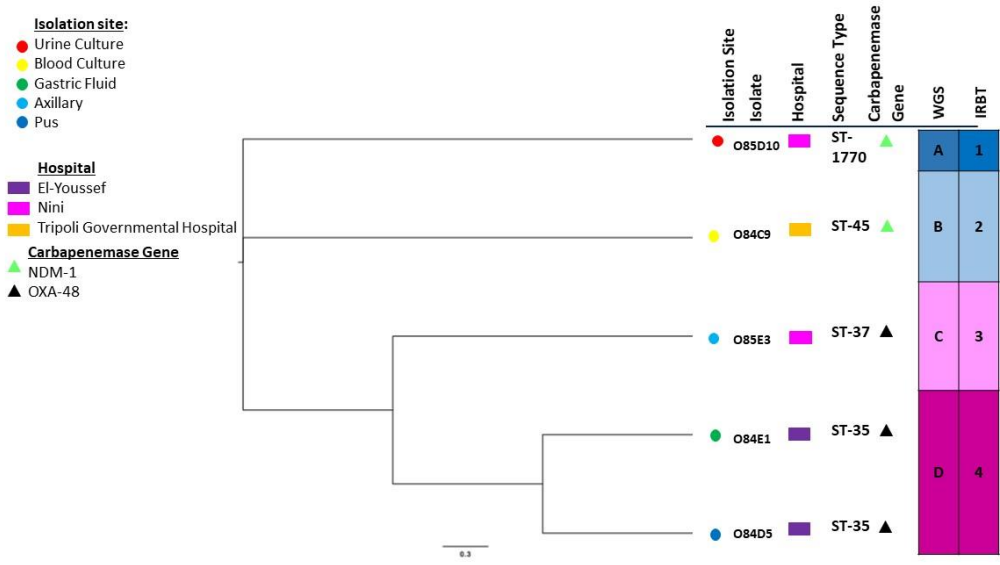
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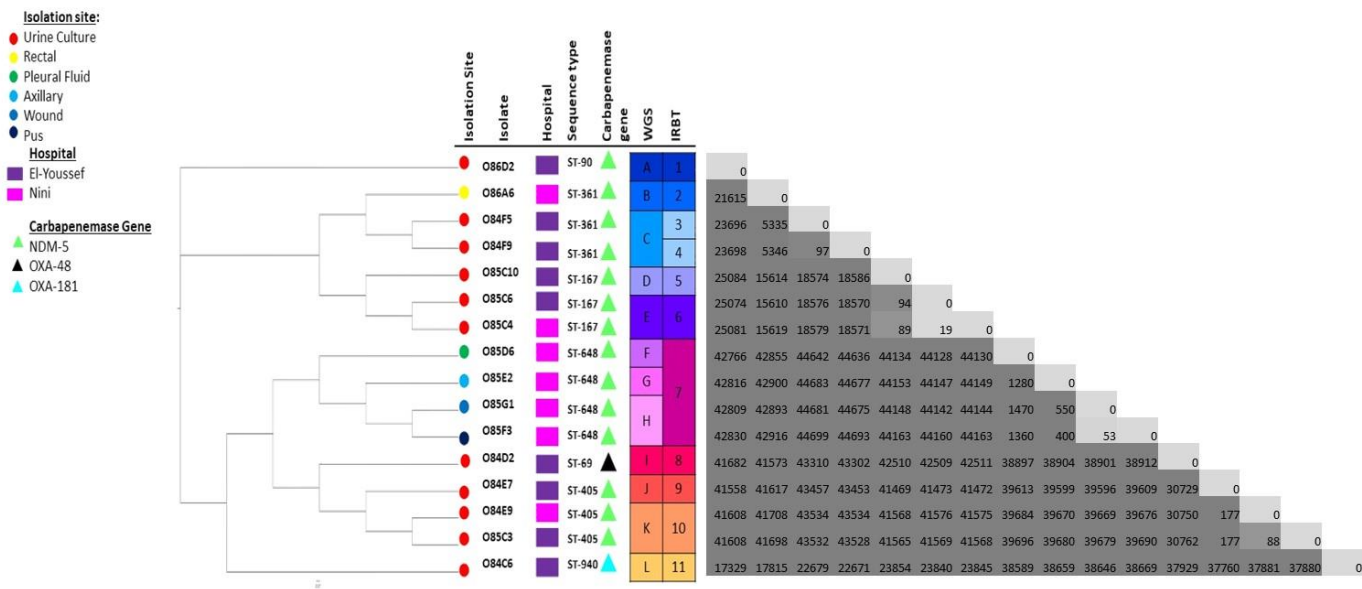
## Supplementary Figures



**Figure S1. Antimicrobial Susceptibility Patterns of Carbapenemase-Producing *Escherichia coli* (n=26) clinical isolates.** Distribution of resistance by number of antimicrobial agents (A) and susceptibility patterns of the isolates (B); Antimicrobial category abbreviations: PEN, penicillin; PEI, penicillin/ $\beta$ -lactamase inhibitor; ESC, extended-spectrum cephalosporins; CBP, carbapenems; AMG, aminoglycosides; CHL, chloramphenicol; TET, tetracycline; FQ, fluoroquinolones; SXT, trimethoprim-sulfamethoxazole; FUR, nitrofurantoin. This list excludes the tested antimicrobials in Table 2.



**Figure S2.** Phylogenetic tree of five carbapenemase-producing *Klebsiella pneumoniae*. A dendrogram was obtained by SNP-based analysis, using CSI Phylogeny 1.4 available online at the center for genomic epidemiology-CGE (<http://www.genomicepidemiology.org/>). The dendrogram was rooted with branch transform using FigTree software. IRBT and WGS types were assigned for each isolate and presented in different colors. The STs, hospital, isolate ID, isolation site, and carbapenemase genes are also shown.



**Figure S3.** Phylogenetic tree of 16 carbapenemase-producing *Escherichia coli* isolates. A dendrogram was obtained by SNP-based analysis, using CSI Phylogeny 1.4 available online at the center for genomic epidemiology-CGE (<http://www.genomicepidemiology.org/>) (1). The dendrogram was rooted with branch transform using Figtree software. IRBT and WGS type was determined for each isolate and presented as different colors. The STs, hospital, isolate ID, isolation site, and carbapenemase genes are also shown.

## Supplementary Tables

**Table S1.** Distribution of extended-spectrum  $\beta$ -lactamase genes carried by ESBL-producing *Enterobacterales* isolates.

Biochemical and Immunochromatographic tests	Species	Extended-spectrum $\beta$ -lactamase gene	MLST type
$\beta$ LACTA (+) / NG-Test CTX-M MULTI (+) (n=55)	<i>Escherichia coli</i> (n=43)	<i>bla</i> <sub>CTX-M-15</sub> (n=40)	ND <sup>1</sup>
		<i>bla</i> <sub>CTX-M-55</sub> (n=2)	ND
		<i>bla</i> <sub>CTX-M-3</sub> (n=1)	ND
	<i>Klebsiella pneumoniae</i> (n=7)	<i>bla</i> <sub>CTX-M-15</sub> (n=7)	ND
	<i>Enterobacter</i> spp. (n=4)	<i>bla</i> <sub>CTX-M-15</sub> (n=4)	ND
<i>Proteus mirabilis</i> (n=1)	<i>bla</i> <sub>CTX-M-15</sub> (n=1)	ND	
$\beta$ LACTA (+) / NG-Test CTX-M MULTI (-) (n=2)	<i>Escherichia coli</i> O86A3 (n=1)	<i>bla</i> <sub>SHV-12</sub> (n=1)	ST167 (Isolated in Nini Hospital)
	<i>Klebsiella pneumoniae</i> O85A6 (n=1)	<i>bla</i> <sub>SHV-187</sub> (n=1)	ST14 (Isolated in Nini Hospital)

<sup>1</sup>ND, not determined.

**Table S2.** Comparison between disk diffusion (DD) and E-test (bioMérieux, Marcy-l'Étoile, France) results for ertapenem, imipenem, and meropenem, and broth microdilution method for temocillin. Major discordance (S/R and R/S) are shown in dark grey and minor discordance (S/I, I/S, R/I, and I/R) are shown in light grey. Results were interpreted according to the EUCAST guidelines

Isolate code	CarbaNP	NG-Test Carba5	$\beta$ LACTA	NG-Test CTX-M MULTI	Ertapenem		Imipenem		Meropenem		Temocillin	
					DD (diameter mm)	MIC (mg/l)	DD (diameter mm)	MIC (mg/l)	DD(diameter mm)	MIC (mg/l)	DD (diameter mm)	MIC (mg/l)
<i>Escherichia coli</i>												
O84C6	+	OXA-like	+	-	R (15)	0.5	S (22)	0.5	S (22)	0.75	R (6)	2048
O84C10	-	OXA-like	+	+	R (15)	0.5	S (33)	0.25	S (22)	0.25	R (10)	64
O84D2	+	OXA-like	-	ND	R (19)	0.25	S (25)	0.25	S (23)	0.125	R (8)	256
O84D6	-	OXA-like	+	+	R (6)	>32	S (35)	0.25	S (22)	0.25	R (12)	64
O84E5	-	OXA-like	-	ND	R (6)	0.38	S (25)	4	S (22)	0.25	R (12)	16
O84E7	+	NDM	-	ND	R (6)	>32	R (10)	6	R (6)	>32	R (6)	512
O84E8	-	OXA-like	-	ND	R (6)	0.25	S (35)	0.25	S (22)	0.25	R (15)	16
O84E9	+	NDM	+	+	R(6)	>32	R(12)	3	R (6)	>32	R (6)	1024
O84F2	-	OXA-like	+	+	R (10)	0.75	S (35)	0.25	S (22)	0.25	R (12)	32
O84F5	+	NDM	+	-	R(6)	>32	R (10)	16	R (6)	>32	R (6)	512
O86D2	+	NDM	+	+	R (8)	4	R (18)	2	R (12)	3	R (6)	64
O84F9	+	NDM	-	ND	R (6)	>32	R (10)	>32	R (6)	>32	R (6)	1024
O84G8	-	OXA-like	-	ND	R (6)	0.25	S (35)	0.25	S (22)	0.25	R (15)	8
O85A1	-	OXA-like	+	+	R (10)	1	S (35)	0.25	S (22)	0.25	R (15)	32
O85A3	-	OXA-like	-	ND	R (12)	0.5	S (35)	0.25	S (23)	0.125	R (16)	32
O85C3	+	NDM	+	+	R (6)	>32	R (18)	1	R (8)	8	R (6)	512
O85C6	+	NDM	+	+	R (8)	6	R (16)	2	R (13)	1	R (6)	512
O85C10	+	NDM	+	+	R (8)	3	R (12)	0.5	R (17)	0.75	R (6)	256
O85D6	+	NDM	-	ND	R (6)	>32	R (8)	>32	R (6)	>32	R (6)	512
O85D8	-	OXA-like	-	ND	R (10)	0.75	S (28)	0.5	S (22)	0.75	R (8)	256
O85E2	+	NDM	-	ND	R (6)	>32	R (6)	>32	R (12)	>32	R (6)	2048
O85F3	+	NDM	-	ND	R (6)	>32	R (12)	12	R (6)	12	R (6)	256
O85G1	+	NDM	-	ND	R (6)	>32	R (12)	8	R (6)	16	R (6)	256
O86A2	-	OXA-like	-	ND	R (6)	0.25	S (32)	0.25	S (22)	0.25	R (14)	16
O85C4	+	NDM	+	+	R(8)	4	R(12)	2	R(6)	2	R (6)	256
O86A6	+	NDM	+	+	R (6)	>32	R (16)	2	R (8)	3	R (6)	32
<i>Klebsiella pneumoniae</i>												
O84C9	+	NDM	+	+	R (8)	6	S (22)	3	R (10)	3	R (6)	64
O84D5	+	OXA-like	+	-	R (10)	3	S (22)	1	R (18)	1	R (6)	512
O84E1	+	OXA-like	+	+	R (20)	0.5	S (22)	0.5	S (22)	0.5	R (8)	256
O85D10	+	NDM	+	+	R	2	S (22)	0.5	S (22)	0.75	R (6)	32
O85E3	+	OXA-like	+	+	R(12)	4	S (22)	0.5	R (15)	1	R (6)	1024
<i>Enterobacter</i> spp.												
O84D8	+	NDM	+	-	R (6)	>32	S (22)	4	R (6)	12	R (6)	128
O84F3	+	OXA-like	+	-	R (1)	6	S (22)	1	R (15)	1.5	R (6)	512



### 3. Article 3: Emergence of NDM-1, VIM-4, and CTX-M-15-co-harboring *Enterobacter xiangfangensis* in bloodstream

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## Abstract

Multidrug resistant *Enterobacter xiangfangensis* are increasingly reported worldwide. Here, we conducted an in-depth characterization of a *bla*<sub>NDM-1</sub>-, *bla*<sub>VIM-4</sub>-, and *bla*<sub>CTX-M-15-CO</sub>-harboring *E. xiangfangensis* isolated from the blood of a patient in Lebanon. The isolate belonged to ST114 and carried potentially transmissible *bla*<sub>VIM-4</sub> on an IncC plasmid and two copies of *bla*<sub>NDM-1</sub> on two different IncFII plasmids. To our knowledge, this is the first report of a *bla*<sub>NDM-1</sub>-, *bla*<sub>VIM-4</sub>-, and *bla*<sub>CTX-M-15-CO</sub>-harboring *E. xiangfangensis* in clinical settings worldwide.

The *Enterobacter cloacae* complex (ECC) is highly adapted to the hospital environment and is frequently responsible for hospital-acquired infections (including but not limited to bacteremia, pneumonia, and urinary tract infections). ECC has been associated often with multidrug resistance (MDR), including reduced susceptibility to critically important antibiotics (1). ECC shows genomic heterogeneity; with 13 clusters based on *hsp60* sequences, and it currently comprises seven different species (2, 3), including *Enterobacter asburiae*, *E. cloacae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, *Enterobacter mori*, and *Enterobacter xiangfangensis*. These species can acquire plasmid-borne genes that encode resistance to extended-spectrum cephalosporins and carbapenems, which complicates treatment regimens (4). Additionally, ECC naturally produces an inducible chromosomally-encoded Ambler class C  $\beta$ -lactamase (cAmpC) characterized by the ability to hydrolyze cephalosporins without being inhibited by clavulanic acid or tazobactam (5). Notably, acquired resistance to broad-spectrum cephalosporins can occur through the acquisition of extended-spectrum  $\beta$ -lactamases (ESBL) or by chromosomal mutations, mostly in *ampD* or *ampR* that lead to cAmpC overproduction (6). Carbapenem resistance can arise by the acquisition of carbapenemases (e.g., KPC, NDM, VIM, IMP, OXA-48) or through the alteration or loss of non-specific porins associated with the hyperexpression of the cAmpC gene and/or production of ESBLs (7).

*E. xiangfangensis* has been described as an emerging pathogen across the globe (8). Here, we report an in-depth characterization of a *bla*<sub>NDM-1</sub>-, *bla*<sub>VIM-4</sub>-, and *bla*<sub>CTX-M-15-CO</sub>-harboring *E. xiangfangensis* (Exn692) isolated from the blood of a male patient at the Nini Hospital (Tripoli, Lebanon) in April 2021. The isolate was whole-genome sequenced using the Illumina technology (NextSeq 500) (9) and plasmids were evaluated by long-read sequencing using the Nanopore

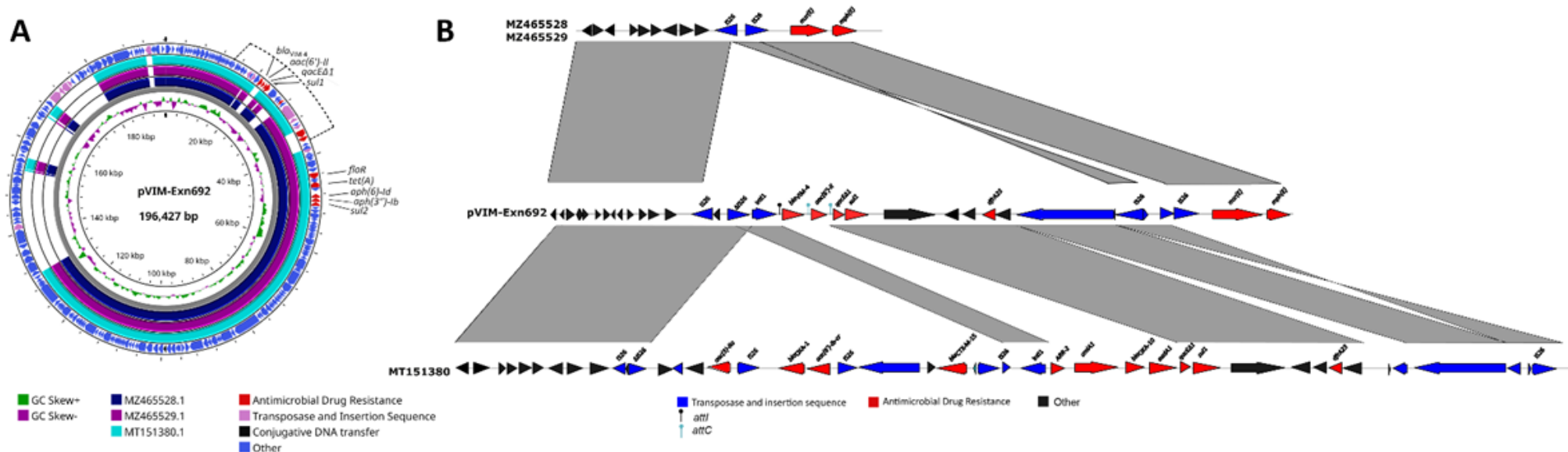
MinION technology (10). Exn692 was identified as *E. xiangfangensis* using the MALDI-TOF VITEK MS Version 3.0 (bioMérieux, Marcy-l'Etoile, France), followed by confirmation using average nucleotide identity and *hsp60* analysis, and clustering with *E. xiangfangensis* LMG27195 strain (CP017183.1) (**Figure S1**).

The Kirby-Bauer disk diffusion and the Sensititre broth microdilution assays (ThermoFisher Scientific, Grenoble, France) showed that Exn692 exhibited resistance to 37 antibiotics that belonged to more than three antibiotic classes; confirming that Exn692 was MDR. The Carba NP and the Lateral Flow Immunoassay NG-Test Carba5 (NG-Biotech, Guipry, France) assays (11) detected the production of both NDM and VIM, which corresponded to *bla*<sub>NDM-1</sub> and *bla*<sub>VIM-4</sub> identified by WGS, respectively. Analysis using ResFinder v4.1 (12) showed that Exn692 carried 21 acquired AMR genes, encoding resistance to different clinically important antibiotic classes (**Table 1**). The isolate was susceptible to aztreonam-avibactam (MIC= 0.12 µg/mL), ceftiderocol (MIC= 2 µg/mL), and colistin (MIC= 1 µg/mL). Of these antibiotics, only colistin is currently available in Lebanon. Additionally, the MIC for ceftiderocol was high (i.e., at the susceptibility breakpoint), which is most likely due to the presence of MBLs and absence of mutations in the *ftsI* that encodes PBP3 (13). Moreover, using breakpoints established for *Escherichia coli* urinary tract infections, Exn692 would also be susceptible to fosfomycin (Ø= 30 mm) and nitrofurantoin (MIC≤ 32 µg/mL). Despite being resistant to all tested aminoglycosides associated with the presence of the 16S rRNA methyltransferase *rmtC*, Exn692 was susceptible to neomycin (MIC≤ 4 µg/mL) and apramycin (MIC= 2 µg/mL), both of which are restricted for veterinary use. Interestingly, the use of apramycin in humans is currently under evaluation in a phase I clinical trial (ClinicalTrials.gov NCT04105205) (14).

The isolate belonged to ST114, which represents a global epidemic *Enterobacter* clone previously reported in humans and animals and associated with various AMR determinants, particularly *bla*<sub>NDM-1</sub> and *bla*<sub>CTX-M-15</sub> (15-17). Notably, an MDR *E. xiangfangensis* LAU\_ENC1 belonging to ST114 and carrying only *bla*<sub>NDM-1</sub>- and *bla*<sub>CTX-M-15</sub> was recently reported in a patient's feces in Lebanon (18). Furthermore, we detected an average of 96 SNPs between Exn692 and the previously reported ST114 *E. xiangfangensis* in Lebanon (18, 19) (Figure S2). Plasmidfinder v2.0 identified several distinct plasmids in Exn692, namely IncC, IncFII, IncX3, and repB. These

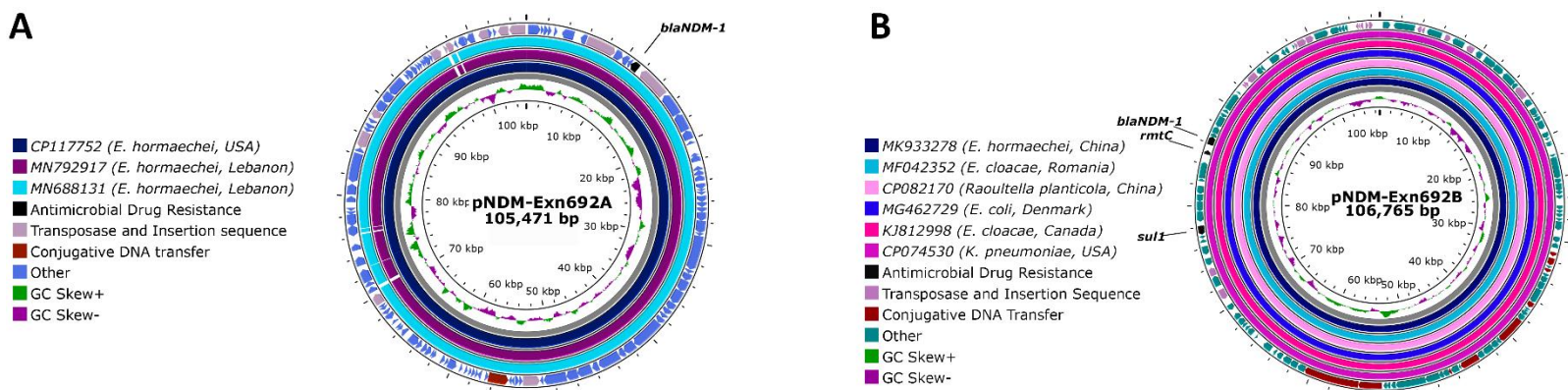
plasmids underwent comprehensive reconstruction via CLC Genomics Workbench 12.0 and subsequent annotation using Prokka 1.14.5 (<https://galaxy.pasteur.fr/>). It was found that the IncC plasmid (pVIM-Exn692) harbored *bla*<sub>VIM-4</sub> along with different AMR genes (*sul1*, *sul2*, *dfrA23*, *qacE*, *floR*, *aph(3'')*-*Ib*, *aac(6')*-*II*, *aph(6)*-*Id*, *tet(A)*, *msr(E)*, and *mph(E)*). The *bla*<sub>VIM-4</sub> resided within a class 1 integron, flanked by two IS26 copies. The analysis of the integron structures showed the presence of the *bla*<sub>VIM-4</sub> gene cassette as the first cassette, followed by *aac(6')*-*II* and *qacEΔ1* (**Figure 1**).





**Figure 1. Circular map of the IncC plasmid (196,427 bp) and comparative analysis with the three closest plasmids.** The BLAST search analysis did not identify any plasmid displaying an alignment coverage of more than 75%. (A) A generated map using the CGView Server (<https://proksee.ca/>) showing a full view of pVIM-Exn692A. The outer ring shows the coding sequences. Each coding sequence is color-coded according to its predicted function, as shown in the figure. The inner ring represents the GC skew graph. The three middle rings show BLAST comparison results (BLASTn) with the primary sequence. (B) The genetic structure surrounding the *bla*<sub>VIM-4</sub>. BlastN comparison of the region delineated in panel 1A was done using Artemis Comparison Tool (ACT) (insertion sequences (IS) that matched in various locations of the genomes were removed to reduce figure complexity) and was then modified by Inkscape. The cassette structure shows *bla*<sub>VIM-4</sub>, followed by the *aac(6')-II* and *qacEΔ1* allele typical of the 3'-CS of *sul1*-associated class 1 integrons. Notably, our analysis of the integron revealed an absence of identical cassette arrangement (100% identity and coverage).

Interestingly, we identified two copies of *bla*<sub>NDM-1</sub> carried by two different IncFII plasmids (pNDM-Exn692A and pNDM-Exn692B). The former carried *bla*<sub>NDM-1</sub> in proximity to a truncated ISAb<sub>a</sub>25 situated within a Tn3-like transposon cassette. Additionally, pNDM-Exn692B harbored *bla*<sub>NDM-1</sub> within a ~6-Kb region flanked by two copies of 256-bp Tn3-derived inverted-repeat transposable element (TIME), which potentially contributed to the mobilization of *bla*<sub>NDM-1</sub> (20, 21) (**Figure 2**). Subsequently, plasmids were extracted using the Kieser technique (22) and successfully transformed into electro competent *Escherichia coli* TOP10 as described elsewhere (22). Furthermore, conjugation experiments (23) using sodium azide-resistant *E. coli* J53 as the recipient strain confirmed the ability of the three plasmids to transfer the *bla*<sub>VIM-4</sub> and *bla*<sub>NDM-1</sub> genes horizontally. The *bla*<sub>CTX-M-15</sub> was found integrated into the chromosome within a phage genome, being 99.98% identical to the phage LAU1 (MN688132.1) previously described in *E. xiangfangensis* LAU\_ENC1 (18).



**Figure 2. Circular map of the IncFII plasmids (pNDM-Exn692A and pNDM-Exn692B) and comparative analysis with the closet plasmids carrying the *bla*<sub>NDM-1</sub>.** A generated map using the CGView Server (<https://proksee.ca/>) shows a full view of pNDM-Exn692A (105-Kb) and pNDM-Exn692B (106-Kb). The outer ring shows the coding sequences. Each coding sequence is color-coded according to its predicted function. The inner ring represents the GC skew graph. The middle rings show BLASTn comparison results with the primary sequence. (A) pNDM-Exn692A was highly similar (>99% identity and >97% alignment) to plasmid from *E. hormaechei* (CP117752) isolated in the United States in 2022 and to plasmids pLAU\_ENM30 (MN792917) and pLAU\_ENC1 (MN688131), both identified in *E. xiangfangensis* strains ST114 in Lebanon isolated between 2016 and 2018. (B) Closely related plasmids (>99% identity over 100% alignment) carrying *bla*<sub>NDM-1</sub> region have been detected across diverse species, isolated from different countries and settings (20, 21, 24-26).

*E. xiangfangensis* ST114 is already recognized as an epidemic clone that causes human infections worldwide and has been associated with *bla*<sub>NDM-1</sub> and *bla*<sub>CTX-M-15</sub> (17, 19). However, the presence of an additional carbapenemase gene, *bla*<sub>VIM-4</sub>, perhaps further elevates the public health

importance of this pathogen and sheds light on its ability to evolve and acquire important AMR determinants. To our knowledge, this is the first report of a *bla*<sub>NDM-1</sub><sup>-</sup>, *bla*<sub>VIM-4</sub><sup>-</sup>, and *bla*<sub>CTX-M-15</sub><sup>-</sup> co-harboring *E. xiangfangensis* in clinical settings worldwide. Notably, NDM-1 and VIM-4 were encoded by plasmid-borne genes and were transmissible to otherwise naïve recipient strains. The coexistence of these genes with the capacity for horizontal spread raises significant concerns regarding the emergence of MDR and highly virulent ECC clones in healthcare systems. Therefore, there is a paramount need to rigorously monitor the evolution of ECC and establish accurate identification tools for *E. xiangfangensis* to understand its epidemiology, pathogenesis, and AMR properties, especially in low- and middle-income countries that are facing public health challenges like Lebanon. This is critical because AMR is not limited by national borders and can expand rapidly to affect vulnerable populations across the globe. Despite the ongoing economic crisis in Lebanon, we highlighted the significance of integrating reliable identification and genomic methods in routine microbiology laboratories. Here, we argue that there is a peremptory need to invest and establish resilient surveillance systems that actively monitor the emergence of AMR in important pathogens in LMICs like Lebanon.

#### **Data availability**

The genome assembly (JAUTYX000000000) and the sequence of the plasmids pVIM-Exn692 (OR497351), pNDM-Exn692A (OR497352), and pNDM-Exn692B (OR497353) were submitted to GenBank database.

#### **Ethical approval**

This investigation received the approval (CE-EDST-1-2020) of the ethical committee of the Doctoral school of Science and Technology/Lebanese University, authorized by the Lebanese Ministry of Public Health. The data were analyzed anonymously.

#### **Conflict of interest**

The authors declare no conflict of interest.

#### **Acknowledgments**

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**Funding information**

The authors declare no specific funding

**Table 1.** Genome analyses and antimicrobial susceptibility patterns of the *bla*<sub>NDM-1</sub>, *bla*<sub>VIM-4</sub>, and *bla*<sub>CTX-M-15</sub>-co-harboring *Enterobacter xiangfangensis* (Exn692) strain from a bloodstream infection.

Isolate ID	GenBank accession number of assembled genome	Non-susceptibility to selected antimicrobials <sup>a,*</sup>	Susceptibility to selected antimicrobials <sup>b,*</sup>	Acquired antimicrobial resistance genes <sup>c</sup> (alignment length/gene length)	QRDR <sup>d</sup> mutations	Plasmid replicons detected by WGS (identity %)	Sequence type	Virulence genes <sup>e</sup>
Exn692	JAUTYX000000000	AMX, AMC, TIC, TCC, PIP, TZP, TEM, CXN, CXM, FOX, CTX, TIO, CAZ, CAZ/AVI, FEP, CFT, BPR, ETP, IMP, IMP/REL, MEM, MEM/VAB, MEM, MEM/VAB, MEC, ATM, STR, GMN, TMN, AMK, TET, TIG, ERV, CHL, CIP, LVX, SXT	ATM/AVI, FDC, CST, FSF, FUR, NEO, APR	<i>bla</i> <sub>NDM-1</sub> (813/813), <i>bla</i> <sub>VIM-4</sub> (801/801), <i>bla</i> <sub>OXA-1</sub> (831/831), <i>bla</i> <sub>CTX-M-15</sub> (876/876), <i>msr(E)</i> (1476/1476), <i>mph(E)</i> (885/885), <i>aac(6')-Ib-cr</i> (600/600), <i>aac(3)-IIa</i> (861/861), <i>aph(6)-Id</i> (837/837), <i>rmtC</i> (846/846), <i>aph(3'')-Ib</i> (804/804), <i>aac(6')-II</i> (459/459), <i>qnrB1</i> (645/645), <i>dfrA14</i> (474/474), <i>dfrA23</i> (561/561), <i>sul1</i> (840/840), <i>sul2</i> (816/816), <i>tetA</i> (1260/1275), <i>catB3</i> (442/663), <i>erm(42)</i> (906/906), <i>qacE</i> (282/333)	GyrA S83L ParC S80I	IncC (100%), IncFII (PkpX1) (100%), IncFII (YP) (99.13%), IncX3 (100%), repB (99.2%)	ST114	<i>rpoS</i> , <i>csqD</i> , <i>flgG</i> , <i>flgH</i> , <i>cheZ</i> , <i>cheB</i> , <i>cheR</i> , <i>motA/B</i> , <i>flhC/D</i> , <i>fliZ</i> , <i>fliC</i> , <i>fliG</i> , <i>fliN</i> , <i>cheY</i> , <i>motB</i> , <i>fliY</i> , <i>fliJ</i> , <i>fliM</i> , <i>fliQ</i> , <i>ompA</i> , <i>nmpC</i> , <i>entA</i> , <i>entB</i> , <i>entE</i> , <i>entS</i> , <i>fepA/C/D</i> , <i>clpB</i> , <i>gnda</i> , <i>galF</i> , <i>resB</i> , <i>arcA</i> , <i>arcB</i> , <i>clpB</i> .

<sup>a</sup> AMX, amoxicillin; AMC, amoxicillin-Clavulanate; TIC, ticarcillin; TCC, ticarcillin-clavulanate; PIP, piperacillin; TZP, piperacillin-tazobactam; TEM, temocillin; CXN, cephalixin; CXM, cefuroxime; FOX, cefoxitin; CTX, cefotaxime; TIO, ceftiofur; CAZ, ceftazidime; CAZ/AVI, ceftazidime-avibactam; FEP, cefepime; CFT, ceftaroline; BPR, ceftobiprole; ERT, ertapenem; IMP, imipenem; IMP/REL, imipenem-relebactam; MEM, meropenem; MEM/VAB, meropenem-vaborbactam; MEC, mecillinam; ATM, aztreonam; STR, streptomycin; GMN, gentamicin; TMN, tobramycin; AMK, amikacin; TET, tetracycline; TIG: tigecycline; ERV, eravacycline; CHL, chloramphenicol; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulfamethoxazole.

<sup>b</sup> ATM/AVI, aztreonam-avibactam; FDC, cefiderocol; FSF, fosfomicin; CST, colistin; NEO, neomycin; APR, apramycin; FUR, nitrofurantoin.

<sup>c</sup> Acquired antimicrobial drug resistance genes detected by ResFinder V4.1 using 99% identity as cut-off. The intrinsic antimicrobial drug resistance gene, *bla*<sub>ACT-16</sub>, was also detected (alignment length=1140/gene length=1140). No mutations in the *ftsI* encoding PBP3 were identified, which explains the susceptibility of the isolate to cefiderocol (MIC=2 µg/mL) and aztreonam-avibactam (MIC=0.12 µg/mL).

<sup>d</sup> QRDR, quinolone resistance-determining region.

<sup>e</sup> Virulence genes were screened using the Virulence Factor Database (VFDB) with a cutoff of 70% identity and 60% alignment length. This was chosen due to the absence of *Enterobacter* virulence genes in VFDB. The identified candidates were compared against RAST annotations and were subjected to a BLASTx search. Only those annotated by RAST and/or exhibiting 100% identity and alignment length in the BLAST search were retained.

\* Clinical breakpoints were adopted according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (<https://www.sfm-microbiologie.org/>) and the Clinical and Laboratory Standards Institute (CLSI-M100 and CLSI VET01S ED6:2023). As apramycin and neomycin are only for veterinarian use, the breakpoints set by the CA-SFM for veterinarian recommendations (<https://www.sfm-microbiologie.org/2021/12/10/casfm-veterinaire-2021/>) and CLSI VET01S ED6:2023 were used. *Escherichia coli* clinical breakpoints were used for nitrofurantoin and fosfomicin, as no breakpoints for ECC exist for these molecules

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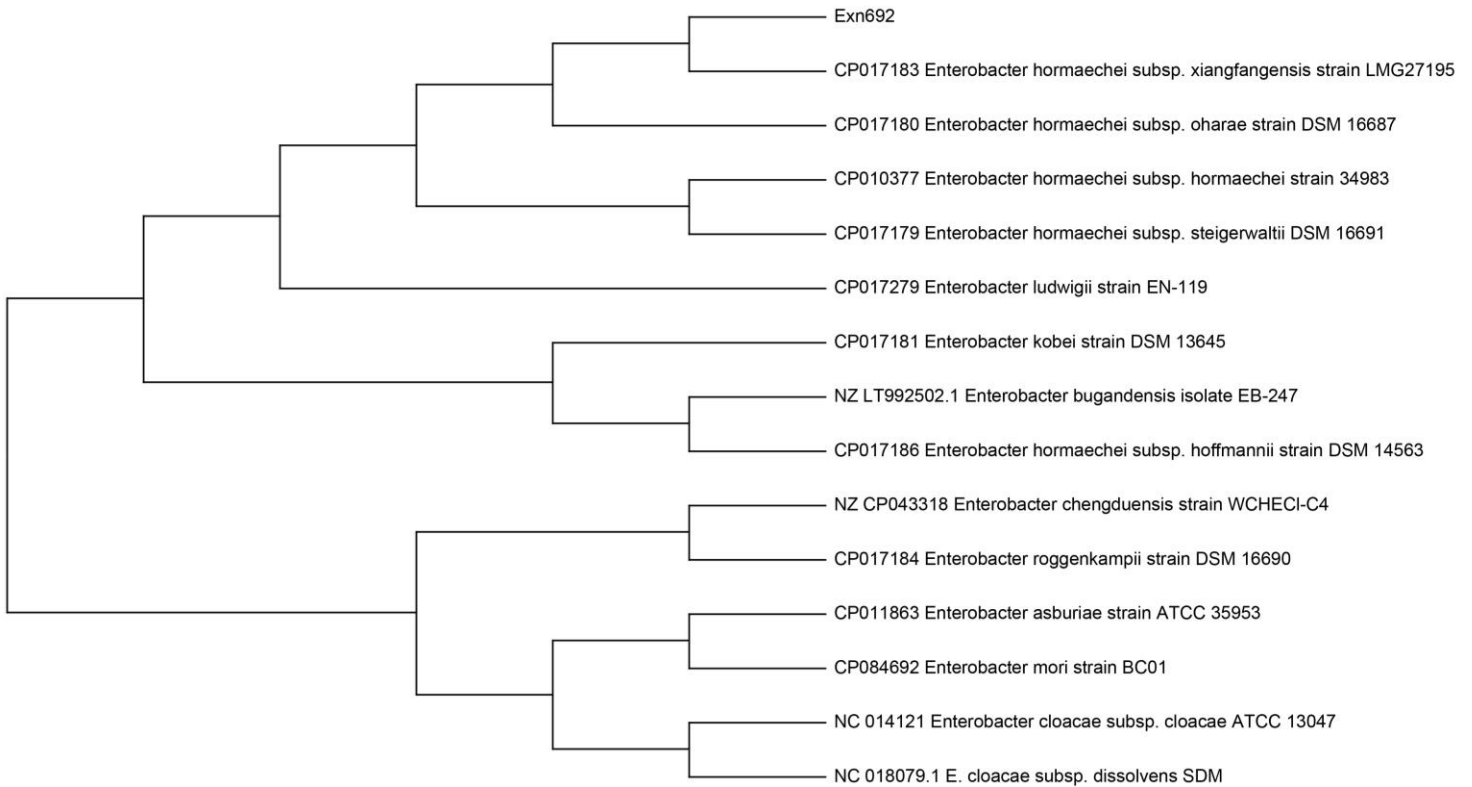
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## Supplementary materials

### Supplementary figures



**Figure S1. Phylogenetic tree constructed by the neighbor-joining method using MEGA 11 software.** The *hsp60* gene of the *Enterobacter xiangfangensis* (Exn692) isolate was compared to 14 reference strains (*Enterobacter hormaechei* subsp. *xiangfangensis* CP017183; *Enterobacter hormaechei* subsp. *oharae* CP017180; *Enterobacter hormaechei* subsp. *hormaechei* CP010377; *Enterobacter hormaechei* subsp. *steigerwaltii* CP017179; *Enterobacter ludwigii* CP017279; *Enterobacter kobei* CP017181; *Enterobacter bugandensis* NZ\_LT992502.1; *Enterobacter hormaechei* subsp. *hoffmannii* CP017186; *Enterobacter chengduensis* NZ\_CP043318; *Enterobacter roggenkampii* CP017184 ; *Enterobacter asburiae* CP011863; *Enterobacter mori* CP084692; *Enterobacter cloacae* subsp. *cloacae* NC\_014121; and *Enterobacter cloacae* subsp. *dissolvens* NC018079.1) used for species identification of the *Enterobacter cloacae* complex (ECC) isolate.



**4. Article 4: Neonatal sepsis due to NDM-1 and VIM-2 co-producing *Pseudomonas aeruginosa* in Morocco**

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Keywords: carbapenem resistance, *Pseudomonas aeruginosa*, MBLs, XDR, neonatal infection

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## Abstract

We isolated an extremely drug-resistant (XDR) *Pseudomonas aeruginosa* that co-produced NDM-1 and VIM-2 from a blood culture of a new-born hospitalized in the neonatal intensive care unit in Morocco. The isolate was only susceptible to colistin and apramycin. This is of great concern due to limited treatment options in neonates.

The resistance of *Pseudomonas aeruginosa* to carbapenems is worryingly increasing, leading to ever-restricted therapeutic choices that mainly rely on colistin, an old repurposed but toxic drug, and a few relatively newer molecules that are not yet available in low and middle income countries (LMICs) (1). *P. aeruginosa* is intrinsically resistant to many antimicrobials through the constitutive expression of several efflux pumps, low permeability of the outer membrane, and production of the chromosome-encoded cephalosporinase (PDC) (1). While carbapenem resistance is frequently associated with membrane impermeability, acquired transferrable carbapenemases have been increasingly described (2). Among the latter, VIM- and IMP-type metallo- $\beta$ -lactamases (MBLs) are the most common worldwide, with VIM-types encountered more frequently in Europe and IMP-types in Southeast Asia (3). Both confer resistance to all beta-lactams except monobactams and are characterized by their high carbapenem hydrolysis compared to other non-MBL carbapenemases (4). Notably, the co-expression of two MBLs in *P. aeruginosa* is thought to be rare, likely due to fitness costs associated with the production of two carbapenemases (5).

In Morocco, VIM-2-producing *P. aeruginosa* has been described previously (6), and two NDM-VIM- and one VIM-IMP-co-producing *P. aeruginosa* isolated from two women (49 and 70 years-old) and a man (62 years-old) and characterized enzymatically (7). However, previous studies in Morocco have focused largely on carbapenem resistance in Enterobacterales, perhaps resulting in less attention to *P. aeruginosa* (8). Here, we report an extremely drug-resistant (XDR) *P. aeruginosa* that co-produced NDM-1 and VIM-2 and was notably resistant to novel last-resort antibiotics, including cefiderocol and aztreonam-avibactam. These drugs are recommended to treat infections by XDR *P. aeruginosa*.

*P. aeruginosa* O82J1 was isolated from a blood culture of a newborn hospitalized in the neonatal intensive care unit (NICU) in Ibn Rochd University Hospital Center (Casablanca, Morocco) in December, 2019. The isolate was identified by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Antimicrobial susceptibilities were determined by the Kirby-Bauer disk diffusion technique (Bio-Rad, Marnes-La-Coquette, France) and by broth microdilution (Sensititre, Thermofisher, Grenoble, France) and interpreted according to the guidelines of EUCAST 2023 ([https://www.eucast.org/clinical\\_breakpoints](https://www.eucast.org/clinical_breakpoints)) and the Antibiogram Committee of the French Society for Microbiology (CA-SFM, 2023) for veterinary use (<https://www.sfm-microbiologie.org/>). Several novel molecules were also tested including, imipenem/relebactam, meropenem/vaborbactam, aztreonam/avibactam, ceftazidime/avibactam, cefiderocol, eravacycline, apramycin, and delafloxacin. The antibiograms revealed that this isolate was XDR. MICs confirmed that the isolate was resistant to all  $\beta$ -lactams, including carbapenems, cefiderocol, novel  $\beta$ -lactam-inhibitor combinations, chloramphenicol, ciprofloxacin, levofloxacin, tetracycline, eravacycline, amikacin, tobramycin, gentamicin, and neomycin. The isolate remained susceptible to colistin, which is not recommended for treating neonatal infections, and apramycin, a veterinary-restricted antibiotic which is currently under evaluation in human clinical studies (**Table 1**). Carbapenem-hydrolyzing activity was detected *P. aeruginosa* O82J1 using the Carba NP test. Furthermore, the NG-Test Carba5 lateral flow immunoassay (NG-Biotech, Guipry, France) detected two MBLs; NDM- and VIM-type.

**Table 1.** Resistome and the antimicrobial susceptibility pattern of *Pseudomonas aeruginosa* O82J1.

ID	Hospital	Sampling date	MLST <sup>1</sup>	$\beta$ -lactam resistance genes <sup>1</sup>	Non $\beta$ -lactam resistance genes <sup>1</sup>	Non-susceptible profile <sup>2</sup>	MIC values (mg/L)	Susceptible Profile	MIC values (mg/L)
O82J1	Ibn Rochd University Hospital Center	Dec 2019	ST773	<b>Chromosome-borne (intrinsic)</b> <i>bla<sub>PAO</sub></i> , <i>bla<sub>OXA-395</sub></i>	<b>Chromosome-borne (intrinsic)</b> <i>aph(3')-IIb</i>	FSF	>256	APR	16
						AMC, CXN	>128	CST	2
						TIC, PIP, TPZ, FOX, CAZ, CFT, CAZ/AVI, IMP, MEM, MEM/VAB, AMK, NEO, STR	>32		
						MEC, ATM, ATM/AVI, FEP, CHL, IMP/REL, TET	>16		
						CFDC	8		
						CTX, ETP, GEN, SXT, TOB	>8		
						ERV, LEV	>4		
						CEF, CIP, TIG, DLX	>2		
						BPR	>1		

1 MLST and antimicrobial drug resistance genes detected by MLST 2.0 and ResFinder V4.3.3, respectively (<http://www.genomicepidemiology.org/services/>).

2 AMC, amoxicillin-clavulanate; AMK, amikacin; APR, apramycin; ATM, aztreonam; ATM/AVI, aztreonam-avibactam; BPR, ceftobiprole; CAZ, ceftazidime; CAZ/AVI, ceftazidime-avibactam; CEF, ceftiofur; CFT, ceftaroline; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; CXN, cephalexin; DLX, Delafloxacin; ETP, ertapenem; ERV, eravacycline; CFDC, cefiderocol; FEP, cefepime; FSF, fosfomicin; FOX,

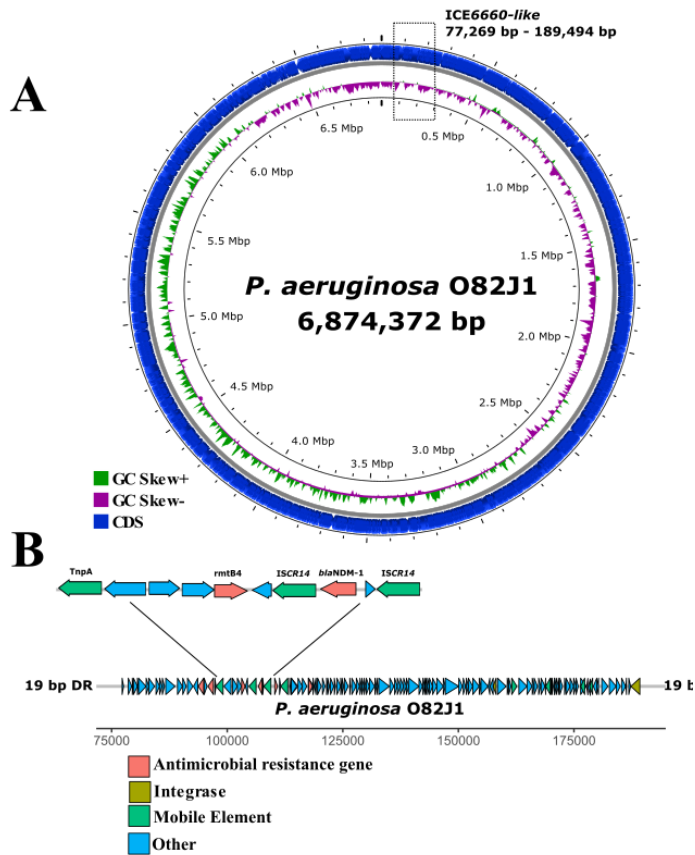
cefoxitin; GEN, gentamicin; IMP, imipenem; IMP/REL, imipenem-relebactam; LEV, levofloxacin; MEC, mecillinam; MEM, meropenem; MEM/VAB, meropenem-vaborbactam; NEO, neomycin; PIP, piperacillin; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TIC, ticarcillin; TIG: tigecycline; TOB, tobramycin; TPZ, piperacillin-tazobactam. Clinical breakpoints were adopted according to the guidelines of EUCAST 2023 ([https://www.eucast.org/clinical\\_breakpoints](https://www.eucast.org/clinical_breakpoints)) and the Antibioqram Committee of the French Society for Microbiology (CA-SFM) for veterinary use (<https://www.sfm-microbiologie.org/>).

*P. aeruginosa* O82J1 was further evaluated by whole-genome sequencing using the Ultra II DNA Library Prep Kit for Illumina (NEB, Évry-Courcouronnes, France) on a NextSeq 500 sequencer (Illumina, San Diego, USA) and MinION sequencer (Oxford Nanopore, Oxford, UK), as described previously (9). MinION contigs were corrected using Illumina short reads using CLC Genomics Workbench v12 (Qiagen, Les Ulis, France) (10). Assembled genome and plasmid were annotated using RAST server (<https://rast.nmpdr.org/>). Sequence type (ST), acquired antimicrobial resistance genes, and plasmid incompatibility types were determined using the CGE server (<http://www.genomicepidemiology.org/services/>). The assembled genome and plasmid were deposited in GenBank database (Accession numbers JAUTDQ000000000 and OR682658, respectively).

*P. aeruginosa* O82J1 belonged to ST773, which is considered one of the most frequently reported high-risk *P. aeruginosa* internationally. ST773 is detected worldwide and frequently associated with mobile genetic elements, which play a key role in the emergence and spread of resistance genes (11).

Genomic analysis of antimicrobial resistance determinants showed that *bla*<sub>NDM-1</sub>, the natural *bla*<sub>P<sub>AO</sub></sub>, and *bla*<sub>OXA-395</sub> genes were located on the chromosome. Additionally, genes associated with resistance to aminoglycoside (*aph*(3')-IIb, *aadA10*, and *rmtB*), sulfonamide (*sul1*), fosfomycin (*fosA*), quinolones (*qnrVC1*), tetracycline (*tet*(G)), and amphenicol (*catB7*) were also carried on the chromosome (**Figure 1**). Of note, *P. aeruginosa* O82J1 carried the 16S-rRNA methyltransferase gene *rmtB* that confers resistance to all aminoglycosides and has been previously reported to colocalize with genes encoding MBLs, especially NDM. *P. aeruginosa* O82J1 harbored an integrative conjugative element (ICE) of c.a. 112-kb that was identical (100% coverage, 100% identity) to the ICE6660-like carrying the *bla*<sub>NDM-1</sub> gene in a previously sequenced *P. aeruginosa* strain PSE6684 isolated from a urine sample in South Korea (Accession number:

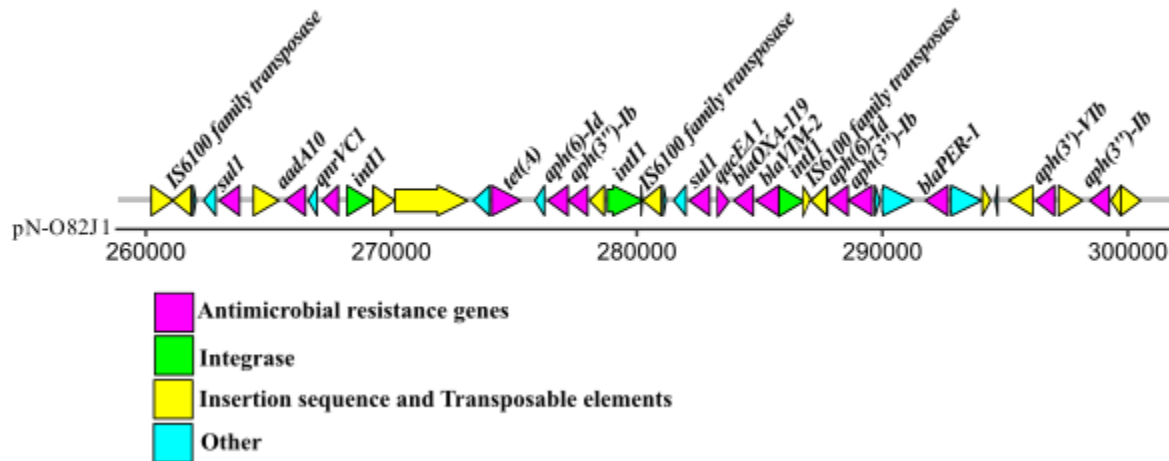
CP053917.1) (12). This region was inserted into the *tRNA<sub>gly</sub>* gene by a P4 prophage integrase. The 19 bp direct repeat (DR), corresponding to the ICE6660-like *attL* site-specific recombination site was TTGGAGCGGGAAACGAGAC (77798-77816 bp) and the paired 19 bp ICE6660-like *attR* site was TTGGAGCGGGAAACGAGAC (189476-189494 bp). The chromosomal location of *bla<sub>NDM-1</sub>* was previously reported in a few *P. aeruginosa* isolates from India and in some Enterobacterales (13).



**Figure 1:** Schematic representation of the genetic context of *P. aeruginosa* O82J1 strain. (A) Circular genome map representation. The blue ring show *P. aeruginosa* O82J1 strain's coding sequences. G + C positive skew, green peak; G + C negative skew, purple peak. ICE6660-like region from 77,269 to 189,494 bp is also marked within the dotted rectangular. (B) Genetic context of *bla<sub>NDM-1</sub>* gene within the ICE6660-like. The 19-bp direct repeat (DR), ICE6660-like *attL* site-specific recombination site was TTGGAGCGGGAAACGAGAC (77798-77816 bp) and the paired 19-bp ICE6660-like *attR* site was TTGGAGCGGGAAACGAGAC (189476-189494 bp).



The *bla*<sub>VIM-2</sub> gene was located on plasmid pN-O82J1 and integron-borne together with *bla*<sub>OXA-119</sub>, which encodes a narrow spectrum oxacillinase belonging to the OXA-46 family (14). The integron was surrounded by two IS6100, forming a likely composite transposon inserted into a multi-drug resistance region that contained other determinants that encoded resistance to aminoglycosides (three copies of *aph*(3'')-Ib, two copies of *aph*(6)-Id), and one copy of *aph*(3')-VIIb), sulfonamides (*sulI*), tetracyclines (*tet*(A)), quinolones (*qnrVC1*), quaternary ammonium compounds (*qacE*), and β-lactams (*bla*<sub>PER-1</sub>) (**Figure 2**). The latter encodes a minor extended-spectrum β-lactamase (ESBL) which was frequently described in *P. aeruginosa* from Turkey. Although the *bla*<sub>VIM-2</sub> gene was previously described in NDM-1-producing *P. aeruginosa* isolates from India, it was suggested to be horizontally transferred via an IncF plasmid and associated with different integron-borne gene cassettes (3). However, mating out experiments to show the transferability of plasmid pN-O82J1 could not be performed due to the XDR phenotype of our isolate.



**Figure 2:** Genetic context of the *bla*<sub>VIM-2</sub> gene.

## Conclusions

Here, we have characterized the complete genome sequence of *P. aeruginosa* O82J1, which carried two MBLs genes; a chromosome inserted *bla*<sub>NDM-1</sub> and plasmid encoded *bla*<sub>VIM-2</sub>. Our study suggest that dual MBLs-producing isolates may have already been emerging in Moroccan hospitals as early as 2019.

The isolation of XDR *P. aeruginosa* isolates expressing several carbapenemases in a neonatal intensive care unit is of great concern due to the reduced treatment options that could rely on colistin, but not recommended in neonates, and apramycin, not yet approved for human therapy. Concerns were further elevated due to the resistance of our isolate to ceftiderocol, a novel last-resort antibiotic recommended to treat infections caused by Gram-negative bacteria, particularly drug-resistant *P. aeruginosa* in adults (1). To date, information about the use of ceftiderocol in pediatric cases is limited, despite various studies that have reported *in vitro* data suggesting its potential effectiveness in neonates infected with carbapenem-resistant bacteria (15). However, our findings highlight that the prospects of using ceftiderocol in treating infections caused by dual MBL-producing strains might be jeopardized. Moreover, another MBL-specific antibiotic combination aztreonam/avibactam had also MICs in the resistance range, when using the breakpoints of aztreonam. Notably, the isolate carried a plasmid-borne antiseptic resistance gene (*qacE*), which potentially allows the persistence of this bacterium in the NICU and hospital environment. Taken together, the occurrence of these strains in NICUs and beyond is a significant concern because treatment options are severely limited.

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### **Competing interests**

The authors declare no conflict of interest.

### **About the Author**

Ms. Daaboul is a PhD student with supervision shared between Université Paris-Saclay and the Lebanese University. Her work focuses on antimicrobial resistance and One Health.

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## II. Résistance aux carbapénèmes en milieu extra-clinique au Liban

Dans cette partie notre travail visait à identifier et à caractériser les Entérobactéries productrices de carbapénémase en milieu extra-clinique au Liban. Dans nos études précédentes menées dans le secteur clinique, nous avons mis en évidence la dissémination d'isolats de *E. coli* NDM-5-positifs.

Le travail présenté ci-dessous se divise selon 2 axes :

En premier, une étude est menée dans des fermes au Nord du Liban. Les échantillons sont collectés des différents animaux dans les fermes ainsi que des sources d'eau utilisées. Les résultats de cette étude nous ont permis d'identifier pour la première fois la colonisation d'un chien de ferme par une souche d'*Escherichia coli* productrices de *bla*<sub>NDM-5</sub>. Ainsi, que la colonisation d'un chien et d'une vache dans une ferme, par le gène *mcr-1* codant pour la résistance à la colistine.

Ensuite, une étude est menée au sein de 4 camps de réfugiés Syriens, à Akkar au Nord du Liban. La crise humanitaire provoquée par le conflit syrien est considérée comme l'une des plus grandes crises mondiales de réfugiés de l'histoire moderne. Le conflit en Syrie, comme tout autre conflit, offre des conditions idéales pour le développement et l'émergence de la résistance aux antimicrobiens. De plus, des infections causées par des agents multirésistants ont été documentées, soulignant la nécessité d'utiliser des antibiotiques de dernier recours pour traiter ces populations. Les camps de réfugiés syriens sont souvent caractérisés par la surpopulation, des installations sanitaires inadéquates et un accès limité à l'eau potable. Ces conditions créent un environnement propice à la propagation de maladies infectieuses, notamment d'infections bactériennes multirésistantes. Les réfugiés originaires de pays présentant une forte prévalence de résistance aux antimicrobiens pourraient être porteurs d'isolats résistants vers leur nouveau pays de résidence.

# 1. Article 5: The occurrence of carbapenemase gene *bla*<sub>NDM-5</sub>, on a transmissible IncX3 plasmid in multidrug-resistant *Escherichia coli* isolated from a Farm Dog

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Genome Note

## The occurrence of the carbapenemase gene, *bla*<sub>NDM-5</sub>, on a transmissible IncX3 plasmid in multidrug-resistant *Escherichia coli* isolated from a farm dog



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### ABSTRACT

**Objectives:** In-depth phenotypic and genomic analyses on a carbapenem-resistant *Escherichia coli* isolate, recovered from the faeces of a farm dog in Lebanon, focusing on its antimicrobial resistance (AMR) patterns and the underlying resistome.

**Methods:** *E. coli* strain EC-106 was identified using MALDI-TOF-MS. Analyses using Carba NP, immunochromatographic assay NG Carba5, and other antimicrobial susceptibility testing were performed. Whole-genome sequencing (WGS) using the Illumina technology and different software available at the Center of Genomic Epidemiology were used to predict the resistome, sequence type (ST), plasmid types, and virulence genes.

**Results:** Susceptibility testing revealed that *E. coli* EC-106 was multi-drug resistant, including against newer antimicrobials such as imipenem-relebactam (MIC = 16 µg/mL), meropenem-vaborbactam (MIC = 16 µg/mL), and ceftazidime-avibactam (MIC > 32 µg/mL), but remained susceptible to aztreonam (MIC = 0.12 µg/mL), aztreonam-avibactam (MIC = 0.06 µg/mL), and cefiderocol (MIC = 0.5 µg/mL). WGS analyses showed that *E. coli* EC-106 carried 13 acquired resistance genes associated with resistance to β-lactams (*bla*<sub>NDM-5</sub> and *bla*<sub>TEM-1B</sub>), aminoglycosides (*aac(3)-IId*, *aph(3')-Ia*, *aadA1*, and *aadA2*), tetracyclines (*tetA*), amphenicols (partial *catA1*), macrolides (*mphA*), sulphonamides (*sul1* and *sul3*), trimethoprim (*dhfrA12*), and quaternary ammonium compounds (partial *qacE*). The *bla*<sub>NDM-5</sub> was located on an IncX3 plasmid. The isolate was predicted to be a human pathogen (92.9%) and belonged to ST1011.

**Conclusion:** To our knowledge, this is the first report of the detection of an IncX3 plasmid carrying the *bla*<sub>NDM-5</sub> gene in animals in Lebanon, highlighting the severe AMR challenges in the country. Taken together, our current and previous findings suggest that *bla*<sub>NDM-5</sub> might be spreading in different hosts and genetic backgrounds across clinical and non-clinical settings.

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The inappropriate use of carbapenems has played an important role in the global emergence of carbapenem-resistant bacteria, which has posed a public health threat. The latter is especially pertinent in low- and middle-income countries (LMICs) where surveillance programs and antimicrobial stewardship pro-

grams are typically inefficient and/or not well developed. Furthermore, the spread of bacteria that are resistant to high-priority antibiotics in LMICs has drastically limited treatment options, leading to adverse clinical outcomes and elevated costs of medical care [1].

In Lebanon, a Mediterranean country with increasing antimicrobial resistance (AMR) and a plethora of other public health challenges [2–5], carbapenemase-producing Enterobacterales have been reported in healthcare settings [6], the community [5,7], and the environment [8] in the past decade. Despite companion and farm animals being common carriers of multidrug-resistant (MDR) bacteria and AMR determinants, little is known about the epidemiology of carbapenem resistance in these hosts in Lebanon. To date, only a few sporadic reports showed the emergence of carbapenemase-encoding determinants in food-producing animals [9]. Notably, AMR surveillance programs in animals are generally unavailable in Lebanon. This existing knowledge gap poses an additional and considerable obstacle to the understanding of the burden of carbapenem resistance in Lebanon. To address this gap, we report here the isolation and in-depth characterisation of a *bla*<sub>NDM-5</sub>-harboring *Escherichia coli* isolated from the faeces of a farm dog, further highlighting the importance of companion animals as reservoirs and potential sources of important AMR determinants.

In July 2022, during a national screening effort, a fresh faecal sample was collected from a pet dog on a dairy farm in North Lebanon. The sample was placed on ice, transferred to the laboratory, and analyzed within 4 h of collection. The sample was resuspended in 0.9% sterile saline water and homogenised by pipetting. As previously described [7], an aliquot (100 µL) was spread onto an Enterobacterales selective medium, RAPID'E. coli 2 agar (Bio-Rad, Hercules, California) plates, supplemented with 1 µg/mL ertapenem (Sigma-Aldrich, St. Louis, Missouri). After incubation for 24 h at 37 °C under aerobic conditions, *E. coli* colonies were selected and purified.

The identity of the isolates was further confirmed as *E. coli* using MALDI-TOF VITEK MS Version 3.0 (bioMérieux, Marcy L'Etoile, France). For one isolate (EC-106), the Carba NP test was positive and the immunochromatographic assay NG Carba5 (NG Biotech, Guipry, France) [6] detected the production of the New Delhi metallo-beta-lactamase (NDM) that was confirmed as *bla*<sub>NDM-5</sub> after whole-genome sequencing (WGS; Illumina). Antimicrobial susceptibility was evaluated using the Kirby Bauer disk diffusion method and the Sensititre broth microdilution system (ThermoFisher Scientific, Grenoble, France), which showed that EC-106 was MDR, exhibiting resistance to 33 antimicrobials. Notably, EC-106 was resistant to most of the β-lactams tested, including carbapenems with or without inhibitors (Table 1). However, the isolate remained susceptible to aztreonam, aztreonam-avibactam, ceftiderocol, fosfomycin, colistin, amikacin, apramycin, tigecycline, eravacycline, and nitrofurantoin.

WGS analysis was conducted as described previously [10] and showed that EC-106 belonged to ST1011, which has been previously detected among *mcr-1*-positive *E. coli* isolated in clinical settings [11] and the poultry sector [12] in Lebanon. Analysis using ResFinder v4.1 revealed that EC-106 carried 13 acquired AMR genes, encoding resistance to clinically- and veterinary-important antimicrobials of different classes such as beta-lactams, aminoglycosides, tetracyclines, amphenicols, folate pathway antagonists, and quaternary ammonium compounds (Table 1). Notably, neither ESBL genes nor mutations in the *ftsI* encoding PBP3 were identified [13], which explains the high susceptibility of the isolate to ceftiderocol (MIC = 0.5 µg/mL), aztreonam (MIC = 0.12 µg/mL), and aztreonam-avibactam (MIC = 0.06 µg/mL).

Additionally, analysis using Plasmidfinder v2.1 only detected IncFIB and IncX3 plasmids. *bla*<sub>NDM-5</sub> was found to be located on the IncX3 plasmid, revealing a notable potential source for this impor-

**Table 1**  
Genomic analysis and antimicrobial susceptibility patterns of the *bla*<sub>NDM-5</sub>-carrying *Escherichia coli* EC-106 isolated from the faeces of a farm dog.

Isolate ID	GenBank accession number of assembled genome	Genome size (bp)	No. of contigs	N50 (bp)	L50	GC (%)	Non-susceptibility to selected antimicrobials <sup>a</sup> *	Susceptibility to antimicrobials <sup>b</sup> *	Acquired antimicrobial resistance genes <sup>c</sup>	Quinolone resistance-determining region (QRDR) mutations	Plasmid replicons detected by WGS (identity %) <sup>d</sup>	Sequence type <sup>e</sup>	Virulence genes <sup>f</sup>
EC-106	JAFEXY000000000	5139219	157	172471	10	50.4	AMX, AMC, TIC, TCC, PIP, TZP, TEM, CXN, CXM, FOX, CTX, TIO, AMK, APR, TGC, CAZ, CAZ/AVI, FEP, CFT, BPR, ERT, IMP, IMP/REL, MEM, MEM/VAB, MEC, STR, GMN, TMN, NEO, TET, CHL, FLO, CIP, LVX, SXT	ATM, ATM/AVI, FDC, FSF, CST, AMK, APR, TGC, ERV, FUR	<i>bla</i> <sub>NDM-5</sub> ; <i>bla</i> <sub>TEM-1B</sub> ; <i>aac(3)-IId</i> ; <i>aph(3')-Ia</i> ; <i>aadA1</i> ; <i>aadA2</i> ; <i>tetA</i> ; <i>catA1</i> (partial); <i>mphA</i> ; <i>sufI</i> ; <i>suI3</i> ; <i>dfrA12</i> ; <i>qacE</i> (partial)	<i>gyrA</i> (S83L), <i>gyrA</i> (D87N), <i>parC</i> (S80I)	IncX3 (100%); IncFIB (99.82%)	ST1011	<i>fimH</i> ; <i>chuA</i> , <i>hlyF</i> ; <i>cb</i> , <i>cm</i> , <i>cyoC</i> , <i>iroN</i> , <i>sitA</i> , <i>iss</i>

<sup>a</sup> AMX, amoxicillin; AMC, amoxicillin-clavulanate; TIC, ticarcillin; TCC, ticarcillin-clavulanate; PIP, piperacillin; TZP, piperacillin-tazobactam; TEM, temocillin; CXN, cephalaxin; CXM, cefuroxime; FOX, cefoxitin; CTX, ceftaxime; TIO, ceftiofur; CAZ, ceftazidime; CAZ/AVI, ceftazidime-avibactam; FEP, cefepime; CFT, ceftazidime; BPR, ceftibiprole; ERT, ertapenem; IMP, imipenem; IMP/REL, imipenem-relebactam; MEM, meropenem; MEM/VAB, meropenem-vaborbactam; MEC, mecillinam; STR, streptomycin; GMN, gentamicin; TMN, tobramycin; NEO, neomycin; TET, tetracycline; CHL, chloramphenicol; FLO, florfenicol; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulfamethoxazole.  
<sup>b</sup> ATM, aztreonam; ATM/AVI, aztreonam-avibactam; FDC, ceftiderocol; FSF, fosfomycin; CST, colistin; AMK, amikacin; APR, apramycin; TGC, tigecycline; ERV, eravacycline; FUR, nitrofurantoin.  
<sup>c</sup> Acquired antimicrobial drug resistance genes were detected by ResFinder v4.1 (<http://genepi.food.dtu.dk/resfinder>) using 99% identity as a cut-off.  
<sup>d</sup> Plasmid types were detected by PlasmidFinder v2.1 (<https://cge.food.dtu.dk/services/PlasmidFinder/>) using 95% identity as cut-off.  
<sup>e</sup> Sequence type (ST) was determined by MLSTFinder v2.0.9 (<https://cge.food.dtu.dk/services/MLST/>).  
<sup>f</sup> Virulence genes detected by VirulenceFinder v2.0 (<https://cge.food.dtu.dk/services/VirulenceFinder/>) using 100% identity as a cut-off.  
\* Clinical breakpoints were adopted according to the guidelines of the AntibioGram Committee of the French Society for Microbiology (CA-SFM) (<https://www.sfm-microbiologie.org/>). Notably, no clinical breakpoints are available in the CA-SFM for neomycin and florfenicol, so we adopted those breakpoints for these antimicrobials from the guidelines of the veterinary CA-SFM.

tant gene. The reads were aligned to a *bla*<sub>NDM-5</sub>-harboring *E. coli* reference genome (BioProject: PRJNA288601), revealing an exact match between the IncX3 plasmid of our isolate and the previously identified 46kb IncX3 plasmid (CP117206.1) in the United States. Plasmids were extracted using the Kieser technique [14], and successfully transformed into electrocompetent *E. coli* NCTC 50192. Additionally, conjugation experiments [15] using sodium azide-resistant *E. coli* J53 as the recipient strain, confirmed the ability of IncX3 to transfer *bla*<sub>NDM-5</sub> horizontally. IncX3-type plasmids have high stability, low fitness cost, and self-transferability, with a conjugation frequency ranging from 10<sup>-9</sup> and 10<sup>-2</sup> [16,17]. These plasmids are also frequently associated with the transmission of NDM variants (including *bla*<sub>NDM-1</sub>, *bla*<sub>NDM-4</sub>, *bla*<sub>NDM-5</sub>, *bla*<sub>NDM-7</sub>, *bla*<sub>NDM-13</sub>, *bla*<sub>NDM-17</sub>, *bla*<sub>NDM-19</sub>, *bla*<sub>NDM-20</sub>, and *bla*<sub>NDM-21</sub>) [16]. Importantly, IncX3 might play a crucial role in the global dissemination of AMR genes in Enterobacterales, conferring resistance to critically important antimicrobials.

Using PathogenFinder v1.1 and Virulence Finder v2.0, EC-106 was predicted to be a human pathogen (92.9%) that carried several virulence factors, including type I fimbriae (*fimH*), outer membrane protein (*chuA*), hemolysin (*hlyF*), bacteriocin (*cib* and *cma*), colicins (*cvaC*), enterobactin siderophore receptor protein (*iroN*), aerobactin (*sitA*), and serum resistance (*iss*).

To our knowledge, this is the first report of the detection of an IncX3 plasmid carrying *bla*<sub>NDM-5</sub> in animals in Lebanon. Our findings highlight concerns about the potential spread of *bla*<sub>NDM-5</sub> and other determinants in companion and farm animals in Lebanon. Taken together, our previous and current observations suggest that *bla*<sub>NDM-5</sub> might be spreading in different hosts and genetic backgrounds. The close contact between pets and farm animals and humans is known to facilitate the transmission of AMR in the human-animal interface. Therefore, comprehensive antimicrobial stewardship programs that adopt the One Health approach are needed to monitor and control AMR in Lebanon, with special consideration to its potential impact on local communities, particularly disenfranchised populations, and its ability to spill over across national borders.

**Competing interests:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this study.

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**Data availability:** The WGS sequence of EC106 has been submitted to the GenBank database under the accession number JAS-FXY000000000.

**Ethical approval:** This investigation received the approval (CE-EDST-1-2020) of the ethical committee of the Doctoral School of Science and Technology/Lebanese University, authorised by the

Lebanese Ministry of Public Health. The data were analyzed anonymously.

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## 2. Article 6: Multidrug-Resistant Pathogens Contaminate River Water Used in Irrigation in Disenfranchised Communities

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## Abstract

**Objectives:** The contamination of surface and irrigation waters pose a significant burden on human health and prosperity, especially in marginalized communities, where limited resources and inadequate infrastructure can easily spread the contamination to crops. Previously, we conducted a nationwide study that showed widespread contamination of fresh surface waters used in irrigation in Lebanon, a Mediterranean country that has been experiencing unprecedented economic and public health challenges. Our data indicated disproportionate pollution in impoverished areas, which has also been experiencing elevated incidences of enteric disease. Based on previous our findings, we targeted a polluted river (Al-Oueik) which is used for irrigation of agricultural fields in a disenfranchised area that also hosts a makeshift camp of Syrian refugees. Our objective was to perform in-depth genomic analyses of the multidrug resistant bacterial contaminants in the water.

**Methods:** A composite freshwater sample was collected from the Al- Oueik river. The water was filtered using a membrane that was used to enumerate fecal coliforms and isolate extended-spectrum cephalosporins or ertapenem resistant bacteria on selective agar plates, respectively. Non-duplicate isolates were identified using MALDI-TOF MS analysis followed by whole-genome sequencing to identify the genetic determinants underlying the resistance in these isolates as well as other properties such as sequence types, plasmid types, and virulence genes.

**Results:** The density of fecal coliforms detected in the water was approximately for  $10^6$  CFU/100 ml. Four non-duplicate drug-resistant Gram-negative bacteria were identified; namely *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter hormaechei*, and *Pseudomonas otitidis*. Notably, the *E. coli* isolate harbored *bla*<sub>NDM-5</sub> and the YRIN-inserted PBP3, representing an emerging therapeutic and public health challenge. The *K. pneumoniae* isolate carried both *bla*<sub>CTX-M-15</sub> and *bla*<sub>SHV-38</sub> as well as mutations in the gene encoding the OmpK37 porin. *E. hormaechei* and *P. otitidis* harbored *bla*<sub>ACT-16</sub> and *bla*<sub>POM-1</sub>, respectively.

**Conclusion:** To our knowledge, this is the first report that provides in-depth genomic analysis of antibiotic resistant bacteria in irrigation water in Lebanon. Our results confirmed that fecally contaminated irrigation water can be a reservoir of important pathogens and resistance which can spread to affect the adjacent agricultural fields and the Mediterranean Sea, posing both a public health and food safety concerns. Therefore, there is an urgent need to implement effective water quality monitoring and management programs to control the proliferation of antibiotic resistant pathogens in irrigation water in Lebanon.

## Introduction

Fresh surface waters are invaluable resources that have a substantial impact on human health and prosperity, affecting food production, safety, and security. Polluted rivers can act as reservoirs for various etiologic agents of infectious diseases, including those that can cause cholera, diarrhea, dysentery, typhoid fever, cryptosporidiosis, and hepatitis among others [1, 2]. Additionally, contaminated waters can spread agents of disease to humans via direct (e.g., recreational activities and drinking water) and indirect (e.g., contamination of food via irrigation) exposure. Notably, the World Health Organization has estimated that 80% of diseases in developing countries are waterborne, highlighting waterborne diseases as a leading cause of human illness [3]. These diseases have been associated with approximately 3.5 million deaths across the globe annually, with children comprising most of these losses [4]. In the USA, The CDC estimates that 7.2 million individuals become ill every year due to waterborne diseases [5]. However, it has been argued that waterborne diseases disproportionately affect marginalized communities, where limited resources and inadequate infrastructure can easily spread the contamination from water to other critical resources such as crops. In addition, it well-established that these communities are also prone to infectious diseases, including foodborne and waterborne illnesses. Therefore, the pollution of water, including irrigation water, is of paramount importance in marginalized communities.

Previously, we conducted a nationwide study that showed widespread fecal contamination of rivers used for recreation and irrigation in Lebanon, a Mediterranean country that has been experiencing unprecedented economic and public health challenges [6, 7]. Specifically, based on densities of fecal indicators, it was found that ~61-73% of river water samples exceeded the microbiological acceptability standards for irrigation, while ~46% of the *E. coli* isolated from the water were classified as multidrug resistant (MDR) [6]. Additionally, in a separate study, we isolated MDR *E. coli* that harboured the colistin resistance gene, *mcr-1*, as well as genes associated with resistance to cephalosporins (*bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>CTX-M</sub>) and carbapenems (*bla*<sub>NDM-1</sub> and *bla*<sub>OXA-48</sub>) from irrigation water channels [8, 9]. Taken together, these findings suggested that contaminated irrigation water might spread MDR bacterial pathogens and/ or AMR determinates to crops, soil, and other water resources in Lebanon. A major point of concern is the proximity of the irrigation water channels to disenfranchised and agricultural communities, especially in the Beqaa and North

region of Lebanon, which also includes refugee camps. The latter is very important, because Lebanon hosts currently over 1.5 million refugees (fleeing civil war across the border in Syria); many of whom reside in makeshift camps located in the vicinity of rivers in agricultural areas [2, 10, 11] . These camps lack adequate infrastructure, which results in the contamination of the domestic water in the camps as well as nearby water bodies, including irrigation waters, with sewage and waste generated by the refugees. Indeed, many camps had shallow and compromised sewage conduits that overflowed, especially after rainfall, and it was found that camp sewage was discharged directly to the environment and irrigation canals in some instances [2]. Subsequently, we showed that both domestic and sewer waters in the camps harbored MDR *E. coli* and pathogens (*Proteus mirabilis*) that carried genes encoding resistance to essential antibiotics [12-14]. Therefore, these unsanitary conditions pose elevated risks to water quality and the health of both the refugees and the hosting communities [15, 16]. Notably, both the refugee and vulnerable hosting communities are susceptible to disease, which creates a cycle of pathogen amplification (in hosts), cross contamination (water and crops), and infections. Therefore, water plays a central role in spreading pathogens and resistance determinants; potentially serving as a major catalyst in the aforementioned cycle.

Lebanon has been experiencing an unprecedented economic collapse; with the currency being devalued by over 95%, food and water prices skyrocketing, and more than 80% of the population currently live in poverty [17]. These unfortunate conditions have complicated and exacerbated water pollution across the country. For example, the few available waste-water-treatment plants shutdown frequently due to lack in funds and power (electricity/ fuel). Furthermore, weak in sanitation services resulted in the accumulation and overflow of waste to water bodies, including rivers and irrigation channels, especially from camps, and disenfranchised agricultural areas that cannot afford private waste disposal services. Consequently, water adjacent to these areas might be heavily contaminated with MDR bacteria and other contaminants.

Here, we expanded our previous investigations to specifically target *al.-Oueik* river in North Lebanon, because 1) the river is used as an irrigation source for large and diverse agricultural area, 2) it is in close proximity to a sizable refugee camp, 3) it resides in a historically disenfranchised area, 4) it has been reported anecdotally to be heavily polluted with sewage and to flood refugee

and Lebanese residences in winter, and 5) it empties to the Mediterranean Sea. Additionally, we performed in-depth analysis using whole genome sequencing and an arsenal of other assays to evaluate AMR determinants in potential bacterial pathogens in the water.

## **Materials and methods**

### **Ethics**

This study is a component of a larger research initiative that has been granted approval (CE-EDST-4-2021) by the ethical committee of the Doctoral School of Science and Technology at the Lebanese University (authorized by the Lebanese Ministry of Public Health).

### **Study design, bacterial isolation, and identification**

We targeted the Al-Oueik river (**Figure 1a**) which runs from Ain-Mashrah spring to the Mediterranean Sea and is primarily used for irrigation. We specifically sampled water at the location of the river that is adjacent to a Syrian refugee camp (125 tents and ~1000 refugees). A composite water sample (~1 Liter) was aseptically collected by submerging a sterile container 20–30 cm underwater without disrupting the sediment as recommended by the US Environmental Protection Agency (EPA).[18] The sample was transported to the laboratory in a cooler (2–10°C) and processed within 6h of collection. The sample was homogenized by shaking and left to settle for 5 min. Water was then filtered (100 mL) through a 0.22-µm Millipore® membranes (Sigma-Aldrich, St. Louis, MO, USA). The membranes were transferred onto RAPID'E. coli 2 agar plates (BioRad, USA) supplemented or not with ceftazidime (2 mg/L) or ertapenem (2 mg/L) (Sigma-Aldrich, USA), respectively. The plates were incubated at 36°C for 24 h. Typical colony forming units (CFU) of fecal coliforms (blue) and *E. coli* (violet to pink) colonies were counted on RAPID'E. coli 2 agar without antibiotics and reported as CFU/100 mL. Presumptive drug-resistant Gram-negative bacteria colonies were randomly selected and purified. The identification of the isolates was performed using a MALDI-TOF MS-based Vitek MS system version 3.0 (bioMérieux, Marcy L'Etoile, France).

### **Antibiotic susceptibility analysis and screening for carbapenemase activity**

Antibiotic susceptibility was evaluated using the Kirby Bauer disc diffusion assay as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. The *E. coli* ATCC 7624 strain was used for quality control. A total of 13  $\beta$ -lactam (ampicillin, amoxicillin/clavulanic acid, cefazolin, cefuroxime, cefoxitin, cefixime, cefotaxime, ceftazidime, cefepime, aztreonam, ertapenem, imipenem, meropenem) and 13 non- $\beta$ -lactam (fosfomycin, tetracycline, tigecycline, kanamycin, tobramycin, gentamicin, streptomycin, chloramphenicol, trimethoprim-sulfamethoxazole, norfloxacin, levofloxacin, ciprofloxacin, and nitrofurantoin) antibiotics were tested. Susceptibility to colistin (minimum inhibitory concentration; MIC) was evaluated using the broth microdilution method as described in the guidelines. The Carba NP and NG-test Carba 5 (NG Biotech, France) assays were used to screen for carbapenemase production as described in the manufacturer's instructions.[20]

### **Whole-Genome Sequencing (WGS)**

All non-duplicate isolates with resistance to either extended-spectrum cephalosporins or carbapenems were sequenced. DNA was extracted using the QIAamp DNA minikit (Qiagen, USA) as described in the manufacturer's protocols. The Nextera XT DNA library preparation kit and the Qubit dsDNA high-sensitivity (HS) assay kit (Invitrogen) were used to prepare and determine the concentrations of the sample libraries, respectively [8, 21].

The libraries were diluted and denatured according to the Illumina "Denature and Dilute Libraries Guide" protocol A ([https://support.illumina.com/content/dam/illumina-support/documents/documentation/system\\_documentation/miseq/miseq-denature-dilute-libraries-guide-15039740-10.pdf](https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-denature-dilute-libraries-guide-15039740-10.pdf)) and loaded into the MiSeq reagent cartridge (MiSeq reagent kit v2, 300 cycles). Sequencing was performed using the paired-end sequencing strategy with a MiSeq sequencer (Illumina). Low-quality reads were removed with Trimmomatic v0.36. Draft genome sequences were assembled using the "–careful" option in SPAdes v. 1. 3.15.5, and the quality of the draft genomes was evaluated with QUAST v4.5. Sequence types (STs) and resistance genes were determined using the CGE online tools MLSTFinder 2.0.4 and ResFinder 4.1, respectively (<http://www.genomicepidemiology.org/>), while Virulence genes and plasmid types were identified using VirulenceFinder v2.0 and PlasmidFinder v2.1, respectively.

### Accession number(s)

The genome sequences were deposited in GenBank under accession numbers JAQOLY000000000, JARBGB000000000, JARVGF000000000, and JARVGG000000000.

### Results and Discussion

The density of fecal coliforms in the water sample was approximately 106 CFU/100 ml. This was consistent with the gross examination of the river (**Figure 1b**) which revealed an unusual watercolor (brownish) and unpleasant odor, consistent with sewage contamination. Furthermore, the river banks were littered with debris and household waste. Four non-duplicate isolates were selected and identified as *E. coli*, *Pseudomonas otitidis*, *Klebsiella pneumoniae*, and *Enterobacter hormaechei*, which are all potentially opportunistic pathogens. Furthermore, phenotypic and genotypic analyses revealed that *E. coli*, *K. pneumoniae* and *E. hormaechei* were MDR (resistance  $\geq 3$  antibiotic categories) (**Table 1**). These results are notable and concerning, because the waters of the river are used for irrigation and empty into the sea, posing a risk for the contamination of produce and to recreational activities. It should be noted that most farming hands are refugees and marginalized Lebanese communities who might collect some of the produce for consumption. Given that these individuals do not have sustainable access to clean water, electricity, and cooking tools and fuel, it can be argued that their risk of infection from the consumption of contaminated produce is disproportionately high. The latter corroborates our previous findings that highlighted high gastrointestinal infections and a diversity of pathogens in disenfranchised rural populations in North Lebanon [22].

The *E. coli* isolate (KLW3) exhibited a high level of resistance to carbapenems (ertapenem, meropenem, and imipenem), with positive Carba NP and NG-test Carba 5 (NDM) test results. The isolate was also resistant to another (n=16) clinically and agriculturally relevant antibiotics, including aminoglycosides, tetracycline, fluoroquinolones, and nitrofurantoin. The WGS analysis showed that the isolate belonged to ST1284 and was *bla*<sub>NDM-5</sub>- and *bla*<sub>CMY-42</sub>-positive. In Lebanon, different *bla*<sub>NDM-5</sub>-positive *E. coli* strains have recently emerged in clinical settings (ST167, ST617, and ST648 isolated from COVID-19 patients), community (ST6836 carried by a food handler) [23], and the environment (unreported ST from irrigation water; ST361 recovered from

Orontes River) [9, 24]. Additionally, *E. coli* KLW3 harbored the YRIN-inserted PBP3 that consisted of a four amino acid insertion after position P333; which is associated with higher minimum inhibitory concentrations against carbapenems and new  $\beta$ -lactam combinations (i.e., aztreonam/avibactam, ceftazidime/avibactam, and imipenem/relebactam).[25] Similar *E. coli* ST1284 strains with the YRIN insertion have been reported in Turkey and Italy, but these isolates harbored *bla*<sub>OXA-181</sub>, *bla*<sub>CTX-M-15</sub>, and/or *bla*<sub>CMY-42</sub> genes.[26]. Taken together, our findings suggest a potential dissemination of this *E. coli* ST with important resistance determinants in the Mediterranean basin, representing an emerging public health challenge. Furthermore, mutations in chromosomally-encoded genes (*gyrA* mutations S83L and D87N) have been found in *E. coli* KLW3, conferring resistance to fluoroquinolones, including norfloxacin, ciprofloxacin, and levofloxacin. The isolate also carried several virulence genes, including *astA*, *capU*, *sgA*, *gad*, *hlyE*, *iss*, *iucC*, *iutA*, *nlpI*, *sitA*, *traJ*, and *traT* (**Table 1**).

A *bla*<sub>SHV-138</sub>-positive *K. pneumoniae* (ST4110) isolate was also recovered from the water. Mutations Gln31-Leu31 in the *bla*<sub>SHV-11</sub> broad spectrum  $\beta$ -lactamase gene results in SHV-187  $\beta$ -lactamase. To our knowledge, this is the first detection of SHV-187 in Lebanon, this variant was previously identified in *K. pneumoniae* isolated from WWTPSs effluent samples in Romania and was among most frequently detected variants of *bla*<sub>SHV</sub> in Enterobacterales retrieved from sepsis patients in Ethiopia [27, 28]. Additionally, the isolate was phenotypically resistant to ertapenem but sensitive to imipenem and meropenem, which constitutes a first step in the selection of higher-level carbapenem resistance [29]. Interestingly, mutations in the OmpK37 porin associated with the expression of ESBL genes might have contributed to decreasing susceptibility to carbapenems (Table 1).

A *bla*<sub>ACT-16</sub>-producing *E. hormaechei* (ST894) was also isolated from the water. ACT-16 has been recently described among *E. hormaechei* isolated from patients in Lebanon between 2013 and 2018 [30]. Both the *K. pneumoniae* and *E. hormaechei* isolates carried *fosA* confirming their resistance to fosfomycin. These finding are important, because fosfomycin is considered a last-resort antibiotic to control antimicrobial-resistant Gram-negative infections, and a low level of fosfomycin resistance was previously reported in Lebanon [31, 32].



We also isolated a *bla*<sub>POM-1</sub>-positive *P. otitidis*, which showed a wild-type genotype (**Table 1**). Although the Carba NP test was negative, the isolate appeared to be resistant to imipenem and meropenem. The expression of the POM-1, a subclass B3 metallo- $\beta$ -lactamase commonly found in *P. otitidis*, is typically constitutive but may confer a carbapenem-resistant phenotype in some strains.[33, 34].



**Figure 1**

**Table 1:** Genome properties and antibiotic susceptibility profile of the drug-resistant Gram-negative bacteria isolated from the water sample of the Al-Oueik river.

Isolate	Strain code	Genbank accession number of assembled genome	Genome size (bp)	Number of Contigs	N50 (bp)	L50	Genome Coverage (x)	GC content (%)	Resistance pattern	Intermediate resistance pattern	Susceptibility pattern	Antibiotic resistance genes detected by WGS	Plasmids detected by WGS	Sequence type (ST)
<i>Enterobacter hormaechei</i>	KLW1	JAQOLY000000000	4693641	91	156861	10	100X	55.3	AMP-AMC-CFZ-CTX-FOX-CXM-CFM-FSF	FEP-CAZ-ATM-TET	ERT-IPM-MEM-CST-GMN-TMN-KMN-STR-TGC-CIP-LVX-NOR-SXT-CHL-FUR	<i>bla</i> <sub>ACT-16</sub> , <i>fosA</i>	Col(pHAD28)	894
<i>Klebsiella pneumoniae</i>	KLW2	JARBGB000000000	7779238	6327	124502	-	100X	53.2	AMP-CFZ-FEP-CTX-CXM-CAZ-CFM-ATM-KMN-STR-TET-SXT-FSF-FUR	ERT	AMC-FOX-IPM-MEM-CST-TGC-GMN-TMN-CIP-LVX-NOR-CHL	<i>bla</i> <sub>SHV-187</sub> , <i>ompK37</i> ( <i>I128M</i> ); <i>I70M</i> ), <i>fosA</i> , <i>dfrA14</i> , <i>aph(3')-Ia</i> , <i>sul1</i> , <i>oqxA6</i> , <i>oqxB19</i> .	IncFIB(K), IncFII(K), IncFII(pSF O)	4110
<i>Escherichia coli</i>	KLW3	JARVGF000000000	4862068	270	77119	21	50X	50.7	AMP-AMC-CFZ-FEP-CTX-FOX-CXM-CAZ-CFM-ATM-ERT-IMP-MEM-STR-TET-CIP-LVX-NOR-FUR	-	CST-FSF-GMN-TMN-KMN-TGC-SXT-CHL	<i>bla</i> <sub>NDM-5</sub> , <i>bla</i> <sub>CMY-42</sub> , <i>aadA2</i> , <i>tet(B)</i> , <i>gyrA</i> ( <i>S83L</i> ; <i>D87N</i> ), <i>sul1</i> , <i>dfrA12</i>	Col(BS512), IncFIA, IncFII, IncI(Gamma a)	1284
<i>Pseudomonas otitidis</i>	KLW4	JARVGG000000000	6068461	291	84300	24	50X	67.2	AMP-AMC-CFZ-CTX-FOX-CXM-CFM-ERT-IMP-MEM-KMN-STR-TET-FSF-FUR	-	FEP-CAZ-ATM-CST-TGC-GMN-TMN-CIP-LVX-NOR-SXT-CHL	<i>bla</i> <sub>POM-1</sub>	-	-

\*AMP, ampicillin; AMC, amoxicillin plus clavulanic acid; CFZ, cefazolin; FEP, cefepime; CTX, cefotaxime; FOX, ceftaxime; CXM, cefuroxime; CAZ, ceftazidime; CFM, cefixime; ATM, aztreonam; ERT, ertapenem; MEM, meropenem; IPM, imipenem; CST, colistin; GMN, gentamicin; TMN, tobramycin; KMN, kanamycin; STR, streptomycin; TET, tetracycline; TGC, tigecycline; CIP, ciprofloxacin; LVX, levofloxacin; NOR, norfloxacin; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; FSF, fosfomicin; FUR, nitrofurantoin. The antibiotics in the resistance profiles are arranged according to the order of antibiotics/classes listed in the CLSI guidelines; WGS, whole-genome sequencing.

Although this study was limited to targeted water samples, the findings confirm the severity of water pollution in Lebanon. The sewage management infrastructure suffers from many limitations across the country. Furthermore, infrastructure in refugee settlements is non-existent or weak at best; comprised of makeshift sewage conduits that are very shallow and exposed. Moreover, people have been increasingly disposing of their waste in the rivers severe due to the impoverishment and weak services that were amplified by the ongoing economic crisis in Lebanon. Taken together, there is a critical need to address water pollution in the country via One Health interventions. We argue that even in a country that is facing challenges, ensuring the safety and sustainability of water resources, and preventing the dissemination of MDR determinants to other niches is essential for recovery efforts and is economically profitable in the long term. The latter will reduce infections and associated medical expenses while reducing lost work hours and enhancing marketability of the agricultural commodities. Finally, we urge the adoption of clear and rigorous guidelines and standards for water quality that include continuous monitoring of river and irrigation waters, which are crucial contributors to both public health and the economy.

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### 3. Article 7: Emergence of ST617 *Escherichia coli* harbouring *bla*<sub>NDM-5</sub> carbapenemase gene in Lebanon

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Research Letter

Research Letter

## Emergence of *bla*<sub>NDM-5</sub>-harbouring *Escherichia coli* ST617 in refugee and host communities and their environment

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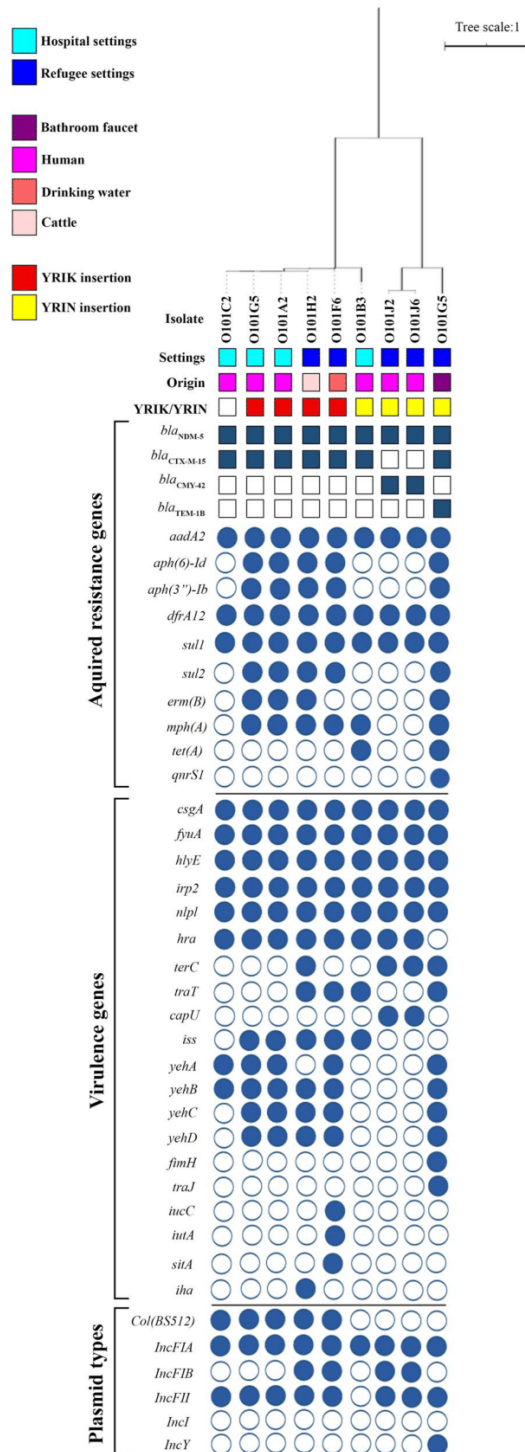
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The global spread of carbapenem-resistant *Escherichia coli* has become a serious challenge to public health. Of particular concern are the genetic determinants that encode New Delhi metallo-beta-lactamase (NDM) variants, which can rapidly disseminate between related bacterial species and confer resistance to extended-spectrum cephalosporins and carbapenems.<sup>1</sup> NDM-5 differs from its precursor (i.e. NDM-1) by only two amino acids (Val-88-Leu and Met-154-Leu) and can also be carried by different conjugative plasmid types.<sup>2</sup> Although *bla*<sub>NDM-5</sub>-harbouring Enterobacterales in some countries, such as China, appear to be endemic, reports on these strains remain sporadic in the Mediterranean region.<sup>3–5</sup>

Disenfranchised communities can serve as potential hotspots for the emergence of antimicrobial resistance (AMR). Notably, carbapenem resistance can have more severe consequences in vulnerable populations residing in low- and middle-income countries. The latter is associated with multiple factors, including weak access to adequate medical care, self-medication and availability of relatively cheap drugs without prescription. Indeed, the

burden of carbapenem resistance has been difficult to estimate in Lebanon, a lower middle-income country of the Mediterranean rim, due to the weakness of national surveillance programmes and an increase in vulnerable individuals associated with a steep economic crisis. Additionally, Lebanon has been hosting ~1.5 million refugees displaced by the ongoing armed conflict in Syria.<sup>6</sup> Furthermore, available data on carbapenem resistance are often limited and suffer from a lack of representativeness. Here, through a national screening effort, we performed in-depth characterization and investigated genetic relatedness of emerging *bla*<sub>NDM-5</sub>-harbouring *E. coli* ST617 isolated from vulnerable hospitalized Lebanese individuals, Syrian refugees, and livestock and the environment of the refugee camps. *Escherichia coli* ST617 is an emerging single-locus variant of the ST10 lineage of *E. coli*, which is ubiquitous at the human–animal interface. This lineage has emerged by the complete loss of the *wca* operon that encodes elements involved in colanic acid biosynthesis in the lipopolysaccharide biosynthesis pathway.<sup>5</sup> Interestingly, *bla*<sub>NDM-5</sub>-producing *E. coli* ST617 has been reported in hospital



**Figure 1** Maximum likelihood phylogeny of *bla<sub>NDM-5</sub>*-harbouring *E. coli* ST617 isolates. The phylogenetic tree was constructed based on pairwise SNP distances calculated from core-genome alignments. Metadata columns include resistome, virulome and plasmid type patterns

settings in China and Germany and was an etiologic agent of large and persistent outbreaks in healthcare settings in Italy.<sup>5,7</sup> The subtype was also isolated from wastewater treatment plant effluent in Switzerland.<sup>7</sup>

Between October 2021 and July 2022, nine carbapenem-resistant *E. coli* isolates were identified and included in this study. Four clinical isolates (two isolates from stool samples, one from peritoneal fluid, and one from urine) were recovered from different Lebanese patients admitted to the Nini Hospital (Tripoli, North Lebanon) as part of routine clinical care. In parallel, two carbapenem-resistant *E. coli* were isolated from the stool samples of two Syrian refugees, while additional three isolates were collected from cattle (stool samples), drinking water, and a bathroom faucet from three different refugee camps that were also located in North Lebanon (Figure S1, available as Supplementary data at *JTM* online). The sample collection and the isolation of *E. coli* were carried out as previously described.<sup>3</sup> The bacterial identification was further confirmed using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Vitek MS, bioMérieux, Marcy L'Etoile, France). Antimicrobial susceptibility profiles of the isolates were determined using the Kirby Bauer disc diffusion method as described in the guidelines of the Clinical and Laboratory Standards Institute (CLSI-M100). The Carba NP and the Lateral Flow Immunoassay NG-Test Carba5 (NG-Biotech, Guipry, France) assays were used to detect the production of NDM, which supported the presence of *bla<sub>NDM-5</sub>* that was identified by whole-genome sequencing (WGS) using the Illumina technology (NextSeq 500, Illumina, Les Ulis, France).<sup>1</sup> Multilocus sequence typing analysis showed that the nine isolates belonged to ST617, which has been rarely identified and associated with carbapenem resistance genes in Lebanon.<sup>8,9</sup> To date, only four *E. coli* ST617 isolates have been reported previously in this country, including two isolates harbouring *bla<sub>CTX-M-15</sub>* from a river,<sup>8</sup> a clinical isolate co-harboring *bla<sub>CTX-M-15</sub>* and *bla<sub>CMY-42</sub>*,<sup>9</sup> and another clinical isolate that carried *bla<sub>NDM-5</sub>* in a coronavirus disease-2019 patient.<sup>4</sup>

Analysis of the genomes using ResFinder v4.3.3 (<http://ge.nepi.food.dtu.dk/resfinder>) revealed that the isolates carried 5–11 acquired AMR genes that encoded resistance to critically important antimicrobials. All the isolates harboured *bla<sub>NDM-5</sub>* with either *bla<sub>CTX-M-15</sub>* or *bla<sub>CMY-42</sub>* (Table 1). Moreover, eight isolates also showed mutations (YRIN and YRIK insertions) in *ftsI* that encodes PBP3, which suggested that the strains had lower susceptibility against new-generation  $\beta$ -lactam agents (i.e. cefiderocol, aztreonam/avibactam, ceftazidime/avibactam and imipenem/relebactam). The isolates remained susceptible to fosfomicin, tigecycline, chloramphenicol, gentamicin, tobramycin and amikacin.

Pairwise single nucleotide polymorphism (SNP) distances were calculated from core-genome alignments and revealed potential dissemination of related *bla<sub>NDM-5</sub>* carrying strains in different hosts and niches in the human–animal–environment continuum, yet the possibility of polyclonal transmission cannot be dismissed at this point due to the relatively limited number of isolates in Lebanon (Figure 1). Notably, three clinical isolates (O101C2, O101A2 and O100G5) along with that of the cattle (O101H2) displayed an average of 23 SNP differences



**Table 1** Genome analyses and antimicrobial susceptibility patterns of the *bla*<sub>NDM-5</sub>-harbouring *E. coli* ST617 isolates from clinical and non-clinical samples in disenfranchised Lebanese population, Syrian refugees, and refugee camp settings

Isolate ID	Origin (location)	Sex (age)	Sample	Non-susceptibility to selected antimicrobials <sup>a</sup>	Susceptibility to selected antimicrobials <sup>a</sup>	Acquired antimicrobial resistance genes <sup>b</sup> (alignment length/gene length)	QRDR <sup>c</sup> mutations	Human pathogenicity (%) <sup>d</sup>
O101F6	Drinking water (Al-Qlayaat camp, Akkar)	NA	Water	AMX, AMC, TIC, TCC, PIP, TPZ, TEM, CXN, CXM, FOX, CAZ, CRO, FEP, ATM, ERT, IMP, MEM, MEC, NA, LVX, CIP, SXT	TGC, FSF, CHL, GEN, AMK, TOB	<i>bla</i> <sub>NDM-5</sub> (813/813), <i>bla</i> <sub>CTX-M-15</sub> (876/876), <i>aadA2</i> (792/792), <i>aph(6)-Ib</i> (837/837), <i>aph(3'')-Ib</i> (804/804), <i>dfrA12</i> (498/498), <i>sul1</i> (840/840), <i>sul2</i> (523/816), <i>mph(A)</i> (906/906)	<i>gyrA</i> (S83L) <i>gyrA</i> (D87N) <i>parC</i> (S80I) <i>parE</i> (S458A)	93.6
O101G5	Bathroom faucet (Al-Qlayaat camp, Akkar)	NA	Swab	AMX, AMC, TIC, TCC, PIP, TPZ, TEM, CXN, CXM, FOX, CAZ, CRO, FEP, ATM, ERT, IMP, MEM, MEC, NA, LVX, CIP, SXT	TGC, FSF, CHL, GEN, AMK, TOB	<i>bla</i> <sub>NDM-5</sub> (813/813), <i>bla</i> <sub>CTX-M-15</sub> (876/876), <i>bla</i> <sub>TEM-1B</sub> (861/861), <i>aadA2</i> (792/792), <i>aph(6)-Ib</i> (837/837), <i>aph(3'')-Ib</i> (804/804), <i>dfrA12</i> (498/498), <i>sul1</i> (840/840), <i>sul2</i> (816/816), <i>mph(A)</i> (903/906), <i>qnrS1</i> (657/657), <i>tet(A)</i> (1200/1200)	<i>gyrA</i> (S83L) <i>gyrA</i> (D87N) <i>parC</i> (S80I) <i>parE</i> (S458A)	93.3
O101H2	Cattle (Al-Mida camp, Akkar)	NA	Stool	AMX, AMC, TIC, TCC, PIP, TPZ, TEM, CXN, CXM, FOX, CAZ, CRO, FEP, ATM, ERT, IMP, MEM, MEC, NA, LVX, CIP, SXT	TGC, FSF, CHL, GEN, AMK, TOB	<i>bla</i> <sub>NDM-5</sub> (813/813), <i>bla</i> <sub>CTX-M-15</sub> (876/876), <i>aadA2</i> (792/792), <i>aph(6)-Ib</i> (837/837), <i>aph(3'')-Ib</i> (804/804), <i>dfrA12</i> (464/498), <i>sul1</i> (840/840), <i>sul2</i> (816/816), <i>erm(B)</i> (738/738), <i>mph(A)</i> (787/906)	<i>gyrA</i> (S83L) <i>gyrA</i> (D87N) <i>parC</i> (S80I) <i>parE</i> (S458A)	93.1
O101J2	Human (Syrian refugee; Al-Mhamra camp, Miniyyeh)	Male (3y)	Stool	AMX, AMC, TIC, TCC, PIP, TPZ, TEM, CXN, CXM, FOX, CAZ, CRO, FEP, ATM, ERT, IMP, MEM, MEC, NA, LVX, CIP, SXT	TGC, FSF, CHL, GEN, AMK, TOB	<i>bla</i> <sub>NDM-5</sub> (813/813), <i>bla</i> <sub>CMY-42</sub> (1146/1146), <i>aadA2</i> (792/792), <i>dfrA12</i> (498/498), <i>sul1</i> (840/840)	<i>gyrA</i> (S83L) <i>gyrA</i> (D87N) <i>parC</i> (S80I) <i>parE</i> (S458A)	92.4
O101J6	Human (Syrian refugee; Al-Mhamra camp, Miniyyeh)	Female (50y)	Stool	AMX, AMC, TIC, TCC, PIP, TPZ, TEM, CXN, CXM, FOX, CAZ, CRO, FEP, ATM, ERT, IMP, MEM, MEC, NA, LVX, CIP, SXT	TGC, FSF, CHL, GEN, AMK, TOB	<i>bla</i> <sub>NDM-5</sub> (813/813), <i>bla</i> <sub>CMY-42</sub> (1146/1146), <i>aadA2</i> (792/792), <i>dfrA12</i> (498/498), <i>sul1</i> (840/840)	<i>gyrA</i> (S83L) <i>gyrA</i> (D87N) <i>parC</i> (S80I) <i>parE</i> (S458A)	92.3
O100G5	Human (Lebanese patient <sup>e</sup> ; Miniyyeh)	Male (68y)	Stool	AMX, AMC, TIC, TCC, PIP, TPZ, TEM, CXN, CXM, FOX, CAZ, CRO, FEP, ATM, ERT, IMP, MEM, MEC, NA, LVX, CIP, SXT	TGC, FSF, CHL, GEN, AMK, TOB	<i>bla</i> <sub>NDM-5</sub> (813/813), <i>bla</i> <sub>CTX-M-15</sub> (876/876), <i>aadA2</i> (792/792), <i>aph(6)-Ib</i> (837/837), <i>aph(3'')-Ib</i> (804/804), <i>dfrA12</i> (498/498), <i>sul1</i> (840/840), <i>sul2</i> (816/816), <i>erm(B)</i> (738/738), <i>mph(A)</i> (903/906)	<i>gyrA</i> (S83L) <i>gyrA</i> (D87N) <i>parC</i> (S80I) <i>parE</i> (S458A)	93.3

Table 1 Continued

Isolate ID	Origin (location)	Sex (age)	Sample	Non-susceptibility to selected antimicrobials <sup>a</sup>	Susceptibility to selected antimicrobials <sup>a</sup>	Acquired antimicrobial resistance genes <sup>b</sup> (alignment length/gene length)	QRDR <sup>c</sup> mutations	Human pathogenicity (%) <sup>d</sup>
O101A2	Human (Lebanese patient <sup>e</sup> ; Miniyeh)	Male (1y)	Stool	AMX, AMC, TIC, TCC, PIP, TPZ, TEM, CXN, CXM, FOX, CTX, FEP, CAZ, ATM, ERT, IMP, MEM, MEC, NA, LVX, CIP, SXT	TGC, FSF, CHL, GEN, AMK, TOB	<i>bla</i> <sub>NDM-5</sub> (813/813), <i>bla</i> <sub>CTX-M-15</sub> (876/876), <i>aadA2</i> (792/792), <i>aph(6)-Id</i> (837/837), <i>aph(3'')-Ib</i> (804/804), <i>dfrA12</i> (498/498), <i>sul1</i> (840/840), <i>sul2</i> (816/816), <i>erm(B)</i> (738/738), <i>mph(A)</i> (903/906)	<i>gyrA</i> (S83L) <i>gyrA</i> (D87N) <i>parC</i> (S80I) <i>parE</i> (S458A)	93.3
O101B3	Human (Lebanese patient <sup>e</sup> ; Tripoli)	Female (7y)	Peritoneal fluid	AMX, AMC, TIC, TCC, PIP, TPZ, TEM, CXN, CXM, FOX, CTX, FEP, CAZ, ATM, ERT, IMP, MEM, MEC, NA, LVX, CIP, SXT	TGC, FSF, CHL, GEN, AMK, TOB	<i>bla</i> <sub>NDM-5</sub> (813/813), <i>bla</i> <sub>CTX-M-15</sub> (876/876), <i>aadA2</i> (792/792), <i>dfrA12</i> (498/498), <i>sul1</i> (840/840), <i>mph(A)</i> (906/906), <i>tet(A)</i> (1200/1200)	<i>gyrA</i> (S83L) <i>gyrA</i> (D87N) <i>parC</i> (S80I) <i>parE</i> (S458A)	93.7
O101C2	Human (Lebanese patient <sup>e</sup> ; Tripoli)	Female (84y)	Urine	AMX, AMC, TIC, TCC, PIP, TPZ, TEM, CXN, CXM, FOX, CTX, FEP, CAZ, ATM, ERT, IMP, MEM, MEC, NA, LVX, CIP, SXT	TGC, FSF, CHL, GEN, AMK, TOB	<i>bla</i> <sub>NDM-5</sub> (813/813), <i>bla</i> <sub>CTX-M-15</sub> (876/876), <i>aadA2</i> (792/792), <i>dfrA12</i> (498/498), <i>sul1</i> (840/840)	<i>gyrA</i> (S83L) <i>gyrA</i> (D87N) <i>parC</i> (S80I) <i>parE</i> (S458A)	93.4

<sup>a</sup>AMX, amoxicillin; AMC, amoxicillin-clavulanate; TIC, ticarcillin; TCC, ticarcillin-clavulanate; PIP, piperacillin; TPZ, piperacillin-tazobactam; TEM, temocillin; CXN, cephalaxin; CXM, cefuroxime; FOX, ceftaxime; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; ERT, ertapenem; IMP, imipenem; MEM, meropenem; MEC, mecillinam; ATM, aztreonam; TGC, tigecycline; NA, nalidixic acid; LVX, levofloxacin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; FSF, fosfomicin; CHL, chloramphenicol; GEN, gentamicin; AMK, amikacin; TOB, tobramycin. Clinical breakpoints were adopted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI-M100). <sup>b</sup>Acquired antimicrobial drug resistance genes detected by ResFinder v4.3.3 (<http://genepi.food.dtu.dk/resfinder>) using 99% identity as cut-off. <sup>c</sup>QRDR, quinolone resistance-determining region. <sup>d</sup>Using PathogenFinder v1.1 (<https://cge.food.dtu.dk/services/PathogenFinder/>), *E. coli* isolates were predicted to be a human pathogen. <sup>e</sup>*E. coli* was isolated at the Nini Hospital (Tripoli, North Lebanon).

in the genomes, indicating a high degree of genetic similarity among these isolates (Table S1, available as Supplementary data at JTM online). Importantly, these isolates also exhibited genetic relatedness to a previously reported clinical isolate from Beirut (the capital of Lebanon), with an average of 108 SNP differences.<sup>4</sup> Furthermore, the isolates from refugees at Al-Mhamra camp (O101J2 and O101J6) shared the same acquired resistome and virulome patterns and showed a remarkably low SNP distance, with 17 SNP differences identified, suggesting a potential transmission of related strains and perhaps evolution within the camp. These isolates were detected in refugees that belonged to different families and have resided in the refugee camp for the past 3 years. The other two camp-associated *E. coli* isolated from drinking water (O101F6) and a bathroom faucet (O101G5) at the Al-Qlayaat camp were found to be potentially of different *E. coli* lineages, with 12 044 SNP differences. Taken together, these observations raise different hypotheses about the transmission route, which can happen through direct contact with human or animal hosts or via environmental reservoirs such as contaminated food and water sources among others.

Notably, all the isolates were predicted to be potential human pathogens, carrying multiple virulence-associated factors,

including type I fimbriae (encoded by *fimH*), YHD fimbriae cluster (*yehA/B/C/D*), capsule (*capU*), biofilm formation (*csxA*), hemolysin (*blyE*), heat-resistant agglutinin (*bra*), siderophores (*iutA*, *fyuA*, *irp2*, *iba*), aerobactin (*sitA*, *iucC*), tellurite resistance (*terC*) and/or serum resistance (*traT*, *iss*). Analysis using PlasmidFinder v2.0 detected IncFIA in all the isolates and IncFII in eight isolates only (Figure 1). Other plasmid types, including ColB5512, IncFIB, IncI and IncY, were also identified in different isolates. Given that previous reports from the USA, China, India and Egypt showed that plasmids might play an essential role in the transmission of *bla*<sub>NDM-5</sub>,<sup>10</sup> analysis of our isolates using long-read sequencing technologies as well as plasmid mating/transformation experiments is required to determine the localization of *bla*<sub>NDM-5</sub> and associated mobile genetic elements.

Our findings have highlighted active AMR acquisition and transmission dynamics in disenfranchised populations and settings, raising concerns about the dissemination of critical AMR genes, including *bla*<sub>NDM-5</sub>, in these vulnerable populations in Lebanon and beyond. Of particular concern is the occurrence of *bla*<sub>NDM-5</sub>-harbouring *E. coli* ST617 in otherwise healthy Syrian refugees, their animals, and the camp environment, because

this represents a health risk for the refugees and their hosting community, who both pose a suitable niche for evolving AMR strains. We believe that our data strongly highlight the risk of AMR in vulnerable and disenfranchised communities, while emphasizing the overlooked role of war, mass migration and displacement in the spread of AMR. Therefore, integrated surveillance programmes addressing antimicrobial use and resistance are of paramount importance and urgency in these scenarios. These programmes must rely on comprehensive data collection and analysis, collaborations between stakeholders across various sectors in the human–animal–environment nexus, and efforts to strengthen both local and global risk assessment systems. One Health studies adopting WGS-based approaches are also critical to characterize the dynamics of AMR transmission, which will greatly benefit robust mitigation measures.

### Supplementary data

Supplementary data are available at *JTM* online.

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**Conflict of interest:** The authors declare no conflict of interest.

### Data availability

The genome sequences were deposited in GenBank under accession numbers JAWWLL000000000 (O100G5), JAWWLK000000000 (O101A2), JAWWLJ000000000 (O101B3), JAWWLI

000000000 (O101C2), JAWWLQ000000000 (O101F6), JAWWL P000000000 (O101G5), JAWWLO000000000 (O101H2), JAWWLN000000000 (O101J2), and JAWWLM000000000 (O101J6).

### Ethical considerations

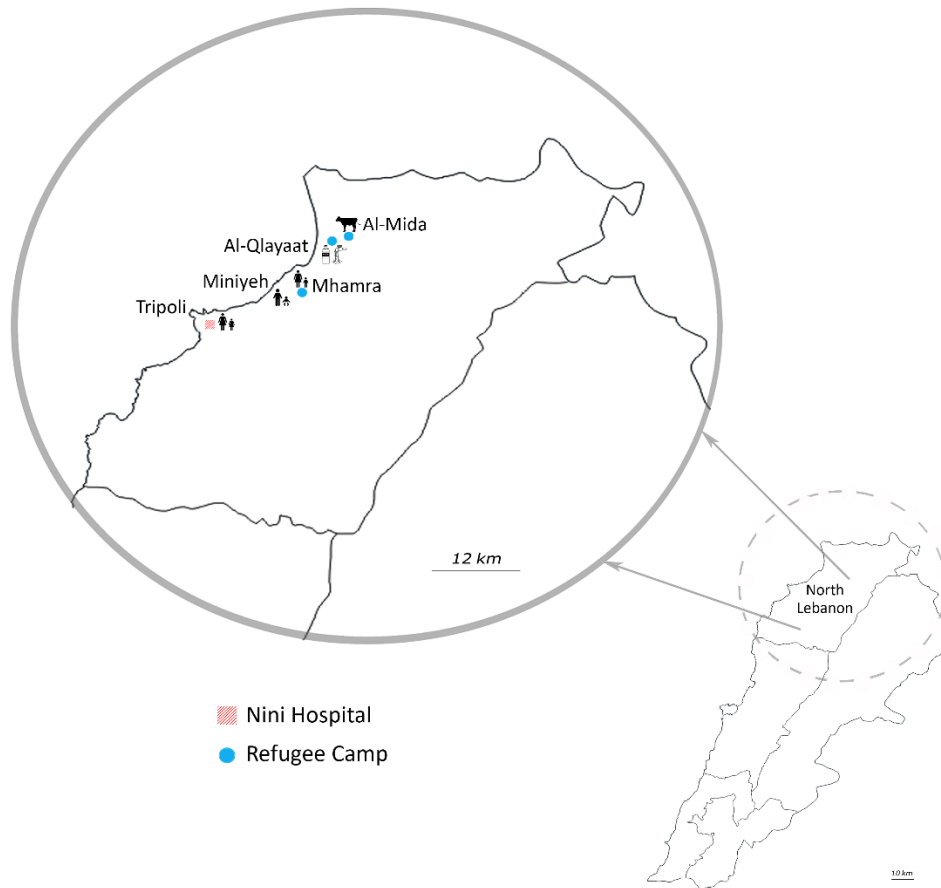
This study was approved by the ethics committee of the Doctoral School of Science and Technology at the Lebanese University (document no. CE-EDST-1-2020/CE-EDST-4-2021) and authorized by the Lebanese Ministry of Public Health.

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## Supplementary materials

### Supplementary figures



**Figure S1.** Map showing the geographic distribution and origin of the isolates identified in this study. Four clinical carbapenem-resistant *Escherichia coli* isolates were recovered from Lebanese patients admitted to the Nini Hospital (Tripoli, North Lebanon). Two carbapenem-resistant *E. coli* were isolated from two Syrian refugees, while another three isolates were collected from cattle, drinking water, and a bathroom faucet from three different refugee camps (Al-Mhamra, Al-Mida, and Al-Qlayaat) that were also located in North Lebanon.

## Supplementary tables

**Table S1:** Matrix of core genome single nucleotide polymorphisms (SNPs) between carbapenem-resistant *Escherichia coli* ST617 isolates reported in Lebanon, revealing that the isolates does not constitute a sole epidemic cluster.

	O101C2	O101A2	O100G5	O101H2	ECOL 66‡	AUH_IMP202‡	O101G5	O101J2	O101J6	O101F6	O101B3
O101C2	0										
O101A2	24	0									
O100G5	22	14	0								
O101H2	32	22	22	0							
ECOL 66‡	115	107	105	105	0						
AUH_IMP202‡	378	372	370	374	403	0					
O101G5	12021	12011	12009	12013	12022	11819	0				
O101J2	11597	11585	11585	11587	11626	11443	5306	0			
O101J6	11602	11592	11590	11594	11633	11444	5301	17	0		
O101F6	601	595	593	593	638	857	12044	11797	11794	0	
O101B3	2474	2468	2464	2470	2485	2538	12542	12283	12280	2365	0

\*Matrix was obtained by SNP-based analysis, using CSI Phylogeny 1.4 available online at the Center for Genomic Epidemiology-CGE (<http://www.genomicepidemiology.org/>).<sup>1</sup>

‡ECOL66 (*bla*<sub>NDM-5</sub>-producing)<sup>2</sup> and AUH\_IMP202 (*bla*<sub>CTX-M-15</sub> and *bla*<sub>CMY-42</sub>-co-producing)<sup>3</sup> are clinical carbapenem-resistant *Escherichia coli* ST617 previously reported in Lebanon. The genome assemblies were downloaded from the *Escherichia coli* genome database Enterobase (<http://enterobase.warwick.ac.uk/species/index/ecoli>) and the NCBI database, respectively (Genome Assembly Accession numbers: ESC\_CC4734AA\_AS, GCF\_002155825.1).

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## **Chapitre 4 : Discussion et Perspectives**

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La menace du problème de la résistance aux antibiotiques apparaît désormais comme l'une des crises sanitaires internationales majeures du 21<sup>ème</sup> siècle. Cependant, et malgré l'attention mondiale, cette crise continue à s'aggraver, affectant notre capacité à lutter contre les maladies infectieuses chez l'homme et l'animal (263). Des études récentes ont révélé une augmentation rapide et accélérée des taux de résistance dans de nombreux pays que ce soit à haut niveau de ressources, mais surtout dans les pays à ressources moyennes ou faibles, y compris le Liban.

Le problème de la résistance aux antibiotiques au Liban reste flou, en raison de manque de données officielles de surveillance nationale, lié en grande partie à une absence d'études épidémiologiques moléculaires nationales. Les rares données disponibles sont largement issues d'enquêtes régionales limitées. Bien que le Liban soit inscrit dans le GLASS (Global Antimicrobial Resistance and Use Surveillance System) de l'OMS en 2017, les données épidémiologiques restent encore faibles (216). Outre leur rareté, ces données concerne exclusivement le monde humain, un peu animal, mais quasiment pas le monde agricole et environnemental (264-266). En effet, les pratiques agricoles ne bénéficient pas d'aucune attention des pouvoirs publics. À cause de ces lacunes évidentes, les taux de résistance aux antibiotiques augmentent et les gènes de résistance aux antimicrobiens nouvellement découverts apparaissent rapidement. De nombreuses études récentes réalisées au niveau régional soulignent le problème lié à la résistance aux antibiotiques (201, 264, 265). Par ailleurs, l'afflux de réfugiés en provenance de Syrie et d'Irak a probablement contribué à l'aggravation du problème de la résistance aux antibiotiques au Liban (239, 265, 267).

Trois facteurs principaux déterminent les taux élevés de résistance au Liban : (i) la promotion de l'automédication et de l'auto-prescription d'antibiotiques ; (ii) l'utilisation abusive des antibiotiques comme facteurs favorisant la croissance du bétail et de la volaille ; et (iii) le manque des programmes de gestion des antibiotiques et les pratiques inadéquates de contrôle des infections dans les secteurs de santé. Malheureusement, plusieurs facteurs favorisent l'automédication: la disponibilité en vente libre, les faibles revenus, le coût élevé des consultations médicales (268). La situation dans le domaine vétérinaire n'est guère mieux, avec de nombreux cocktails à base d'un ou plusieurs antibiotiques légalement et facilement et disponibles sur le marché libanais sans prescription du vétérinaire (269). Théoriquement, ces médicaments ne doivent être administrés

qu'après prescription vétérinaire pour le traitement des maladies animales, mais en pratique ils sont excessivement utilisés à long terme par les agriculteurs comme stimulateurs de croissance et traitement prophylactique.

Le Liban est devenu la terre d'accueil de nombreux réfugiés politiques. Selon Médecins Sans Frontières, plus de 1,5 millions de réfugiés syriens ont fui vers le Liban depuis le début de la guerre civile syrienne, faisant du Liban le pays accueillant le plus grand pourcentage de réfugiés au monde. Malgré les efforts du Ministère de la Santé Publique au Liban, dans le soutien aux établissements de santé pour les réfugiés, le coût des soins médicaux restent un obstacle pour une grande partie des réfugiés (267). L'utilisation non-contrôlée des antibiotiques par les communautés résidentes au Liban est la principale raison qui conduit à une forte pression de sélection et qui est à l'origine de la réponse évolutive dans le monde bactérien. De même, malgré que les lois au Liban imposent l'accessibilité aux antibiotiques sur ordonnance uniquement, ces médicaments sont fréquemment fournis sans prescription médicale dans la majorité des zones libanaises. De plus, plusieurs études transversales ont montré une mauvaise connaissance et une mauvaise attitude des pharmaciens (201, 270), concernant l'utilisation abusive des antibiotiques et la résistance. En résumé, le système médical au Liban souffre du manque de formation médicale sur l'utilisation des antibiotiques pour les prescripteurs et la faible efficacité des mesures de contrôle des infections et programmes de gestion des antibiotiques dans les établissements de soins de santé.

Les actions politiques régionales et nationales, telles que la mise en œuvre des plans d'action nationaux, sont désormais essentielles. Au niveau communautaire, des campagnes de sensibilisation étendues ciblant le public et les pharmaciens communautaires, utilisant une approche interactive et centrée sur l'humain sont nécessaires afin d'atteindre des niveaux élevés d'accord et les changements de comportement souhaités. Au niveau médical, la formation des microbiologistes, des pharmaciens cliniciens, des médecins et des vétérinaires est la première étape pour atteindre nos objectifs. En outre, un programme complet d'approche One Health, devrait être mis en œuvre pour : (i) établir un système national solide de surveillance de la résistance ; (ii) garantir l'utilisation adéquate des antibiotiques chez les humains et les animaux, (iii) interdire l'utilisation d'antibiotiques comme stimulateurs de croissance dans l'alimentation animale, et (iv) interdire l'utilisation d'antibiotiques sans ordonnance clinique ou vétérinaire.



Dans ce travail, nous avons confirmé que la résistance aux antibiotiques est devenue une préoccupation au Liban à l'image de ce qui est observé à l'échelle mondiale et constitue un problème de santé publique. Un traitement prolongé aux antibiotiques peut entraîner le développement d'une résistance chez un micro-organisme qui est initialement sensible aux antibiotiques, mais qui peut ensuite s'adapter progressivement et développer une résistance aux antibiotiques (271).

A travers cette thèse, nous avons réalisé plusieurs études qui ont montré la résistance des bactéries aux antibiotiques d'origine humaine, animale et environnementale y compris les carbapénèmes et la colistine au Liban. Cela impose la mise en œuvre des mesures d'urgence pour le contrôle et l'utilisation des antibiotiques. La surconsommation de ces médicaments dans de multiples secteurs (humain, animal, agriculture) est le principal problème. En effet, les micro-organismes confrontés à la pression de sélection des antimicrobiens améliorent leur aptitude en acquérant et en exprimant des gènes de résistance, puis en partageant ces gènes avec d'autres bactéries. Ainsi, l'utilisation et la surutilisation des antibiotiques sont des facteurs importants du phénomène de résistance ; les autres principaux facteurs sont des facteurs qui favorisent la propagation de bactéries résistantes et leurs gènes au niveau local et mondial. Ceux-ci incluent un mauvais contrôle des infections, une contamination de l'environnement et des mouvements géographiques des humains et des animaux infectés (250).

Notre travail a mis en évidence l'émergence des bactéries résistantes aux carbapénèmes en milieu clinique au Liban. Les carbapénèmes font partie des médicaments de choix pour le traitement des infections nosocomiales. Cependant, leur efficacité est de plus en plus compromise en raison de l'émergence mondiale d'isolats résistants (272). Nos résultats ont montré que les souches d'*E. coli* résistante aux carbapénèmes sont productrices du gène *bla<sub>NDM-5</sub>*. A noter que les enzymes de type OXA-48 étaient les carbapénémases les plus répandues parmi les Entérobactéries au Liban au cours de la dernière décennie, tandis que le gène *bla<sub>NDM-5</sub>* était rare en milieu hospitalier et communautaire (220, 273). Dans notre étude, le gène *bla<sub>NDM-5</sub>* est devenu le gène prédominant des carbapénémases. Le gène *bla<sub>OXA-244</sub>* arrive en deuxième position. Ce gène est de plus en plus décrit dans le monde (274), et la dissémination des OXA-244 représente une menace pour la santé

publique en raison des difficultés de détection à l'aide des tests de dépistage classiques basé sur la sélection sur des milieux contenant des carbapénèmes et/ou de la témocilline (275).

Dans cette recherche doctorale, nous avons également mis en évidence la présence de *E. coli* résistante aux carbapénèmes, productrices de *bla*<sub>NDM-5</sub> chez les animaux de compagnie. Ces résultats sont très importants, du fait que nos observations précédentes et actuelles suggèrent que ce gène pourrait diffuser chez différents hôtes. De plus, le contact étroit entre les animaux de compagnie avec d'autres animaux ou avec les humains, peut faciliter la transmission des gènes de résistance à l'interface homme-animal.

De plus, durant notre étude, des bactéries multirésistantes, y compris des bactéries résistantes aux céphalosporines à large spectre ou aux carbapénèmes, ont été signalées dans une rivière au Nord du Liban. Ces résultats sont très préoccupantes, car les eaux de cette rivière sont utilisées pour l'irrigation et se déversent dans la mer Méditerranée, posant un risque de dissémination dû aux activités récréatives et agricoles.

En conclusion, nous espérons que nos travaux pourront sensibiliser d'avantage les autorités scientifiques et politiques afin que des études épidémiologiques nationales dans un contexte 'One health' soient diligentés et que des solutions concrètes puissent être apportées pour endiguer ce fleau. La combinaison de toutes les interventions et initiatives, telles que les lois, les politiques de suivi, les interventions de sensibilisation et d'éducation, et la surveillance nationale officielle de la résistance et des connaissances et attitudes à l'égard de l'usage abusif des antibiotiques dans la communauté et le corps médical, contribuera certainement à réduire le l'utilisation d'antibiotiques, et ainsi minimiser le développement de la résistance et à éviter des conséquences négatives sur la santé publique et l'économie.

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