



OXA-48-producing Enterobacterales and novel molecules to combat them

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Mariam Rima. OXA-48-producing Enterobacterales and novel molecules to combat them. Life Sciences [q-bio]. Paris Saclay University, 2023. English. NNT : 2023UPASQ061 . tel-04542635v1

HAL Id: tel-04542635

<https://hal.science/tel-04542635v1>

Submitted on 11 Apr 2024 (v1), last revised 8 Oct 2024 (v2)

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OXA-48-producing Enterobacterales and novel molecules to combat them

Aspects de la lutte contre le problème de la résistance aux antimicrobiens

Thèse de doctorat de l'université Paris-Saclay – Faculté de pharmacie

École doctorale n° 569, Innovation thérapeutique : du fondamental à l'appliqué

Doctorat en microbiologie médicale

Graduate school : Santé et médicaments

Thèse préparée dans l'unité de recherche « Structure, dynamique, fonction et expression de β -lactamase à large spectre » (Université Paris-Saclay, Inserm UMR1184) sous la direction du docteur Thierry NAAS, et la co-supervision du docteur Saoussen OUESLATI

Thèse soutenue à Paris-Saclay, le 30 novembre 2023, par

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Acknowledgements

Words cannot express my gratitude to **Dr. Thierry Naas** who gave me the opportunity to be part of his laboratory team. Thank you for your confidence, your availability and especially the autonomy you offered me during my PhD journey. Your constructive advices helped me grow and achieve my goals.

I would also like to thank **Dr. Saoussen Oueslati**, for having the patience to teach me all technical strategies to succeed. Thank you for guiding me and being there whenever I needed your help.

My special acknowledgements goes for the jury members, **Pr. Imad Kansau, Pr. Monzer Hamzeh, Pr. Richard Bonnet, Pr. Corinne Arpin, Pr. Francesca Spyrakis** and **Pr. Marie-Frederique Lartigue** for the precious time and effort they put to review my thesis manuscript.

I wish to express my sincere appreciation for all UMR1184 members, who helped me at one time or another. Thanks to you, I was able to discover and share the daily life of a friendly, rigorous and dynamic team, which allowed me to work with confidence in a pleasant and relaxed atmosphere.

I would like to express my special appreciation to **Drs. Laura Dabos, Agnès Jousset, Delphine Girlich, Rémy Bonnin** and **Laurent Dortet** for their help.

Elodie Creton, Oceane Vanparis, Camille Gonzalez, you have made working in the lab a great experience. **Laura Biez**, thank you for your kindness, **Dina Daaboul**, thank you for being there whenever I needed you, thank you for your unfailing support, I feel so fortunate to have a friend like you.

This work couldn't have been accomplished without the support of my family.

I dedicate this work....

To my superhero, my father, Ahmad No words can express my respect, my eternal love and my consideration for the sacrifices you have made for my well-being, may God bless you with good health.

To my dearest in the world, to my mother, Souheir, who accompanied me with her precious advices and prayers, I put in your hands, the fruit of long years of studies, of difficult times I spent away from you. May this modest work be the fulfillment of your wishes, fruit of your innumerable sacrifices, though I cannot fulfill you enough.

To my dear brother Karim and my dear sister Jana, I cannot express through these lines all my feelings for you. May love and fraternity unite us forever.

At the end, I would like to dedicate this work to the love of my life, the one who stood by my side no matter what, the one who believed in me and encouraged me from the very first day, Omar, I don't know how I would have pulled through my PhD journey without your support.

This work is also dedicated to my little princess, Maria, you have made me stronger, better and more fulfilled than I could have ever imagined. I love you to the moon and back.

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Acronyms

AMR	Antimicrobial resistance
MDR	Multi-drug resistant
BLs	β -lactamases
CPE	Carbapenemase producing Enterobacterales
GNB	Gram-negative bacilli
LMICs	Low and middle income countries
DTI	Difficult to treat infections
WHO	World health organization
OM	Outer membrane
IM	Inner membrane
LPS	Lipopolysaccharide
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
PBP	Penicillin binding protein
MRSA	Methicillin resistant staphylococcus aureus
ESC	Extended spectrum cephalosporins
ST	Sequence type
DHP	Dehydropeptidase
MBL	Metallo β -lactamase
EDTA	Ethylenediamine tetra-acetic acid
BLDB	B-lactamase database
CHD	Chelocardin
CDCHD	Amidochelocardin
AMP	Antimicrobial peptide

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Abstract

The fight against bacteria is turning into one of the greatest challenges faced by our society, especially with the spread of multi-drug resistant (MDR) bacteria that become increasingly difficult to treat. In some cases, resistance extends to the entire repertoire of available therapeutic agents. β -Lactams due to their safety, reliable killing properties and clinical efficacy are among the most frequently prescribed antibiotics, but their clinical use is currently threatened by the worldwide spread of β -lactamases (BLs) capable of hydrolyzing even the newest and most powerful β -lactams, the carbapenems. The dissemination of carbapenemase-producing *Enterobacterales* (CPE) is a great matter of clinical concern given the major role of *Enterobacterales* (GNB) as causes of nosocomial infections (and, for *Escherichia coli* (*E. coli*), also of community-acquired), and of carbapenems in the treatment of these infections.

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. Through several epidemiological studies conducted in hospital and community settings we were able to highlight the emergence and broad dissemination of *bla*_{NDM-5} and *bla*_{OXA-244} genes in Lebanese clinical settings.

As in most countries of the Mediterranean rim OXA-48-like carbapenemases are the most prevalent. The increasing isolation of OXA-244 variants is a scary scenario, as these enzymes are difficult to detect using classical diagnostic tests, thus resulting in under detection and silent spread. Using steady state kinetic determinations and 3D modelling were able to show, that the hydrolytic properties of OXA-244, a R214G point mutant derivative of OXA-48, were impaired likely as the result of the interruption of a salt bridge between D159 and R214. The characterization of OXA-933, another OXA-48 variant, with an D159G confirmed our results. Finally, we also confirmed the key role of R214 in carbapenem and temocillin hydrolysis by studying the kinetic properties of OXA-484, an R214G derivative of OXA-181.

The spread of *bla*_{NDM-5} gene in *E. coli* is even more threatful, as these bacteria are resistant to

most antibiotics, including newly released or promising and still in clinical evaluation β -lactam/inhibitor combinations. Thus, newly antibiotics, with novel targets are urgently needed to address the increasing prevalence of NDM-producers. Cystobactamids and chelocardins, two novel antibiotics, have shown interesting results as they were able to inhibit the growth of highly drug resistant Gram-positive and Gram-negative bacteria, including ESKAPE pathogens except *Klebsiella pneumoniae* (*K. pneumoniae*). To identify the targets of these molecules, mutants were selected and sequenced, and RNA seq experiments performed. Cystobactamids induced many SNIPs but consistently in *in vitro* generated mutants, single nucleotide changes in *qseBC*, coding for a transcriptional regulator, *ArnF* and *pmrB* genes, involved in colistin resistance were observed. No cross resistance could be observed however with colistin resistant bacteria. RNAseq experiments revealed that upon cystobactamids exposure upregulation of DNA repair response and SOS responses occurred, while upon chelocardin exposure, most of the upregulated genes were linked to the bacterial cell wall. In addition, iron metabolism was also affected in both conditions suggesting a critical role of the iron homeostasis.

Our findings highlight an urgent need for transformative approaches to combat antimicrobial resistance in both community and hospital settings in Lebanon, especially with emergence of highly drug resistant and difficult to detect isolates. Finally, we were able to demonstrate the potential of two novel antibiotic families with novel targets and no crossing resistance with other antibiotic families.

La lutte contre les bactéries devient l'un des plus grands défis de notre société, en particulier avec la propagation des bactéries multirésistantes (MDR) qui deviennent de plus en plus difficiles à traiter. Dans certains cas, la résistance s'étend à l'ensemble des agents thérapeutiques disponibles. Les β -lactames, en raison de leur sécurité, de leurs propriétés de destruction fiables et de leur efficacité clinique, comptent parmi les antibiotiques les plus fréquemment prescrits, mais leur utilisation clinique est actuellement menacée par la propagation mondiale de β -lactamases (BLs) capables d'hydrolyser même les β -lactames les plus récentes et les plus puissantes, les carbapénèmes. La dissémination des entérobactéries productrices de carbapénémases (EPC) est un sujet de préoccupation clinique majeur étant donné le rôle majeur des entérobactéries (GNB) comme causes d'infections nosocomiales (et, pour *Escherichia coli* (*E. coli*), également d'infections communautaires), et des carbapénèmes dans le traitement de ces infections.

Par conséquent, il est essentiel de surveiller et d'évaluer en permanence la propagation de la résistance aux carbapénèmes, en particulier dans les pays à revenu faible et intermédiaire (PRFI) qui sont confrontés à des défis importants en matière de gestion des antimicrobiens et de systèmes de santé publique. Les observations disponibles suggèrent que la résistance aux antimicrobiens (RAM) a augmenté de façon précipitée au Liban, un pays confronté à une pléthore de problèmes résultant d'un effondrement économique sans précédent. Grâce à plusieurs études épidémiologiques menées en milieu hospitalier et communautaire, nous avons pu mettre en évidence l'émergence et la large diffusion de la résistance aux antimicrobiens au Liban.

Comme dans la plupart des pays du pourtour méditerranéen, les carbapénémases de type OXA-48 sont les plus répandues. L'isolement croissant des variantes OXA-244 est un scénario inquiétant, car ces enzymes sont difficiles à détecter à l'aide des tests de diagnostic classiques, ce qui entraîne une sous-détection et une propagation silencieuse. En utilisant des déterminations cinétiques en régime permanent et la modélisation 3D, nous avons pu montrer que les propriétés hydrolytiques d'OXA-244, un dérivé mutant ponctuel R214G d'OXA-48, étaient altérées, probablement en raison de l'interruption d'un pont salin entre D159 et R214. La caractérisation d'OXA-933, un autre variant d'OXA-48, avec un D159G a confirmé nos

résultats. Enfin, nous avons également confirmé le rôle clé de R214 dans l'hydrolyse des carbapénèmes et de la témocilline en étudiant les propriétés cinétiques d'OXA-484, un dérivé R214G d'OXA-181.

La propagation du gène *bla*_{NDM-5} chez *E. coli* est encore plus menaçante, car ces bactéries sont résistantes à la plupart des antibiotiques, y compris les combinaisons β -lactamines/inhibiteurs récemment réalisées ou prometteuses et encore en cours d'évaluation clinique. Il est donc urgent de mettre au point de nouveaux antibiotiques, avec de nouvelles cibles, pour faire face à la prévalence croissante des producteurs de NDM. Les cystobactamides et les chélocardins, deux nouveaux antibiotiques, ont donné des résultats intéressants, car ils ont pu inhiber la croissance de bactéries Gram-positives et Gram-négatives hautement résistantes aux médicaments, y compris les pathogènes ESKAPE, à l'exception de *Klebsiella pneumoniae* (*K. pneumoniae*).

Pour identifier les cibles de ces molécules, des mutants ont été sélectionnés et séquencés, et des expériences de séquençage de l'ARN ont été réalisées. Les cystobactamides ont induit de nombreux SNIP, mais dans les mutants générés in vitro, des changements de nucléotides simples ont été observés dans les gènes *qseBC*, codant pour un régulateur transcriptionnel, *ArnF* et *pmrB*, impliqués dans la résistance à la colistine. Aucune résistance croisée n'a cependant pu être observée avec des bactéries résistantes à la colistine. Les expériences RNAseq ont révélé que lors de l'exposition aux cystobactamides, la réponse de réparation de l'ADN et les réponses SOS étaient régulées à la hausse, tandis que lors de l'exposition à la chélocardine, la plupart des gènes régulés à la hausse étaient liés à la paroi cellulaire bactérienne. En outre, le métabolisme du fer a également été affecté dans les deux conditions, ce qui suggère un rôle critique de l'homéostasie du fer.

I- Background

The discovery of penicillin, and more general of antibiotics has revolutionized Medicine by enabling efficient treatment of many life threatening bacterial infections. However, Alexander Fleming warned during his Nobel Laureate allocution in 1945 about the emergence of antimicrobial resistance (AMR), e.g. the ability of microbes to resist to these potent medicines by different mechanisms resulting from their misuse and overuse: “Penicillin should only be used if there is a properly diagnosed reason and, if it needs to be used, use the highest possible dose for the shortest time necessary. Otherwise antibiotic resistance will develop”.

The fight against bacteria is turning again into one of the greatest challenges faced by our societies, especially with the spread of multi-drug resistant (MDR) bacteria. In some cases, resistance extends to the entire repertoire of available therapeutic agents (the so-called pan-drug resistant phenotypes), resulting into difficult to treat infections (DTI) posing a formidable challenge to the antimicrobial therapy. This situation brings us back to the pre-antibiotic era and thus threatens many achievements of modern medicine that rely on antibiotic therapies. An extremely worrying situation was described in a recent report by the World Health Organization (WHO) stating that the resistance phenomena exhibited by infectious agents represented a global threat for humanity and could lead to a "post-antibiotic era" (1). β -lactams are among the most prescribed antibiotics worldwide mainly due to their weak toxicity and good efficacy. However, their clinical use is currently threatened by the worldwide spread of β -lactamases (BLs) capable of hydrolyzing them, especially among MDR Gram-negatives (GNB) (2).

According to recent reports, at least 700,000 people were estimated to die in 2016, and 1.3 millions in 2019 from infections caused by AMR microbes (3). This problem is expected to get worse, if nothing is done, and it is estimated that mortality due to AMR could reach 10 million deaths per year by 2050, and would become the world's first cause of mortality before cancer and cardiovascular diseases (3). Besides the human burden of AMR, it has also been suggested that heavy economic impact are to be predicted, which in terms will affect, economies, health systems and generate poverty. It is estimated that AMR may result in 3-5% drop of gross domestic product and a huge global economic loss of about \$100 trillion in total by 2050 (3).

The over-use and mis-use of available antibiotics, along with the lack of diagnostics are among

the main causes of AMR emergence and dissemination. Moreover, the random use of antibiotics in agriculture and veterinarian fields, worsens the problem, especially in LMIC. Many strategies were implemented to tackle this issue, or at least to slow its progression down. Those actions include better understanding of AMR emergence, reinforcement of public awareness campaigns and surveillance systems, improvement of hygiene to prevent dissemination in hospital settings, and development of novel therapies, including novel antibiotics and vaccines (3).

1. *Enterobacterales*

1.1. *Characteristics*

Enterobacterales, a group of GNB, represents an order that includes several species, sharing similar biochemical and morphological characteristics (4). Bacteria belonging to this order are non spore-forming, facultative anaerobes, showing an oxidase negative reaction, with the majority of them being motile by means of peritrichous flagella. They are able to reduce nitrate to nitrite and ferment sugars to produce lactic acids (5). Most grow well at 22-35 °C (6). This order includes many harmless isolates, in addition to a large number of opportunistic pathogens such as *Escherichia* spp., *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *Serratia* spp. and others, but also true pathogens such as *Salmonella* spp., some *Escherichia* spp., *Shigella* spp., and *Yersinia* isolates such as *Y. pestis* (5, 6).

Enterobacterales are ubiquitous in nature and can be found in soil, water, sewage as well as in the human and animal digestive tract. The opportunistic pathogens take advantage from the reduced host immunity to induce infections such as cystitis, pyelonephritis, peritonitis, meningitis, bloodstream infections, gastrointestinal infections, and nosocomial pneumonia (7, 8).

1.2. *Envelope*

The cell envelope of gram-negative bacteria is a 3-layered structure consisting of (i) the outer membrane (OM), (ii) the peptidoglycan cell wall, and (iii) the cytoplasmic membrane also known as inner membrane (IM). Between the 2 membranes, a periplasmic space exist .

The outer membrane (OM) constitutes the outermost layer of gram negative bacteria providing thus additional protection as a selective permeability barrier. It's a phospholipid bilayer with the phospholipids confined to the inner leaflet of this structure whereas the outer leaflet is characterized by the presence of lipoproteins and glycolipids (9)

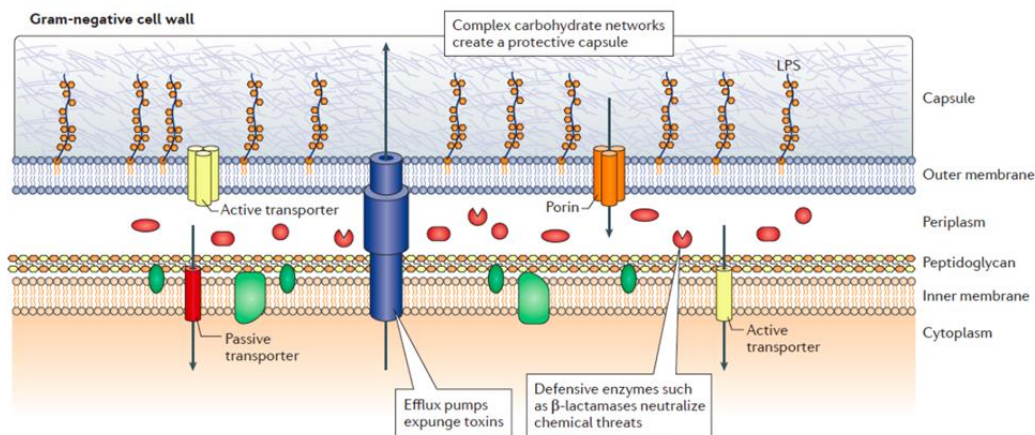


Figure 1: Structure of gram negative bacteria's cell wall – Tommasi et al. 2015 (10)

Lipopolysaccharides (LPS), is another important structure that is specific to gram negative bacteria. LPS is heterogeneous in the various bacterial groups, and is composed of lipid A, core oligosaccharide and O-antigen (11). This endotoxin is responsible for toxic shock in gram negative bacteremia. LPS is considered a real barrier for gram negative bacteria since it protects them from deleterious hydrophobic substances such as bile salts as well as high molecular weight substances like proteases and lipases.

The OM has also integral transmembrane proteins with a β -barrel conformation, known as outer membrane proteins or OMPs. They include a variety of transport proteins such as porins. Porins ensure the transport of low molecular weight hydrophilic molecules such as sugars, amino acids and antibiotics (e.g. hydrophilic β -lactams) (12, 13). Porins such as OprF facilitates the transport of large molecules such as tri- and tetra-saccharides, OprB supports the transport of sugars and saccharides, while OprD ensures the passage of basic amino acids and peptides (14). Most importantly, these porins constitute the entry point for β -lactam antibiotics. Larger molecules require specific transporters and energy to cross the outer membrane.

The peptidoglycan is a rigid exoskeleton made up of repeated units of the disaccharides N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) crosslinked by pentapeptide side chains. Peptidoglycans provide structural strength and allow for the counteraction of the osmotic pressure of the cytoplasm as well as giving the bacteria its shape. Cell wall is the target of many antibiotics since any damage in it, causes bacterial cell lysis.

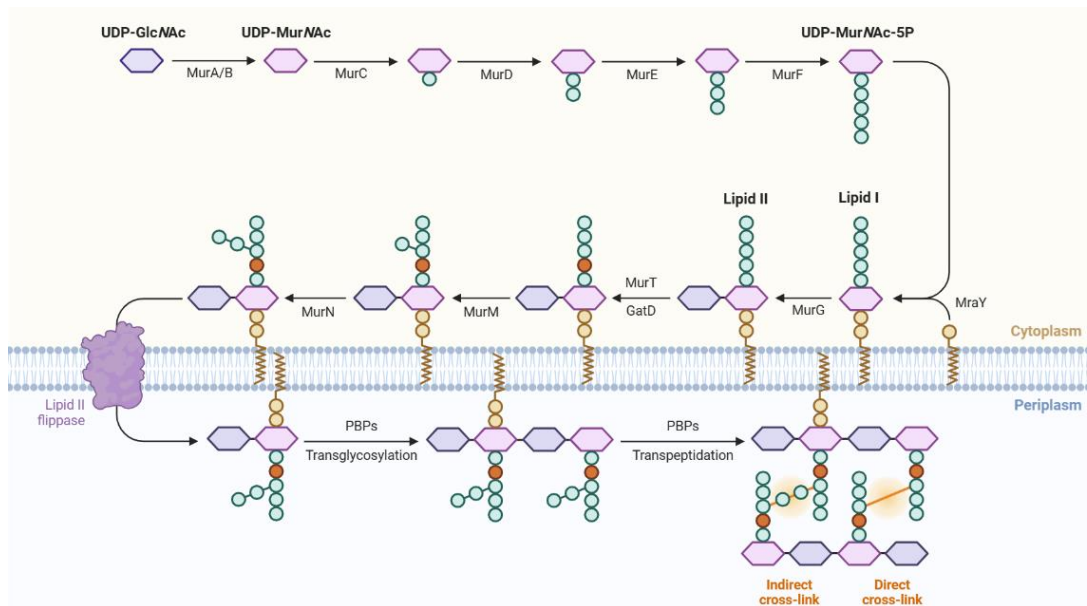


Figure 2: Cell wall biogenesis – N-acetyl-glucosamine (NAG) (represented by the blue hexagone) and N-acetyl-muramic acid (NAM) (represented by the purple hexagone) are peptidoglycan precursors. NAM binds to L-Ala-D-Glu-DAP-D-Ala-D-Ala pentapeptide. This structure is then translocated through the cytoplasmic membrane where it binds to NAG, transfers to the periplasm, polymerize and become stable by peptide bridges through the action of transpeptidase, carboxypeptidase and transglycosylase also called penicillin binding proteins (PBPs) (15).

The synthesis of peptidoglycan is done in several stages (figure 3): the first step takes place in the bacterial cytoplasm, each unit of NAM is bound to a pentapeptide (NAM-PP) usually composed of L-Ala-D-Glu-DAP-D-Ala-D-Ala, with DAP representing diaminopimelic acid. The NAM-PP is then shipped to the inner side of the cytoplasmic membrane, where the NAG becomes attached to it (NAG-NAM-PP). This complex is then translocated to the outer side of the cytoplasmic membrane. Finally, there is polymerization of precursors between them in the intermembrane space (or periplasm) to form carbohydrate chains, which will be connected to each other by peptide bridges (16).

The bridging reaction between two glycan chains is catalyzed by enzymes called DD peptidases, also called penicillin-binding proteins (PBPs). Transpeptidases, carboxypeptidases and transglycosylases are all considered PBPs (17).

PBPs are very important enzymes since they are responsible for the assembly of peptidoglycan, the latter ensuring the rigidity of the wall and the protection of bacteria against the external environment. The number and nature of PBPs vary from one bacterial species to another.

Two main groups are distinguished according to their molecular weight:

- High molecular weight PBPs having transpeptidase and transglycosidase activity

- Low molecular weight PBPs having carboxypeptidase activity only.

All PBPs are active serine enzymes, with the exception of *Streptomyces albus* having DD-peptidase, which is an enzyme involving zinc ions (Zn^{2+}) at its active site (18). PBPs are anchored in the cytoplasmic membrane with their active site directed to the periplasmic space.

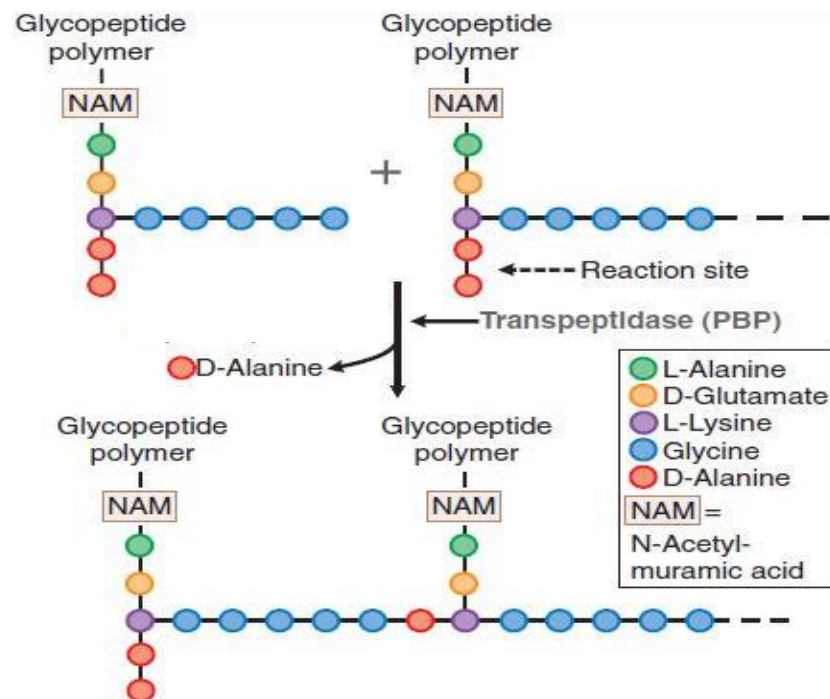


Figure 3: Transpeptidation reaction by the intermediate of PBPs – Goodman et al. (19)

Both the inner and outer membranes delimit an aqueous cellular compartment named **the periplasm**, it's densely packed with proteins and it's more viscous than the cytoplasm. It comprises a variety of proteins having different roles in sugar transport, amino acid (a.a.) transport, chemotaxis, envelope biogenesis ...

2. Antibiotics

2.1. History

Alexander Fleming was at the origin of the antimicrobial drug era, in 1928, at St. Mary's hospital in London. Before leaving on a holiday, he forgot freshly inoculated plates with *Staphylococcus aureus* at room temperature, and upon his return he observed that several cultures were contaminated with molds. The presence of these molds seemed to influence the

colony morphologies. The colonies of *S. aureus* located near the colonies of molds were transparent and appeared to be undergoing lysis. Fleming cultivated the mold, named *Penicillium notatum*, developed a procedure for extracting the active compound produced by this fungi, and called it penicillin. After its discovery, several years were necessary to obtain a penicillin commercially manufactured to treat infections. It was Florey and Chain that could industrialize the entire process so that Penicillin could be used in large scale in 1943 during the second world war. Penicillin was not the first antibiotic available, in the 1930s, a sulfonamide called Prontosil was introduced in human therapy. The word « Antibiotic » was introduced for the first time by Selman Waksman back in 1940 : « An antibiotic is a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms » (20).

The discovery of antibiotics continued and most of the families we know today were introduced between 1940 and 1962. They consisted in chloramphenicol, and streptomycin, and to novel generations of already existing antibiotic families (Figure 4). In 1990s and after the release of all these molecules, discoveries of new antimicrobial agents slowed down (21).

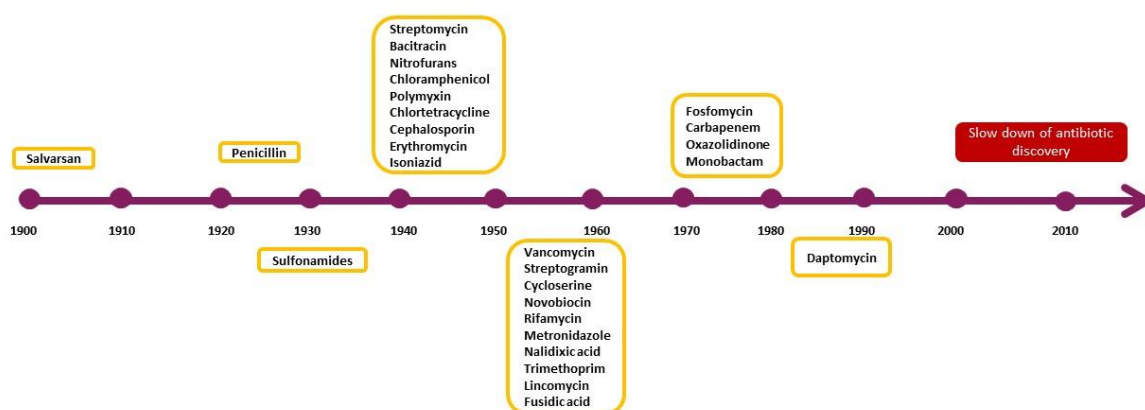


Figure 4: Timeline of antibiotic discovery

2.2. Targets

Antibiotics can be divided into two groups according to their effect on bacteria. We distinguish bactericidal agents, which usually work by killing bacteria at human compatible doses,

and bacteriostatic agents that inhibit bacterial growth when used at human compatible doses (22).

Each antibiotic family acts on a specific target, that must ideally be specific to prokaryotic cells ensuring a selective-toxicity without harming the host. Targets include (i) cell wall synthesis inhibition, (cycloserine ; vancomycine ; bacitracin ; penicillins ; cephalosporins ; monobactams ; carbapenems), (ii) cell membrane disruption, (polymyxine ; daptomycine) (iii) protein synthesis inhibition, (macrolides ; chloramphenicol ; clindamycin ; lincomycin ; tetracyclines ; spectinomycin ; gentamicin ; kanamycin ; amikacin ; nitrofurans) (iv) nucleic acid synthesis inhibition, (quinolones ; novobiocin) (v) folate metabolism inhibition (trimethoprim ; sulfonamides) (Figure 5) (23).

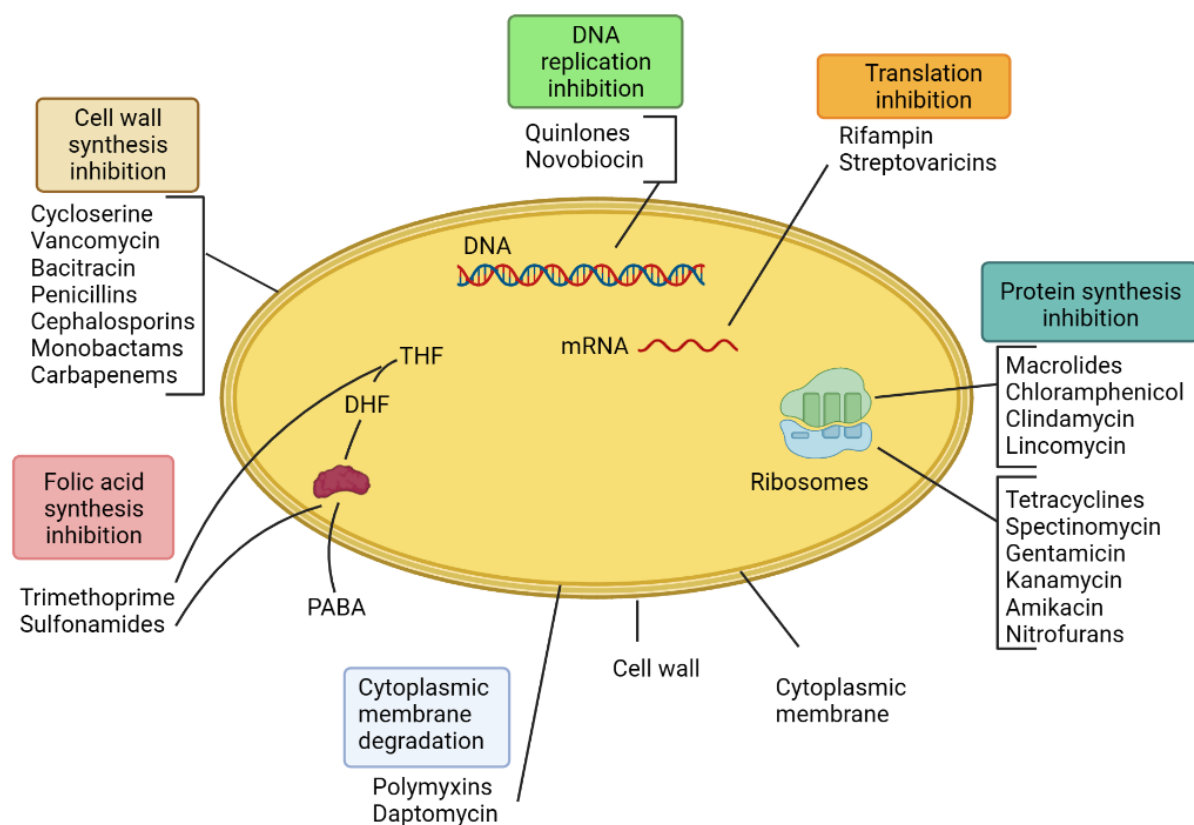


Figure 5: Mode of action of different antibiotic families – Created in BioRender.com

3. β -lactam antibiotics

β -lactam is the most widely used family of antibiotics in prophylaxis and therapy. The importance of their use results from their wide spectrum of action, their low toxicity, therapeutic efficacy and low cost.

3.1. Structure and mode of action

The structure of β -lactams is characterized by a four-membered β -lactam ring that serves as a substrate for the transpeptidase target enzymes, involved in the synthesis of peptidoglycan (24). Transpeptidases, involved in cell wall synthesis by recognizing the D-ala D-ala, are inhibited by the β -lactam ring that has a homologous structure (Figure 1) (25). Thus, a competitive inhibition of the transpeptidases occurs, which prevents the formation of the peptide bridges between the peptidoglycan chains of the bacterial wall and leads to cell death by bursting due to cell wall weakening (25, 26).

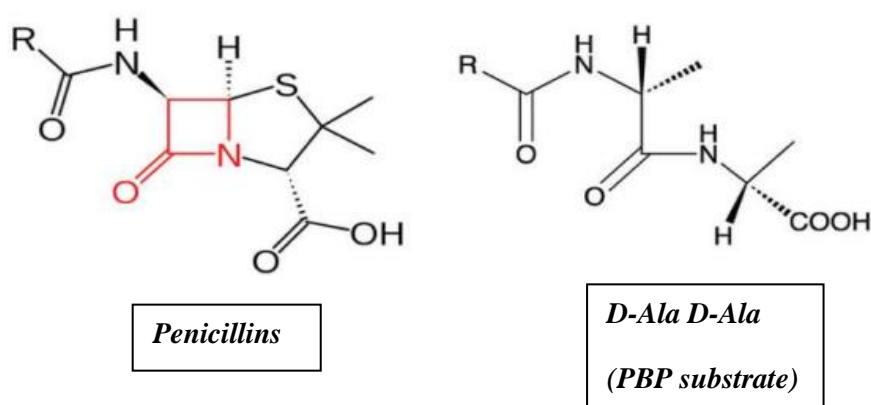


Figure 6: The structural homology between penicillins and D-alanine-D-alanine, the substrate of PBPs.

3.2. β -lactam families

The β -lactams, as their name suggests, are characterized by the presence of a β -lactam nucleus attached, or not, to a heterocycle of 5 to 6 atoms. Thus, depending on the nature of the heterocycle, 4 β -lactam antibiotics sub-families exist: penicillins, cephalosporins, monobactams and carbapenems (Figure 7).

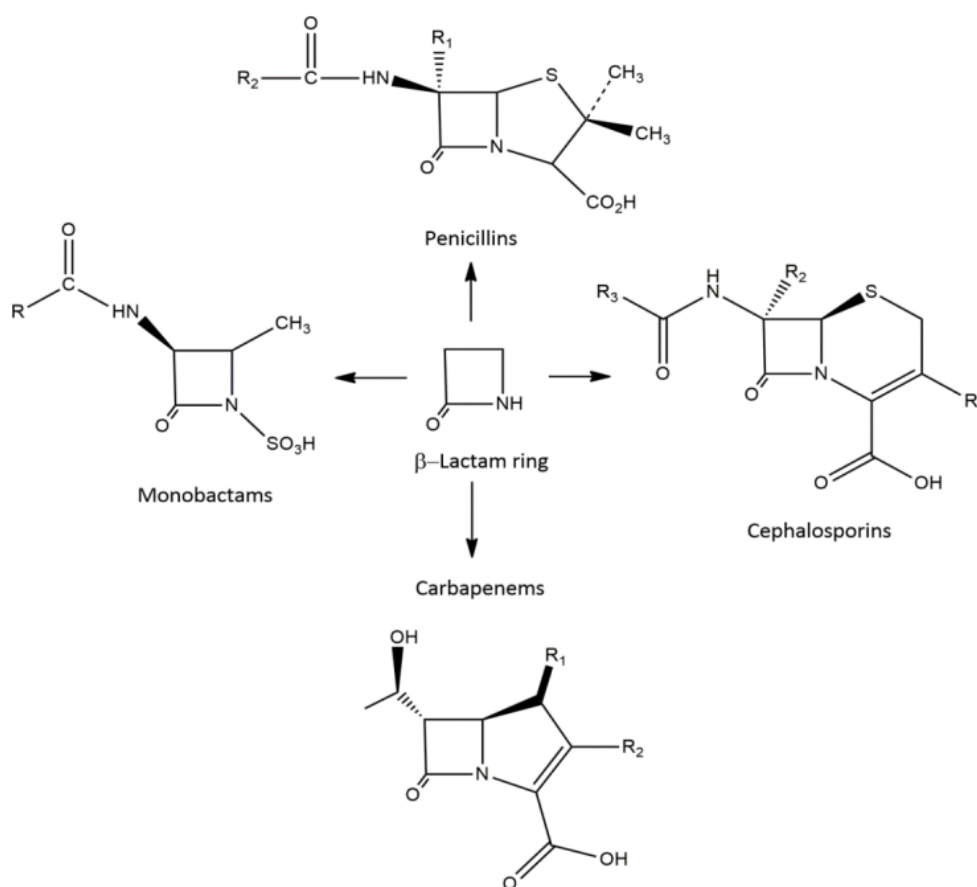


Figure 7: Structures of β -lactam antibiotics – adapted from Bush et al. 2016 (27)

- Penicillins

Penicillins are β -lactams with a penam nucleus associated with a thiazolidine ring as penicillin G discovered in 1940s. The spectrum of activity of penicillin G is broad as it encompasses gram positive cocci, gram positive rods, anaerobes and some gram negative cocci but spare gram negative rods as penicillin G cannot enter into the periplasm of these bacteria the bacteria. In addition penicillin G could not be administrated per os, as it was labile in presence of then stomach acidity. Several derivatives of penicillin G were developped to over come certain limitations. Thus, molecules with moderate antibacterial spectrum but resistant to acidity such as penicillin V, were developped and were still mainly active on gram-positive bacteria. Similarly, penicillin M such as methicillin was developped in response to the emergence of penicillin G resistant *Staphylococci*. Finally molecules

active on Gram-negative rods were also developed such as aminopenicillins (amoxicillin, ampicillin), ureidopenicillins (piperacillin), and carboxypenicillins (ticarcillin) (28), temocillin (29) .

- **Cephalosporins**

They are β -lactams with a cephem nucleus, that is made of a β -lactam cycle and a dihydrothiazine cycle. After initial discovery in 1940s, from a mold *Cephalosporium acremonium*, several derivatives were synthesized. Thus, today several generations of cephalosporins exists (1st, 2nd, 3rd, 4th, and 5th). They act in the same way as penicillins, their effective bactericidal activity is known to kill gram positive as well as gram negative bacteria and their toxicity is relatively low.

First generation cephalosporins show great activity against gram positive cocci except Enterococci and methicillin resistant *Staphylococcus aureus* (MRSA). This class acts moderately on some gram negative rods including *E. coli*, *Proteus mirabilis*, *Klebsiella* and anaerobic cocci. Cefalothin, cephalexin and cefadroxil belong to this group.

Second generation cephalosporins cover same species with some additional gram negative rods (*Klebsiella* and *Proteus*). This generation does not cover *P. aeruginosa*. Cefoxitine and cefuroxime are some examples of this group.

Third generation cephalosporins act on gram negative rods, *P. aeruginosa*, and are very useful in the management of hospital acquired gram-negative bacteremia and are characterized by their ability to reach the central nervous system and cerebrospinal fluid, which makes them a good treating option for meningitis (30). Some examples are cefotaxime, ceftriaxone, ceftazidime and ceftizoxime.

Fourth generation cephalosporins represent an enhanced version of the previous classes with an additional activity on cephalosporinase producing Gram negatives such as *Enterobacter* spp, *Citrobacter* spp, and *Streptococci*. Among this generation, we cite cefepime.

Finally, a fifth generation of cephalosporins comprising ceftaroline and ceftobiprol, two cephalosporins active on MRSA and gram positive bacteria.

- **Carbapenems**

Carbapenems are the β -lactams with the broadest spectrum of activity. They are considered

as last resort antibiotics and the first line therapy for critical infections, especially those caused by Enterobacterales producing β -lactamases capable of hydrolysing all the other β -lactams (31). They differ from penicillins by the presence of a carbon atom in the place of a sulfur in position 1 and an unsaturated bond of C2-C3, which is also present in cephalosporins. They were developed from thienamycin naturally produced by *Streptomyces cattleya* (32). This natural molecule being unstable, a more stable derivative has been developed: N-formimidoyl thienamycin or imipenem. However, imipenem is degraded by a human enzyme present in the renal tubules, the dehydropeptidase-1 (DHP-1) (33), therefore, imipenem is usually administered with an inhibitor of this enzyme, cilastatin. Other carbapenems: ertapenem, meropenem and doripenem remain insensitive to DHP-1 activity. Carbapenems, have the widest spectrum of activity including gram-positive bacteria (such as *S. aureus*), aerobic and anaerobic gram-negative bacteria (34). Ertapenem has a reduced spectrum of activity as it doesn't include *P. aeruginosa* and *A. baumannii* (33).

Because of their strong antibacterial power, they have become an important therapeutic option for severe nosocomial infections (35). Carbapenems are used for the treatment of infections caused by gram-negative bacteria resistant to expanded-spectrum cephalosporin (ESC). Because of their short half-life, imipenem and meropenem are administered several times during the day. The emergence of resistance to carbapenems is worrying since it limits the treatment options and can lead to real therapeutic impasses.

- **Monobactams**

Despite their narrow spectrum, they are highly active on aerobic gram negative bacilli, Those antibiotics are so named due to their monocyclic β -lactam ring (36). Aztreonam, the representative of this family is used primarily as an alternative to aminoglycosides for the treatment of aerobic gram-negative infections, since it's active on gram negative rods, especially *Enterobacterales* and *P. aeruginosa*, but remains inactive on gram positive and anaerobic bacteria (37). Their spectrum of activity is relatively comparable to that of ESCs.

- **Inhibitors**

Clavulanic acid, tazobactam, and sulbactam

The first β -lactamase inhibitor to be introduced into clinical medicine is clavulanic acid, it

was isolated from *Streptomyces clavuligerus* in 1970s, hence it's name (38). This molecule is known to significantly reduce amoxicillin MICs against *S. aureus*, *K. pneumoniae*, *P. mirabilis* and *E. coli*, however, it shows little antimicrobial activity on it's own (39). In 1978 and 1980, sulbactam and tazobactam, two penicillin sulfones were synthetically developed (40, 41).

Clavulanic acid, sulbactam and tazobactam differ from β -lactam antibiotics as they possess a leaving group at position C-1 of the five membered ring (sulbactam and tazobactam are sulfones, while clavulanate has an enol ether oxygen at this position). The better leaving group allows for secondary ring opening and β -lactamase enzyme modification. Compared to clavulanate, the unmodified sulfone in sulbactam is a relatively poor leaving group, a property reflected in the high partition ratios for this inhibitor (42). Tazobactam possesses a triazole group at the C-2 β -methyl position. This modification leads to tazobactam's improved IC₅₀s, partition ratios, and lowered MICs for representative class A and C β -lactamases (43, 44).

Boronic acid

In the 1970s, boronic acid compounds were described as forming reversible, dative covalent bonds with serine proteases and inhibiting these enzymes by assuming tetrahedral reaction intermediates (45-47). Boronic acid works as a competitive inhibitor, by forming a reversible dative bond with the β -lactamase. Glycylboronates are based on β -lactam substrate homology and contain side chains of penicillins and cephalosporins. More recently, new modifications to the β -lactam analog boronate were added by replacing the carboxamide group, conserved in all penicillin and cephalosporins, with a sulfonamide (48, 49). These novel derivatives are more potent than their carboxamide analogs. Combined with ceftazidime, the lead compounds lowered MICs up to 64 folds.

RPX7009 known as vaborbactam was presented in combination with a carbapenem antibiotic (RPX2003; biapenem) (50). Alone, RPX7009 did not exhibit antibacterial activity, but the combination showed strong potentiation of biapenem against class A carbapenemase producing Enterobacterales (51).

Avibactam and derivatives

Avibactam is another potent inhibitor of β -lactamases, with an inhibition spectrum that was considerably broadened when compared to that of clavulanate. Indeed, avibactam

efficiently inhibit most class A β -lactamases, including the inhibitor-refractory KPC-type carbapenemases, both the chromosome and plasmid encoded class C enzymes and several class D β -lactamases, including the OXA-48 carbapenemase (52-54). The crystal structure of different β -lactamase-avibactam covalent adducts provided a structural basis for the broad spectrum of inhibition of the inhibitor (55). The avibactam molecule bound to the catalytic serine, in which the oxygen carbonyl is located in the oxyanion hole, creates a network of interactions that's very similar in both class A and class C enzymes and closely resembles that of acylated β -lactam substrates. The sulfonate moiety interacts with residues of the conserved KTG motif, while the carboxamide side chain is stabilized by interactions with Asn/Gln residues. The mechanism of inhibition of avibactam is significantly different from that of clavulanate or penam sulfones, in that avibactam acts, with most β -lactamases, as a reversible inhibitor, while being a covalent inhibitor (53).

Relebactam, another inhibitor with a piperidinium substituent on the carboxamide side chain of the diazabicyclooctane (DBOs) scaffold, was developed in combination with imipenem-cilastatin (56). It shows properties very similar to that of avibactam except a lower activity on the OXA-48 carbapenemase (27).

Nacubactam is also a DBO showing a substituent on the carbaxamide side chain, resulting in a potent inhibition of β -lactamases as well as PBP2 (57).

Zidebactam, currently developed in combination with cefepime, is another example of substituted DBO with a similar effect due to PBP2 inhibition (58).

MBL inhibitors

Moving to MBLs, they are insensitive to most of the clinically available β -lactamase inhibitors and hence represent one of the most worrying bacterial resistance factors. They are characterized by a broad substrate specificity, being able to hydrolyse all β -lactam antibiotics, most importantly carbapenems with the exception of monobactams (59). The difficulty in finding a universal inhibitor of MBLs arises from the structural and mechanistic differences spread among MBL's subclasses (60). MBLs inhibitors can be grouped depending on their chemical determinants able to interfere with the MBLs active site.

ZN chelating inhibitors

Strong chelating agents as for example EDTA, can sequester and extract zinc from MBLs.

Although effective, such compounds strongly suffer from being aspecific, and thus potentially associated with cytotoxicity issues. However, the potential clinical application of Ca-EDTA was demonstrated in a murine sepsis model of infection (61).

MBL covalent inhibitors

An unusual type of inhibitors is the selenium-bearing compound ebselen (2-phenyl-1,2-benzoselenazol-3-one) that has been shown to efficiently inactivate NDM-1 and restore meropenem activity on NDM-1 expressing *E. coli* cells (62). It appears that the activity of this molecule is due to the opening of selenazol ring and formation of a covalent Se-S bond with the zinc binding Cys221 residue. Along this line of research 4-oxo-4H-1-benzopyran-3-carboxaldehyde (3-formylchromone) has also been shown to be a covalent inhibitor of NDM-1 by binding to the active site Lys244 (63).

4. Mechanisms of resistance to β -lactams

Since the discovery of antibiotics, bacterial resistance occurred and scientists started questioning the causes of this emerging phenomenon. The first cases of penicillin G resistant *Staphylococcus* isolates were described in 1941, with a prevalence of less than 1%. In 1947, three years after penicillin G was marketed, the number of cases rose from 1% to 38% (64).

According to the WHO, bacterial resistance to antibiotics, occurs when bacteria resist to the effects of these drugs at treatment compatible doses. Multidrug-resistance (MDR) is defined when the bacteria becomes resistant to the cytogenic and cytotoxic activity of three or more classes of antimicrobial agents. Many factors have contributed to the emergence of AMR, such as misuse and overuse of antibiotics in human (self medication, over-counter sales, treatment of non bacteriological infections), and veterinary practices (especially their use as growth promoting factors) (65, 66). AMR may be the result of a natural resistant trait in certain bacteria (intrinsic bacterial resistance) or of acquisition by the intermediate of genetic mutations or of external genetic material through horizontal transfer (67). Acquired bacterial resistance to antibiotics may be related to four distinct genetic mechanisms (figure 8).

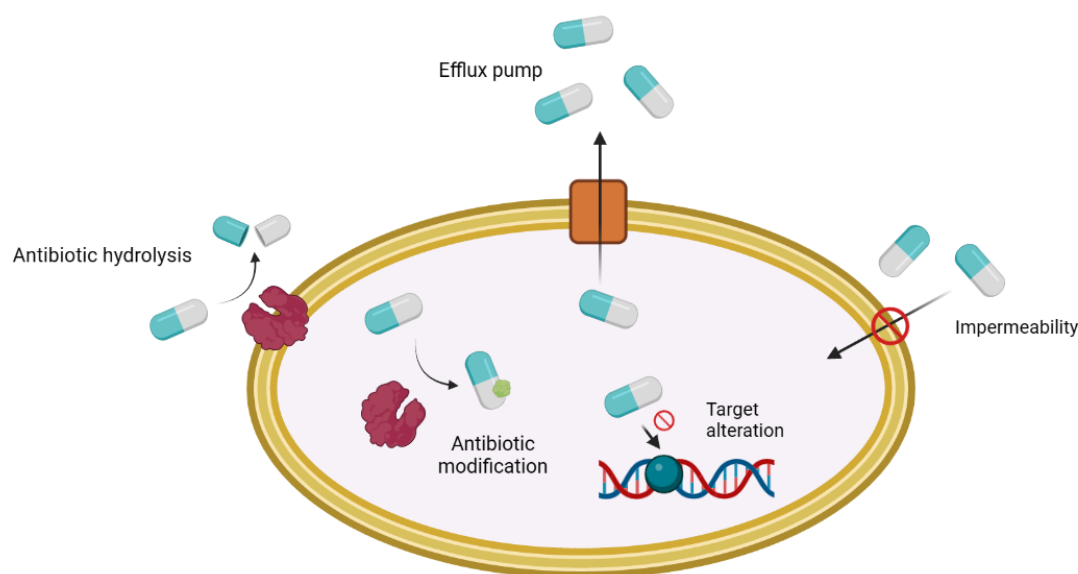


Figure 8: Main mechanisms of antibiotic resistance. Adapted from E. Wistrand-Yuen (68)

i. Impermeability

Unlike Gram positives, with Gram negatives, the β -lactams have to cross the outer membrane (OM) to reach their targets, the PBPs. Resistance or reduced susceptibility by impaired OM permeability occurs when the antibiotic cannot reach its target anymore. As porins ensure the passive entry of β -lactams into the periplasmic space, their alteration is the main cause of resistance by impermeability. Mutations of the genes encoding the porins may have a qualitative effect, preventing β -lactams from entry, or of the genes involved in the regulation of porin synthesis may have a quantitative effect, with the reduction of the number of porins present in the OM, and thus in a reduction of the antibiotics that can reach the periplasm. This resistance mechanism was reported in several gram-negative isolates including *P. aeruginosa*, *A. baumannii*, *E. coli*, *Klebsiella sp*, *Enterobacter sp*, *Serratia*, *Salmonella*, *Shigella* and others (69).

In *P. aeruginosa*, one single porin, OprD, is responsible for carbapenem uptake, and its loss results in carbapenem-resistance alone. OprD mutations account for the main carbapenem resistance mechanism in *P. aeruginosa* and is present in c.c. 15% of clinical isolates in France (cnr-resistance-antibiotiques.fr). In most other Gram-negatives, OM impermeability results often in reduced susceptibility to β -lactams, and require additional resistance mechanisms, such as production of enzymes inactivating β -lactams to reach high

level resistance. In *E. coli* and in *K. pneumoniae*, the alteration of OmpA, OmpC and OmpF and OmpK35 and OmpK36 porins, respectively, is frequently described in multidrug-resistant isolates and in carbapenem resistance (70, 71).

ii. Efflux

Another resistance mechanism affecting antibiotics is active efflux through the up-regulation of efflux pumps, which are transmembrane transporters that actively eliminate toxic molecules, including antibiotics, outside the bacteria.

Several efflux pumps families have been described, including ATP-Binding cassette transporters ABC, small multidrug resistance SMR, multidrug and toxic compound extrusion MATE, major facilitator superfamily MSF, and resistance nodulation cell division RND (Figure 9) (72).

High level resistance could be observed as a result of mutations causing overexpression in efflux pumps. Usually this overexpression will cause resistance to more than one family of antibiotics (73, 74). For instance, the primary multidrug resistance efflux pump in *E. coli* is AcrAB-Tol C which is made of three proteins spanning the inner and outer cell membrane (AcrA: linker protein ; AcrB: inner membrane efflux transporter ; TolC: outer membrane channel) (75). Another example of efflux pump is the MexAB-OprM, a major actor in *P. aeruginosa* antibiotic resistance (76). It exports a large variety of antimicrobial compounds.

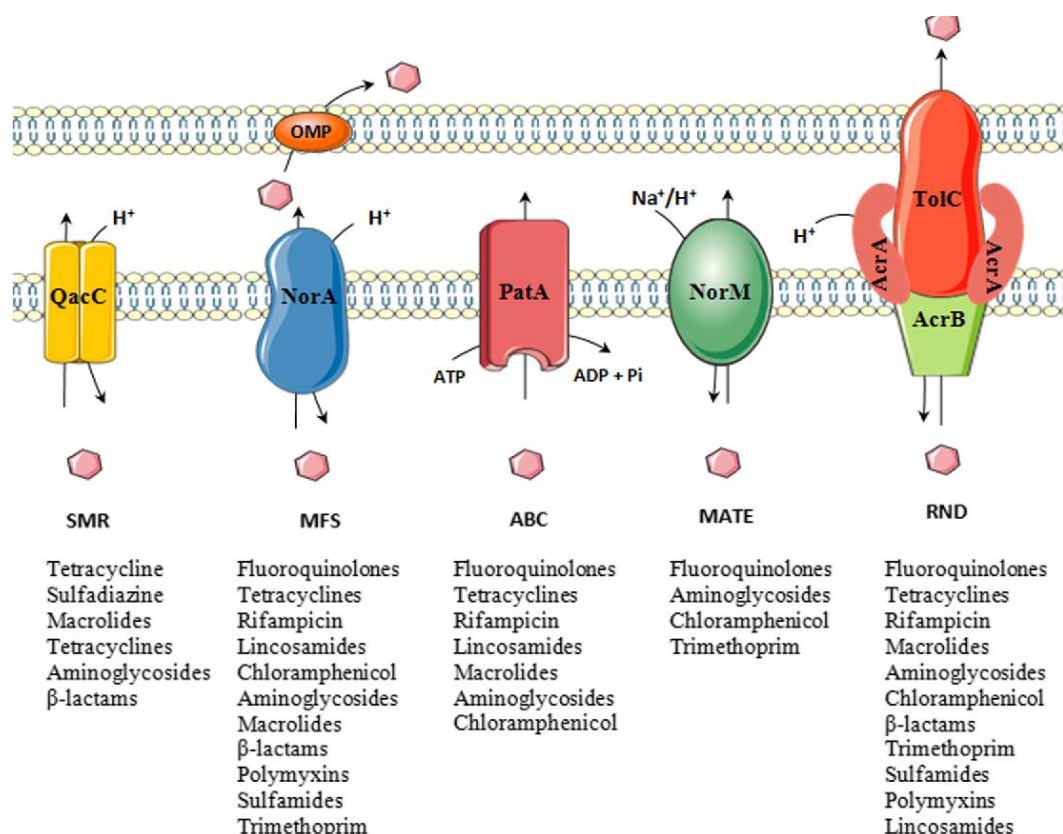


Figure 9: Efflux pump families. Schematic representation of the five efflux pump families with their known antibiotic substrates. SMR family "Small Multidrug Resistance" family, MFS family « Major Facilitator Superfamily, ABC Family "ATP-binding cassette", MATE Family "Multidrug and Toxic" compound extrusion ", RND family "Resistance-Nodulation-Division. OMP (outer membrane protein): outer membrane protein. From Yilmaz and Özcengiz (77)

iii. Target site alteration

A third mechanism by which bacteria may resist to the action of antibiotics is the alteration of the target site. Once mutations occur in the target molecules of antibiotics, a decrease of binding affinity of the antibiotics, will result in reduced susceptibility to the latter. However, most target sites are vital for bacteria (PBPs, RNA polymerase, DNA gyrase, etc.), which means that their total inactivation to evade antimicrobial activity will harm the bacteria. Thus mutations in these targets lead to reduced the susceptibility to antibiotic. For β -lactams, in addition to the alteration of chromosomally-encoded PBPs, as seen for gram-negatives (78), acquisition of through horizontal tranfer of PBPs, such as PBP-2A in MRSA, may lead to hich level resistance to all β -lactams (79). Talking about PBPs, it was shown that insertions in PBP3 lowers susceptibility of bacteria to 3rd generation cephalosporins and aztreonam (80). Thoroughly, a significant increase in aztreonam, avibactam and cefiderocol MICs was shown when four-amino-acid inserts

YRIK and YRIN are present in PBP3 (81, 82).

iv. Enzymatic inactivation

Inactivation of antibiotics through specific enzymes is the main mechanism of resistance to β -lactams in Gram-negatives(83). Bacteria may produce enzymes, β -lactamases that are capable of inactivating β -lactam molecules by opening the β -lactam ring and thus rendering the antibiotic ineffective. Some species are characterized by a constitutive expression of enzymes e.g. *K. pneumoniae* expressing SHV, while some enzymes may be acquired through horizontal gene transfer. β -lactamase production, constitute the primary resistance mechanism in GNB (83).

5. β -lactamases

5.1. Classification

Although many mechanisms of resistance exist, β -Lactamase production remain the most common one enabling resistance to almost all β -lactam antibiotics. Since the early 1940s, β -lactamases, encoded on mobile genetic elements, or on chromosomes have played a major role in emergence and dissemination of resistance (84). Two classifications of β -lactamases are currently in use (83, 85): Ambler classification, which classifies the β -lactamases according similarity in their a.a. sequences , and the functional classification of Bush, Medeiros and Jacoby, which classifies the β -lactamases according to their substrate specificities (86). The most widely used in that of Ambler that contains four classes of enzymes. Class A β -lactamases, also called penicillinases, represent the most important group of serine β -lactamases, they hydrolyze penicillins, and are inhibited by clavulanic acid, sulbactam and tazobactam, initially characterized as penicillinases. Class B, known also as metallo β -lactamases (MBLs), and are inhibited by EDTA, which is a divalent ion chelator. MBLs hydrolyze all β -lactams except aztreonam. Class C, also so-called cephalosporinases, comprise serine β -lactamases, they hydrolyze aminopenicillins and cephalosporins, and are inhibited by cloxacillin. Finally, Class D β -lactamases, also called oxacillinases, are composed of serine β -lactamases, they hydrolyze oxacillins at a higher rate than penicillinG (86).

Table 1: Ambler's classification

Ambler class	Class A	Class B	Class C	Class D
-Type of enzyme based on the active site	Serine β -lactamase	Metallo β -lactamase	Serine β -lactamase	Serine β -lactamase
-Enzyme	TEM, SHV, CTX-M, KPC, GES, IMI, NMC	NDM, IMP, VIM, GIM	AmpC	OXA
-Inhibitors	Clavulanic acid, sulbactam, tazobactam	EDTA	Cloxacillin	Avibactam, Relebactam, Vaborbactam

5.2. Structure and mode of action

Serine β -lactamases

Classes A, C and D of Ambler are serine active enzymes. Serine β -lactamases interfere with the binding of PBPs to β -lactam antibiotics and induce their hydrolysis due to highly conserved amino acid sequences in common with PBPs (87, 88). Serine β -lactamases consists of two structural domains and a serine based active site located at the groove between them (89). Active serine β -lactamases all have three highly preserved common motifs (90). The motifs 1 and 3 are identical in the three classes of Ambler while motif 2 varies and it is characteristic of the enzyme class (Table 2).

Table 2: Conserved motifs of serine β -lactamases in their active site. The active serine is marked in red.

Motifs	Class A	Class C	Class D	DD-peptidase
1	S XXK	S XXK	S TFK	S (T/S)FK
2	SDN	YAN	SXV	(Y/S)X(N/C)
3	(K/R)(S/T)G	KTG	K(T/S)G	(H/K)(T/S)G

The acylation reaction marks the beginning of the enzymatic reaction, where serine β -lactamases acylates β -lactam antibiotics and hydrolyzes the acylated β -lactam ring by the means of a water molecule then the enzyme regenerates to act on other β -lactam molecules (Figure 10).

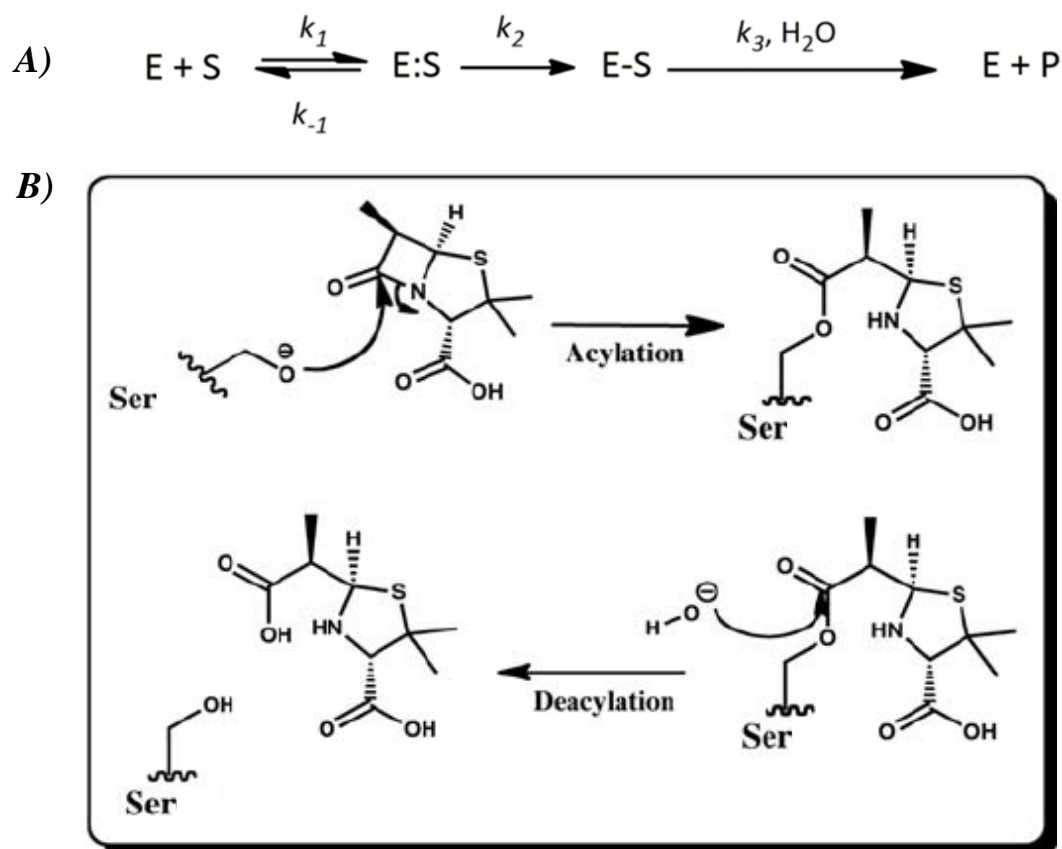


Figure 10: Mechanism of action of serine β -lactamases (A) Catalytic model of active serine β -lactamase. E: enzyme, S: substrate, P: product, ES: Michaelis complex of pre-acylation. The speed constants for each steps are represented by k_1 (association rate constant), k_{-1} (dissociation rate constant), k_2 (acylation rate constant) and k_3 (desacylation rate constant). (B) Detailed acylation and desacylation reactions. In the first half-reaction, the nucleophilic Ser residue attacks the carbon of the carbonyl of the β -lactam ring, which causes the cutting of the cycle and the formation of a stable enzyme acylate. In the second step, a water molecule attacks the same carbon, the bond breaks and the inactivated ligand is released from the active site. Adapted from Szarecka et al. (91).

The kinetic model of these enzymes is based on three steps characterized by different interactions between the enzyme and the substrate (Figure 10A). The first step is the binding of the enzyme to the substrate via a non-covalent bond forming a complex called the Michaelis complex (E:S). This complex is characterized by two constants: the constant association (k_1) and the dissociation constant (k_{-1}). In a second step, this complex is transformed into a covalent complex (called acyl-enzyme complex: E-S) through the establishment of an ester bond

between the active serine and the carbonyl function of the β -lactam nucleus (91).

From a molecular point of view, the serine, once activated, performs a nucleophilic attack at carbon level of the carbonyl function of the β -lactam nucleus. This results in the rupture of the amide (C-N) bond of the β -lactam nucleus. This reaction is called acylation (characterized by the acylation constant k_2) allows the formation of the covalent stable complex: the acyl-enzyme. In a second step, a water molecule attacks this same carbon causing the rupture of the covalent enzyme-substrate bond and releasing the inactive product, this affinity on reaction is characterized by constant k_3 .

Steady state kinetics are mainly based on several constants among which K_m , so-called Michaelis Menten constant, represents the affinity of enzyme to substrate and is defined by the following equation : $K_m = (k_3 \times k_s / k_1) / (k_2 + k_3)$, with $k_s = (k_{-1} + k_2) / k_1$. K_m is the substrate concentration at which the reaction rate is half of its maximal reaction rate. The lower the value of K_m , the higher is the affinity. Another important determinant is k_{cat} , which is the turnover number representing the number of enzymes hydrolyzed per second and is defined as $k_{cat} = k_2 k_3 / (k_2 + k_3)$. $V_{max} = k_{cat} I$, represents also an important parameter, it is the maximum velocity of an enzymatically catalyzed reaction when the enzyme is saturated with its substrate, it could allow for the estimation of enzyme's concentration. This hydrolysis serves as a regeneration step of the enzyme and results in the release of β -lactam antibiotic devoid of its bacterial activity.

Metallo β -lactamases

MBLs are Ambler class B β -lactamases that contain one or two zinc ions in the active site. Within this family, three subgroups are distinguished according to their amino acid sequences and hydrolysis profiles: subgroups B1, B2 and B3. Although there is low sequence homology between these different subgroups, these enzymes exhibit a high similarity in their folding. Subgroup B1 and B3 have a hydrolytic profile including all β -lactams with the exception of monobactams, while B2 hydrolysis mostly carbapenems. MBLs are not inhibited by classical serine β -lactamase inhibitors (such as clavulanic acid, or even newer molecules such as avibactam), but are inactivated by metal chelators that deprive them from the Zinc cationic ions and thus they become non-functional (92-94). MBLs have a different mode of

action as compared to serine β -lactamases. In the case of MBLs, the nucleophilic attack of the amide bond of the β -lactam ring is mediated by a water molecule (hydroxyl group -OH) coordinated by one or two zinc ions. Thus, no covalent intermediate gets formed, and the substrate is released from the active site (89, 95).

Subclass B1 contains the most clinically-relevant MBLs, such as Verona Integron-encoded Metallo-beta-lactamase (VIM), New Delhi Metallo- β -lactamase (NDM), IMiPenemase (IMP) are monomeric and are characterized by one high affinity zinc binding site and another one with low affinity (96). Subclass B2 enzymes are all produced by species of *Aeromonas* spp. (97). They are all in the form of mono-zinc with a high affinity site for the zinc ion. The binding of a second zinc ion inhibits their enzymatic activity (98). Whereas, enzymes of subclass B3, even though mostly chromosomally-encoded and naturally-produced by certain species, some variants such as AIM, LMB-1 have been described as plasmid-encoded in Enterobacterales and *P. aeruginosa* (99, 100). They are predominantly monomeric (101, 102) and essentially in the di-zinc form. MBLs are usually encoded on genetic structures such as integrons and plasmids which increases the rate of their dissemination and make them an important source of resistance (92).

II- Carbapenemase

Since antibiotics discovery, bacteria developed many ways to evade bacterial killing among which the production of specific enzymes capable of degrading, or modifying them (103). Soon after the commercialisation of imipenem in 1985, the β -lactamase IMP was described for the first time in Japan in 1990. Subsequently, several other carbapenem-hydrolyzing enzymes have been discovered across the world (Figure 11).

Carbapenemases correspond to β -lactamases that are able to hydrolyze efficiently carbapenems (104). They comprise the greatest variety of enzymes belonging to classes A, B and D of Ambler (105). Each enzyme presents its own characteristics, but their spectrum of action is, in general, quite wide and can include, in addition to carbapenems, penicillins, cephalosporins and monobactams (Table 3).

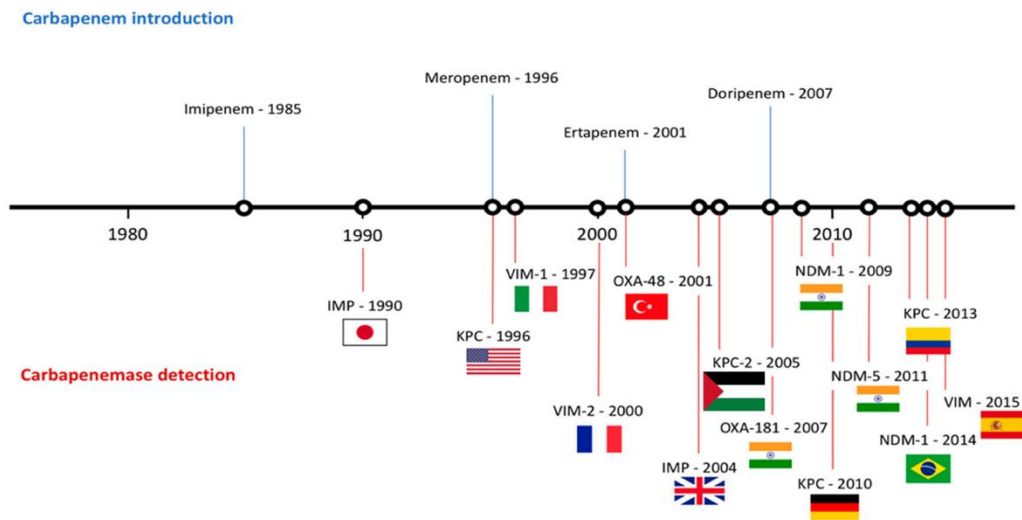


Figure 11: Timeline of introduction of the different carbapenems and the discovered carbapenemases Adapted from Suay-Garcia et al. (106).

The first natural carbapenemases, belonging to class B of Ambler, were identified in *S. maltophilia*, *Aeromonas* sp., *B. cereus* and *Bacteroides fragilis* (107). It was in the early 1980s when the first chromosomal class A carbapenemases were described in Enterobacterales, with SME-1 in *S. marcescens* in London in 1982 (108, 109), IMI-1 in *E. cloacae* complex in the United States in 1984 (110), and NMC-A in *E. cloacae* from France in 1994 (111).

Since the 2000s, an increase in the number of carbapenem-resistant Enterobacterales was witnessed all over the world. This increase is mainly linked to the spread of the 5 main carbapenemases: KPC (class A), NDM, VIM, IMP (class B), and OXA-48 (class D).

Table 3: Characteristics of carbapenemases

Carbapenemases	Number of variants according to BLDB	Class of Ambler	Most frequently identified in	Genetic background	Activity
KPC	157	A	<i>Enterobacterales</i> , <i>P. aeruginosa</i> , <i>A. baumannii</i>	Plasmid encoded	hydrolyze aminopenicillins, uereidopenicillins, 1st and 2 nd generation cephalosporins, aztreonam, imipenem and meropenem
SME	5	A	<i>S. marcescens</i>	Chsomosomally encoded	hydrolyze aminopenicillins, uereidopenicillins, 1st and 2 nd generation cephalosporins, aztreonam, imipenem and meropenem
IMI/NmcA	25	A	<i>E. cloacae</i>	Chromosomally and plasmid encoded	hydrolyze aminopenicillins, uereidopenicillins, 1st and 2 nd generation cephalosporins, aztreonam, imipenem and meropenem
NDM	50	B	<i>Enterobacterales</i> , <i>P. aeruginosa</i> , <i>A. baumannii</i>	Plasmid encoded	hydrolyze aminopenicillins, uereidopenicillins, 1st and 2 nd generation cephalosporins, aztreonam, imipenem and meropenem
IMP	98	B	<i>Enterobacterales</i> , <i>P. aeruginosa</i> , <i>A. baumannii</i>	Plasmid encoded	hydrolyze aminopenicillins, uereidopenicillins, 1st and 2 nd generation cephalosporins, aztreonam, imipenem and meropenem
VIM	86	B	<i>P. aeruginosa</i> mostly but also in <i>Enterobacterales</i> , and <i>A.</i>	Plasmid encoded	hydrolyze aminopenicillins, uereidopenicillins, 1st and 2 nd

			<i>baumannii</i>		generation cephalosporins, aztreonam, imipenem and meropenem
GIM	3	B	<i>P. aeruginosa</i> , <i>E. cloacae</i> , <i>Acinetobacter pittii</i>	Plasmid encoded	hydrolyze aminopenicillins, ureidopenicillins, 1st and 2 nd generation cephalosporins, aztreonam, imipenem and meropenem
SPM	1	B	<i>P. aeruginosa</i>	Plasmid encoded	hydrolyze aminopenicillins, ureidopenicillins, 1st and 2 nd generation cephalosporins, aztreonam, imipenem and meropenem
OXA-48-like	50	D	Enterobacterales	Chromosomally or plasmid encoded	hydrolyze aminopenicillins, ureidopenicillins, slightly carbapenems and poorly 3 rd generation of cephalosporin
OXA-23	44	D	<i>A. baumannii</i>	Chromosomally or plasmid encoded	hydrolyze aminopenicillin, ureidopenicillins, does not hydrolyze the ESC ceftazidime
OXA-24	11				
OXA-58	8				

1. Class A carbapenemase

The first Ambler Class A carbapenemases were described in the 80s in clinical isolates of *E. cloacae* and *S. marcescens* likely of environment source (112). These enzymes were able to hydrolyze imipenem and penicillins but had no activity against ESCs (113). The addition of clavulanic acid could partially restore the activity of these molecules. Enzymes responsible for this resistance were NMC-A (non-metallo carbapenemase), SME (*Serratia marcescens* enzyme) (108) and IMI (imipenem-hydrolyzing β -lactamase)(114, 115). SFC-1 has been only reported once to date, in a *Serratia fonticola* strain described in 2003 in Portugal (116).

Subsequently other enzymes have been described, such as BKC-1, for Brazilian *Klebsiella* carbapenemase, BIC-1 carbapenemase which was identified in an environmental *Pseudomonas*, it hydrolyzes carbapenems, penicillins, cephalosporins, and monobactams (117, 118), fluorescens isolate (PF-1) resistant to carbapenems and recovered from water of the Seine river in Paris (France) (118), FRI Fribourg resistance to imipenem, (119). These carbapenemases remain rare because they are chromosomally-encoded (*bla*_{SME}, *bla*_{NMC-A}, *bla*_{IMI-1}) (112), not usually associated with mobile genetic elements to facilitate their dissemination. Among them, some IMI variants (IMI-2) are plasmid encoded and thus likely to spread to other species, as revealed in *E. coli* in Spain or in China (120, 121). Recent years have witnessed an increasing number of variants described worldwide, suggesting an acceleration of the rapid spread of these enzymes.

GES (Guiana extended-spectrum) -type β -lactamases (also named IBC) are increasingly reported in gram-negative rods, including in *P. aeruginosa*, *A. baumannii*, *E. coli*, and *K. pneumoniae* (114, 122). GES enzymes are peculiar ESBLs as they can by single amino-acid changes extend their spectrum of hydrolysis towards carbapenems, azteonom and cefoxitin. Among the 53 known GES variants 32 (60,4%) with either Gly170Asn (n=2; 3.8%) or Gly170Ser (n=30; 56.6%) mutations are carbapenemases, while the others were categorized as minor ESBLs without any carbapenem hydrolytic activity (112, 123, 124). GES-carbapenemases have a moderate hydrolytic activity towards carbapenems resulting in reduced susceptibility to carbapenems in Enterobacterales expressing them, thus rendering their detection difficult (125). *bla*_{GES} genes are carried by integrons themselves inserted into plasmids and are therefore more able to be transmitted horizontally (122). Among other class A carbapenemases that can be encoded on plasmids we find IMI-2 and IMI-3 (imipenem-

hydrolyzing β -lactamase).

Currently, the most common class A carbapenemase is KPC (*Klebsiella pneumoniae* Carbapenemase) (104, 126). KPC has been initially described in 1996 in the United States (127, 128). Until 2005, KPC production was confined to the eastern part of the United States (129). Nowadays, these enzymes went global and are largely found in nosocomial infections worldwide (130, 131). This spread is mainly due to the dissemination of a high risk *K. pneumoniae* ST258 clone, the plasmidic location of *bla*_{KPC} and its frequent association with a 10 kb Tn3 type transposon, Tn4401, capable of high frequency transposition without target site specificity (132, 133). In addition, biochemical data revealed that KPC-like enzymes are capable of hydrolyzing all β -lactams (penicillins, cephalosporins, carbapenems and monobactams) but is poorly inhibited by conventional β -lactamase inhibitors (clavulanic acid and tazobactam) (127).

2. Class B carbapenemase

Class B carbapenemases are characterized by their resistance to the conventional inhibitors of β -lactamases (clavulanic acid and tazobactam) and by their inhibition by ethylenediamine tetraacetic acid (EDTA), a chelator of Zn^{2+} ions necessary for the hydrolysis of β -lactams. The clinically most important enzymes belong to the subgroup B1, which are capable of hydrolyzing all β -lactams, except aztreonam. Within this group, VIM, IMP and NDM carbapenemases are the most common.

IMP-1 was the first described enzyme to confer transferable resistance to carbapenems. The first bacterium to express this carbapenemase, was *P. aeruginosa* described in the early 90s in Japan (134). This enzyme was later described in many Enterobacterales species, (135-138). IMP-2 has also been described in *A. baumannii* (139) and, in strains of *Achromobacter xylosoxidans* (140). Today more than 100 IMP variants (141). have been described in more than 30 countries including Japan, China, United States, Canada, and Italy (142-146).

VIM-1 has been described for the first time in 1997 in a strain of *P. aeruginosa* isolated from Verona, Italy (147). Soon after, VIM-2, another variant was reported from France (148). VIM-enzymes have been described worldwide, especially in *P. aeruginosa*, and *A. baumannii*, but increasing reports underline spread in Enterobacterales (143, 149-152). More than 80 variants have been described, signalling an actively spreading carbapenemase (141).

The MBL NDM-1 has been described for the first time in Sweden, in 2009, in a *K. pneumoniae* strain isolated from a patient repatriated from an Indian hospital (153). Very rapidly infections with NDM-1 gram-negatives were described worldwide, with a link to the Indian subcontinent (154, 155). Now autotochthonous cases of NDM carriage or infections are described. The rate of NDM-1 producing GNB from India and China account for 58.15% of the total number of NDM-1-producing bacteria in the world and 16.8% of all NDM-producing bacteria are found in Europe, with a wide dissemination in Balkan countries (156). More than 60 NDM variants have been described, with NDM-5, NDM-7 and NDM-9 increasingly isolates, as they have slightly higher carbapenem hydrolysis (82, 141). The genes encoding NDM-1 and its variants are carried on different plasmids like IncFII, IncL/M, IncN, IncR, IncHIB-M/FIB-M, with IncFI being the most prevalent (157). As for the other MBLs (IMP and VIM), NDM hydrolyzes all β -lactam except Aztreonam.

Other MBLs, are of lesser importance since they have disseminated either locally (SPM in Brasil, GIM in Germany) (158, 159), or have been only reported sporadically (TMB-1 in Lybia, LMB-1 in Austria and Argentina, KHM-1 in Japan, SIM in South Korea, FIM-1 in Florence) (100, 160-163).

3. Class D carbapenemase

Class D β -lactamases are serine active enzymes also called oxacillinases, as they hydrolyze oxacillin and cloxacillin faster than benzylpenicillin (83). However, this definition is no longer valid because some oxacillinases have been described to have little or no effect on cloxacillin or oxacillin (164). In contrast, all oxacillinases hydrolyze aminopenicillins and carboxypenicillins. Unlike class A β -lactamases, oxacillinases are generally not inhibited by clavulanic acid, tazobactam and sulbactam, but can be inhibited in vitro by sodium chloride (NaCl) (165). According to the β -lactamase database (141), more than one thousand oxacillinases have been identified to date. Their hydrolysis spectrum vary from narrow (penicillinase) to very broad spectrum, including ESC and/or carbapenem. Class D β -lactamases having a carbapenemases activity are named CHDLs for Carbapenem Hydrolyzing class D β -Lactamases (166). The hydrolysis of carbapenems by these enzymes remains low as compared to other carbapenemases such as KPC-2 or NDM-1. In addition, most of these CHDLs display a low or no hydrolysis of ESCs (164).

The first CHDL described, OXA-23, was isolated from an *A. baumannii* strain in the 90s (167). Since then, CHDL-producing *A. baumannii* strains have spread worldwide and became responsible for a large number of outbreaks of difficult to treat infections (DTI) (168, 169). Today 11 sub groups of *A. baumannii* borne CHDL have been described based on their amino acid sequence: OXA23, OXA-24/40, OXA-51, OXA-58, OXA-134a, OXA-143, OXA-211, OXA-213, OXA-214, OXA-229, and OXA-235 (170). CHDLs have also been found in Enterobacterales. OXA-48 and its variants are the most frequently described enzymes, but some minor enzymes have been described such as OXA-198, OXA-427, OXA-372 in Enterobacterales, and OXA-198 in *P. aeruginosa* (171-174).

4. OXA-48 β -lactamases

4.1. History

OXA-48, CHDL initially identified in a carbapenem-resistant *K. pneumoniae* isolate from Turkey in 2001, has now spread globally in Enterobacterales (175). Currently, Enterobacterales harboring *bla*_{OXA-48} genes are endemic in Turkey, in Middle Eastern countries (i.e., Lebanon, Jordan, Oman, Iran, and Saudi Arabia), and North Africa (Morocco, Algeria, Tunisia, and Egypt) (Figure 12) (175, 176). Their rapid dissemination is linked to an epidemic IncL plasmid and/or association with specific high-risk clones (175).

OXA-48 and OXA-181 that differ from each other by 4 amino acid substitutions (T103A, N110D, E169Q, and S171A) remain the most important OXA-48-like enzymes. Several variants of these two enzymes have been reported, differing by a few amino acid substitutions or deletions (175). For a complete list of variants refer to the β -Lactamase DataBase (141). OXA-48 hydrolyzes penicillins at a high level, carbapenems at a low level, and lacks significant hydrolytic activity toward ESCs (177). While some OXA-48-variants with single amino acid substitutions have similar hydrolytic profiles as OXA-48, others, such as OXA-163 or OXA-405, carry a four-amino-acid deletion in the β 5– β 6 loop that results in the loss of carbapenemase activity and a gain of the ability to hydrolyze ESCs (178, 179). Two OXA-48-variants, OXA-438 and OXA-517, with two AA deletions in the β 5– β 6 loop, have been described with ability to hydrolyze carbapenems and oxymino-cephalosporins (180, 181). Dabos et al. has shown by introducing one to six a.a. deletions in the β 5– β 6 loop

in both directions, that loss of carbapenem-hydrolysis and gain of cephalosporin hydrolysis occurred after four-amino acid deletions (180). Finally, single point mutants in the $\beta 5$ – $\beta 6$ loop such as OXA-232, an R214S variant of OXA-181 have been described with reduced overall activities (178). Substitutions at position 214 in the $\beta 5$ – $\beta 6$ loop, results in impaired carbapenem-hydrolyzing activity suggesting a pivotal role in the hydrolysis of carbapenems (178). Cristallography reaveled that the conformation of the $\beta 5$ – $\beta 6$ loop in OXA-48 is promoted by a salt bridge between Arg214 within the Ω -loop and Asp159 residue (177, 178) The $\beta 5$ – $\beta 6$ loop extends into the outer portion of the active site crevice and defines a rather hydrophilic cavity filled with several water molecules, one of which will perform the nucleophilic attack on the acyl-enzyme intermediate in the deacylation step (177, 178) .

Among OXA-48 variants that are currently been increasingly isolated is OXA-244, a single amino-acid variant of OXA-48 (R214G) with reduced activity against carbapenems and temocillin (182) posing diagnostic challenges (183, 184). Since 2013 an increase in OXA-244-producing *E. coli* has been observed in several countries worldwide. Genomic studies have shown that the dissemination of OXA-244-producing *E. coli* is polyclonal, but dominated by *E. coli* ST 38 (80, 183). Recently, hospital outbreak of OXA-244-producing *E. coli* ST38 involving three hospitals in the Western region of Norway, a country with a very low prevalence of CPE has been described (185).

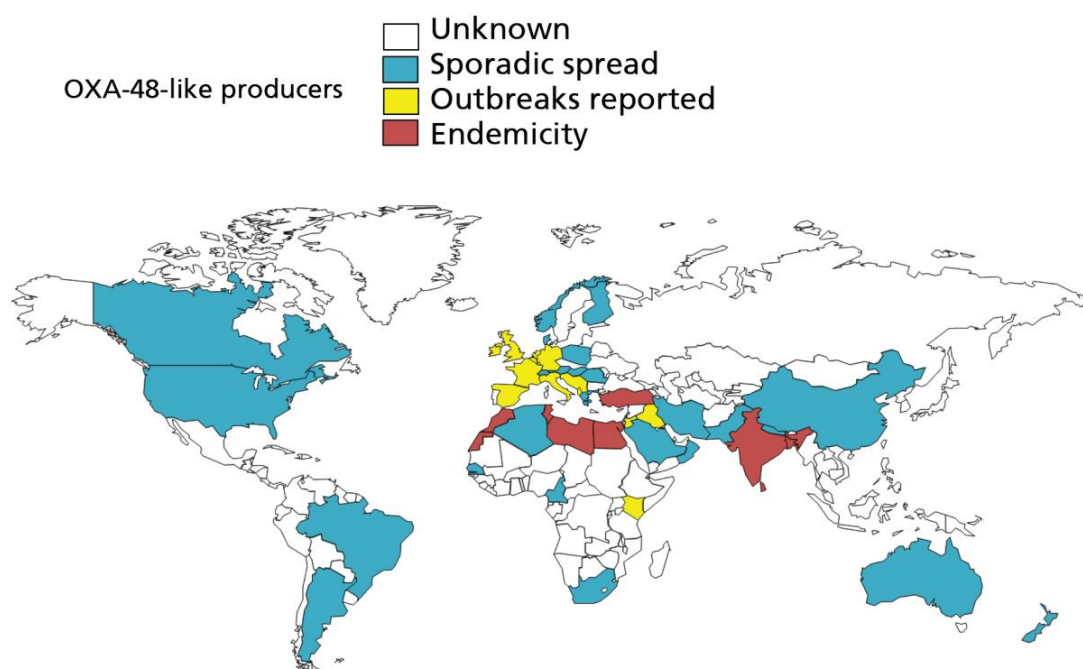


Figure 12 : Geographical distribution of OXA-48 producers worldwide – Bonomo et al 2017 (186)

4.2. Genetic environment and support

Initially, the *bla*_{OXA-48} gene was described on a 62-kb IncL self-transmissible plasmid (187) and bracketed by two insertion sequences, IS1999, forming a composite transposon, called Tn1999 (188) (Figure 13). Since, several variants of Tn1999, have been described with additional insertion sequences : *IS1R* upstream *bla*_{OXA-48} gene in Tn1999.2, and two *IS1R* flanking *bla*_{OXA-48} gene in Tn1999.3 (Figure 13). The 62-kb IncL plasmid that carries *bla*_{OXA-48} gene doesn't carry any other antibiotic resistance genes, (189, 190), however, the co-production of OXA-48 with ESBLs has reported, resulting in pan-β-lactam resistance (191).

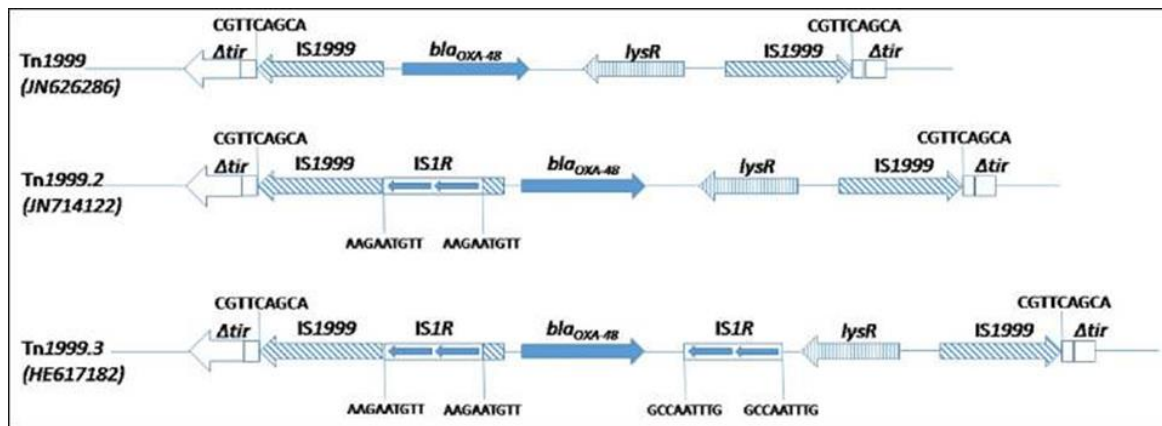


Figure 13: Genetic environment of *bla*_{OXA-48} bearing Tn1999 like transposon in Enterobacterales - Adapted from Yamuna Devi Bakthavatchalam et al (191).

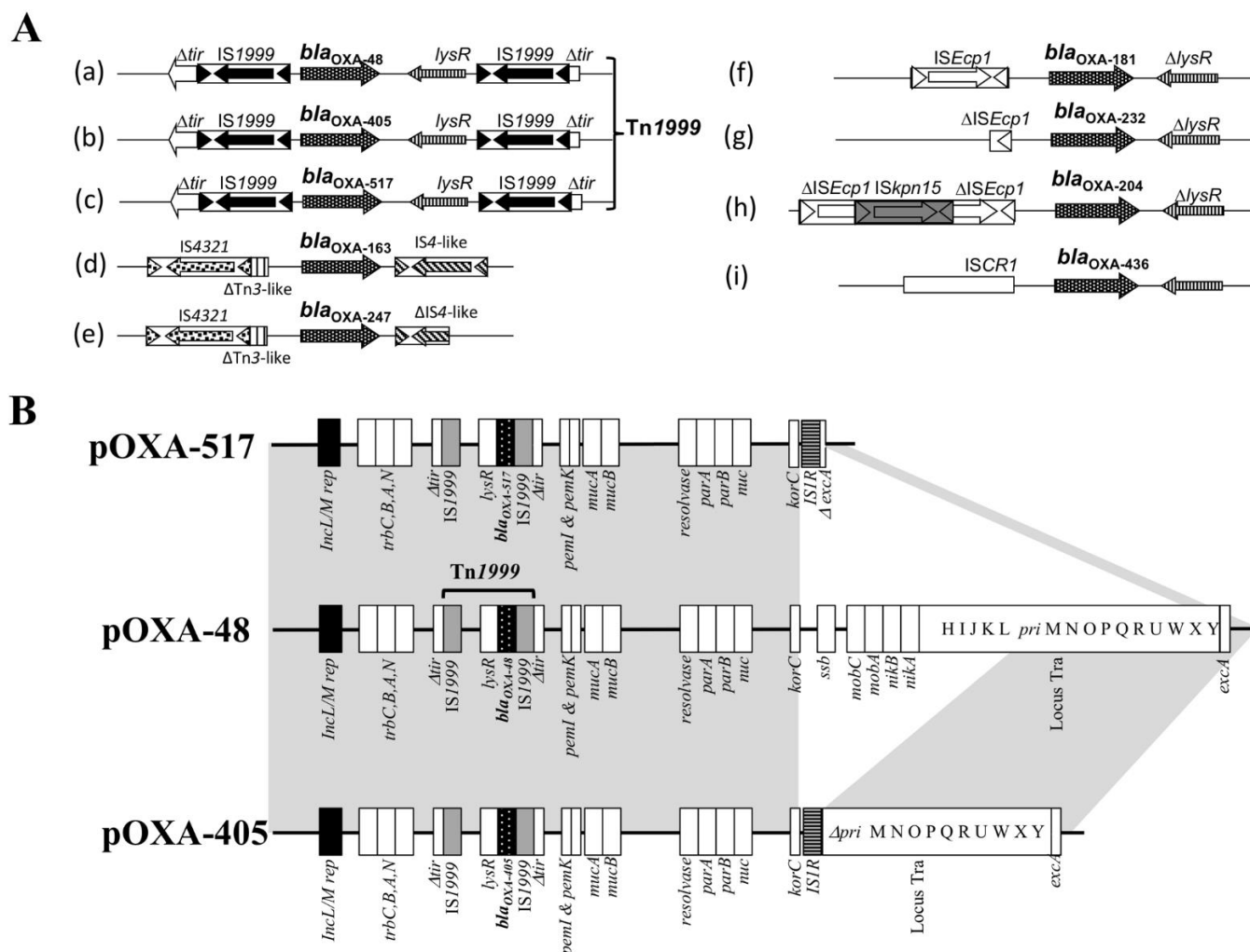


Figure 14: Plasmid structures and genetic environments of various OXA-48 Like enzymes. Schematic representation of the genetic environments of the bla_{OXA-48} (a), $bla_{OXA-405}$ (b), $bla_{OXA-517}$ (c), $bla_{OXA-163}$ (d), $bla_{OXA-247}$ (e), $bla_{OXA-181}$ (f), $bla_{OXA-232}$ (g), and $bla_{OXA-204}$ (h) genes. The Tn1999 composite is formed by two copies of the IS1999 insertion sequence, bracketing a fragment containing the bla_{OXA-48} , $bla_{OXA-405}$ and $bla_{OXA-517}$ genes. (B) Comparison of the major structural features of the plasmid pOXA-517 from *K. pneumoniae* 1219 with the prototype IncL bla_{OXA-48} plasmid pOXA-48 (GenBank accession number JN626286) and pOXA-405. Common structures are highlighted in gray.

4.3. Phenotype and hydrolysis spectrum

OXA-48 confers resistance to penicillins (AMX, TIC, TEM), it has a moderate activity on carbapenems (IMP, ETP, MEM), and C1G, weak activity against cefotaxime, and no detectable hydrolysis against Ceftazidim (CAZ) and Cefepim (FEP) (Figure 15).

As, the carbapenemase activities of OXA-48-like enzymes are moderate to low, additional chromosomal mutations in porins/efflux pumps are necessary to achieve high levels of carbapenem resistance (177, 192).

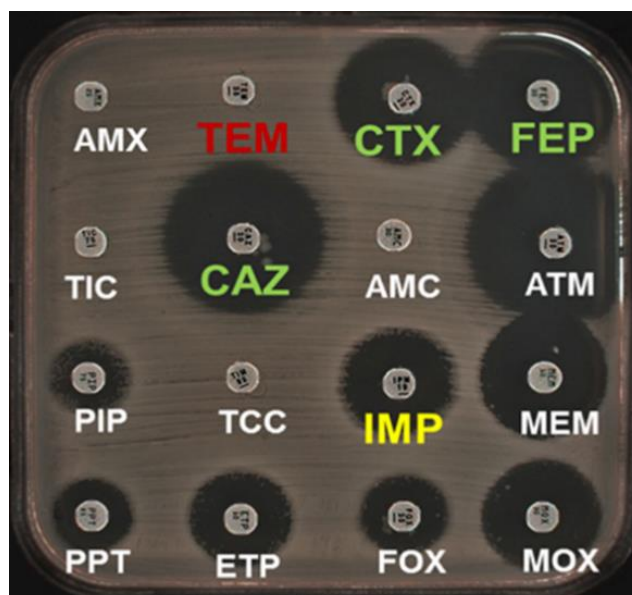


Figure 15 : Antibiogram of *E. coli* TOP10 – p-TOPO – *bla*_{OXA-48}. AMX: amoxicillin ; TEM: temocillin ; CTX: cefotaxime ; FEP: cefepime ; TIC: ticarcillin ; CAZ: ceftazidime ; AMC: amoxicillin with clavulanic acid ; ATM: aztreonam ; PIP: piperacillin ; TCC: ticarcillin with clavulanic acid ; IMP: imipenem ; MEM: meropenem ; PPT: piperacillin with tazobactam ; ETP: ertapenem ; FOX: ceftiofime and MOX:moxalactam

Kinetic parameters of OXA-48, determined by Docquier et al. in 2009 (177) revealed a catalytic efficiency (k_{cat}/K_m) 100 folds greater for imipenem as compared to ertapenem and meropenem (Table 4). This difference is the result of an increase in k_{cat} , accompanied with an increase in K_m . Class A β -lactamase inhibitors are ineffective against OXA-48, IC_{50} for clavulanic acid, sulbactam and tazobactam of 16 μ M, 50 μ M and 1.7 μ M respectively (192).

Table 4: Kinetic parameters of OXA-48

Substrate	K_m (μ M)	k_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1}/s^{-1})	Substrate	K_m (μ M)	k_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1}/s^{-1})
Ampicillin	395	955	2418	Ceftazidime	NH	NH	ND
Piperacillin	410	75	180	Cefotaxime	>900	>9	10
Oxacillin	95	130	1368	Cefepime	>550	>0.6	1.1
Temocillin	45	0.3	6.6	Imipenem	13	4.8	369
Cephalothin	195	44	226	Meropenem	11	0.07	6.2
Ceftiofime	>200	>0.05	0.26	Ertapenem	100	0.13	1.3

Adapted from Docquier et al (177)

4.4. Structure of OXA-48

OXA-48 consists of 265 a.a. encoded by a 798 bp gene. This OXA-48 3D structure was determined by Docquier et al. in 2009 (177). The active site of the enzyme is located between two domains and closed on one side by the Arg 214 ($\beta 5$ - $\beta 6$ loop), and by the glutamine 124, and on the other side, by an isoleucine 102 and a serine 244. The active site is characterized by the presence of the three conserved motifs that are found in class D oxacillinases: the first one is made of **Ser-70, Thr-71, Phe-72 and Lys-73** (usually carbamoylated), the second one is made of **Ser-118, Val-119 and of the Val-120** and the third one, **Lys-208, Thr-209 and Gly-210**.

The particular conformation of OXA-48's active site could be stabilized by an interaction between arginine 214 and the aspartic acid residue at position 159. Furthermore, it was found that arginine 214 could have a functional role in OXA-48 (177, 178).

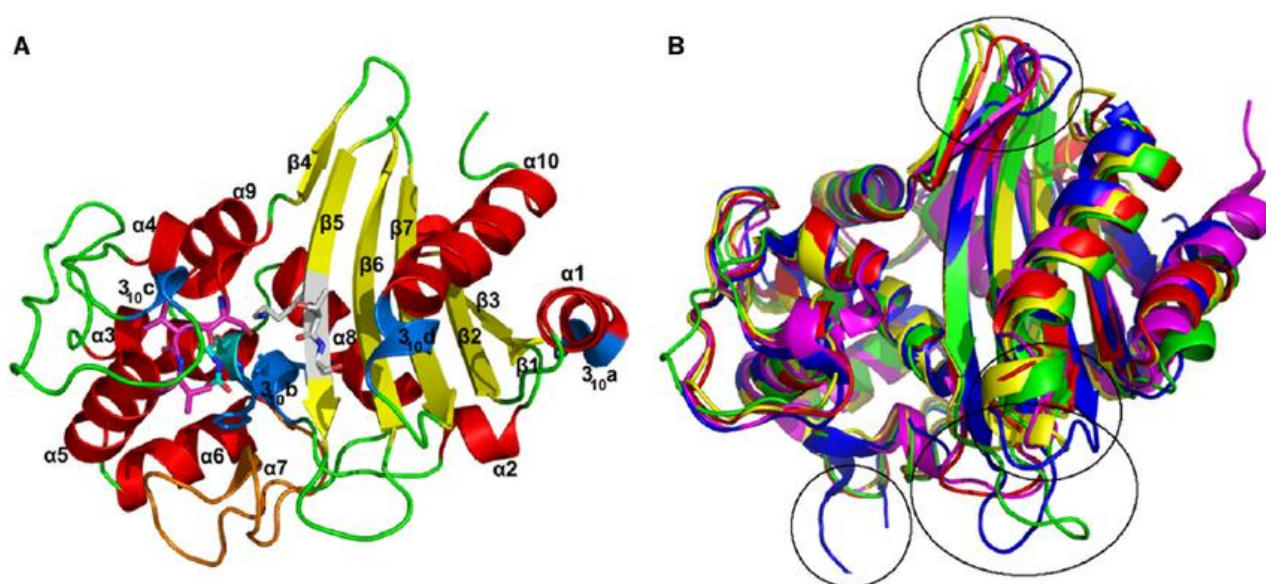


Figure 16 : OXA-48 tertiary structure: (A) Ribbon representation of OXA-48 subunit, showing the secondary structure elements assigned as follows: $\beta 1$: 26–28; $3_{10}a$: 29–31; $\alpha 1$: 31–35; $\beta 2$: 42–48; $\beta 3$: 53–56; $\alpha 2$: 59–62; $3_{10}\beta$: 69–72; $\alpha 3$: 73–82; $3_{10}c$: 103–105; $\alpha 4$: 110–115; $\alpha 5$: 120–130; $\alpha 6$: 132–142; $\alpha 7$: 156–159; $\alpha 8$: 166–177; $\alpha 9$: 185–194; $\beta 4$: 196–199; $\beta 5$: 204–212; $\beta 6$: 219–227; $\beta 7$: 232–240; $3_{10}d$: 244–246; $\alpha 10$: 245–260. α helices are shown in red; β strands in yellow and turns in green. The three DBL motifs are shown with the respective residues as sticks: motif I (containing the catalytic residues Ser-70, Thr-71, Phe-72, and carbamylated Lys-73), cyan; motif II (Ser-118, Val-119, and Val-120), magenta; motif III (Lys-208, Thr-209, and Gly-210), white. (B) Least-squares superposition of representative members of the DBL family. The loops where different conformations are observed are framed as well as the catalytically relevant portions of the molecule. Color legend and PDB codes are as follows: OXA-48 (3HBR), red; OXA-1 (1M6K), blue; OXA-2 (1K38), yellow; OXA-10 (1K55), green; OXA-24 (2JC7), magenta. Docquier et al. 2009 (177).

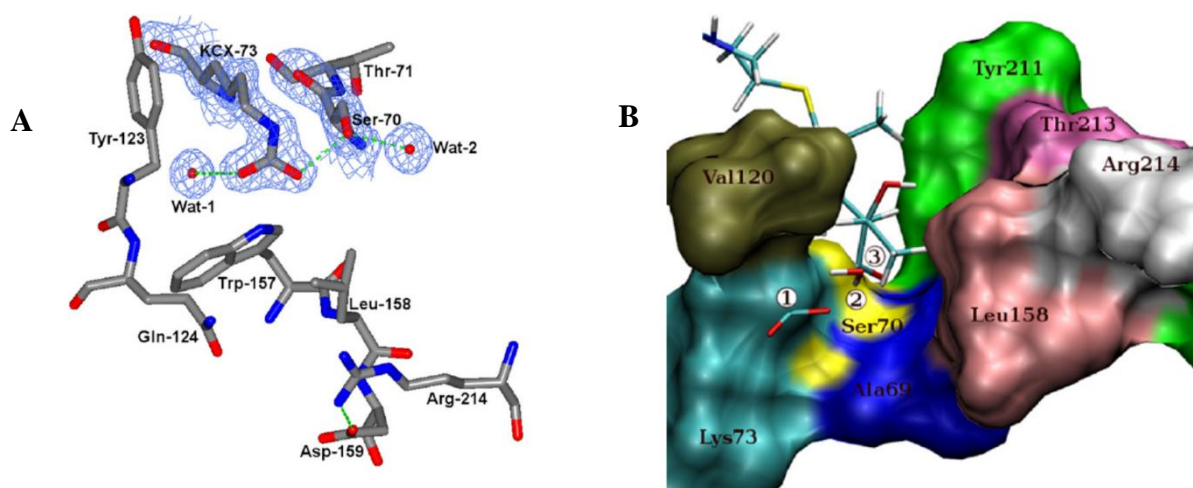


Figure 17: The active site of OXA-48. (A) shows the carbamylated side chain of Lys73 conserved in OXA active site. Water molecules and other relevant residues are shown as well. (B) Docking of meropenem into OXA-48's active site. the meropenem adopts a conformation in which the methyl group of the hydroxyethyl substituent points toward Leu-158 (pink) and the hydroxyl group is more exposed to the solvent. In this conformation the methyl group obstructs the access of water molecule 2 to the carbonyl carbon 3. Docquier et al. (177).

The $\beta 5$ - $\beta 6$ loop is an important functionally relevant structure of OXA-48, it extends to the outer portion of the active site crevice, and have an open conformation that might be due to the interaction between Arg-214 and Asp-159. Crystallography showed that this active site is actually a hydrophilic cavity with water molecules located close to the substrate binding site. It is believed that this conformation is important for the occurrence of the nucleophilic attack during the hydrolysis reaction (192).

5. OXA-48 Like variants

As of September 2023, 54 variants of OXA-48 are identified according to BLDB, they differ by few amino acids substitutions and/or deletions (141). OXA-48 like variants don't share same hydrolytic profile, rather, they can be grouped in four categories :

- Group one**, comprises variants having the same activity as OXA-48 e.g. OXA-162, OXA-181
- Group two**, in which we find variants lacking carbapenemase activity but able to hydrolyze cephalosporins e.g. OXA-405, OXA-163
- Group Three**, with enzymes having both carbapenemase and ESC hydrolytic activity, such as OXA-517, OXA-793, OXA-438 (181, 193).
- Group four**, comprises variants having reduced carbapenem and temocillin hydrolytic activity

e.g. OXA-244, OXA-232

Most of the differences between OXA-48's variants affecting hydrolysis are located in the β 5- β 6 loop (141). OXA-181, that differs from OXA-48 by four amino acid substitutions all located outside the loop (T103A, N110D, E169Q, and S171A), has similar hydrolytic activity as OXA-48. OXA-232, a R214S variant of OXA-181 shows significantly lower hydrolytic activity against carbapenem and temocillin (194). It differs from OXA-181 by an additional substitution (R214S) in the β 5- β 6 loop (178). In contrast, OXA-163, with a four AA deletion variant in the β 5- β 6 loop effectively hydrolyses cephalosporins (ceftazidime, cefotaxime, and cefepime) and aztreonam (195) but has lost carbapenem hydrolysis. Phenotypically, OXA-163 resembles more to an ESBL than to carbapenemase.

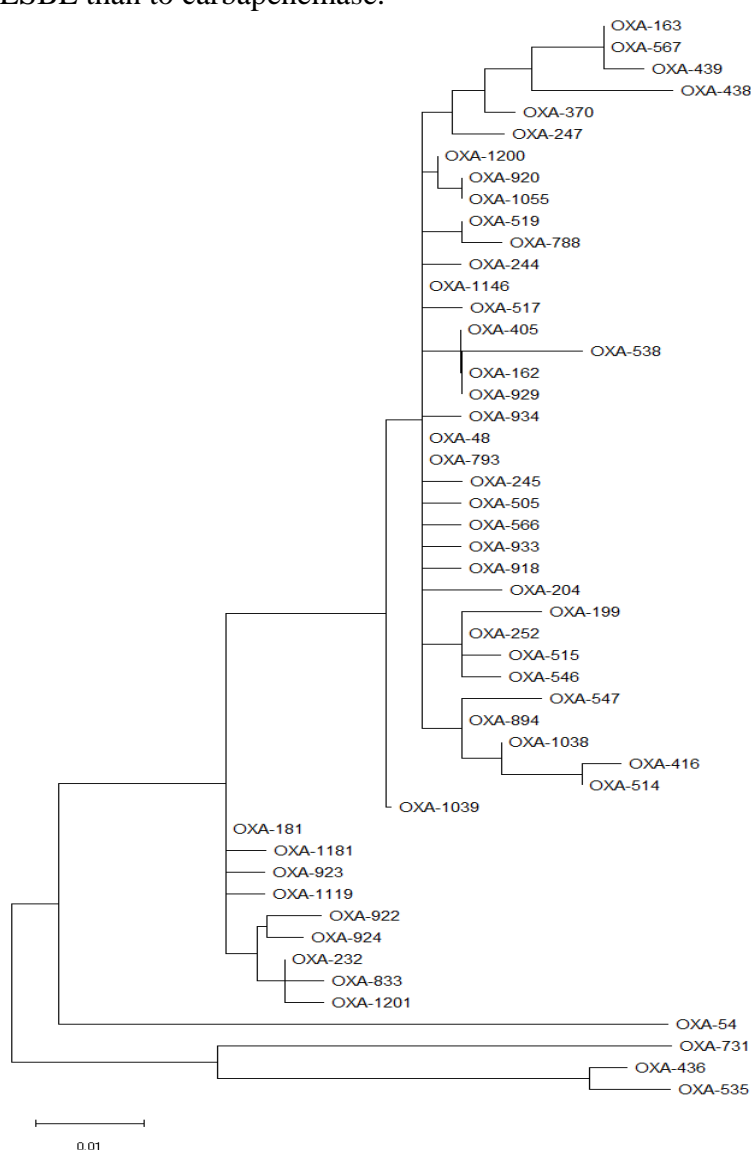


Figure 18: Phylogenetic tree of OXA-48 like variants. Tree constructed using MEGA7

As previously mentioned, the global prevalence of carbapenemases is widespread, and the Middle East is no exception to this issue. The specific occurrence and distribution of bacteria that produce carbapenemases in the Middle East can vary between countries and even within different healthcare facilities. Efforts to address the spread of carbapenemase-producing bacteria in the Middle East and other regions involve enhancing infection control measures, implementing antibiotic stewardship programs, and conducting surveillance to monitor the prevalence and distribution of these bacteria.

Monitoring antibiotic resistance through the detection of carbapenemase production is vital for patient care, infection control, public health, and the ongoing effectiveness of antibiotics. This comprehensive approach requires the collaboration of healthcare providers, researchers, and public health authorities to tackle this growing threat.

For researchers, it is essential to continuously track the various enzyme variants contributing to the epidemiology of carbapenemases in the MENA region. The following review summarizes recent publications that describe the current status of carbapenemase dissemination in Middle Eastern countries and emphasizes the significance of surveillance as the initial step in addressing the issue of antimicrobial resistance.

Carbapenemase Dissemination in the Middle East and North Africa: Alarming Situation or Delusion?

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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 β -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 East and North African (MENA) regions. We focused on the different variants of OXA-48 in each country of the MENA region to provide an assessment of the current situation..

Keywords: *Antimicrobial resistance, carbapenemase, Enterobacterales, P. aeruginosa, A. baumannii, Middle east, oxacillinases*

1- 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 anti-microbial 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 pre-scribed 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).

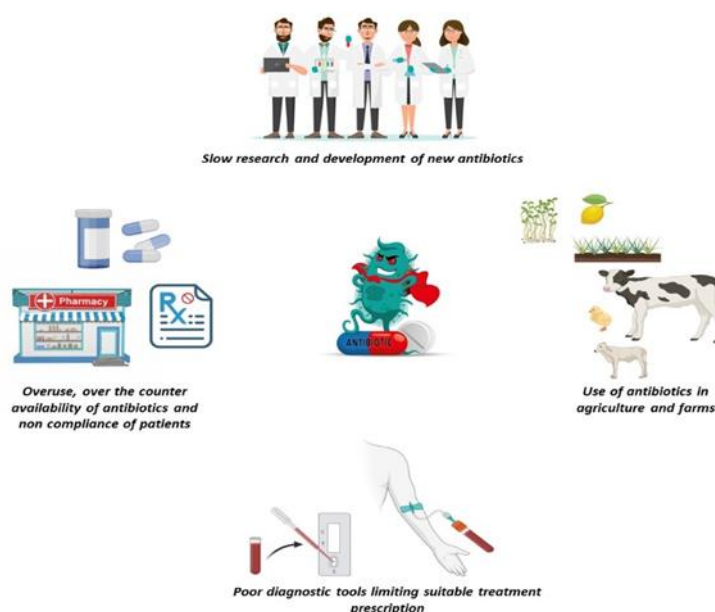


Figure 1: Various causes behind the exacerbation of antimicrobial resistance (AMR)

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 β -lactams by the production of β -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 β -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 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 β -lactam ring, while class B carbapenemases are metallo- β -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 β -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, NMC-A, PenA ...), on plasmids (KPC, GES) or on both (IMI).

Class B carbapenemases, also known as metallo- β -lactamases and represented mainly by IMPenase (IMP), Verona integron-borne metallo- β -lactamase (VIM), Sao Paulo metallo- β -lactamase (SPM), German imipenemase (GIM), and Seoul imipenemase (SIM) and New Delhi Metallo- β -lactamase (NDM) families (9). This class of carbapenemases is able to hydrolyze all β -lactams except aztreonam, resist to the action of commercially available β -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).

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	PI	<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	PI	<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	PI/Ch	<i>Enterobacteriaceae</i> , <i>P. aeruginosa</i> , and other <i>GNNFB</i>						-	+	S
Class D	OXA-23 group (OXA-23, OXA-27, OXA-49)	PI/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

Figure 7: Characteristics of carbapenemases - PI: Plasmidic, Ch: Chromosomal, CLA: Clavulanic acid, ATM: Aztreonam, R: Resistant, S: Susceptible

2. Carbapenem-hydrolyzing 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 β -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
Subgroup 1	OXA-48, OXA-162, OXA-181, OXA-204	<ul style="list-style-type: none"> - Poor carbapenem hydrolysis - Poor cefotaxime hydrolysis - Strong penicillin hydrolysis - No ceftazidime hydrolysis - Temocillin strongly resistant
Subgroup 2	OXA-163, OXA-252, OXA-405	<ul style="list-style-type: none"> - Loss of carbapenemase activity - Gain of cephalosporinase activity
Subgroup 3	OXA-244, OXA-232	<ul style="list-style-type: none"> - Reduced carbapenemase activity
Subgroup 4	OXA-517, OXA-733, OXA-438	<ul style="list-style-type: none"> - Carbapenem hydrolysis - Gain of cephalosporinase activity

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

Subgroup	Subgroup name	Carbapenemase variants
Ia	OXA-23-like β -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
Ib	OXA-24/40-like β -lactamases	OXA-24, -25, -26, -72, -139, -160, -207, and -437
Ic	OXA-51-like β -lactamases	OXA-64, -65, -66, -67, -68, -69, -70, -71, -75, -76, -77, -78, -79, -80, -82, -83, -84
Id	OXA-58-like β -lactamases	OXA-58, -96, -97, -164, -198, -372 -397, -420, and -512

OXA-48 β -lactamases are by far the most important enzymes. Initially described in *Klebsiella pneumoniae* isolates in Turkey in 2004, *bla*_{OXA-48-like} genes have spread in several countries, especially of the Mediterranean rim and subsequently all over the world (31-33). 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 β -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 β 5- β 6 loop located close to the substrate binding site, which represents 22/45 variants described have significant changes in the hydrolytic spectrum of β -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 β 5- β 6 loop, have been described with the ability to hydrolyze carbapenems and oxy-imino-cephalosporins equally well (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 (6,41).

3. What are the carbapenemases identified in the Middle East and North Africa?

The predominance of *bla*_{OXA-48} 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 key-words 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.

4. MENA Countries

4.1 IRAN

According to the latest studies, health care associated infections with MDR bacteria in Iran, are drastically rising, requiring special emphasis in detection and reporting cases, to set up 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*_{NDM}, *bla*_{IMP}, *bla*_{VIM}, being the most widespread genes (49, 51), especially, NDM-1 (52). A recent study, showed that among 236 carbapenem-resistant *P. aeruginosa* (CRPA), 116 isolates harbored MBL genes and 29 isolates were found positive for *bla*_{NDM-1} (50), which ensures the high prevalence of class B MBLs among *P. aeruginosa* in Iran. According to the same study, CRPA, harbored *bla*_{IMP-1}, or *bla*_{VIM-2} and *bla*_{OXA-10} in 27.5%, or 21.1% and 32.2% of isolates respectively, and interestingly, a co-production of *bla*_{NDM-1}, with either *bla*_{IMP-1}, *bla*_{VIM-2}, were determined in 11 (4.6%), and 27 (11.4%) of isolates, respectively (50). In this study infections caused with imipenem resistant *P. aeruginosa* isolates that harbor *bla*_{OXA-23} and *bla*_{OXA24/40} genes, normally encountered in *A. baumannii*, have also been 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*_{OXA-23}-like, *bla*_{OXA-58}-like, *bla*_{OXA-24/40}-like and

*bla*_{OXA-143-like} (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*_{OXA-23-like} genes were 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*_{OXA-317}, *bla*_{OXA-64}, and *bla*_{OXA-69}, 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 chromosomally encoded *bla*_{OXA-68}, *bla*_{OXA-66}, *bla*_{OXA-71}, *bla*_{OXA-90} 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 MBLs, 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*_{OXA-48} (24%) and *bla*_{IMP} (13%) genes had the highest prevalence, while *bla*_{KPC} and *bla*_{GIM} 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* ST 13 and ST 392, were the most disseminated among NDM-1 producers in south Iran (90). More-over, 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 re-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 ensure 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*_{KPC}, while *bla*_{IMP}, *bla*_{VIM}, and *bla*_{OXA-48} 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*_{CTX-M-15}, *bla*_{NDM-1}, *bla*_{OXA-48} and *bla*_{IMP} 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).

4.2. 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 iso-lates, 70% have OXA-48 (31/44) (15 *K. pneumoniae*, 8 *E. coli*, 4 *S. marcescens*, 3 *E. cloacae*, 1 *M. morganii*) (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*_{OXA-23}, *bla*_{OXA-24}, *bla*_{GES-11} or *bla*_{OXA-24} with *bla*_{GES-11}. 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*_{NDM-1} or the *bla*_{OXA-72} in 2015 (124). Dual-carbapenemase secretion of GES-6 and VIM-2 or IMP-15, was reported in carbapenem-resistant *P. 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 worsen 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.

4.3. 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).

4.4. 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*_{KPC} (63.5%) followed by *bla*_{OXA-48} (55.7%) and *bla*_{VIM} (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*_{OXA-48} and *bla*_{NDM-1} 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*_{IMP}, *bla*_{VIM}, *bla*_{GIM}, *bla*_{SPM} 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 out-break 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*_{OXA-24/40} (65.2%), followed by *bla*_{OXA-23} (30.4%) and *bla*_{OXA-58} (17.4%). *bla*_{IMP}, *bla*_{VIM}, and *bla*_{NDM} 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*_{NDM}, *bla*_{OXA-23-like}, and *bla*_{KPC} genes (143). Similarly in 2019 a large variety of acquired carbapenem-hydrolyzing β -lactamases have been described, including *bla*_{KPC}, *bla*_{GES}, *bla*_{NDM}, *bla*_{SIM}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{OXA-23-like} (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*_{NDM-1} and *bla*_{OXA-48} genes (146). Between 2018 and 2019, among carbapenemase-producing *K. pneumoniae*, *bla*_{KPC-2} and/or *bla*_{NDM-1} 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*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{IMP-1} 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*_{NDM-1}, *bla*_{NDM-5}, *bla*_{OXA-48}, *bla*_{OXA-181} and *bla*_{KPC2} were also detected among geographically diverse randomly selected MDR *K. pneumoniae* isolates from nine Egyptian hospitals (150). In 2018, a study that *bla*_{OXA-48} gene was the most identified carbapenemase followed by *bla*_{NDM}, *bla*_{VIM} and *bla*_{KPC} 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. *bla*_{VIM-1} and *bla*_{VIM-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*_{VEB} together with *bla*_{GES} in Egypt (154). *bla*_{VIM} and *bla*_{IMP} were also detected among *P. aeruginosa* clinical isolates (155). OXA-48 and IMP are the most prevalent carbapenemase genes among *E. 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*_{IMP-4} and *bla*_{VIM-2} genes was reported for the first time in 2015 in the neonatal intensive care unit of Cairo University Hospital (157). *bla*_{OXA-51}, *bla*_{OXA-23} and *bla*_{VIM} were detected among all CRAB isolates from Egypt. *bla*_{OXA-58} and *bla*_{NDM-1} 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*_{KPC}, *bla*_{OXA-48}, *bla*_{NDM} were described in Enterobacterales strains isolated from fish farms in Egypt (160). Among carbapenem resistant, shiga toxin-producing *E. coli*, isolated from food samples and diarrheic samples (cattle and human), the *bla*_{VIM} gene was detected in all isolates, and one human isolate carried the *bla*_{NDM-1} gene (161).

4.5. 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 *Klebsiella 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 *Escherichia 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 Abdul-lah 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*_{OXA-23-Like} (88.3%) and *bla*_{NDM-1} (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*_{VIM} (n= 75/109; 68.8%) followed by *bla*_{NDM} (n= 12/109; 11%), and *bla*_{KPC} (n= 5/109; 4.6%)(166).

4.6. TURKEY

OXA-48 was reported initially from 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*_{VIM} 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*_{OXA-23} gene has become the most prevalent carbapenemase in Turkey (182). Generally, *bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{OXA-51}, *bla*_{OXA-58}, *bla*_{IMP}, *bla*_{NDM}, *bla*_{KPC}, *bla*_{OXA-48} 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*_{NDM-1} and *bla*_{VIM-48} metallo- β -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*_{OXA-23}, 5 (2.9%) *bla*_{OXA-58} and one isolate (0.6%) was positive for both genes. *bla*_{IMP}, *bla*_{KPC}, *bla*_{NDM} and *bla*_{OXA-48} genes were not detected (182).

4.7. 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*_{OXA-48} (n= 26/29; 90%) and *bla*_{NDM-1} (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*_{VIM} 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 (196). Unfortunately, the occurrence of carbapenem resistant bacteria is rising in vegetables also, such as bacteria encoding *bla*_{OXA-48} and *bla*_{VIM-4} genes (197).

4.8. 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*_{OXA-51} and *bla*_{OXA-23} 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*_{OXA-23} and *bla*_{NDM} genes was also detected in Morocco. *P. aeruginosa* is well known to carry MBLs especially *bla*_{VIM-2} (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.

4.9. 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 is 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*_{OXA-23-like}, *bla*_{OXA-24-like}, and *bla*_{OXA-48-like} 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- β -lactamase, designated TMB-1 (211).

4.10. 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 *A. 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 *K. pneumoniae* and *E. coli* strains but is still uncommon (216, 217). Metallo- β -lactamases

are described in Tunisia, for instance, IMP metallo- β -lactamase, was described only in *K. pneumoniae* environmental isolate (218, 219). VIM enzyme was also frequently reported among *P. aeruginosa*, *K. pneumoniae* and scarcely among *E. coli* and *E. 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- β -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 β -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*_{GES-5} and *bla*_{VIM-11} carbapenemase genes occurred in 2022 (229).

4.11. 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 *K. 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*_{NDM-1}, *bla*_{NDM-4}, *bla*_{NDM-5}, *bla*_{NDM-7}, *bla*_{OXA-181} or *bla*_{KPC-2} 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*_{OXA-181} insertion, in a *K. pneumoniae* isolate, was the cause of resistance to carbapenems (240).

In Saudi Arabia, Abd El Ghani et al. showed the presence of *bla*_{NDM-5}, *bla*_{OXA-1} and *bla*_{OXA-181} (241).

In particular, among carbapenem-resistant *E. coli* clinical isolates, from Iraqi patients with urinary tract infection, *bla*_{OXA-48} was the most frequently identified carbapenemase gene, followed by *bla*_{PER}, *bla*_{KPC}, *bla*_{VEB} and *bla*_{VIM} (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*_{OXA-51-like} genes and *A. calcoaceticus* complex isolates additionally carried the *bla*_{OXA-23-like} gene *bla*_{OXA-24-like} (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*_{NDM-1} 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*_{VIM} and *bla*_{IMP} gene were detected among *P. aeruginosa* isolated from diabetic patients (247). Ismail et al. also aimed to detect the prevalence of *bla*_{NDM} variants metallo- β -lactamase among clinical *P. aeruginosa*. *P. aeruginosa* isolates carrying *bla*_{NDM-1} and *bla*_{NDM-2} were reported for the first time in Iraq (248).

In Kuwait, several articles reported the presence of *bla*_{NDM-1} along with *bla*_{OXA-48} (249). The enzymes *bla*_{OXA-23}, *bla*_{IMP}, *bla*_{OXA-24/40}, *bla*_{GES}, *bla*_{OXA-48}, and *bla*_{OXA-58}, are also found in Kuwait, but a lack of *bla*_{KPC}, *bla*_{VIM}, *bla*_{SIM}, and *bla*_{NDM} 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*_{OXA-181} and *bla*_{NDM-5} carrying plasmids were also detected in some strains (252).

In Oman, double carbapenemase genes were identified among *K. pneumoniae* isolates in Oman, where *bla*_{NDM-1} carried on IncHI1b plasmid was identified with a chromosomally encoded *bla*_{OXA-181} (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.

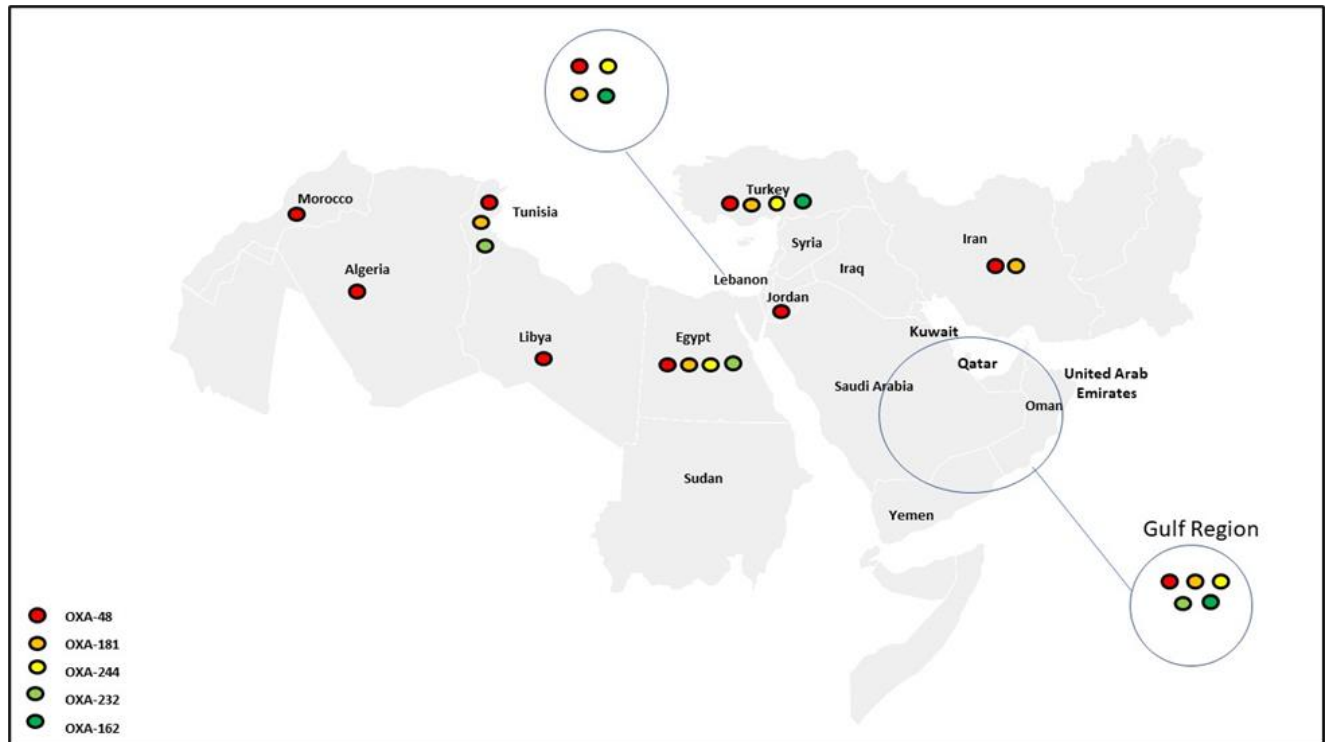


Figure 3: Distribution of OXA-48 Like variants in the Middle East

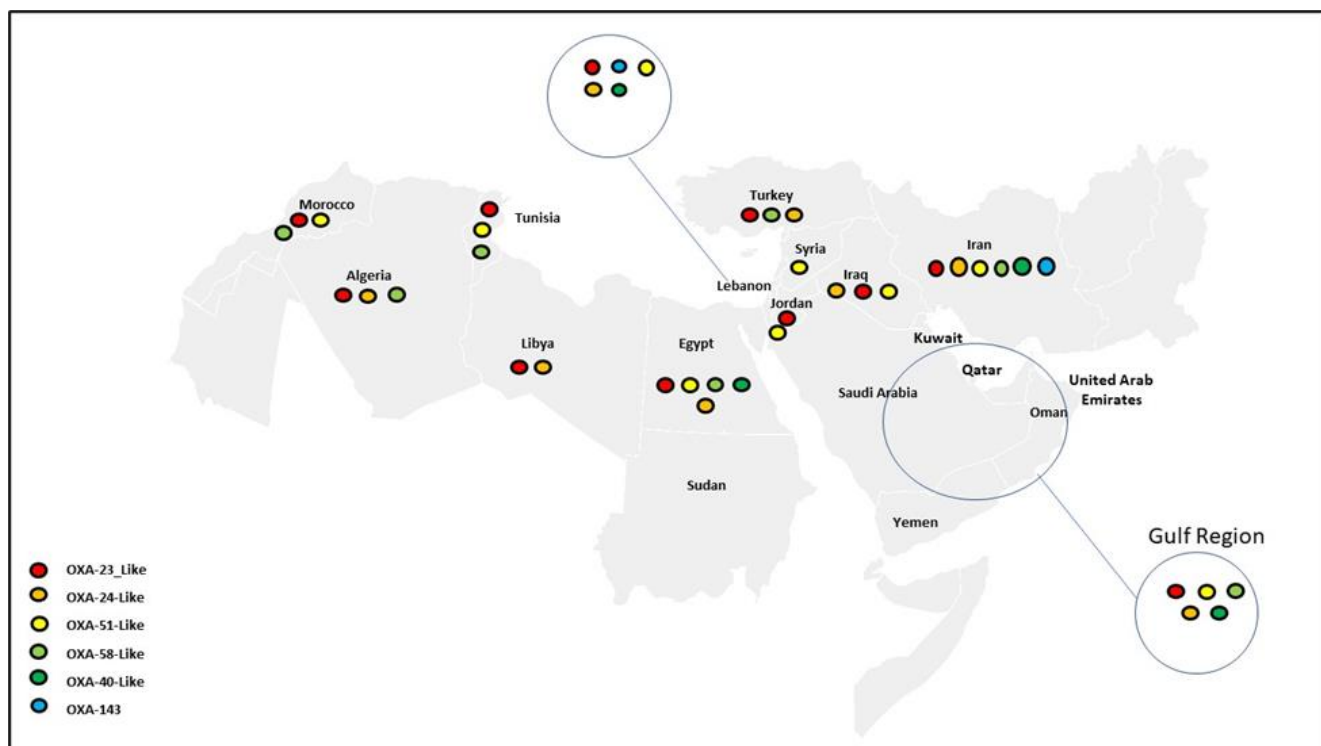


Figure 4: Repartition of various oxacillinases in the Middle East

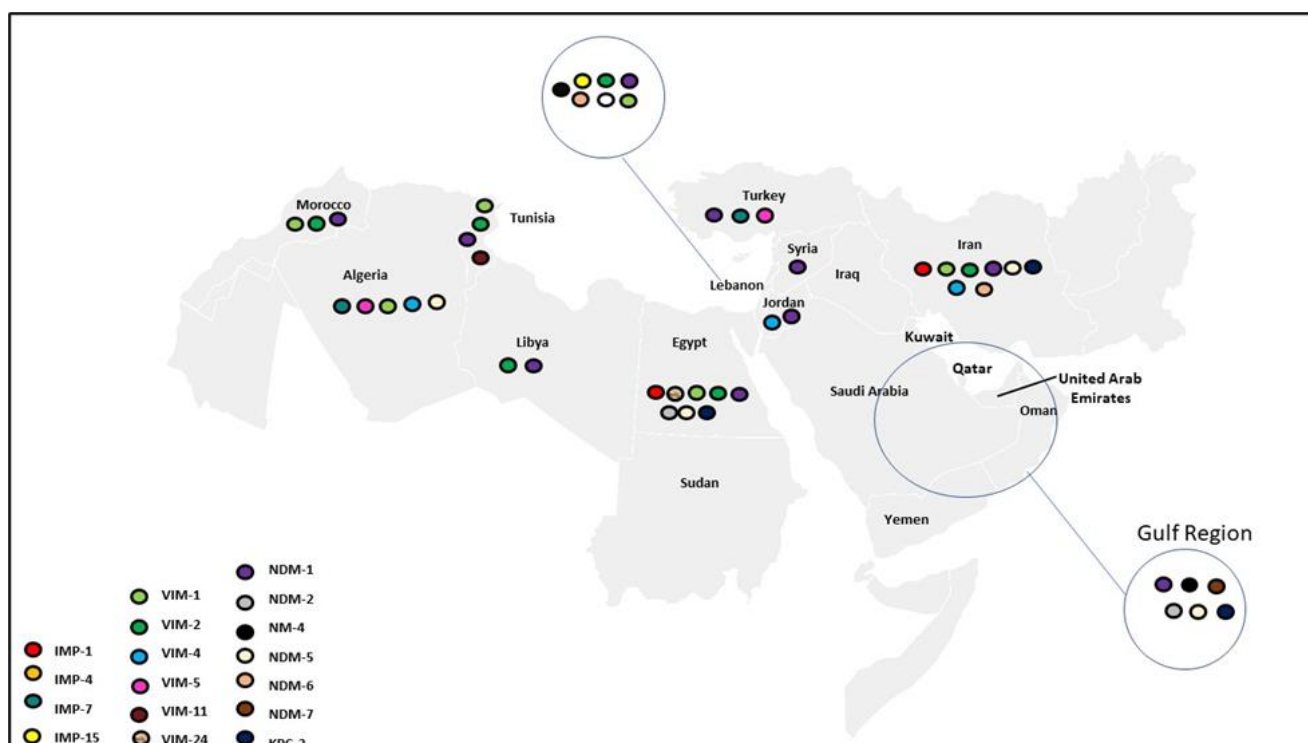


Figure 4: Distribution of some carbapenemase variants in the Middle East

5. Conclusions

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 β -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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III- Novel molecules to overcome AMR

1. Novel antibiotic candidates

Unfortunately, bacteria are rapidly acquiring the capacity to escape the action of broad spectrum antibiotics by point mutations and/or efficient horizontal gene transfer, mediated by transformation, phage transduction, and conjugation. Antibiotic resistant bacteria that survive antibiotic treatments are becoming more and more common, turning available antibiotic regimens ineffective, and resulting in therapeutic dead-end situations and high mortality rates (196). Thus, the need for the development of novel antibiotics with ideally novel targets is unprecedented. Screening natural products to identify novel compounds with interesting antibacterial activity has been neglected. Thus, the number of antimicrobial compounds isolated from natural sources is in continuous drop, which results in only very few novel antibiotics under development.

The battle against AMR will require a better understanding of the emergence, and dissemination routes of MDR bacteria in a one health aspect, development of rapid diagnostic tests to limit the spread in hospital settings where the most vulnerable are found, and of course the development of novel antibiotics able to act on already highly drug resistant bacterial isolates. In this context, two novel compounds have shown promising activity against Gram positive and Gram negative bacteria: Cystobactamids and chelocardins (197, 198)

1.1. Cystobactamids

1.1.1. History

Following the golden antibiotic discovery era of 60s and 70s of the last century, a major scarcity of newly developed antibacterial agents resulted in drastic reduction of therapeutic choices in the light of high AMR. Recently, research on novel antibiotics has gained interest again, and several projects focusing on the isolation of new compounds in natural products along with the enhancement of already existing agents have been initiated.

In 2014, cystobactamids, which are novel myxobacterial natural products were isolated from *Cystobacter* spp. and showed good activity against gram-negative bacteria (199). It was suggested that they are promising candidates that could help in the elimination of infections

caused especially by gram negative bacteria. Their bactericidal activity against gram negative pathogens including clinical isolates of *P. aeruginosa* and fluoroquinolone resistant Enterobacterales was shown recently (197). Since then, many derivatives of the natural compound were elaborated in order to improve the activity profile (197).

1.1.2. Structure

A special feature of cystobactamids is an unusual aromatic oligopeptide structure, composed of five para-aminobenzoic acid (PABA)-derived moieties and a central aliphatic amino acid (Figure 19) (200).

Derivates of cystobactamid share a hexapeptidic structure composed of three para-aminobenzoic acid motifs and two para-nitro aminobenzoic acids connected together through different linkers (201). Novel analogues were found to have a higher stability than the natural compound. CN-DM-861, is one of these derivatives, and was found to be more potent in terms of *in vivo* efficacy (200). From a structural point of view, CN-DM-861 lacks the methoxy group in the central linker region (Figure 19) (200).

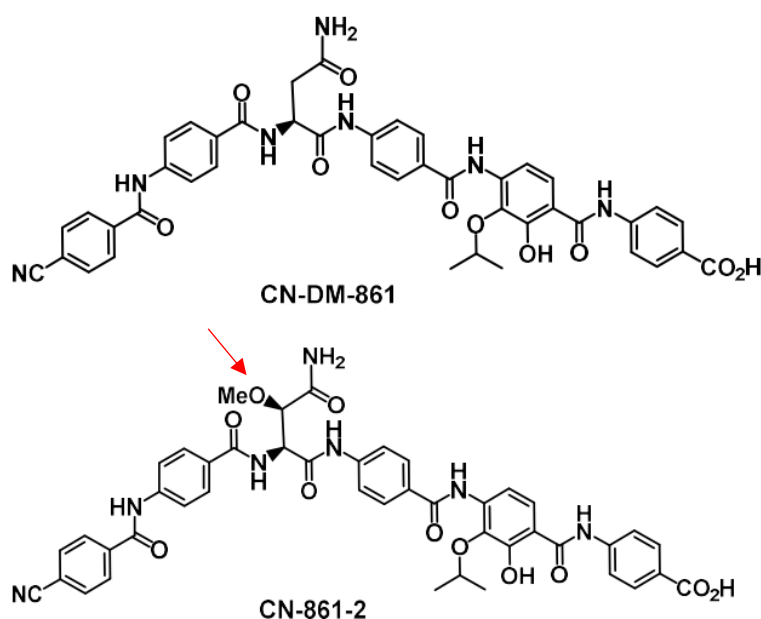


Figure 19: CN-DM-861 compared to the natural derivative CN-861-2. Red arrow indicates the difference between both derivatives.

1.1.3. Mode of action and activity

Cystobactamids have been shown to inhibit gyrase through stabilization of the covalent enzyme-DNA complex with double-stranded DNA breaks resulting in an accumulation of linear DNA (202). Cystobactamids are bactericidal compounds believed to kill bacteria by inhibiting the bacterial type IIa topoisomerases, which recalls the mode of action of fluoroquinolones (197). Cystobactamids action is mainly mediated by DNA minor- groove binding rather than intercalation (201).

When talking about cystobactamids, it's crucial to mention albicidins, which are potent DNA gyrase inhibitors produced by *Xanthomonas albilineans* (203). Albicidins consist of polyaromatic oligopeptides mainly composed of p-aminobenzoic acids and cyanoalanine which makes them similar structurally to cystobactamids (197, 203).

1.2. Chelocardins

1.2.1. History

Chelocardins (CHD) were isolated from *Amycolatopsis sulphurea* and *Nocardia sulphurea* in 1962. They were considered novel atypical tetracycline-like molecules, based on structural similarities (Figure 20) (204). In 1970, CHD was tested in vivo in a phase II clinical trial aiming to treat patients from UTIs including infections caused by tetracycline resistant pathogens, and it showed effectiveness (205, 206). It showed some gaps in its activity spectrum especially against *P. aeruginosa*, that's why researchers generated a new more potent version of this molecule, the amido-chelocardin CDCHD (207).

1.2.2. Structure

As shown below, a tetracycline's core structure consists of a four-ring system where the ring D on the left side is aromatic but the three rings on the right A, B and C include saturated carbon centers. Even though, the tetracyclines appear to have a planar conformation, they in fact have a significant bend and a unique 3D-structure (208, 209).

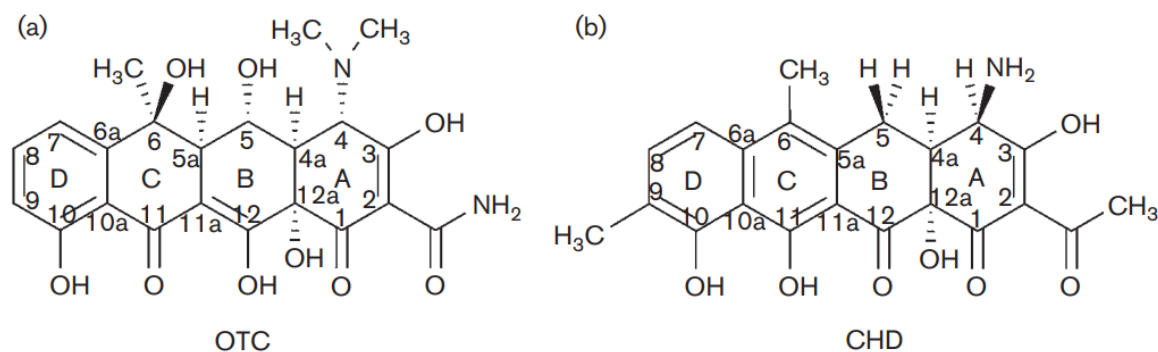


Figure 20: Structures of oxytetracycline (OTC) (a) and chelocardin (CHD) (b). Adapted from Lukezic et al. 2013 (209).

From a comparative point of view, a chemical modification is present in chelocardin structure, with the top part of the molecule at positions 4 to 6 being affected and the bottom half at the positions 9 to 12. CHD has an acetyl rather than a carboxamido moiety at C2, in addition, different aromatization pattern is seen on C9. CHD has also less functional groups that could explain the difference in the mode of action between oxytetracycline OTCs and chelocardins and might be the reason behind the effectiveness of chelocardins on tetracycline resistant isolates (210). In addition, these modifications in CHD lead to enhanced antibacterial properties over OTC (208).

Structural modifications in CHD led to the production of derivatives having a more potent activity. For instance, 2-carboxamido-2-deacetyl-chelocardin (CDCHD), is one of those derivatives (211). CDCHD was obtained after biosynthetic engineering of CHD, and its structure is shown below (Figure 21) (212, 213).

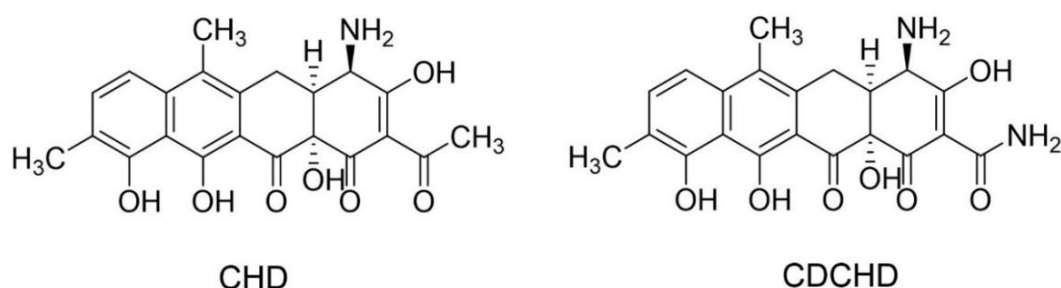


Figure 21: Chemical structure of chelocardin (CHD) and its amidated derivative 2-carboxamido-2-deacetyl-chelocardin (amidochelocardin, CDCHD) – Hennessen et al. 2020 (205).

1.2.3. Mode of action and activity

This class of compounds not only showed activity towards clinical isolates of ESKAPE complex, but also did not select resistance in *K. pneumoniae*. Despite the fact that their resemblance to tetracyclines suggests an interaction of chelocardins with the ribosomes or elimination through bacterial efflux pumps, it was suggested, that they may interact also with the bacterial cytoplasmic membrane (206, 207, 214).

A few studies showed activity of CHD on Enterobacterales including *E. coli*, and *K. pneumoniae* (215), and CDCHD revealed additional activity on *P. aeruginosa* (205). CHD and CDCHD are thought to act in a bactericidal way (198).

CHD and its derivatives are promising candidates for urinary tract infections treatment since it acts on MDR uropathogenic isolates (206, 216), including tetracycline resistant bacteria (216).

Until now, there's a controversial debate in the literature about the mode of action of chelocardins and other atypical tetracyclines. On one hand, structural similarities between CHD and tetracyclines suggest similar mode of actions. This observation is conformed by a significant binding of chelocardins to rRNA of ribosomal subunits , along with activation of biosensors sensing protein synthesis inhibition (198). On the other hand, chelocardins show bactericidal activity, unlike tetracyclines that are bacteriostatic, and they result in membrane depolymerisation after exposure to chelocardins. Thus, it is likely that a dual mechanism of action for chelocardins involving protein synthesis inhibition and bacterial membrane depolarization is involved (198).

Resistance to CHD has been shown to rely mainly on the up-regulation of AcrAB-TolC multidrug efflux pump in *E. coli*. However, CDCHD a weak substrate for AcrAB-mediated efflux, is not affected by this resistance mechanism, confirming the absence of cross-resistance between both derivatives (216).

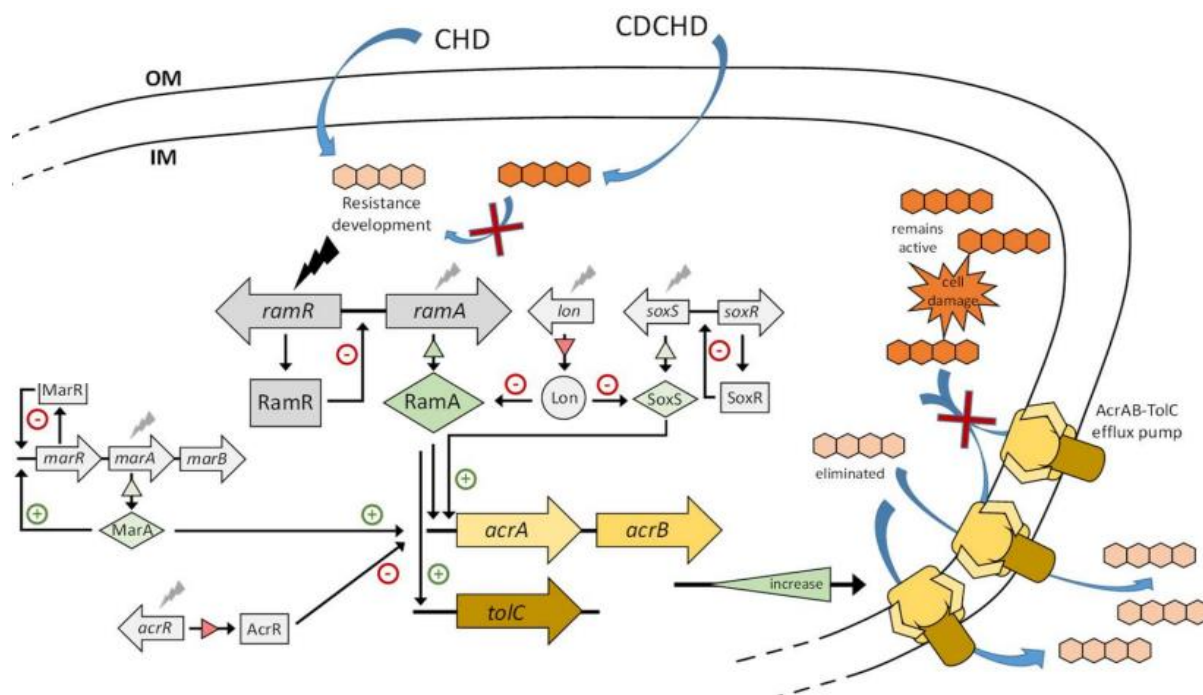


Figure 22: Model of regulatory cascade and genes implicated in resistance against CHD in *K. pneumoniae* mediated by AcrAB-TolC efflux system. CDCHD, is unlike CHD and does not lead to the resistance development and is able to overcome efflux (Red cross). In grey triangles are mentioned the gene products, and in green diamonds, transcriptional repressors and activators are listed. Amounts of gene products are indicated through red and green triangles. The genes or operons affected by CHD-mediated resistance include ram (resistance antibiotic multiple), sox (sulfur-oxidizing operon), mar (multiple antibiotic resistance), lon (encoding a serine protease), and acrAB-tolC (encoding the antibiotic efflux pump). OM, outer membrane; IM, inner membrane. Adapted from Hennessen et al. 2020 (205).

2. Antimicrobial peptides

In order to exert their activity, antibiotics must be able to penetrate through the outer membrane of gram negative bacteria. In many cases highly potent molecules have been found in vitro on purified enzymes, but due to weak penetrations, they could not show the activity on bacteria. Therefore, finding a way to reduce the permeability of bacterial membrane through destabilizing agents that could ease penetration of novel compounds, or simply restore antibiotic activity in bacteria with impaired OM permeability resulting from mutated porines, or in very impermeable bacteria.

Antimicrobial peptides (AMPs) are short peptides of few amino acids, mostly components of the innate immune system for various organisms (217). For most AMPs, the interaction with the plasma membrane has been demonstrated as an indicator of their bactericidal activity (218). Those molecules are thought to act on their own or in synergy with conventional antibiotics. AMPs allow the restoration of the activity of antibiotics, which lost their potential due to impermeability problems. The evaluation of the activity of several AMPs remains of a particular

interest in view of the membrane destabilizing properties of AMPs. Among AMPs, Cathelicidin-BF, Frenatin 2.3S, SAAP-148 were evaluated on their own and in association with cystobactamids and chelocardins.

A detailed overview taking into account the classification, mode of action of AMPs, their role in the diverse health fields, and their potential to replace antibiotics is presented in the following review (219).



Review

Antimicrobial Peptides: A Potent Alternative to Antibiotics

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Citation: Rima, M.; Rima, M.; Fajloun, Z.; Sabatier, J.-M.; Bechinger, B.; Naas, T. Antimicrobial Peptides: A Potent Alternative to Antibiotics. *Antibiotics* **2021**, *10*, 1095. <https://doi.org/10.3390/antibiotics10091095>

Academic Editor: Giovanna Batoni

Received: 18 August 2021
Accepted: 6 September 2021
Published: 10 September 2021

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Abstract: Antimicrobial peptides constitute one of the most promising alternatives to antibiotics since they could be used to treat bacterial infections, especially those caused by multidrug-resistant pathogens. Many antimicrobial peptides, with various activity spectra and mechanisms of actions, have been described. This review focuses on their use against ESKAPE bacteria, especially in biofilm treatments, their synergistic activity, and their application as prophylactic agents. Limitations and challenges restricting therapeutic applications are highlighted, and solutions for each challenge are evaluated to analyze whether antimicrobial peptides could replace antibiotics in the near future.

Keywords: antimicrobial peptides; antibiotic resistance; multidrug resistance; ESKAPE

1. Introduction

There was collective enthusiasm about the advent of antibiotic therapy, and this optimism prevailed in the light of the constant discoveries of new classes of antibiotics, despite the very early description of therapeutic failures and resistance to treatments with these drugs [1]. At the present, in 2021, multidrug-resistant (MDR) and even pan-drug-resistant (PDR) bacteria have spread widely around the world and are currently responsible for increasing morbidity and mortality rates, as well as the significant cost to society [2]. The low frequency of discoveries of new classes of antibiotics, and the rapid emergence of resistance to novel antibiotics, show the need for novel therapeutic alternatives to antibiotics, such as lysine-based small molecules, vaccines, anti-virulence strategies, phage therapy and antimicrobial peptides.

Antimicrobial resistance (AMR) is an emerging global health problem that results, in some cases, in difficulties to treat bacterial infections. It was listed by the World Health Organization (WHO) among the top ten global public health threats facing humanity, as it is predicted to cause about 10 million deaths each year by 2050 [3]. Therefore, efforts to slow down the propagation of AMR have been implemented worldwide. As such, a Global Action Plan on Antimicrobial Resistance (GAP) was created in 2015, aiming to implement national action plans to limit the progress of AMR. In addition, the WHO reports call

for urgent action to avert an antimicrobial resistance crisis and insists on the importance of discovering and developing new antibiotics. Thus, new compounds that are active against pathogens, especially those which cause nosocomial infections and tend to adopt multidrug resistance, are needed. To curb this problem, several alternative therapies have been proposed, among which antimicrobial peptides (AMPs) were suggested to be very promising more than 20 years ago, as they have existed in nature for millions of years with almost no or limited resistance development [4]. This makes them very attractive compared to antibiotics that develop resistance relatively fast. This absence/slow development of resistance against microbes may be attributed to the presence of various modes of action of AMPs against the bacteria in comparison to the fixed targets used by the antibiotics [5]. In addition, AMPs are considered less toxic, as they are broken down into amino acids unlike other therapeutics, which might generate potentially harmful metabolites. This review is to highlight where these molecules stand now in the overall scheme to curb MDR bacterial infections. Their potential to counteract AMR, to replace traditional antibiotics, to evaluate their benefits and to describe the challenges faced by R&D will be discussed in this review.

AMPs are small polypeptide molecules, typically made up of around 12 to 50 amino acids, found in all classes of living organisms [6]. These molecules are produced as secondary metabolites, are part of the innate immunity and are, in mammals, usually ribosomally produced by epithelial cells, but also by phagocytes (cells of the immune system). These peptides can be found in tissues or mucous membranes; in fact, the latter harbor a multitude of pathogenic or commensal microorganisms. Among these peptides, some have a broad spectrum of antimicrobial activity, capable of inhibiting or killing different types of microorganisms (MO) (Gram-positive or -negative bacteria and/or fungi) but also protozoans, and viruses [7,8]. According to The Antimicrobial Peptide Database, more than 3257 antimicrobial peptides have been described to date [9]. They originate from six kingdoms, where 365 come from bacteria, 5 from archaea, 8 from protists, 22 from fungi, 360 from plants, and 2414 from animals, in addition to some synthetic peptides. Recently, another webserver with several subsections that screen among 40,000 entries has been made available. It summarizes all available data for desired peptide properties, thus allowing further structural and functional studies [10].

2. Classification and Mode of Action of Antimicrobial Peptides

2.1. Classification

AMPs are usually classified according to several criteria: First, based on their biological source. In this classification, we distinguish AMPs from human and mammalian sources, such as cathelicidin and defensin, which make up the most important families of AMPs [11]; AMPs from amphibians [12], fish [13], insects [14], and plants [15] are also classified in this category. Classification could be based on the AMP's biological functions; for example, antibacterial, antiviral, antifungal, antiparasitic peptides were described. Third, based on their biochemical properties (amino acid sequence, composition, length, hydrophobicity, charge), where conformation and structure serve as criteria for classification as well [7].

2.2. Mode of Action

Some factors can modulate the activity and specificity of AMPs, such as size, charge, hydrophobicity, secondary structure, or amphiphilic character. The conformation of AMPs may also play a role in antimicrobial activity. Indeed, it has been shown that peptides possessing amphipathic structures interact better with the membrane of pathogens. AMPs can have a membrane permeabilization action and/or act on certain intracellular functions (Figure 1) [16].

2.2.1. Membrane Permeabilization

The bacterial membrane is the most important target of AMPs as they act by disrupting the integrity of the pathogen's membrane [17]. Several models have been described in the literature, all of which result in osmotic lysis. Membrane disruption may happen through

different models, as recently reviewed in Huan et al. [7]. (i) The “barrel-stave” model indicates that peptides insert into the membrane and direct their hydrophobic regions toward the lipid core of the bilayer, forming a transmembrane pore. Experimental evidence for this model has been obtained from structural and biophysical investigations of very hydrophobic sequences, some being devoid of any charge [18]. (ii) The “carpet” model suggests that AMPs accumulate parallel to the membrane surface, forming a “carpet” [19]. Once a threshold concentration of peptides has been reached, they exert a “detergent” effect, leading to the rupture of the cell membrane. Extensive experimental evidence for this mechanism has been obtained for many cationic amphipathic peptides such as magainins [20]. (iii) The “toroidal pore” model implies that AMPs insert themselves perpendicularly into the membrane through interactions between the lipid bilayer and the hydrophilic region of the peptides. In doing so, the membrane distorts and thus forms a “toroidal pore” [21]. (iv) The “aggregate” model assumes the formation of aggregates of peptides and lipids, allowing the translocation of AMPs across membranes. When considering these models, it should be kept in mind that on the one hand, lipid bilayers are soft and can adapt their shapes and thicknesses to the membrane-inserted peptides. On the other hand, the peptides cover a highly dynamic and flexible conformational space and upon interaction with the membrane also respond. Thereby, many different supramolecular arrangements can be formed depending on the lipid composition, peptide concentration, salt, and buffer. These considerations have been incorporated into the SMART model, which takes into consideration that peptides and lipids adjust their conformation and shape when interacting with each other thereby covering a full range of possibilities of interactions between AMPs and membranes [22].

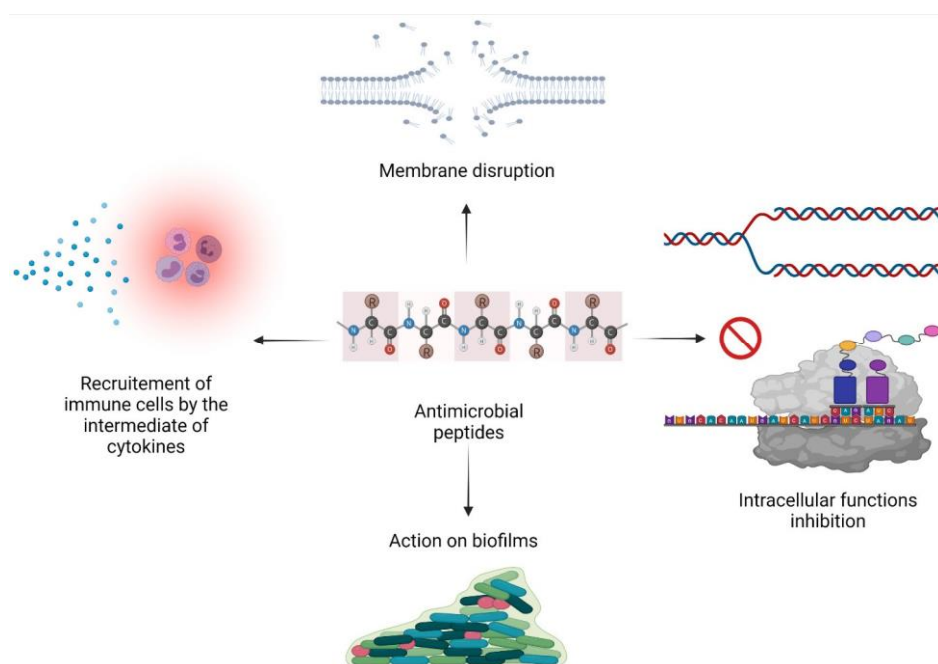


Figure 1. Mechanisms of action of antimicrobial peptides.

2.2.2. Inhibition of Intracellular Functions

Some peptides cross the cytoplasmic membrane and target cellular processes essential for the survival of the pathogen, including inhibition of DNA replication, protein synthesis, interference with nucleic acid biosynthesis, metabolism, cell division, cell wall, and LPS binding proteins [23].

Indeed, some cationic AMPs have been shown to complex and flocculate nucleic acids or other anionic macromolecules due to their affinity for negatively charged phosphodiester bonds [24,25]. For example, Frenatin 2.3S internalizes within bacterial cells after destabilizing their membranes and can bind nucleic acids [26]. Others are able, even at low concentrations, to affect protein synthesis. The examples are countless; for instance, tryptophan-containing AMPs were proven to be efficient at killing *Pseudomonas aeruginosa* by down-regulating the expression of DNA replication-initiating genes [27]. In addition, Tur1A, an AMP found in dolphins, binds to ribosomes, and blocks the translation of mRNA into proteins [28]. Thanatin, an insect-derived AMP targets the bacterial LPS and induces the LPS-mediated aggregation [29]. Tridecaptin blocks ATP synthesis in bacteria and is active on MDR and colistin-resistant Enterobacterales [30].

2.2.3. Immunomodulatory Activity

Well characterized for their antimicrobial activities, AMPs are also known for their immunoregulatory functions. They can thus participate in the recruitment and activation of immune cells.

It has been described in the literature that certain AMPs, such as defensins, can enhance the production of inflammatory cytokines, such as interleukin-1 [31]. To mention another example, cathelicidin BF has been shown to exhibit immunomodulatory activity that, in mice, is able to ameliorate pneumonia caused by *Pseudomonas aeruginosa* [32].

2.2.4. Action on Biofilms

When in the form of biofilms, pathogens escape more easily the action of conventional antimicrobials. Indeed, microorganisms in biofilms are capable of tolerating, in particular, *via* transcriptional regulatory mechanisms, high concentrations of antimicrobials even though they are totally sensitive in planktonic conditions [33].

The mode of action of AMPs on biofilms is not yet fully understood; however, it is recognized that they can prevent different stages of biofilm formation. It has been shown that some AMPs can inhibit the adhesion of bacteria to surfaces by reducing certain types of motilities such as “swarming” or “swimming”. They are also able to stimulate “twitching”, another type of motility known to promote the disassembly of biofilms. AMPs can also down-regulate certain genes involved in quorum sensing, the latter being known to play a role in biofilm formation and/or in the organization and communication of bacteria within the biofilm [34]. Thus, it has been demonstrated that LL-37 cathelicidin targets quorum sensing mechanisms that control *P. aeruginosa* biofilm formation [35].

3. AMPs as Potential Alternatives to Antibiotics

Several advantages were observed for AMPs over classical antibiotics: (i) usual resistance mechanisms observed toward conventional antibiotics are bypassed by AMPs [36,37]; (ii) they are easier to synthesize since they consist usually of short amino acid sequences [38]; (iii) they show rapid killing [39]; (iv) they act on bacteria irrespective of their resistance phenotype, since they are not affected by the known resistance mechanisms [40–42], and (v) they do not affect microbiota, which are often disrupted by conventional antibiotics [43]. These advantages made it possible to have several AMPs that are now in medical use, such as nisin, gramicidin, polymyxins, daptomycin, and melittin (reviewed by Dijksteel et al. [44]).

3.1. Activity against MDR Bacteria and ESKAPE

Hundreds of AMPs have shown antibacterial activity against resistant bacteria in vivo [45]. Among the most recent examples, a novel piscidin-like peptide called cerocin from black sea bass fish was described to exert broad-spectrum antimicrobial activity against several bacteria, especially Gram-positive pathogens [46]. Similarly, jelleine-I, a small AMP formed by eight amino acids, acts against both Gram-negative and Gram-positive bacteria, mainly by disrupting the integrity of the cell membrane [47]. Furthermore, another novel defensin-like peptide was described recently for its antimicrobial activity against Gram-positive bacteria, including *Staphylococcus aureus*, *Staphylococcus carnosus*, *Nocardia asteroides*, and one tested Gram-negative bacterium *Psychrobacter faecalis* [48]. Interestingly, AMPs can act against bacteria of the ESKAPE complex, which is made up of six pathogens responsible for most of the nosocomial life-threatening infections: *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* spp. [49]. ESKAPE pathogens are usually MDR, which limits therapeutic options and puts patients' lives at risk. Many AMPs showed promising activity against ESKAPE pathogens. For example, bip-P-113, dip-P-113 and nal-P-133 are three derivatives of the AMP p-133, that work well against *E. faecium*, showing a low MIC of 4 µg/mL as compared to 64 µg/mL for vancomycin [50].

The resistance of *S. aureus* to methicillin remains a huge drawback associated with high mortality rates [51]. Many AMPs are able to eradicate methicillin-resistant *S. aureus* (MRSA) [52]; for example, poly(2-oxazoline)s, an easy-to-synthesize polymer mimetic of AMPs that displays high and selective antimicrobial activity against MRSA, is considered as a promising antimicrobial candidate due to its low MIC values (12.5 µg/mL) [53]. ΔM3 is a novel synthetic peptide consisting of a short amino acid sequence that has low toxicity coupled to a great biological activity, especially against *S. aureus* strains. The low MICs of ΔM3 (5 µg/mL) against *S. aureus* (ATCC25923) and MRSA (7.5 µg/mL) [54], make it a great AMP candidate. Finally, SAAP-148, a derivative of the human LL37 peptide, is active against several resistant ESKAPE pathogens (*S. aureus*, *P. aeruginosa*, *A. baumannii*), including biofilms that occur in wound infections [55].

K. pneumoniae, is a challenging bacterium with respect to conventional antibacterial therapy, as it may be encapsulated, limiting the antibiotic's capacity to penetrate. However, this bacterium is sensitive to pepW, an AMP that targets, aggregates and disrupts *K. pneumoniae*'s capsules with low MICs (2–4 µg/mL) [56]. PepW is also active against *Escherichia coli* with even lower MICs of 1–2 µg/mL [56]. Similarly, AA139 and SET-M33 may be promising novel drugs against MDR *K. pneumoniae* strains with MICs ranging from 4 to 16 µg/mL, but more interestingly, they remained active even on colistin-resistant *K. pneumoniae* isolates [57].

Aurein 1.2, CAMEL, citropin 1.1, LL-37, Cec4 and omiganan have shown excellent activity against *A. baumannii*, with MICs going from 2 to 16 µg/mL [58,59]. Consequently, AMPs are considered as potential candidates for new treatments since they have relatively low MICs against a bacterial strain for which only limited, if any, treatment options are available. As reviewed by Neshani et al., 46 AMPs exhibit good activity against *A. baumannii*, some of which could be used as preventive and others as therapeutic options [60]. DN4 and DC4 have shown significant activity against *A. baumannii* as well as in a *K. pneumoniae* model, where an MIC of 32 µg/mL was noted [61].

On the other hand, ZY4 permeabilizes the MDR *P. aeruginosa* membrane and exhibits killing with MICs ranging from 2 to 4.5 µg/mL [62]. GL13K was also described as an active AMP showing good activity against MDR *P. aeruginosa*, with an MIC of 8 µg/mL [63,64]. Furthermore, NCK-10 is a small molecule mimetic made of a naphthalene aromatic ring, a decyl chain and a cationic lysine with potent *P. aeruginosa* antibiofilm activity [65].

3.2. Synergistic AMPs

To date, and according to the AMPs database, 47 of the listed AMPs show synergistic activities against several pathogens. This synergistic/additive effect is of interest to find

new efficient therapeutic strategies against the ESKAPE pathogens [66]. Synergies may be observed between different AMPs, AMPs of the same family or AMPs and antibiotics. Table 1 summarizes a few examples of AMPs that showed good synergistic effects.

Table 1. Properties of AMPs showing synergistic activities with one another and/or with antibiotics.

AMPs	Source	Synergistic Molecule	Target	Refs.
PGLa	Frog skin	Magainin 2	<i>E. coli</i> and <i>S. aureus</i>	[67]
Ranalexin	-Bullfrog <i>R. catesbeiana</i> - <i>Staphylococcus simulans</i>	Endopeptidase lysostaphin	<i>S. aureus</i> (MRSA)	[68]
Tridecaptin M	Mud bacterium	Rifampicin, vancomycin, and ceftazidime	Extremely drug-resistant <i>A. baumannii</i>	[69]
Dermaseptin	Amphibians skin	Dermaseptin	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i>	[70,71]
Bactenecin	Lactic acid bacteria	Bactenecin	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. Typhimurium</i>	[72,73]
Lactoferricin	Mammalians	Ciprofloxacin, ceftazidime	<i>P. aeruginosa</i>	[74]
Nisin	<i>Lactococcus lactis</i>	Colistin	<i>Pseudomonas</i> biofilms	[75,76]
P10		Ceftazidim/ doripenem	MDR <i>A. baumannii</i> and colistin-resistant <i>P. aeruginosa</i>	[76]
Gad-1	Fish	Kanamycin, ciprofloxacin	<i>P. aeruginosa</i>	[77]

3.3. AMPs Role in Preventive Healthcare

3.3.1. Prophylaxis

Another potential domain of action of AMPs is preventive healthcare, which deals with the prevention of illness [78]. As such, polycationic peptides were used as prophylactic agents against methicillin-susceptible or methicillin-resistant *Staphylococcus epidermidis* [79]. Antibacterial peptide-based gels were also synthesized to prevent medical implanted device infections [80]. Likewise, coating antimicrobial peptides was used for the prevention of urinary catheter-associated infections [81]. For example, bactericidal activity of chain 201D, an AMP derived from crowberry endophytes, was effective against *E. coli* and *S. aureus*, when immobilized on a model surface where it can bind and kill, by contact, a high percentage of adherent bacteria [81]. This gives AMPs a high potential for the development of antimicrobial surfaces, namely for application in urinary catheters [81]. Another example of AMP-based infection prevention in healthcare is dental caries caused by several pathogens [82]. Some AMPs including α -helical ones prevent bacterial adherence to implant surfaces [83].

3.3.2. Biofilm Treatment

Biofilms are one of the most common spreaders of infectious disease [84]. They form when communities of microorganisms adhere to some surfaces, and they are usually hard to treat [85]. Biofilms can develop on catheters, breast implants, or even on living tissue such as lungs or teeth, putting patients at risk of developing nosocomial infections that are difficult to eradicate with antibiotics [86]. By living in biofilms, bacteria are protected from harmful conditions, and thus can develop resistance to common antibiotics, which makes AMPs promising alternatives for new therapeutic strategies [87,88]. In this context,

as already reviewed by Galdiero et al., many AMPs serve as agents that interfere with bacterial biofilm formation/expansion [89], inhibit bacterial adherence on indwelling medical devices, and therefore can reduce nosocomial infections [81,90]. For example, vancomycin-coated tympanostomy tubes resist MRSA biofilm formation [91]. Interestingly, another AMP of the cathelicidins family D-LL-31 greatly enhances the biofilm-eradicating effects of currently used antibiotics [92,93]. Furthermore, SAAP-148, a synthetic AMP, showed great antibiofilm activity, where it eradicates MRSA and MDR *A. baumannii* [55]. Similarly, KKd-11 is a remarkable AMP that was not only able to inhibit the formation of biofilms but also effectively kill the bacteria within the biofilms [94]. Together, these findings show interesting potential of AMPs, which is a step forward as compared to conventional antibiotic therapy.

3.3.3. Vaccination: AMP-Based Vaccines

In the context of the preventive healthcare field, AMPs could also be used as vaccines or vaccine adjuvants to induce protective immunity or immune response against infections [95]. As such, IC31, a synthetic adjuvant containing the AMP KLKL5KLLK, can induce a protective immune response against the desired antigen [96]. It was also proven to be a potent adjuvant to a DNA vaccine targeting *Mycobacterium tuberculosis* [95]. However, no vaccine exists until today against MDR bacterial isolates [97]. Therefore, in this present scenario of exponentially increasing AMR, using the immuno-modulatory advantages of AMPs could help in alleviating AMR problem. The development of AMP-based vaccines may be able to reduce the burden and to overcome the AMR issue worldwide [98].

4. AMPs for Diagnostic Purposes

Rapid diagnostic tools are the key to prevent the spread of contagious infectious agents by rapidly identifying carriers or infected patients and prompt implementation of adapted infection control measures. The ability of AMPs to sense bacterial presence in potentially infected clinical samples is a major advantage. As already reviewed by Pardoux et al., AMPs could serve as infection selective tracers where they act as probes in biosensors and thus detect pathogens [99]. This is due to the preferential binding of AMPs along with their great number allowing the coverage of a wide range of microorganisms. AMPs were also described as powerful radiotracers and proved to be even better than labeled antibiotics [100]. [^{99m}Tc-HYNIC/EDDA]-MccJ25 is an example of an antimicrobial peptide analog that was used as a potential radiotracer for detection of infection [99]. Interestingly, this imaging agent for *E. coli* infection is also stable against enzymatic and metabolic degradation [101].

5. AMPs Facing 2019 Pandemic: SARS-CoV-2

In addition to activity toward bacteria, according to the database of antimicrobial activity and structure of peptides (DBAASP) [102], 208 AMPs have been reported to show antiviral activities against SARS-CoV-2. Slight changes to glycoicin F and lactococcin G increased their efficacy against SARS-CoV-2 by inhibiting protein synthesis with no side effects [103]. Similarly, mucroporin-M1 (LFRLIKSLIKRLVSAFK), a peptide analog displaying four amino acid changes (G3R, P6K, G10K, and G11R) as compared to the parent peptide mucroporin shows therapeutic activities against SARS-CoV-2 [104]. Brilacidin and LF are additional drug candidates for SARS-CoV-2 treatment [105,106]. Caerin 1.6 and caerin 1.10, two other AMPs, have a very high potential to interact with the spike surface protein of SARS-CoV-2, but low affinity for the ACE2 receptor. The selectivity of these peptides toward viral proteins makes them potential candidates for SARS-CoV-2 therapy [107].

6. Limitations and Challenges of the Therapeutic Use of AMPs

Despite the numerous advantages of AMPs, several problems prevented them from being used as therapeutic agents in human clinics. For instance, one major problem is their

limited stability, especially their susceptibility to the degradation by proteases [108,109]. In fact, the instability toward proteases hampers the development of many AMPs [110], by limiting their use for topical applications and/or requiring sequence modifications, to make them less susceptible to degradation [111]. Other problems include high extraction costs [112,113], poor bioavailability [6], short half-lives [114], cytotoxicity, and lack of specificity [115,116]. In addition, AMPs tend to display lower direct antimicrobial activity as compared to antibiotics. In real, regulatory agencies want AMPs to exhibit similar or even stronger activities than antibiotics [37]. Finally, resistance development by the bacteria toward AMPs cannot be excluded, especially if the microorganism is often exposed to them [117]. So far, only a few resistance mechanisms to AMPs have been described, including sequestration by bacterial enzymes and export by efflux pumps [118]. Taken together, these limitations slow down the successful reach of AMPs into clinical phases and limit their use to a small fraction of their potential (Figure 2) [44].

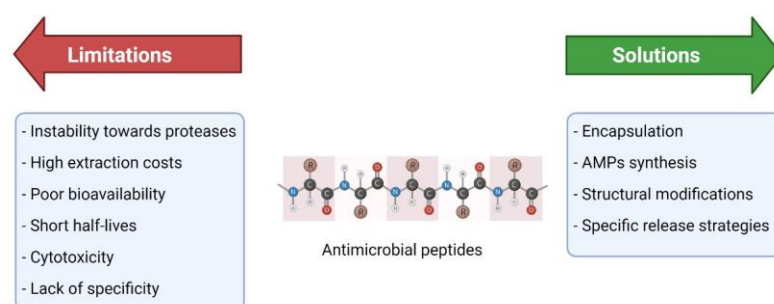


Figure 2. Limitations of AMPs and possible solutions for their therapeutic use.

6.1. Clinical Trials of AMPs

Although a huge number of AMPs have been described, very few reach the market. For example, ghrelin, the endogenous appetite peptide, possessing a good antimicrobial activity against Gram-negative bacteria is still in phase II clinical trial [119,120]. Surotomycin, is another example of AMP that is currently under investigation in phase III clinical trials; it targets *Clostridium difficile* and treats the associated diarrhea. The examples are countless; many AMPs remain under investigation until their efficacy is confirmed and secondary effects are eliminated. Some AMPs fail to reach the last clinical trials, such as (i) frulimicin B, which was terminated after phase I clinical trial due to unfavorable pharmacokinetics [44] and (ii) murepavadin, which was eliminated after a phase III clinical trial since it causes acute kidney injuries [44].

6.2. How to Overcome Some of These Limitations?

Different strategies were explored to overcome the drawbacks described previously. Modifications such as encapsulation of AMPs could be the key to avoiding their degradation by proteases and thus improving stability [121,122]. Several AMPs have been tested before and after encapsulation, and an obvious enhancement was observed: encapsulation of the AMP IK8, with gold nanorods into polyethylene glycol hydrogels protected the AMP from proteolysis and provided a triggered release of the therapeutic molecule [123]. High extraction costs could be limited by synthesizing AMPs in the lab using natural ones as templates where, at the same time, synthetic AMPs are always an enhanced version of the natural ones [124]. For this, genetic programming could be used to save not only money, but also on time and workload, and this could be started in silico through programs that predict the activity of AMPs before even generating them to avoid unnecessary synthetic efforts [125]. To improve bioavailability, increased half-lives of AMPs, and reduced cytotoxicity, structural modifications such as dimerization [126], methylation and

more have been successfully tested [127]. Specificity could be introduced by strategies for site specific release [128], such as specifically targeting AMPs (STAMP), which showed high efficiency in delivering the AMP to the target without harming the natural microbiota [129,130]. This strategy aims to implant AMPs into a drug delivery vehicle composed of the AMP itself in addition to a targeting peptide that allows a specific binding to the desired pathogen [43,129].

6.3. Synthetic AMPs

For decades, research for AMPs was not always successful. As mentioned previously, some AMPs were not introduced into clinics due to their toxicity/ degradation, while others were not very performant. However, none of these efforts were lost as these AMPs played the role of templates for the development of more efficient agents. For example, the introduction of L-phenylalanine into protonection enhanced its selective antibacterial activity against several Gram-positive bacteria and reduced MIC in *S. aureus* from 16 to 4 µg/mL [131]. In addition, the cathelicidin-derived antimicrobial peptide PMAP-23 was further modified by amino acid substitutions to obtain PMAP-23RI (for, Leu5-Arg and Thr19-Ile). The variant exhibited a higher stability and improved antibacterial activity when compared with the original PMAP-23, with MICs reduced by half in some cases from a simple modification to the original AMP [132]. Analogs of mastoparan-C were also designed by amino acid substitution and peptide truncation, which reduced cell toxicity and improved the bioactivity of the original AMP [127,133].

Table 2 provides additional information on many AMPs discussed in this paper.

Table 2. Amino acid sequences, type of synthesis and target groups of the AMPs discussed in this paper. Data were retrieved from the database of antimicrobial activity and structure of peptides [102]. GP: Gram-positive bacteria; GN: Gram-negative bacteria.

AMP	Amino Acid Sequence	Synthesis	Target Group
Cathelicidin-BF	KFFRKLKSKVKRAKEFFKKPRVIGVSIPF	Ribosomal	GP and GN
Defensin	GFGCPDQMQCHRHCQTITGRSGGYCSGPLKTCTCYR	Ribosomal	GP and GN
Nisin-P	VXxKXLxPGXKxGILMXXAIKxAXXGXHFG	Ribosomal	GP and GN
Gramicidin S	VXLfPVXLfP	Nonribosomal	GP, GN, parasites, fungus, cancer cells, and mammalian cells
Polymyxin Colistin	XTXXXLXXT	Synthetic	GN
Mellitin	GIGAVLKWLPAIKRKRQQ	Synthetic	GP, GN, and mammalian cells
Sm-Piscidin	KGARQAWKDYKYNRMQKMNQGYGQGG	Ribosomal	GP, GN, and fungus
Jellein-1	PFKISIH	Ribosomal	GP, GN, and mammalian cells
Aurein-1.1	GLFDIHKIAESI	Ribosomal	GP and GN
Citropin-1.1	GLFDVIKKVASVIGGL	Ribosomal	GP, GN, fungus, mammalian cells, and mollusc
Omiganan	ILRWPPWPPWRRK	Synthetic	GP, GN, fungus, and mammalian cells
ZY4	VCKRWKKWKRKWKWCV	Synthetic	GP, GN, fungus, and mammalian cells
GL13K	GKIIKLKASLKL	Synthetic	GP, GN, insects, and mammalian cells
Chain201D	KWIVWRWRFKR	Synthetic	GP, GN, and fungus
SAAP-148	LKRVRVFKLLKRYWRQLKKPVR	Synthetic	GP and GN
Lactococcin-G	GTWDDIGQIGRVAYWVGKAMGNMDSVNOASRINRKKHKKW GWLAWVEPAGEFLKGFKGAIKEGNKDKWKNI	Ribosomal	GP
Caerin-1.10	GLLSVLGSVAKHVLPVVPVIAEKL	Ribosomal	GP, GN, viruses, and mammalian cells
Murepavadin	LSYXXXXXWASP	Synthetic	GP, GN, cancer cells, and mammalian cells
Protonection	ILGTILPLKGL	Ribosomal	GP, GN, cancer cells, fungus, and mammalian cells

7. Conclusions

AMPs constitute promising agents with good antibacterial activity against a large set of pathogens, including Gram-negative and Gram-positive bacteria. Their ability to kill ESKAPE pathogens, especially those being pan-drug-resistant, represents a clinical added value over antibiotics due to the limited treatment options available to eradicate such infections. In this review, examples of AMPs effective against MDR bacteria that have the potential to replace conventional antibiotics have been reported. Their roles in boosting the activity of therapeutic molecules, eradication of biofilms, and preventive healthcare broaden AMPs' therapeutic potential (Figure 3). However, despite the huge number of AMPs described, and the efforts to optimize them, only a few reached advanced clinical trials. There are several challenges, such as their low stability, high extraction costs, and cytotoxicity, which need to be overcome so AMPs can be used in clinics. Nevertheless, due to increasing research efforts, it is expected that, hopefully, potent alternatives to antibacterial agents with the ability to eradicate untreatable life-threatening infections will be developed soon.

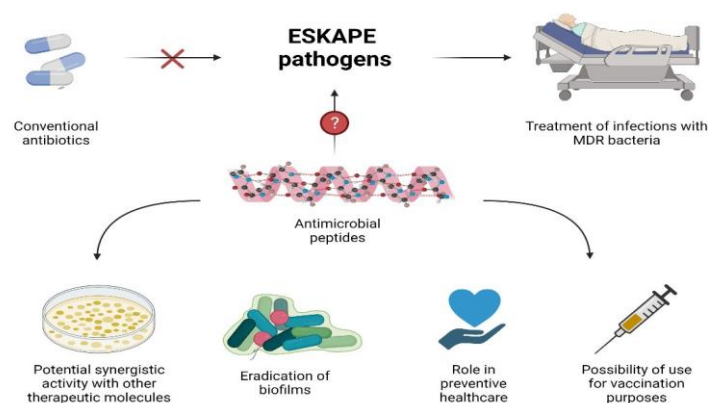


Figure 3. Potential use of AMPs.

Author Contributions: Conceptualization, M.R. (Mariam Rima), M.R. (Mohamad Rima), and T.N.; literature search, M.R. (Mariam Rima), M.R. (Mohamad Rima), and J.-M.S.; validation of search results, M.R. (Mariam Rima), M.R. (Mohamad Rima), Z.F., B.B., J.-M.S. and T.N.; writing—original draft preparation, M.R. (Mariam Rima), M.R. (Mohamad Rima), T.N. and B.B.; writing—review and editing, all. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Assistance Publique-Hôpitaux de Paris, the University Paris-Saclay, the Institut National de la Santé et de la Recherche Médicale (INSERM), the Laboratory of Excellence in Research on Medication and Innovative Therapeutics (LERMIT) through a grant from the French National Research Agency (ANR-10-LABX-33) and by the ANR-BMBF French-German bilateral project Natural-Arsenal “New Antibiotics Tackling mUlti-Resistance by acting on Alternative bacterial tARgets in ynergy with mEmbrane-disruptiNg Antimicrobial peptides” (ANR-19-AMRB-0004).

Acknowledgments: We are grateful to N. D’Amelio for the helpful advice.

Conflicts of Interest: The authors declare no conflict of interest.

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Scientific objectives

A better knowledge of the current epidemiological situation of AMR and the routes of dissemination AMR bacteria are critical to implement efficient intervention strategies to cope with the AMR problem . In addition, these epidemiological data will be very important to inform health authorities, to set the basis of an improved national surveillance systems based on molecular informations and increase the awareness of the general population. Finally, as these multidrug resistant are sometimes resistant to the entire armentarium of antimicrobial molecules, novel antibiotics and novel strategies are required to fight these bacteria.

The inappropriate use of carbapenems has played an important role in the global emergence of carbapenem-resistant bacteria, which poses now a global public health threat (3) The latter is especially pertinent in LMICs where monitoring, surveillance, and antimicrobial stewardship programs 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 (220). In Lebanon, a Mediterranean country with increasing AMR and a plethora of other public health challenges (221). Carbapenemase-producing Enterobacterales have been reported in healthcare settings, the community, and the environment in the past decade. Prior to proposing intervention strategies to curb with the plague, the epidemiological situation needs to be determined, along with the dissemination routes in all the three compartments. Finally, due to frequent patient transfers between Lebanon and France, thorow epidemiological investigations will benefit both countries. Since epidemiologically, OXA-48 and it's variants are widely prevalent enzymes, it's crucial to Understand their emergence and dissemination which requires structural and functional studies identify key residues with impact on the hydrolytic profile of the enzyme, notably the substitution of Arg214 and it's interaction with Asp159. Finally, finding novel therapies,

and most importantly novel antibiotics with unique mode of actions, to avoid cross resistance with current antibiotics is the penultimate goal.

To address these specific aims, we have divided this Thesis in three complementary chapters:

Chapter 1 is dedicated to the molecular epidemiology of carbapenem-resistant Enterobacteriales in Northern Lebanon, with the specific aim to determine the prevalence, the nature and the genetic support of carbapenem resistance in northern Lebanon among clinical isolates collected from 2015 to 2020 from three hospitals.

Chapter 2 is dedicated to explore the role of R214 of OXA-48 in the hydrolysis of carbapenems and temocillin, with the specific aims to : (i) Characterize biochemically and structurally, naturally-occurring, OXA-244 (R214G variant of OXA-48), OXA-484 (R214G variant of OXA-181), and OXA-933 (an D159N variant of OXA-48) and of some laboratory generated variants of them ; and (ii) Explore the role of the interaction between R214 and D159 in the substrate specificity of OXA-48 variants.

Chapter 3 is dedicated to the characterization of two novel antibiotics – cystobactamids and chelocardins, with the specific aims to : (i) define the spectrum of activity of cystobactamids and chelocardins on difficult to treat clinical Gram positive and negative isolates from France and Germany; (ii) explore the potential benefit of association with membrane destabilizing antimicrobial peptides, especially cathelicidins, frenatin, PglA, LH4; (iii) determine the frequency of resistance to cystobactamids and chelocardins and explore the mutational landscape that may lead to resistance; and finally (iv) define the transcriptional changes induced by these compounds and the changes in some mutants affecting regulatory genes using RNA Seq experiments.

Chapter I

“Carbapenem resistant Gram-negative *bacilli* in Lebanon”

Prevalence of carbapenem resistance in Northern Lebanon

Carbapenems, often referred as last resort antibiotics, possess a broad spectrum of activity against most clinically-relevant Gram-negative bacteria. Consequently, it is crucial to continuously monitor and assess the spread of carbapenem resistance, especially 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 (221, 222). 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 have been well-documented in Lebanon, even prior to the economic collapse (223). 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 (223, 224). Nevertheless, studies on AMR in Lebanon are generally scant and, when available, can be limited, in scope (e.g., low sample number) and/or descriptive (e.g., phenotypic AMR evaluation and absence of in-depth genomic analysis).

The first study on isolates from 2015 to 2019 (225), revealed novel several carbapenemase variants, such as NDM-19, VIM-62 and OXA-162, never described so far in Lebanon. Overall, our findings highlighted an alarming situation likely to become a major health issue in Lebanon, and encouraged us to conduct a follow study to see the evolution of this trend, that could be even accelerated due to the unprecedented economic crisis Lebanon is facing.

In the second study, we broaden the scope to include also ESBL producers. Hundred and eighty eight non-duplicate clinical extended-spectrum cephalosporin-resistant Enterobacterales (ESCrE) and carbapenem-resistant Enterobacterales (CRE) isolates from the same three hospitals in Northern Lebanon isolated between June 2019 to December 2020 were included. The collected isolates were characterized phenotypically by automated susceptibility testing and subsequently confirmed by disc diffusion assay. Additionally, CR-E isolates (n=33/188) were further analyzed using Illumina-based WGS to identify resistome, MLST, and plasmid types. The genetic relatedness of the CR-E isolates was also evaluated using an Infrared Biotyper system and compared to WGS. It consisted of a full characterization of CR-E isolates from different Lebanese hospitals revealing the most widespread enzymes especially OXA-48-Like.

These studies highlighted the emergence and broad dissemination of *bla*_{NDM-5} and *bla*_{OXA-244} 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.



Article

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

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Citation: Rima, M.; Oueslati, S.; Dabos, L.; Daaboul, D.; Mallat, H.; Bou Raad, E.; Achkar, M.; Mawlawi, O.; Bernabeu, S.; Bonnin, R.A.; et al. Prevalence and Molecular Mechanisms of Carbapenem Resistance among Gram-Negative Bacilli in Three Hospitals of Northern Lebanon. *Antibiotics* **2022**, *11*, 1295. <https://doi.org/10.3390/antibiotics11101295>

Academic Editor: Simone Simionatto

Received: 12 August 2022
Accepted: 19 September 2022
Published: 22 September 2022

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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*_{OXA-48}-like, *bla*_{NDM}-like and *bla*_{VIM}-like genes and CP-*Pseudomonas* expressed *bla*_{IMP}-like and *bla*_{VIM}-like genes, whereas CP-*Acinetobacter* expressed *bla*_{OXA-23}-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*_{OXA-50}-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 β -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 β -lactam inactivation, hence the importance of studying the various emerging β -lactamases [1]. Over-production of efflux pumps and the production of AmpC-type β -lactamase accompanied by the reduction of porin expression were also reported to play a major role in carbapenem-resistant strains [2]. According to Ambler, β -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- β -lactamases (MBL) that require a divalent cation, usually Zn^{2+} , for their activity [3]. Carbapenemases belong to class A serine β -lactamases, class B MBLs and class D oxacillinases of Ambler's classification, with KPC (*Klebsiella pneumoniae* carbapenemase), NDM (New Delhi metallo- β -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 *Enterobacteriales* 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 (%) ^a	N	Resistant isolates (%) ^a	N	Resistant isolates (%) ^a
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	11,210	311 (2.7%)	1034	155 (15%)	184	106 (58%)

^a 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.

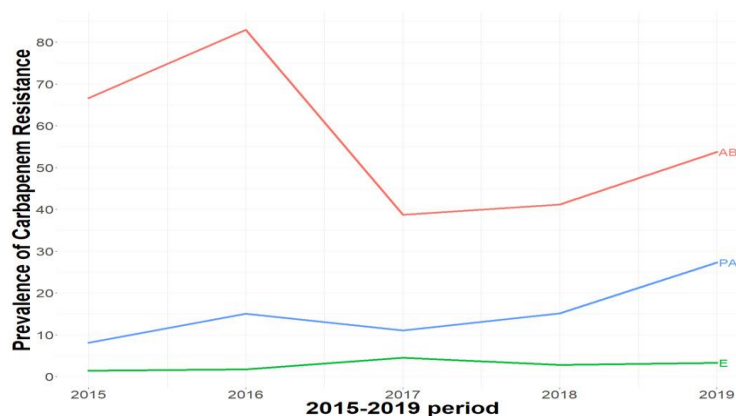


Figure 1. The evolution of carbapenem resistance among *Enterobacterales* (E), *P. aeruginosa* (PA) and *A. baumannii* (AB) isolates in northern Lebanon from 2015 to 2019.

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.

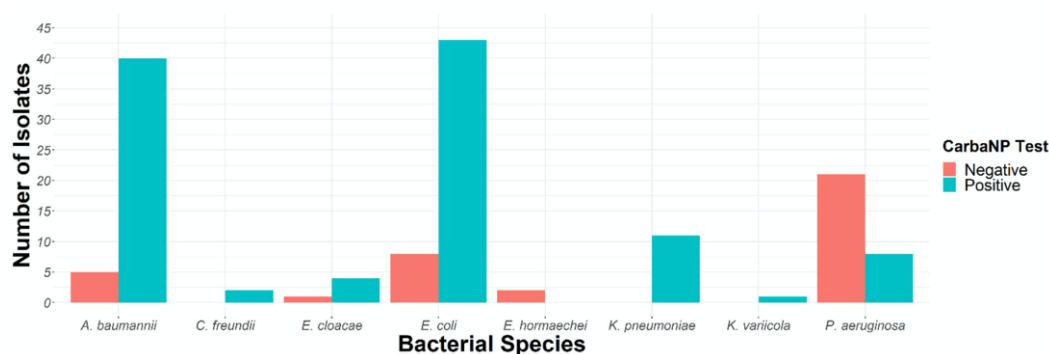


Figure 2. Carba NP test (*Enterobacterales* and *P. aeruginosa*) and CarbAcineto (*A. baumannii*) test results.

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>	<i>n</i> = 11	11 (100%)	OXA-48 (6) OXA-181 (1) NDM-5 (1) (3) ^a
<i>K. variicola</i>	<i>n</i> = 1	1 (100%)	(1) ^a
<i>E. cloacae</i>	<i>n</i> = 5	4 (80%)	OXA-48 (1) VIM-4 (1) VIM-1 (1) NDM-1 (1)
<i>E. hormaechei</i>	<i>n</i> = 2	0	-

Table 2. Cont.

Bacteria	Number of Isolates	CarbaNP-Positive Isolates	Carbapenemases (Sanger Sequencing)
<i>C. freundii</i>	<i>n</i> = 2	2 (100%)	OXA-48 (2)
<i>E. coli</i>	<i>n</i> = 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>	<i>n</i> = 29	8 (27%)	VIM-62 (2) VIM-2 (2) IMP-15 (1) (3) ^a
<i>A. baumannii</i>	<i>n</i> = 45	33 (73%)	OXA-23-LIKE (31) (2) ^a

^a 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*_{CTX-M-15}, *bla*_{SHV-1} and *bla*_{OXA-1} genes and the other one had *bla*_{CTX-M-15}, *bla*_{SHV-81-like} (99.8% nucleotide sequence identity) and *bla*_{TEM-1B} genes, respectively. The *bla*_{NDM-1} 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*_{OXA-50-like} and *bla*_{AmpC-PAO-like}, were found; for *P. aeruginosa* 1, *bla*_{OXA-50-like} mutant (T16A, K112E) and *bla*_{PDC-11} were found, whereas *bla*_{OXA-50-like} (R49C, A133G, A181T) and *bla*_{PDC-45} were noted for the second one, as well as *bla*_{OXA-50-like} (R167H, D109E) and *bla*_{PDC-1-like} for *P. aeruginosa* 3 (Table 3). The WGS of the *A. baumannii* isolate revealed the presence of the natural and chromosome-encoded *bla*_{OXA-64} and *bla*_{ADC-26} genes, together with a *bla*_{OXA-72} 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		Resfinder		MLST	Accession Numbers
	IMP	MER	Carba NP	Carba NP	NG-Test Carba5	β-Lactamases Genes	Other Resistant Genes		
<i>K. pneumoniae</i> 1	0.19	0.032	+	+	-	<i>bla</i> CTX-M-15, <i>bla</i> SHV-1, <i>bla</i> OXA-1	<i>aac</i> (6')-Ib-cr, <i>aac</i> (3)-Iia, <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> CTX-M-15, <i>bla</i> SHV-81, <i>bla</i> TEM-1-B	<i>sul1</i> , <i>sul2</i> , <i>oqx</i> A, <i>df</i> rA17, <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> NDM-1, <i>bla</i> CTX-M-15, <i>bla</i> SHV-106	<i>aac</i> (6')-Ib-cr, <i>msr</i> (E), <i>sul1</i> , <i>OqxA</i> , <i>df</i> rA12, <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> NDM-1, <i>bla</i> SHV-12, <i>bla</i> LEN-9, <i>bla</i> OXA-10, <i>bla</i> OXA-1	<i>aac</i> (6')-Ib-cr, <i>rmf</i> C, <i>rmf</i> H, <i>erm</i> (B), <i>aph</i> (3')-Ia, <i>sul1</i> , <i>sul2</i> , <i>OqxB</i> , <i>OqxA</i> , <i>df</i> rA14, <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>cmiA1</i> , <i>catA2</i>	ST-3195	JALHAY000000000
<i>P. aeruginosa</i> 1	>32	>32	+	+	-	<i>bla</i> OXA-50-like (T16A, K112E), <i>bla</i> PDC-11	<i>sul1</i> , <i>crpP</i> , <i>fosA</i> , <i>catB7</i> , <i>aph</i> (6)-Ia, <i>aph</i> (3')-Ib	ST-357	JALHAX000000000
<i>P. aeruginosa</i> 2	>32	>32	+	+	-	<i>bla</i> OXA-50-like (R39C, A133G, A181T), <i>bla</i> PDC-45	<i>aph</i> (3')-Ib, <i>fosA</i> , <i>catB7</i>	ST-893	JALHAW000000000
<i>P. aeruginosa</i> 3	>32	>32	+	+	-	<i>bla</i> OXA-50-like (R167H, D109E), <i>bla</i> PDC-1-like	<i>Sul1</i> , <i>fosA</i> , <i>aph</i> (3')-Ib, <i>crpP</i> , <i>catB7</i>	ST-277	Pending
<i>A. baumannii</i> 1	>32	>32	+	+	-	<i>bla</i> OXA-72, <i>bla</i> OXA-64, <i>bla</i> ADC-26	<i>aac</i> (6')-Ia, <i>aph</i> (6)-Ia, <i>aph</i> (3')-Ib, <i>aac</i> (3)-Iia, <i>sul2</i> , <i>aac</i> (3)-Iia, <i>tet</i> (B)	ST-229 *	JALHAZ000000000
<i>A. baumannii</i> 2	>32	>32	+	+	-	<i>bla</i> OXA-23, <i>bla</i> OXA-66, <i>bla</i> TEM-1D, <i>bla</i> ADC-73	<i>armA</i> , <i>msr</i> (E), <i>aph</i> (3')-Ia, <i>sul2</i> , <i>tet</i> (B), <i>aph</i> (6)-Ia	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 β -lactam belonging to the cephamycins, is rarely hydrolyzed by β -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 β -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*_{NDM-1} 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.

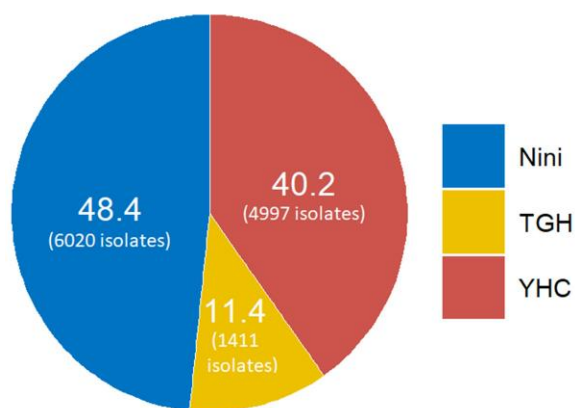


Figure 3. Origin of the isolates (%).

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 ^a	Type of Infection	Year
<i>Klebsiella pneumoniae</i>	11	Nini (7) YHC (4)	– Vaginal secretions (1) – Sputum (2) – Urine (7) – Nasal secretions (1)	2015–2018
<i>Klebsiella variicola</i>	1	YHC (1)	– Urine (1)	2018
<i>Enterobacter cloacae</i>	5	Nini (3) TGH (2)	– Catheter (2) – Ear swab (2) – Wound (1)	2017–2018
<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) – Pus (7) – Blood (2) – Nasal secretions (1) – Gastric liquid (4) – Sputum (1) – Wound (1) – Rectal swab (1)	2016–2018
<i>Pseudomonas aeruginosa</i>	29	YHC (25) TGH (4)	– Urine (18) – Blood (3) – Pus (5) – Wound (2) – Catheter (1)	2016–2018

Table 4. Cont.

Species	N	Hospital ^a	Type of Infection	Year
<i>Acinetobacter baumannii</i>	45	Nini (8) YHC (22) TGH (15)	– Sputum (19)	2016–2019
			– Catheter (5)	
			– Tracheal aspiration (3)	
			– Cavum (1)	
			– Wound (5)	
			– Urine (1)	
			– Blood (2)	
			– Pleural liquid (1)	
			– Bronchoalveolar lavage (4)	
			– Pus (4)	

^a 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 CarbAcineto 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*_{OXA-48}, *bla*_{KPC}, *bla*_{NDM}, *bla*_{VIM} and *bla*_{IMP}, 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.

Author Contributions: Conceptualization, M.R., M.O., M.H. and T.N.; resources, E.B.R., M.A., O.M., M.O., M.H.; methodology, M.R., D.D., S.B., D.G.; statistical analysis, M.O., software, R.A.B.; validation, S.O., L.D. and T.N.; investigation, H.M., E.B.R., M.A., O.M., M.O.; writing, M.R.; review and editing, M.O., M.H., T.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Assistance Publique-Hôpitaux de Paris (AP-HP), the University Paris-Saclay, the Laboratory of Excellence in Research on Medication and Innovative Therapeutics (LERMIT) supported by a grant from the French National Research Agency [ANR-10-LABX-33] and by the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) DesInMBL [ANR-14-JAMR-002]. Marwan Osman is supported by the Atkinson Postdoctoral Fellowship (Cornell University).

Institutional Review Board Statement: This investigation received the approval (CE-EDST-1-2019) 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.

Informed Consent Statement: Not applicable.

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

Acknowledgments: We acknowledge the Genetics department of the Bicêtre hospital for granting access to the WGS Next 500 sequencer.

Conflicts of Interest: The authors declare no conflict of interest.

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RECEIVED 08 September 2023
ACCEPTED 26 October 2023
PUBLISHED 20 November 2023

CITATION
Daaboul D, Oueslati S, Rima M, Kassem I,
Mallat H, Birer A, Girlich D, Hamze M,
Dabboussi F, Osman M and Naas T (2023) The
emergence of carbapenemase-producing
Enterobacteriales in hospitals: a major
challenge for a debilitated healthcare system in
Lebanon.
Front. Public Health 11:1290912.
doi: 10.3389/fpubh.2023.1290912

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

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Background: Carbapenem- and extended-spectrum cephalosporin-resistant *Enterobacteriales* (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*_{NDM-5} (14/33), *bla*_{OXA-244} (10/33), *bla*_{OXA-48} (5/33), *bla*_{NDM-1} (3/33), and *bla*_{OXA-181} (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*_{NDM-5} and *bla*_{OXA-244} 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*_{OXA-48}, *bla*_{NDM-5} and *bla*_{NDM-19}, in *Escherichia coli*, *bla*_{OXA-48}, *bla*_{OXA-181} and *bla*_{NDM-5} in *Klebsiella pneumoniae*, *bla*_{OXA-48}, *bla*_{VIM-1}, *bla*_{VIM-4} and *bla*_{NDM-1} in *Enterobacter cloacae*, and *bla*_{OXA-48} 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 β -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, cefoxitin, ceftazidime, cefepime, temocillin, and imipenem). As for the CR-E isolates, a total of 15 β -lactams (amoxicillin, amoxicillin/clavulanic acid, ticarcillin, ticarcillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cefoxitin, cefotaxime, 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)¹ 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.²

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

The β 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 β 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 β -LACTA™ and NG-Test® CTX-M MULTI tests) were screened by PCR and subsequent Sanger sequencing to identify the *bla*_{CTX-M} allele as described previously (23, 24). For the ESCR-E isolates (positive using the β -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.³ 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 µg/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

² 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 μ L of bacterial colonies selected from the confluent part of the culture was re-suspended in 50 μ L of 70% ethanol solution in an IR Biotyper suspension vial. After vortexing, 50 μ L of deionized water was added, and the solution was mixed by pipetting. The bacterial suspensions (15 μ 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 μ L deionized water and 90 μ L of absolute ethanol was added and mixed. Subsequently, 12 μ 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 tool⁴ 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*_{CTX-M} gene and subsequent sequencing revealed that *bla*_{CTX-M-15} was predominant (91.2%, 52/55), followed by *bla*_{CTX-M-55} (3.5%, 2/55) and *bla*_{CTX-M-3} 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*_{SHV-12} ESBL gene and one *K. pneumoniae* isolate harboring a chromosomally encoded *bla*_{SHV-187} 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 ≤ 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*_{OXA-48-like} had relatively low MICs (≤ 64 μ 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 ≤ 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*_{NDM} and 7 *bla*_{OXA-48-like} producers). All the tested isolates were resistant to ceftazidime 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*_{NDM}-producing isolates (Table 2). Additionally, increased MICs to aztreonam/avibactam (4, 8, and 16 mg/L) were noticed among *bla*_{NDM}-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 β -lactams, aminoglycosides, tetracycline, quinolones, trimethoprim-sulfamethoxazole, fosfomycin, 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%,

⁴ www.comparingpartitions.info

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

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	LXV	CHL	SXT	FUR	TMN	AMK	GMN	CST	
Escherichia coli																											
O84C6	Urine	YHC	+	OXA-like	+	–	R	R	R	R	R	S	S	S	S	S	S	S	S	R	R	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	S	S	S	S	4	
O84D2	Urine	YHC	+	OXA-like	–	ND	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	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	S	S	S	S	1	
O84E5	Urine	YHC	–	OXA-like	–	ND	R	R	R	S	S	S	S	S	S	S	S	S	S	S	R	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	S	R	R	R	2	
O84E8	Urine	YHC	–	OXA-like	–	ND	R	R	R	R	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	R	R	R	S	R	R	R	R	S	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	1	
O84F5	Urine	YHC	+	NDM	+	–	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	S	S	S	S	0.5	
O86D2	Urine	YHC	+	NDM	+	+	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	S	R	S	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	1	
O84G8	Urine	YHC	–	OXA-like	–	ND	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	1	
O85A1	Urine	YHC	–	OXA-like	+	+	S	R	R	R	R	S	S	S	S	S	S	R	R	R	R	S	R	S	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	1	
O85C3	Urine	YHC	+	NDM	+	+	R	R	R	R	R	R	R	R	R	S	S	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	2	
O85C10	Urine	YHC	+	NDM	+	+	R	R	R	R	R	R	R	R	S	S	S	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	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	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	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	1	
O85G1	Wound	NH	+	NDM	–	ND	R	R	R	S	R	R	R	S	R	S	S	R	R	S	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	2	
O85C4	Urine	NH	+	NDM	+	+	R	R	R	R	R	R	R	R	S	S	S	R	R	S	R	S	R	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	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	TMN	AMK	GMN	CST	
Klebsiella pneumoniae																											
O84C9	Blood culture	TGH	+	NDM	+	+	R	R	R	S	R	S	R	R	R	S	S	S	S	S	R	S	R	R	R	1	
O84D5	Pus	YHC	+	OXA-like	+	−	R	R	R	R	R	S	R	S	R	R	S	S	S	R	S	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	1	
O85D10	Urine	NH	+	NDM	+	+	R	R	R	R	R	S	S	S	S	R	S	R	S	R	R	S	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	2	
Enterobacter spp.																											
O84D8 ^a	Urine	NH	+	NDM	+	−	R	R	R	S	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	1	
O84F3 ^b	Urine	YHC	+	OXA-like	+	−	R	R	R	R	R	S	R	S	S	R	S	S	S	S	S	S	S	S	S	1	

^aSusceptibility 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, Ceftiderolol; MEC, Meropenem; 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. ^bO84D8, 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*_{CMY-145} and *bla*_{DHA-1} genes that confer resistance to ESCs were detected in three *E. coli* isolates. ESBL genes such as *bla*_{CTX-M-15} ($n = 15$), *bla*_{CTX-M-27} ($n = 1$), *bla*_{TEM-35} ($n = 1$), *bla*_{SHV-33} ($n = 1$), *bla*_{SHV-12} ($n = 2$), *bla*_{SHV-26} ($n = 1$), were also identified. Specifically, the *bla*_{CTX-M-15} gene was the most frequently detected in *E. coli* ($n = 11$); including one isolate co-harboring two CTX-M alleles: *bla*_{CTX-M-15} and *bla*_{CTX-M-27} and *K. pneumoniae* ($n = 4$) isolates. 16S RNA methylase genes were detected among 6 isolates including 5 *bla*_{NDM-5}-producing *E. coli* and one *bla*_{NDM-1}-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 LR134237) 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*_{NDM-5}-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*_{OXA-48}-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*_{OXA-244} (Table 3). Additionally, *iss* encoding an outer membrane lipoprotein that enhances serum resistance, was detected only in one *bla*_{OXA-244}-positive *E. coli* isolate. The capsular genes, *kpsE* and *kpsM*, were both detected in eight isolates, of which three were *bla*_{NDM-5}-positive (ST648), and 5 were *bla*_{OXA-244}-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 Kiezer plasmid extraction and electro-transformation for 25/33 isolates. For 8 *bla*_{OXA-244}-producing isolates, even with repeated attempts no plasmids were observed on Kiezer gel, and no transformants were

TABLE 2 Antibiotic susceptibility patterns of selected carbapenemase-producing *Escherichia coli* isolates for antibiotics considered last resort molecules using broth microdilution test (Sensititre), and interpreted according to the EUCAST guidelines (18).

Isolate code	MLST	Carbapenemase gene	Amino acids insertion in PBP3	CAZ/AVI	CEF	CFT	BPR	FDC	ATM/AVI ¹	IMP/REL	MER/VAB	ERV	APR	NEO	STR	SA
O84C6	ST940	OXA-181	–	S	R	R	R	S	I	S	S	S	S	S	R	S
O84C10	ST146	OXA-244	–	S	R	R	R	S	S	S	S	S	S	S	R	R
O84D2	ST69	OXA-48	–	S	S	R	R	S	S	S	S	S	S	S	R	R
O84D6	ST10	OXA-244	–	S	R	R	R	S	S	S	S	S	S	S	R	R
O84E7	ST405	NDM-5	YRIK	R	R	R	R	R	I	R	R	S	S	S	R	R
O84E9	ST405	NDM-5	YRIK	R	R	R	R	R	I	R	R	S	S	R	R	R
O84F2	ST38	OXA-244	–	S	R	R	R	S	S	S	S	S	S	S	S	S
O86D2	ST90	NDM-5	–	R	R	R	R	S	S	R	R	S	S	R	R	R
O84F9	ST631	NDM-5	YRIN	R	R	R	R	S	I	R	R	S	S	S	R	R
O85A1	ST8881	OXA-244	–	S	R	R	R	S	S	S	S	S	S	S	R	R
O85C6	ST167	NDM-5	YRIN	R	R	R	R	R	I	R	S	S	S	S	R	R
O85E2	ST648	NDM-5	YRIK	R	R	R	R	R	I	R	R	S	S	S	R	R
O86A2	ST69	OXA-244	–	S	S	R	R	S	S	S	S	S	S	S	S	S

The isolate selection was done according to their resistance phenotype, genotype and MLST type. ¹Dark gray: Resistant; Light gray: Intermediate non-susceptible; No color: Susceptible; Abbreviations: CAZ/AVI, ceftazidime/avibactam; CEF, ceftriaxone; CFT, ceftazidime; BPR, ceftoloprop; FDC: Cefiderocol; ATM/AVI, aztreonam/avibactam; MEM/VAB, meropenem/vaborbactam; IMP/REL, imipenem/relebactam; ERV, eravacyclin; NEO, neomycin; STR, streptomycin; SA, sulfonamides. ²As no breakpoints are yet available, those of ATM were used.

TABLE 3 Virulence genes of the 26 carbapenemase-producing *E. coli* clinical isolates.

Isolate code	Sample	Carbapenemase gene	MLST type	Adhesion				Siderophore			Serum resistance	Invasive	Capsular				Other genes									
				<i>fpA</i>	<i>fimH</i>	<i>afaA</i>	<i>afaC</i>	<i>afgD</i>	<i>afgE</i>	<i>iucC</i>	<i>iutA</i>		<i>capU</i>	<i>kapE</i>	<i>kapM</i>	<i>fydA</i>	<i>terC</i>	<i>chuA</i>	<i>imp2</i>	<i>ompT</i>	<i>traJ</i>	<i>hlyA</i>	<i>hlyE</i>	<i>csa</i>	<i>clb</i>	<i>sat</i>
O84D6**	Urine	<i>bla_{NDM-10}</i>	ST10																							
O83D8**	Urine	<i>bla_{NDM-10}</i>	ST10																							
O84E2**	Urine	<i>bla_{NDM-10}</i>	ST18																							
O84C10**	Gastric fluid	<i>bla_{NDM-10}</i>	ST46																							
O84D2**	Urine	<i>bla_{NDM-10}</i>	ST09																							
O84E2**	Urine	<i>bla_{NDM-10}</i>	ST09																							
O84D8**	Urine	<i>bla_{NDM-10}</i>	ST09																							
O84C2**	Urine	<i>bla_{NDM-10}</i>	ST09																							
O85A3*	Urine	<i>bla_{NDM-10}</i>	ST09																							
O86A2*	Urine	<i>bla_{NDM-10}</i>	ST09																							
O86D2	Urine	<i>bla_{NDM-10}</i>	ST09																							
O85C6	Urine	<i>bla_{NDM-10}</i>	ST167																							
O85C4	Urine	<i>bla_{NDM-10}</i>	ST167																							
O85C10	Urine	<i>bla_{NDM-10}</i>	ST167																							
O84J5	Urine	<i>bla_{NDM-10}</i>	ST301																							
O84I9	Urine	<i>bla_{NDM-10}</i>	ST301																							
O86A6	Rectal	<i>bla_{NDM-10}</i>	ST301																							
O84E7*	Urine	<i>bla_{NDM-10}</i>	ST405																							
O84E9*	Urine	<i>bla_{NDM-10}</i>	ST405																							
O85C3**	Urine	<i>bla_{NDM-10}</i>	ST405																							
O83E2*	Axillary	<i>bla_{NDM-10}</i>	ST648																							
O83J3*	Pus	<i>bla_{NDM-10}</i>	ST648																							
O85G1*	Wound	<i>bla_{NDM-10}</i>	ST648																							
O83D6*	Pleural fluid	<i>bla_{NDM-10}</i>	ST648																							
O85A1**	Urine	<i>bla_{NDM-10}</i>	ST881																							
O84G6**	Urine	<i>bla_{NDM-10}</i>	ST940																							

Virulence genes were detected using the VirulenceFinder 2.0 online tool available at the Center for Genomic Epidemiology-CGE (<http://www.genomicsepidemiology.org/>) (26, 29, 30). Virulence genes were detected using 100% identity as cut-off. *Nim Hospital, **EJ, Youssaf Hospital Center, ***Tripoli Governmental Hospital. Different colors represent different sequence type (ST).

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

Isolate code	MLST type	Carbenapenemase gene	Location (chromosomal or plasmid-borne)	IncX3	IncX4	IncFIA	IncFIB	IncFII	Col	IncI1-I	IncL	IncM
<i>Escherichia coli</i>												
O84D6**	ST110	<i>bla_{OXA-244}</i>	P									
O85D8**	ST110	<i>bla_{OXA-244}</i>	C									
O84F2**	ST38	<i>bla_{OXA-244}</i>	C									
O84C10**	ST46	<i>bla_{OXA-244}</i>	C									
O84D2**	ST69	<i>bla_{OXA-48}</i>	P									
O84E5**	ST69	<i>bla_{OXA-244}</i>	C									
O84E8**	ST69	<i>bla_{OXA-244}</i>	C									
O84G8**	ST69	<i>bla_{OXA-244}</i>	C									
O85A3*	ST69	<i>bla_{OXA-244}</i>	C									
O86A2*	ST69	<i>bla_{OXA-244}</i>	C									
O86D2**	ST90	<i>bla_{NDM-5}</i>	P									
O85C6**	ST167	<i>bla_{NDM-5}</i>	P									
O85C4*	ST167	<i>bla_{NDM-5}</i>	P									
O85C10**	ST167	<i>bla_{NDM-5}</i>	P									
O84F5**	ST361	<i>bla_{NDM-5}</i>	P									
O84F9**	ST361	<i>bla_{NDM-5}</i>	P									
O86A6*	ST361	<i>bla_{NDM-5}</i>	P									
O84E7*	ST405	<i>bla_{NDM-5}</i>	P									
O84E9*	ST405	<i>bla_{NDM-5}</i>	P									
O85C3**	ST405	<i>bla_{NDM-5}</i>	P									
O85E2*	ST648	<i>bla_{NDM-5}</i>	P									
O85T3*	ST648	<i>bla_{NDM-5}</i>	P									
O85G1*	ST648	<i>bla_{NDM-5}</i>	P									
O85D6*	ST648	<i>bla_{NDM-5}</i>	P									
O85A1**	ST8881	<i>bla_{OXA-244}</i>	C									
O84C6**	ST940	<i>bla_{OXA-181}</i>	P									

(Continued)

TABLE 4 (Continued)

Isolate code	MLST type	Carbapenemase gene	Location (chromosomal or plasmid-borne)	IncX3	IncX4	IncFIA	IncFIB	IncFII	Col	Inc11-I	IncL	IncM
<i>Klebsiella pneumoniae</i>												
O84E1**	ST35	<i>bla_{CTX-A48}</i>	P									
O84D5**	ST35	<i>bla_{CTX-A48}</i>	P									
O85E3*	ST37	<i>bla_{CTX-A48}</i>	P									
O84C9***	ST45	<i>bla_{NDM-1}</i>	P									
O85D10*	ST1770	<i>bla_{NDM-1}</i>	P									
<i>Enterobacter</i> spp.												
O84D8*	ST182	<i>bla_{NDM-5}</i>	P									
O84F3**	ST1006	<i>bla_{NDM-48}</i>	P									

Plasmid incompatibility groups present in the 33 clinical carbapenemase-producing Enterobacteriales isolates, were identified using PlasmidFinder 2.1 online tool available at the Center for Genomic Epidemiology - CGE (<http://www.genomic Epidemiology.org>) using 99% identity cut-off (26, 28–30). *Nini Hospital; **El-Youssef Hospital Center; ***Tijoli 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 ^a	7	0.875 (0.813–0.937)
IRBT ^b	11	0.933 (0.857–1.000)
WGS ^c	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). ^aMLST: Multi-locus sequence typing; ^bIRBT: IR Biotyper; ^cWGS: Whole-genome sequencing.

obtained suggesting a chromosomal location. For *K. pneumoniae* and *E. hormaechei*, the *bla_{CTX-A48}* 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 Enterobacteriales (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 Enterobacteriales 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_{CTX-M-15}* 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 Enterobacteriales 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*_{OXA-48-like} variants toward *bla*_{NDM-5} 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*_{NDM-5} gene has become the predominant carbapenemase gene (14 of 26 *E. coli* isolates). The *bla*_{OXA-244} gene (10/26) came in second place, while *bla*_{OXA-48} (1/33), and *bla*_{OXA-181} (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*_{OXA-48} (3/5) and *bla*_{NDM-1} (2/5), while the *E. cloacae* and *E. hormaechei* isolates harbored *bla*_{OXA-48} and *bla*_{NDM-1}, 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*_{NDM-5} gene, rather than a clonal spread of a single clone. Interestingly, we found that the carbapenemase genes were plasmid-borne (except in eight *bla*_{OXA-244}-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 PBP3, respectively, which resulted in a significant increase of MICs to aztreonam/avibactam and cefiderocol as well as to other PBP3-targeting β -lactams (34). Surprisingly, the *bla*_{OXA-244} 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*_{OXA-244} 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*_{NDM-5}-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*_{CTX-M-15} in humans, birds, and companion animals (47). Additionally, ST648 was identified in Lebanon among clinical CRE (48), including *bla*_{NDM-5}-producing *E. coli* (49). Besides, ST167 has been linked to the global spread of *bla*_{NDM} 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*_{CTX-M-15} 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*_{OXA-244}-producing *E. coli*, previously described in estuary water in Lebanon (56). Several European countries have reported increased dissemination of the *bla*_{OXA-244} gene (57, 58), with ST38 being the most common sequence type among *bla*_{OXA-244}-producing *E. coli* isolates (59). Moreover, ST38 *bla*_{OXA-48}-producing *E. coli* has also been found in fowls in Lebanon (60). As observed in our study, ST940 *E. coli* carrying the *bla*_{OXA-181} 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*_{NDM-1} *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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Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Assistance Publique-Hôpitaux de Paris, the transdisciplinary Health and Therapeutic Innovation object (HEALTHI) of the Université Paris-Saclay, through a grant from the French National research Agency (ANR-10-LABX-33), and the ANR-BMBF French-German bilateral project Natural-Arsenal (ANR-19-AMRB-0004). Marwan Osman and Dina Daaboul were supported by the Atkinson Postdoctoral Fellowship (Cornell University) and SAFAR Doctoral Scholarship (Lebanese University and French Embassy in Lebanon), respectively.

Acknowledgments

We are grateful to NG Biotech for providing the NG-Test® CARBA-5 and CTX-M MULTI free of charge.

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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Supplemental Material

1- Supplemental figures

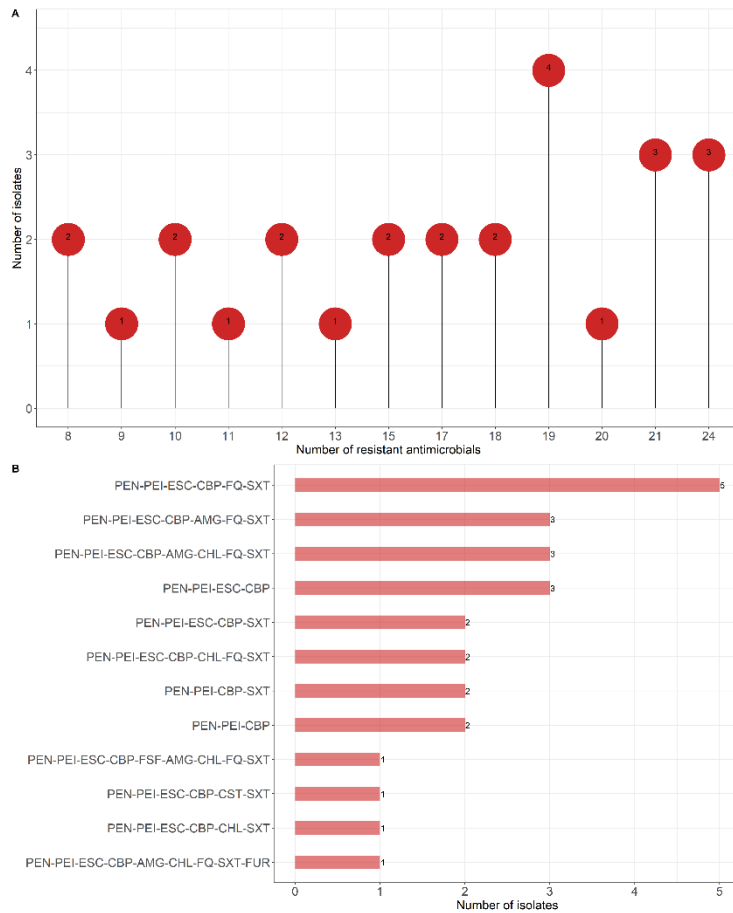


Figure S1. Antimicrobial Susceptibility Patterns of Carbapenemase-Producing *Escherichia coli* (n=26) clinical isolates. Distribution of resistance by number of antimicrobial agents (A) and susceptibility patterns of the isolates (B); Antimicrobial category abbreviations: PEN, penicillin; PEI, penicillin/β-lactamase 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.

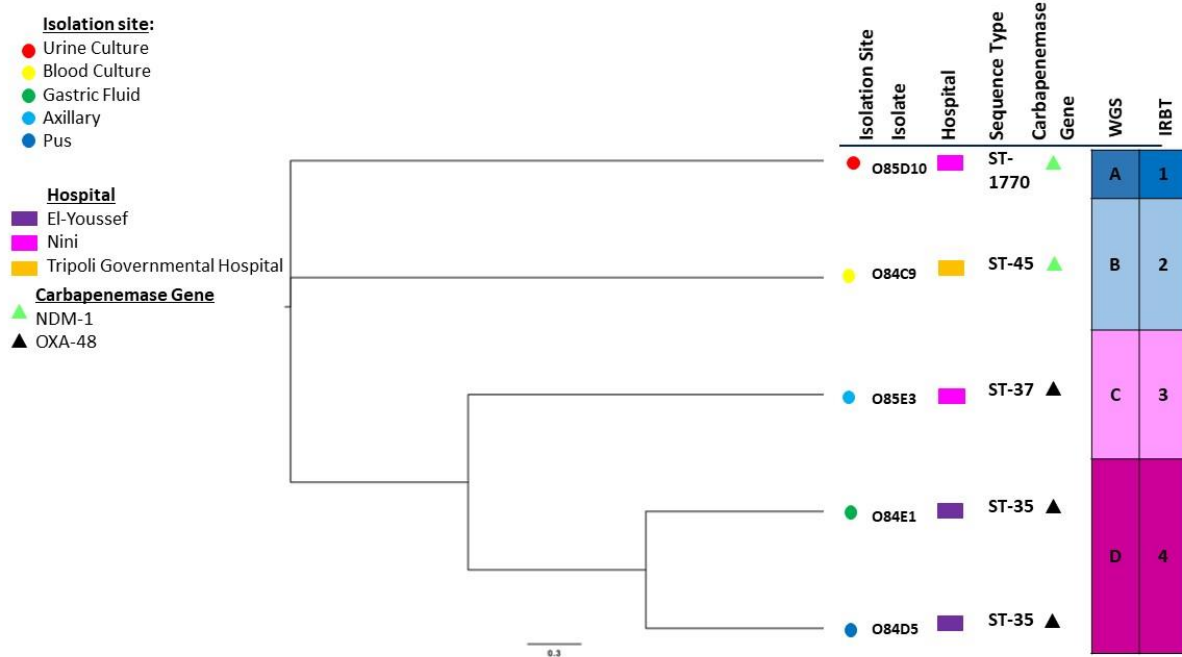


Figure S2. Phylogenetic tree of five carbapenemase-producing *Klebsiella pneumoniae*. A dendrogram was obtained by SNP-based analysis, using CSI Phylogeny 1.4 available online at the center for genomic epidemiology-CGE (<http://www.genomicepidemiology.org/>) (1). The dendrogram was rooted with branch 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.

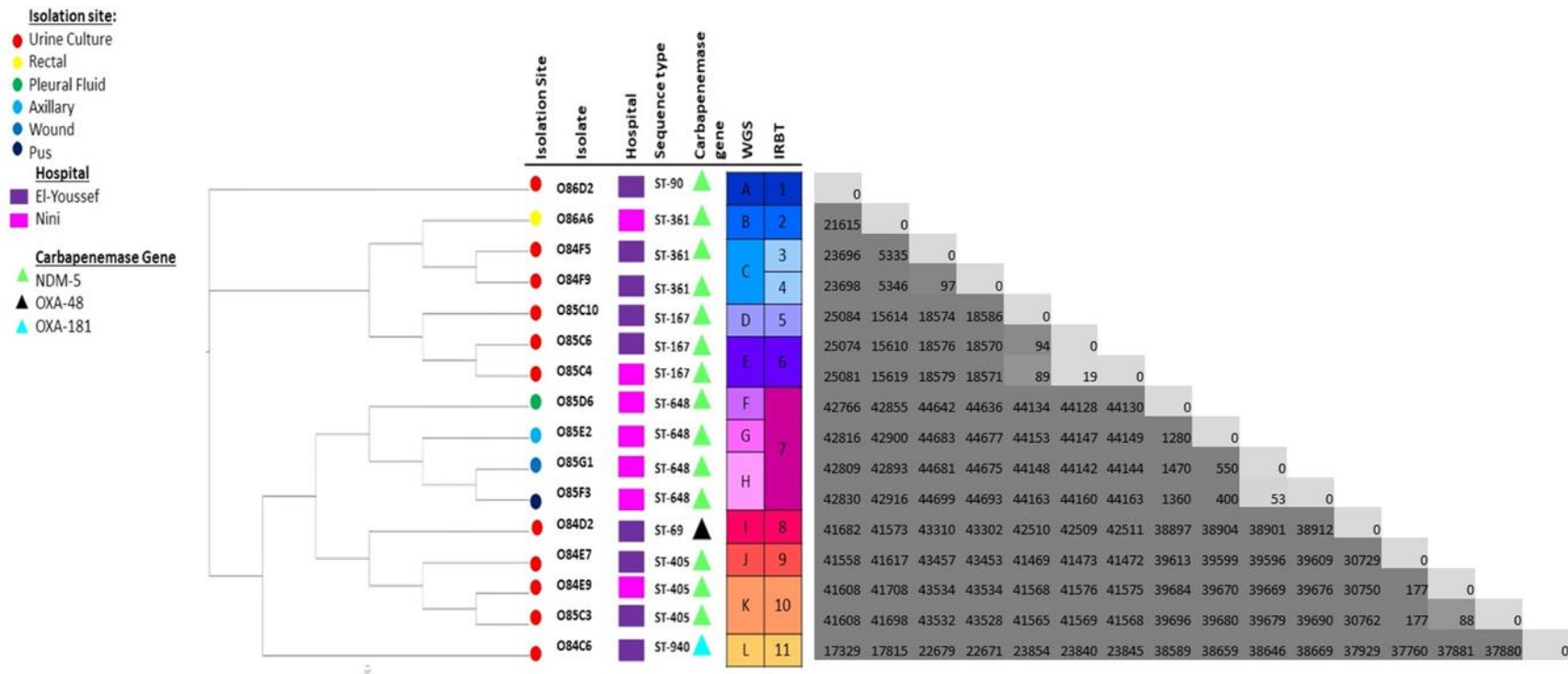


Figure S3. Phylogenetic tree of 16 carbapenemase-producing *Escherichia coli* isolates. A dendrogram was obtained by SNP-based analysis, using CSI Phylogeny 1.4 available online at the center for genomic epidemiology-CGE (<http://www.genomicepidemiology.org/>) (1). The dendrogram was rooted with branch 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.

2- Supplemental tables

Table S1. Distribution of extended-spectrum β -lactamase genes carried by ESBL-producing Enterobacterales isolates.

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

¹ND, 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 (2).

Isolate code	CarbaNP	NG-Test Carba5	β 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)	0.12 5	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-	+	+	R (20)	0.5	S (22)	0.5	S (22)	0.5	R (8)	256

		<i>like</i>										
<i>O85D10</i>	+	<i>NDM</i>	+	+	<i>R</i>	2	<i>S</i> (22)	0.5	<i>S</i> (22)	0.75	<i>R</i> (6)	32
<i>O85E3</i>	+	<i>OXA-like</i>	+	+	<i>R</i> (12)	4	<i>S</i> (22)	0.5	<i>R</i> (15)	1	<i>R</i> (6)	1024
<i>Enterobacter spp.</i>												
<i>O84D8</i>	+	<i>NDM</i>	+	-	<i>R</i> (6)	>32	<i>S</i> (22)	4	<i>R</i> (6)	12	<i>R</i> (6)	128
<i>O84F3</i>	+	<i>OXA-like</i>	+	-	<i>R</i> (1)	6	<i>S</i> (22)	1	<i>R</i> (15)	1.5	<i>R</i> (6)	512

**S*, Susceptible; *R*, Resistant; *ND*, Not determined.

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2. EUCAST, European Society of Clinical Microbiology and Infectious Disease Guidelines. Recommendations 2023

Chapter II

“ Insights in structure function relationship of OXA-48 like variants”

Carbapenem-hydrolyzing class D β -lactamases (CHDLs) have been most frequently found in *Acinetobacter* spp. , while OXA-48-type enzyme have been identified only in Enterobacteriales . Since its initial identification in a clinical *Klebsiella pneumoniae* isolate recovered in Istanbul, Turkey, in 2001 , endemic spread of these bacteria has been reported in countries such as Turkey, Morocco, Libya, Egypt, Tunisia, India and Lebanon. In other countries, such as France, Spain, Italy, Belgium, the Netherlands, the UK, Germany, Switzerland, Lebanon, Kuwait, Saudi Arabia, China, and Japan increasing descriptions are reported (175). In many Western European countries, high prevalences of OXA-48 carbapenemases among carbapenemase-producing Enterobacterales (CPEs) have been reported, best exemplified by Spain and France with 74 and 70%, respectively .

Until today, more than 50 OXA-48-like variants have been reported from clinical Enterobacterales isolates worldwide . For a complete list of variants see the “Beta-Lactamase DataBase” and the “Bacterial Antimicrobial Resistance Reference Gene Database”As displayed on the BLDB database many more variants have been described in metagenomic data (141).

CHDLs of OXA-48-type are very worrisome, given their rapid worldwide spread and their propensity to evolve by mutations leading to various phenotypic expressions . Although OXA-48 hydrolyzes penicillins at a high level, it hydrolyzes carbapenems only at a low level, however, OXA-48 is one of the class D β -lactamase with the highest known catalytic efficiency for imipenem (kcat value of 5 s^{-1}) . In addition, it shows very weak activity against expanded-spectrum cephalosporins such as cefotaxime, but does not significantly hydrolyze ceftazidime and cefepime. Whereas some OXA-48-related enzymes such as OXA-181, and OXA-204

confer similar resistance pattern, e.g. hydrolysis of carbapenems and penicillins with very weak or almost no activity against expanded-spectrum cephalosporins, others have reduced carbapenem- and temocillin-hydrolysis, such as OXA-244 and OXA-232, and others such OXA-163 and OXA-405 compromises the efficacy of broad-spectrum cephalosporins and hydrolyzes carbapenems only marginally.

Among OXA-48 variants that are currently been increasingly isolated is OXA-244, a single amino-acid variant of OXA-48 (R214G) with reduced activity against carbapenems and temocillin (182) posing diagnostic challenges (183, 184). Since 2013 an increase in OXA-244-producing *E. coli* has been observed in several countries worldwide (226-232). Genomic studies have shown that the dissemination of OXA-244-producing *E. coli* is polyclonal, but dominated by *E. coli* ST 38 (183, 184). Recently, hospital outbreak of OXA-244-producing *E. coli* ST38 involving three hospitals in the Western region of Norway, a country with a very low prevalence of CPE has been described (185). To evaluate the impact of R214G substitutions, structural and functional studies have been conducted, using kinetic analysis and molecular modeling.

Using site directed mutagenesis and X-ray crystallography the role of the R214S substititon of OXA-232 was determined (178). The AA214 of OXA-232 was replaced by G, L, D, E, S, R, and K. X-ray structure of OXA-232 along with molecular modelling studies showed that the interaction via a salt bridge between R214 and D159 in OXA-48 is not possible with G214 or S214 mutations. In contrast, with K214 that is also positively charged maintains the interaction with D159. With the E214 mutant an alternative binding conformation of imipenem was evidenced that is not compatible with a nucleophilic attack by S70. Given the importance of R214 revealed in Oueslati et al. study (178), and since it was shown that OXA-244 variant had limited effectiveness against carbapenems and temocillin, we aimed to analyze the kinetic parameters of OXA-244, when interacting with penicillins, cephalosporins, and carbapenems.

OXA-244 displayed a 4-fold lower catalytic efficiency (kcat/Km) for ampicillin hydrolysis compared to OXA-48. Additionally, OXA-244 exhibited a 20-fold lower catalytic efficiency for temocillin compared to OXA-48. Carbapenems were among the favored substrates for OXA-48, with the highest catalytic efficiency observed for imipenem. In contrast, OXA-244 had 6-fold and 14-fold lower kcat/Km values for meropenem and imipenem hydrolysis, respectively, compared to OXA-48. Overall, OXA-232 and OXA-244, despite differing by 5 amino acids (including position 214), exhibited similar catalytic efficiency values. The replacement of S214 with glycine in OXA-232, as in OXA-244, had a similar impact on MICs and kinetics. As stated, the R214 residue, of β 5- β 6 loop, is crucial for the carbapenemase activity, as it stabilizes carbapenems in the active site and maintains the shape of the active site through interactions with D159.

In the frame of the activity of the F-NRC, a novel variant, OXA-933 (D159N) was evidenced. This variant showed a significant decrease of the carbapenemase activity. As D159 is interacting with R214 in OXA-48 and OXA-181, characterization of OXA-933 allows to address the impact of D159 on the hydrolytic profile of the enzyme. To evaluate the importance of D159, point mutations were generated (D159A, D159G, D159K, D159W), kinetics parameters of OXA-933, OXA-48 D159G, and OXA-48 D159K were determined. As expected, MICs values of *E. coli* expressing *bla*_{OXA-933} gene or of its point mutant derivatives were lower for carbapenems. This came along with lower catalytic efficiencies for carbapenems.

These studies confirmed the importance of the R214, and D159 in the conformation of the enzyme's active site where their interaction through a salt bridge affects directly imipenem's affinity.

Biochemical characterization of OXA-244, an emerging OXA-48 variant with reduced β -lactam hydrolytic activity

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Received 1 February 2021; accepted 12 April 2021

Background: OXA-48-producing Enterobacterales have widely disseminated globally with an increasing number of variants identified. Among them, OXA-244 is increasingly reported, despite detection difficulties.

Objectives: To determine the steady-state kinetic parameters of OXA-244.

Methods: The *bla*_{OXA-244} gene was amplified, cloned into plasmids p-TOPO and pET41b+, and transformed into *Escherichia coli* TOP10 for MIC determination and *E. coli* BL21 DE3 for purification. Steady-state kinetic parameters and IC₅₀s of clavulanic acid, tazobactam and NaCl were determined using purified OXA-244. Molecular modelling was also performed.

Results: A reduction in MICs of temocillin and carbapenems was observed in *E. coli* expressing OXA-244 as compared with OXA-48. The kinetic parameters revealed a reduced carbapenemase activity of OXA-244 as compared with OXA-48, especially for imipenem, which was 10-fold lower. Similarly, catalytic efficiency (k_{cat}/K_m) was reduced by 4-fold and 20-fold for ampicillin and temocillin, respectively. Kinetic parameters for cephalosporins were, however, similar. Molecular modelling studies evidenced the key role of R214 in OXA-48, establishing salt bridges with D159 and with the carboxylate group of the R1 substituent of temocillin. These interactions are not possible with G214 in OXA-244, explaining the reduced affinity of temocillin for this enzyme. The R214G mutation in OXA-244 is also likely to induce changes in the active site's water network that would explain the decrease in the hydrolysis rate of carbapenems.

Conclusions: Our data confirm that the R214G mutation (present in OXA-244) results in reduced carbapenem- and temocillin-hydrolysing activity, confirming the crucial role of residue 214 in the hydrolysis of these substrates by OXA-48-like β -lactamases.

Introduction

OXA-48-like enzymes are the most prevalent carbapenemases in many western European countries.^{1,2} Since the initial description in Turkey, more than 30 OXA-48-like variants have been reported worldwide with amino acid (AA) changes mostly located in the β 5- β 6 loop.^{1,3–6} Currently, two main variants, OXA-48 and OXA-181, which differ by 4 AAs, are spreading worldwide.^{1,2} They hydrolyse penicillins at a high level, carbapenems at a low level and have a weak activity against expanded-spectrum cephalosporins.^{3,4} Some derivatives of these variants, such as OXA-244 (OXA-48 R214G) or OXA-232 (OXA181 R214S), are of particular

interest as they confer lower MICs of carbapenems and temocillin as compared with OXA-48 producers.^{6–8} As temocillin and carbapenems are present in several screening media used for carbapenemase-producing Enterobacterales (CPE) detection, these variants may be difficult to detect.^{9,10}

As a consequence, OXA-244 is increasingly reported in several European countries and in the Middle East.^{1,2,8,9,11–14} Initially isolated in Spain in 2012 from a *Klebsiella pneumoniae*,⁸ it is now mainly found in *Escherichia coli* isolates.^{7,9–16} In France, an increasing number of OXA-244 producers have been received at the French National Reference Centre for Carbapenem-Resistant Enterobacterales (CRE) over the last 8 years (0 in 2012 to 72 in

2019, which represents 2.4% of all CPE and 3.4% of all OXA-48-like producers).^{2,9,16} It is likely that these numbers underrepresent the reality, as OXA-244 producers are very difficult to detect using CPE screening media.^{9,10}

The aim of this study was to investigate the molecular mechanisms sustaining the peculiar substrate specificity of OXA-244 using biochemical tools and to compare the results with those of OXA-232 (S214) and OXA-48 (R214).

Materials and methods

Bacterial strains

The OXA-244-producing *E. coli* isolate VAL was used to amplify and clone the *bla*_{OXA-244} gene.⁷ *E. coli* TOP10 (Invitrogen, Saint-Aubin, France) was used for cloning and *E. coli* BL21 (DE3) (Novagen, Fontenay-sous-Bois, France) was used for overexpression experiments. *E. coli* TOP10 (pOXA-232 and pOXA-48) was from Queslati et al.^{6,17}

PCR, cloning, expression and DNA sequencing

Whole-cell DNA of *E. coli* VAL, extracted using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France) and primers PreOXA-48 A (5'-TATATGCATTAAAGCAAGG-3'), preOXA-48 B (5'-CACACAAATACGCGCTAAC-3'), was used to amplify the *bla*_{OXA-244} gene. The resulting PCR product was cloned using a pCR®-Blunt II-TOPO® kit (Invitrogen, Illkirch, France) and electroporated into *E. coli* TOP10 as previously described.^{6,17} The *bla*_{OXA-244} gene sequence without signal peptide (predicted by SignalIP 4.1 Server), encoding the mature protein from AA K23 to P261, was amplified using primers OXA₂₃₋₂₅₆NdeI (5'-aaaaaCATATGaaggaatggcaagaacaaa-3') and OXA-XhoI-Δstop (5'-aaaaaCTCGAGggggaataattttctgtttgag-3') and subsequently cloned in frame with the C-Term His₈-tag into an Nde-I-Xho-I-restricted pET41b vector (Invitrogen®, Life Technologies, Cergy-Pontoise, France), resulting in plasmid pET41b-OXA-244_{23-265His}. Constructs were verified by Sanger sequencing using an automated ABI Prism 3100 sequencer (Applied Biosystems, Les Ulis, France).

Susceptibility testing

Etest® (bioMérieux, Marcy-l'Étoile, France) strips were used for MIC determination and were interpreted according to EUCAST guidelines.¹⁸ Microbroth dilution was used to determine the MICs of temocillin, ampicillin and piperacillin as previously described.^{6,17,19}

Protein purification

The recombinant plasmid pET41b-OXA-244_{23-265His} was transformed into *E. coli* BL21 (DE3) and an overnight culture was used to inoculate 2 L of brain heart infusion broth medium containing 30 mg/L kanamycin. Induction, crude protein extract preparation and His-tag purification were done as previously described.^{17,19}

Steady-state kinetic determinations

Purified β-lactamase was used for kinetic measurements (k_{cat} and K_m), which were determined in 100 mM Tris-H₂SO₄/300 mM K₂SO₄ (pH 7) by analysing β-lactam hydrolysis under initial-rate conditions using the Eadie-Hofstee linearization of the Michaelis-Menten equation as previously described.^{6,17,19} For $K_m < 10 \mu\text{M}$, K_i was determined as previously described.^{4,17} The IC₅₀ was determined by incubating the enzymes with different concentrations of inhibitors for 3 min prior to kinetic parameter determination, using 100 μM benzylpenicillin, as a reporter substrate.^{6,17,19}

Molecular modelling

The three-dimensional structure of OXA-244 was generated by *in silico* mutagenesis from the structure of OXA-48 (PDB code 6P97)²⁰ using the *swapaa* command implemented in the UCSF Chimera software (version 1.14).²¹ Three-dimensional structures of the β-lactam ligands were generated using the Corina (version 3.60) program (Molecular Networks GmbH, Erlangen, Germany). Molecular docking calculations were performed using the Gold program (Cambridge Crystallographic Data Centre, Cambridge, UK)²² and the GoldScore scoring function. The binding site, defined as a sphere with a 20 Å radius, was centred on the oxygen atom of Ser70. All other parameters had default values. Molecular modelling images were generated with UCSF Chimera.²¹

Results and discussion

Susceptibility testing

OXA-244 is a single AA variant of OXA-48 (R214G)⁸ and is an emerging carbapenemase variant in several European countries.^{9-15,20} This variant displays reduced MICs of temocillin and carbapenems (imipenem, meropenem and ertapenem), whereas MICs of benzylpenicillin and cefalotin are not affected (Table S1, available as [Supplementary data](#) at JAC Online).

Arg214 has been shown to be critical for carbapenem hydrolysis.^{17,19} This is the case for OXA-232, where R214 is substituted by a serine, and for other single mutants in this position.¹⁷ When R214 is substituted with an uncharged AA, such as G or L, the MICs were similar to those for OXA-232. The most interesting results were obtained with substitutions by a negatively charged AA at pH 7.0, such as aspartate or glutamate. Indeed, MICs of imipenem and temocillin were remarkably affected, making them similar to those for the *E. coli* TOP10 control isolate.¹⁷

Biochemical properties of OXA-244

Previous findings based on specific activities suggested weak carbapenem and temocillin activities for the OXA-244 variant.⁷ To understand the structural features that may explain these weaker activities,⁷ we purified OXA-244 and determined the steady-state kinetic parameters of penicillins, cephalosporins and carbapenems, and compared them with those for OXA-48 and OXA-232 (Table 1).

The catalytic efficiency (k_{cat}/K_m) of OXA-244 with regard to ampicillin hydrolysis was 4-fold lower ($549 \text{ mM}^{-1} \cdot \text{s}^{-1}$) than that of OXA-48 ($2.4 \times 10^3 \text{ mM}^{-1} \cdot \text{s}^{-1}$) (Table 1). In addition, hydrolysis of temocillin by OXA-244 revealed a K_m and a k_{cat} 100-fold higher and 3-fold lower, respectively, thus resulting in a 20-fold lower catalytic efficiency as compared with OXA-48. Cefalotin is hydrolysed with a 6-fold lower k_{cat}/K_m as compared with the value for OXA-48. Oxyimino cephalosporins, such as cefotaxime, are weakly hydrolysed, but the k_{cat}/K_m could not be determined precisely, as the K_m was $>900 \mu\text{M}$ and so could not be determined experimentally. For ceftazidime, a bulkier oxyimino cephalosporin, no hydrolysis was observed even with 1.92 μM purified enzyme and up to 500 μM substrate, as shown for OXA-48.^{4,17}

Carbapenems were among the preferred OXA-48 substrates, with the highest catalytic efficiency (k_{cat}/K_m) observed for the hydrolysis of imipenem ($0.37 \times 10^3 \text{ mM}^{-1} \cdot \text{s}^{-1}$).^{4,6,17,20} OXA-244 has k_{cat}/K_m values 6-fold and 14-fold lower for meropenem and imipenem hydrolysis, respectively as compared with OXA-48.

Table 1. Steady-state kinetic parameters for hydrolysis of β -lactam substrates by OXA-244, OXA-232 and OXA-48

Substrate	K_m (μ M)			k_{cat} (s^{-1})			k_{cat}/K_m ($mM^{-1}.s^{-1}$)		
	OXA-48	OXA-232	OXA-244	OXA-48	OXA-232	OXA-244	OXA-48	OXA-232	OXA-244
Penicillin G	ND	60	450 \pm 40	ND	125	72 \pm 8	ND	2100	163 \pm 30
Ampicillin	400	220	657 \pm 160	955	132	373 \pm 158	2400	600	549 \pm 130
Temocillin	45	60	364 \pm 20	0.3	0.03	0.11 \pm 0.02	6	0.5	0.31 \pm 0.05
Cefalotin	195	125	88 \pm 30	44	13	3.3 \pm 0.8	225	105	39 \pm 4
Ceftazidime	NH	>1000	NH	NH	>0.6	NH	NH	0.1	NH
Cefotaxime	>900	>1000	>900	>9	>6.5	ND	10	6	ND
Ertapenem	100	110	22.9 \pm 0.3	0.13	0.04	0.02 \pm 0.01	1	0.4	0.1 \pm 0.03
Imipenem	13	9	0.5 \pm 0.2	5	0.2	0.02 \pm 0.001	370	20	38 \pm 10
Meropenem	10	100	23 \pm 4	0.07	0.03	0.02 \pm 0.001	6	0.3	0.8 \pm 0.2

ND, not determined; NH, no detectable hydrolysis was observed with 1.92 μ M purified enzyme and up to 500 μ M substrate. Data are the means of three independent experiments. Data for OXA-48 and OXA-232 were from Docquier et al.⁴ and Oueslati et al.,^{6,17} respectively.

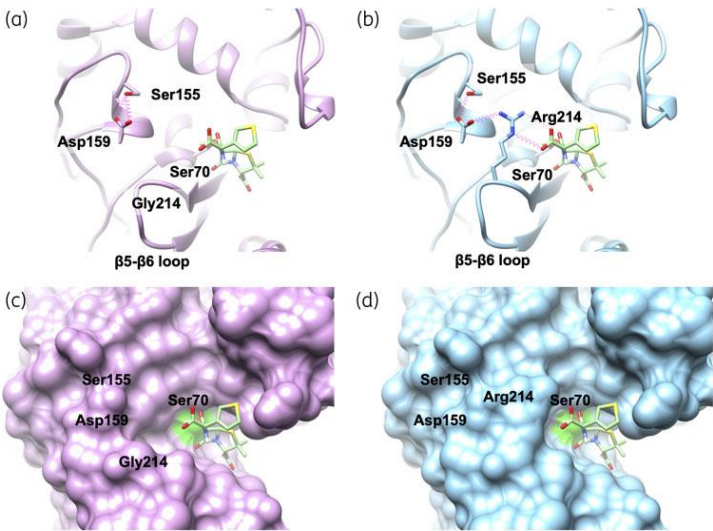


Figure 1. *In silico* model of OXA-244 (a and c) and crystal structure of OXA-48 (b and d; PDB code 6P97) in cartoon (a and b) and surface (c and d) representations, with the docking conformation of temocillin in stick representation. OXA-244, OXA-48 and temocillin are coloured in pink, cyan and green, respectively, and the surface of the active site Ser70 is coloured in green. Hydrogen bonds are shown as springs coloured in magenta.

These reduced catalytic efficiencies for carbapenems in OXA-244 were mainly due to a reduction in the turnover number. Interestingly, as observed for OXA-232, the k_{cat}/K_m of ertapenem for OXA-244 was 370-fold lower than that of imipenem, but not affected as compared with the value for OXA-48 (1 versus 0.94 $mM^{-1}.s^{-1}$). Differences in catalytic efficiencies were due to changes in both K_m and k_{cat} . The steady-state kinetics of OXA-244, which differs by R214G from OXA-48, revealed that the OXA-244 active site can accommodate carbapenem and temocillin substrates, but with

decreased catalytic efficiencies (with a decrease of 7-fold and 10-fold for meropenem and imipenem, respectively, and a decrease of 20-fold for temocillin) as compared with OXA-48. Overall, OXA-232 and OXA-244, even though differing by 5 AAs (including position 214), have similar catalytic efficiency values (Table 1). The replacement of S214 by a glycine in OXA-232, as in OXA-244, showed a similar impact on MICs and on kinetics as in an OXA-181 backbone.^{6,7} Determination of IC_{50} s showed that OXA-244, OXA-232 and OXA-48 are similarly inhibited by clavulanic acid (54, 13.4 and

28.5 μ M, respectively), tazobactam (1.2, 0.75 and 20 μ M, respectively) and NaCl (3.4×10^3 , 97×10^3 and 35×10^3 μ M, respectively). The IC₅₀s of OXA-244 suggest that R214 is not involved in inhibition by tazobactam, clavulanic acid or NaCl.¹⁷

Molecular modelling

An *in silico* study was performed to understand the structural basis for the modification of the hydrolytic profile of OXA-244. The three-dimensional structure of OXA-244 was generated *in silico* by single point mutation starting from the crystal structure of OXA-48 (PDB code 6P97).²⁰

A salt bridge is present in OXA-48 between D159 and R214, that is lost in OXA-244, which has a G in position 214. This mutation induces a significant change in the shape of the active site at its periphery (Figure 1). Moreover, we have shown previously that R214 in OXA-48 also establishes a favourable ionic interaction with the carboxylate group of the R1 substituent of temocillin and that this interaction is lost in OXA-232, which has a serine residue in position 214.¹⁷ Here we show, through molecular docking calculations, that this interaction is also absent in the complex of temocillin with OXA-244, which has a glycine in position 214 (Figure 1). These structural data are in agreement with the lower affinity (higher K_m) of OXA-244 for temocillin as compared with OXA-48. On the other hand, Docquier *et al.*⁴ proposed that the salt bridge between D159 and R214 in OXA-48 is involved in maintaining the conformation of the active site, which leads to the stabilization of the substrates in a conformation compatible with efficient hydrolysis. In OXA-244, the absence of this stabilizing interaction would lead to significant changes in the active site's water network, thus explaining the decrease in k_{cat} values of carbapenems as compared with OXA-48.

Conclusions

In this study, we have characterized the biochemical and structural properties of OXA-244, which can explain its phenotype.¹⁶ The chromosomal location of the *bla*_{OXA-244} gene that results in lower levels of enzyme production as compared with plasmid locations (1 copy versus 10–20 copies), together with its reduced hydrolytic activities, contribute to the difficulties in detecting OXA-244-producing *E. coli* isolates using classical screening media,^{7,9,10} suggesting an even larger underestimation of the real spread of OXA-244 producers in Europe.

Acknowledgements

We are grateful to S. Bernabeu for helpful discussions.

Funding

This work was supported by the Assistance Publique-Hôpitaux de Paris, the University Paris-Saclay, the Institut National de la Santé et de la Recherche Médicale (INSERM), the Laboratory of Excellence in Research on Medication and Innovative Therapeutics (LERMIT) through a grant from the French National Research Agency (ANR-10-LABX-33) and the JPIAMR transnational project DesInMBL (ANR-14-JAMR-0002).

Transparency declarations

None to declare.

Supplementary data

Table S1 is available as [Supplementary data](#) at JAC Online.

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Supplementary data

Table S4. Antimicrobial susceptibility results of *E. coli* TOP10 transformants, and *E. coli* TOP10

	MIC (mg/L)			
	<i>E. coli</i> TOP10 (pTOPO- OXA-48)	<i>E. coli</i> TOP10 (pTOPO- OXA-232)	<i>E. coli</i> TOP10 (pTOPO- OXA-244)	<i>E. coli</i> TOP10
β -Lactam				
Amoxicillin	2048 ^a	2048	>2048 ^a	2
Temocillin	512 ^a	32	16	4
Piperacillin	1024 ^a	32	1024 ^a	1.5
Cefotaxime	0.064	0.047	0.047	0.032
Ceftazidime	0.19	0.125	0.19	0.12
Cefepime	0.19	0.047	0.064	0.023
Meropenem	0.25	0.023	0.032	0.016
Ertapenem	0.25	0.012	0.064	0.003
Imipenem	0.75	0.38	0.25	0.25

^aValues obtained with broth microdilution method.

Role of Amino-Acid 159 in carbapenem-hydrolysis of OXA-933, a novel OXA-48 variant

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Keywords: Resistance carbapenems, Carbapenemase, OXA-48-like, Enterobacterales carbapenemase-producer

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Abstract

OXA-48 has rapidly disseminated worldwide and became one of the most common carbapenemase in many countries with more than 45 variants reported with in some cases significant differences in their hydrolysis profiles. The R214 residue, located in the β 5- β 6 loop, is crucial for the carbapenemase activity, as it stabilizes carbapenems in the active site and maintains the shape of the active site through interactions with D159. In this study, we have characterized a novel variant of OXA-48, OXA-933 with a single D159N change. To evaluate the importance of this residue, point mutations were generated (D159A, D159G, D159K, D159W), kinetic parameters of OXA-933, OXA-48 D159G, and OXA-48 D159K were determined and compared to those of OXA-48 and OXA-244. The *bla*_{OXA-933} gene was borne on Tn2208, a 2696-bp composite transposon made of two IS1 elements surrounded by 9-bp target site duplications and inserted into a non self-transmissible plasmid pOXA-933 of 7872-bp in size. MICs values of *E. coli* expressing *bla*_{OXA-933} gene or of its point mutant derivatives were lower for carbapenems (except for D159G) as compared to those expressing *bla*_{OXA-48} gene. Steady state kinetic parameters revealed lower catalytic efficiencies for expanded spectrum cephalosporins and carbapenems. A detailed structural analysis confirmed the crucial role of D159 in shaping the active site of OXA-48 enzymes by interacting with R214. Our work further illustrates the remarkable propensity of OXA-48-like carbapenemases to evolve through mutations at positions outside the β 5- β 6 loop, but interacting with key residues of it.

Introduction

Carbapenemase producing Enterobacterales constitute a serious health issue limiting the therapeutic options for gram-negative bacterial infections and increasing the morbidity and mortality rates among patients (1). Carbapenemases belong to three molecular classes of β -lactamases (1,2). Class A and D correspond to serine-active β -lactamases and class B enzymes are metallo- β -lactamases that require two Zn^{2+} ions to coordinate a water molecule in the active site for hydrolysis (1,3). Among Carbapenem-Hydrolyzing class D β -Lactamase (CHDL), OXA-48, initially described in 2004, in a carbapenem-resistant *Klebsiella pneumoniae* isolate from Turkey in 2001, has now spread globally in Enterobacterales, to become the most common carbapenemase in many countries (4-6). OXA-48 hydrolyzes penicillins at a high level, carbapenems at a low level and lacks significant hydrolytic activity towards expanded-spectrum cephalosporin (ESC) (6-7). More than 45 variants have since been reported, differing by a few

amino acid substitutions or deletions (Beta-Lactamase DataBase <http://bldb.eu/BLDB.php?class=D#OXA>; (8). 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 of described variants, have significant changes in the hydrolytic spectrum of β -lactams (7,8). 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 (7,9), 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 (10,11). Finally, other variants such as OXA-438, OXA-517 and 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 (12-14). The overall 3D structure of OXA-48 is very similar to that of other class D beta-lactamases (DBLs) (15,16), with however one main difference, e.g. an overall shorter the $\beta 5$ and $\beta 6$ strands (15,16). Consequently, the $\beta 5$ - $\beta 6$ loop has a different orientation from that found in other DBLs. The observed conformation of the loop in OXA-48 is promoted by a salt bridge of R214 with the Ω -loop D159 residue, which itself is stabilized by S155 (15-18). In 16 out the 22 OXA-48 variants with changes in the $\beta 5$ - $\beta 6$ loop, the R214 is changed to S (n=3), G (n=3), N (n=1), D (n=3), K (n=1), E (n=2), and G (n=3) (8). In 5 out of the 6 remaining, R214 is deleted and only in one case, in OXA-438, the R214 is still present, but two amino acids 215-216 have been deleted (12). As shown by Oueslati *et al.* changes at position R214 have a drastic impact on the hydrolysis of carbapenems as a consequence of the loss of the salt bridge with D159 (18). The $\beta 5$ - $\beta 6$ loop extends into the outer portion of the active site crevice and defines a rather hydrophilic cavity filled with several water molecules, one of which will perform the nucleophilic attack on the acyl-enzyme intermediate in the deacylation step (15,17).

In this study, we have characterized a novel natural OXA-48 variant, OXA-933 with a D159N change. To evaluate the impact of this AA change and to further characterize the role of this position and its interaction with R214, susceptibility testing, steady state kinetic parameters together with a detailed structural analysis were performed for OXA-933 (OXA-48-D159N), and several OXA-48 point mutant derivatives (D159A, D159G, D159K, D159W). In addition, the genetic support of this novel OXA-48 variant was determined.

Results

K. pneumoniae 241B9 clinical isolate, antimicrobial susceptibility testing and carbapenemase confirmation tests

The clinical *K. pneumoniae* 241B9 strain was recovered from an urinary tract infection of an hospitalized woman in 2019. Disk diffusion antibiogram revealed that *K. pneumoniae* 241B9 was resistant as expected to amoxicillin, ticarcillin, and piperacillin, but also to amoxicillin/clavulanic acid, ticarcillin/clavulanic acid and piperacillin/tazobactam, likely suggesting the presence of an OXA-type acquired β -lactamase. In addition, the isolate was also resistant to temocillin, but with a zone diameter of 13 mm, which could be compatible with an OXA-48 type variant. *K. pneumoniae* 241B9 remained susceptible to all other β -lactams tested, including carbapenems. The zone diameter of ertapenem was slightly reduced (24 mm) and that of meropenem (28 mm) was at the EUCAST recommended screening cut-off of $>0.125 \mu\text{g/ml}$ (zone diameter $>28 \text{ mm}$). MIC testing using E-tests revealed an MIC of $0.5 \mu\text{g/ml}$ and $0.032 \mu\text{g/ml}$ for ertapenem and meropenem, respectively. The presence of a carbapenem-hydrolyzing enzyme was further searched using the Carba NP, a home-made biochemical test based on imipenem hydrolysis (19), and the immunochromatographic NG-Test Carba5 (NG Biotech, Guipry, France;(20)). While Carba NP test was inconsistently positive, the NG-Test Carba5 was repeatedly positive for OXA-48-like enzyme.

Disc diffusion antibiogram revealed further that *K. pneumoniae* 241B9 was susceptible to all the other tested antibiotic families e.g. aminoglycosides, fluoroquinolones, chloramphenicol, tigecyclines, and colistin (confirmed by MIC testing [$\text{MIC}=0.5 \mu\text{g/ml}$]), except to rifampin.

As *K. pneumoniae* 241B9 has a reduced susceptibility toward carbapenems and also temocillin, we tested its ability to grow on standard chromogenic and selective media, such as ChromID® CARBA SMART. Using an inoculum of 10^3 CFU , *K. pneumoniae* 241B9 strain grew on both sides (Carba and OXA-48) of the ChromID® CARBA SMART plate (BioMérieux).

Genomic features of K. pneumoniae 241B9

WGS sequencing of *K. pneumoniae* 241B9 revealed a genome of 5,339,000-bp with a mean coverage of 90.7 X. The Multi locus sequence typing (MLST) of *K. pneumoniae* 241B9 according to MLST 1.8 software (21), revealed ST-1079 (2-5-1-1-9-1-19), an ST rarely described and with only 10 occurrences in *Klebsiella* Pasteur MLST database over the last 15 years from Argentina, United Kingdom, France and Australia.

Acquired resistance genes and point mutations involved in resistance were analyzed using ResFinder (22). The naturally occurring *bla*_{SHV-1} gene, and a variant of *bla*_{OXA-48} carbapenemase gene with a G₄₇₅ to A mutation that results in D159N AA change, named *bla*_{OXA-933} gene, were identified in this isolate. In addition, *oqx*A and *oqx*B genes were also present in the isolate, likely at the origin of reduced susceptibility to fluoroquinolones (MIC of 0.5 µg/ml to ciprofloxacin) as no chromosomal mutation was present in the QRDR region. *fos*A gene conferring resistance to fosfomycin was also present in the genome (23). In addition, point mutations were found in *ompK36* and *ompK37*, but none has been described to be involved in reduced susceptibility to third generation cephalosporins. According to PlasmidFinder (24), no plasmid replication origin could be evidenced.

Genetic support and environment of bla_{OXA-933} gene

The *bla*_{OXA-933} gene was located on Tn2208, a 2696-bp composite transposon made of two IS1 elements inserted with a 9-bp target site duplication into a non-self-transmissible plasmid resulting in plasmid pOXA-933 of 7872-bp in size (Figure 1).

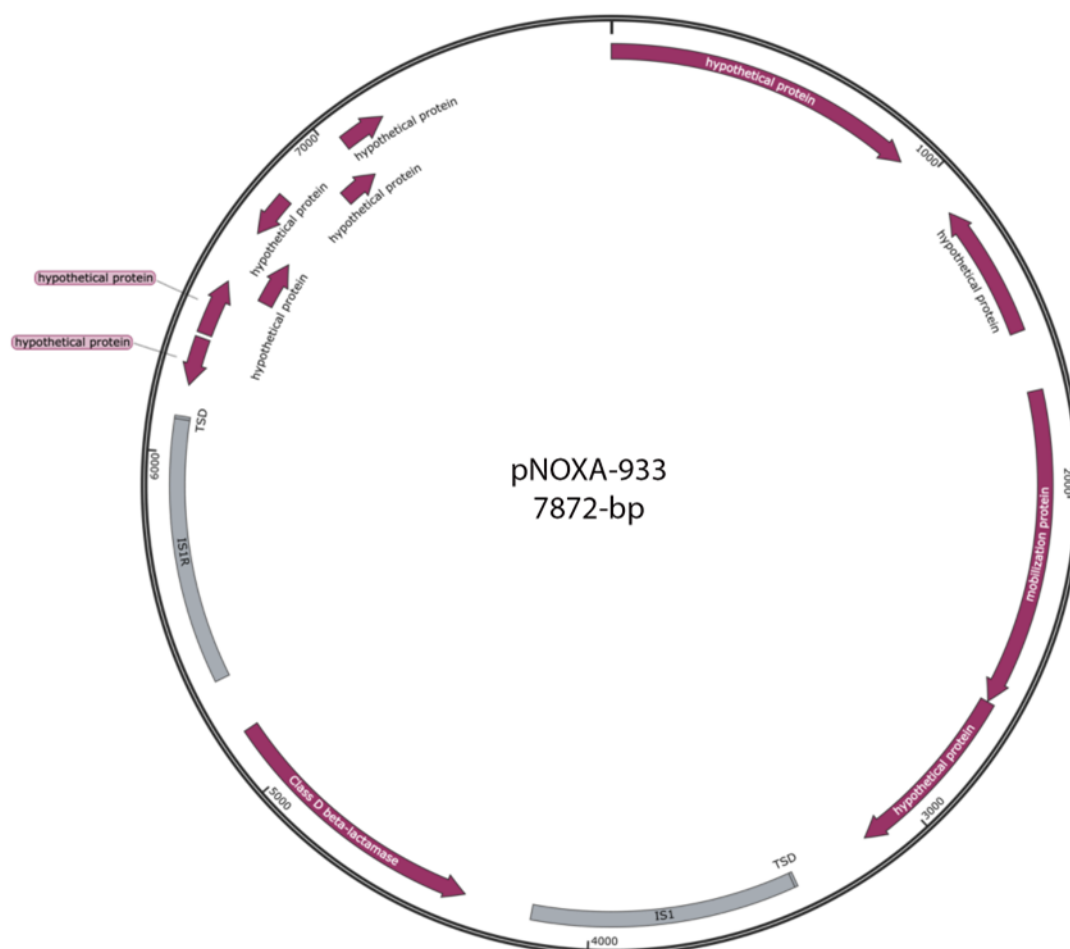


Figure 1: Map of the natural plasmid pOXA-933 carrying *bla*_{OXA-933} gene located on Tn2208.

This plasmid differed only by 5 nucleotides (including the G₄₇₅ to A mutation that resulted in D159N AA change in OXA-933) from plasmid pMTY17816 expressing OXA-48 in an MDR *K. pneumoniae* clinical isolate TUM17816 from Vietnam (AP019554) or from plasmid pIPCEC31_3 expressing OXA-48 in an *E. coli* IPCEC31 isolate from Japan (AP026786), 19 nucleotides from plasmid p53_E-OXA48 expressing OXA-48 in an *E. coli* 53 isolate from Switzerland (CP048364.1), and 24 nucleotides from plasmid pMTY17823_OXA48 isolated in *K. pneumoniae* TUM17823 from Japan (AP019557). Electroporation of this plasmid into *E. coli* TOP10 yielded transformants resistant to β -lactams and with reduced susceptibility to carbapenems (Table 1).

Table 1: CarbaNP assay, NG test Carba5 results and MICs of β -lactams for *K. pneumoniae* 241B9 clinical strain, *E. coli* HB4 with the natural plasmid pOXA-933, with pTOPO-OXA-933, *E. coli* HB4 alone and *E. coli* TOP10 expressing OXA-48, OXA-933, OXA-48-D159K, OXA-48-D159G, OXA-48-D159W and OXA-48-D159A. ^a; OXA-244; ^b; OXA-933.

	MIC (mg/L)											
	<i>K.pneumoniae</i> 241B9		<i>E. coli</i> HB4		<i>E. coli</i> TOP10-pTOPO-						<i>E. coli</i> TOP10	
	OXA-933	pOXA-933	pTOPO-OXA-933	<i>E. coli</i> HB4	OXA-48	OXA-48 R214G ^a	OXA-48 D159N ^b	OXA-48 D159K	OXA-48 D159G	OXA-48 D159W	OXA-48 D159A	
Amoxicillin	>256	>256	>256	24	>256	>256	12	12	24	4	12	2
Piperacillin	128	>256	6	4		>256						
Ticarcillin/clavulanic acid	>256	>256	>256	12								
Temocillin	128				>256	16	12	12	12	12	4	4
Ceftazidime	<0.25	2	0.5	0.5	0.19	0.19	0.125	0.19	0.19	0.19	0.19	0.12
Cefotaxime	<0.25				0.06	0.047	0.06	0.06	0.06	0.06	0.06	0.06
Cefepime	0.094	8	0.5	0.5		0.064						
Imipenem	0.25	>32	0.16	0.125	0.75	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Meropenem	0.016	32	0.25	0.25	0.25	0.016	0.032	0.016	0.023	0.016	0.016	0.016
Ertapenem	0.5	>256	32	1	0.25	0.064	0.003	0.003	0.003	0.003	0.003	0.003
CarbaNP test	+/-				+	+/-	-	-	+	-	-	-
NG testCarba 5	OXA				OXA	OXA	OXA	OXA	OXA	-	OXA	-

Phenotypical characterization of OXA-933 β -lactamase and comparison with in vitro generated point mutant derivatives

MICs, Carba NP test and immunochromatographic assays were performed on *E. coli* TOP10 harboring pOXA-48, pOXA-933 (D159N), and pOXA-48-D159-A,-G,-K, and -W (Table 1). All the OXA-48 variants exhibited lower MICs values for penicillins and carbapenems as compared to OXA-48. In contrast, no change of MIC values for ceftazidime and cefotaxime was observed between OXA-48, OXA-933 and the four mutants. CarbaNP test revealed an inconsistent and weak positive result with the clinical strain but was negative for all the mutants except for D159G suggesting that OXA933 and OXA-48-D159G exhibited a weak imipenem hydrolysis. Finally, NG-test Carba5, an immunochromatographic assay used for detection of the most prevalent carbapenemases such as OXA-48, was positive for the clinical *K. pneumoniae* 249B1 strain, and for *E. coli* harboring pOXA-48, pOXA-933 (D159N), pOXA-48D159-A,-G, and -K, but not for pOXA-48-D159W. Together, the lack of carbapenem-hydrolyzing activity and the absence of detection with specific antibodies suggest that the aspartate replacement with the bulky and hydrophobic tryptophan induced a significant modification of the 3D structure of the enzyme.

MICs assays were also performed in an OmpC/OmpF porin-deficient *E. coli* HB4 strain harboring the natural pOXA-933, and pTOPO-OXA-933 (Table 1). With the natural plasmid pOXA-933, the *E. coli* HB4 was resistant to all β -lactams tested, while *E. coli* HB4 pTOPO-OXA-933, that contains only the open reading frame of OXA-933, only reduced susceptibilities are observed, as transcription and translation are in suboptimal configuration.

Biochemical properties of OXA-933

Steady-state kinetic parameters were determined to compare the catalytic properties of OXA-933 and two in vitro generated mutants (OXA-48-D159G and OXA-48-D159K) with those of OXA-48 and OXA-244 (Table 2).

Table 2: Steady-state kinetic parameters for hydrolysis of β -lactam substrates by OXA-933, OXA-D159G, OXA-D159K, OXA-244 and OXA-48.

	K_m (μM)					k_{cat} (s^{-1})					k_{cat}/K_m ($mM^{-1}.s^{-1}$)				
Enzyme	OXA-48 ^a	OXA-48 R214G ^b	OXA-48 D159N ^c	OXA-48 D159G	OXA-48 D159K	OXA-48 R214G ^a	OXA-48 D159N ^b	OXA-48 D159G	OXA-48 D159K	OXA-48	OXA-48 R214G ^a	OXA-48 D159N ^b	OXA-48 D159G	OXA-48 D159K	OXA-48
<i>Benzylpenicillin</i>	NA	450 ± 40	88 ± 9	64 ± 48	48 ± 5	NA	72 ± 8	126 ± 6	6.2 ± 4	69 ± 17	NA	163 \pm 30	1450 \pm 199	133 \pm 14	1445 \pm 256
<i>Ampicillin</i>	400	657 ± 160	392 ± 103	ND	ND	955	373 ± 158	152 ± 32	ND	ND	2400	549 ± 130	413 ± 147	ND	ND
<i>Temocillin</i>	45	364 ± 20	125 ± 9	86 ± 90	35 ± 6	0.3	0.11 ± 0.02	1.4 ± 0.03	0.74 ± 0.9	0.6 ± 0.03	6	0.31 ± 0.05	11 ± 0.8	7 \pm 2	19 ± 3.6
<i>Cephalothin</i>	195	88 ± 30	788 ± 33	85 ± 19	152 ± 75	44	3.3 ± 0.8	7.4 ± 1.6	2.4 ± 0.4	3.8 ± 1.3	225	39 ± 4	10 ± 2.4	29 ± 7	29 ± 18
<i>Cefotaxime</i>	>900	>1000	321 ± 72	248 ± 155	104 ± 24	>900	ND	11 ± 8	1.2 ± 0.34	0.9 ± 0.2	10	ND	32 ± 21	6 ± 3	9 ± 1.4
<i>Imipenem</i>	13	0.5 ± 0.2	22 ± 7	48 ± 9	5 ± 0.4	5	0.02 ± 0.001	1.8 ± 1.6	2.5 ± 0.4	0.6 ± 0.12	370	38 ± 10	125 ± 35	52 ± 1.5	120 ± 16
<i>Meropenem</i>	10	23 ± 4	160 ± 46	193 ± 104	103 ± 10	0.07	0.02 ± 0.001	0.05 ± 0.002	0.08 ± 0.03	0.04 ± 0.002	6	0.8 ± 0.2	0.3 ± 0.09	0.4 ± 0.1	0.4 ± 0.02
<i>Ertapenem</i>	100	22.9 ± 0.3	200 ± 67	154 ± 110	150 ± 28	0.13	0.02 ± 0.01	0.05 ± 0.007	0.074 ± 0.02	0.11 ± 0.06	1	0.1 ± 0.03	0.26 ± 0.06	0.6 ± 0.2	0.75 ± 0.4

^a OXA-48 values were retrieved from Docquier et al. (15); ^b data for OXA-244 were from Rima et al. (9); ^c correspond to OXA-933; ^d: NA, not available; ^e: ND, not determined. Data are the mean of three independent experiments.

The catalytic efficiency (k_{cat}/K_m) of OXA-933 with regard to ampicillin hydrolysis was 5.8-fold lower ($0.413 \text{ mM}^{-1} \text{ s}^{-1}$) than that of OXA-48 ($2.400 \text{ mM}^{-1} \text{ s}^{-1}$) (Table 2), comparable to OXA-244 ($0.549 \text{ mM}^{-1} \text{ s}^{-1}$). In addition, hydrolysis of temocillin by OXA-933 revealed a K_m and a k_{cat} 3-fold higher and 4-fold higher, respectively, thus resulting in a 2-fold higher catalytic efficiency as compared with OXA-48. This result is very different from that of OXA-244, where a 20-fold decrease was observed (9). Cefalotin is hydrolyzed with a 20-fold lower k_{cat}/K_m as compared with the value for OXA-48. Oxyimino cephalosporins, such as cefotaxime, are weakly hydrolyzed, and the k_{cat}/K_m was comparable with that of OXA-48. For ceftazidime, a bulkier oxyimino cephalosporin, no hydrolysis was observed even with $1.92 \text{ }\mu\text{M}$ purified enzyme and up to $500 \text{ }\mu\text{M}$ substrate (data not shown), as shown previously for OXA-48 (4,7).

Carbapenems were among the preferred OXA-48 substrates (7), with the highest catalytic efficiency (k_{cat}/K_m), observed for the hydrolysis of imipenem ($0.37 \cdot 10^3 \text{ mM}^{-1} \text{ s}^{-1}$) (7). OXA-933 has k_{cat}/K_m values 3-fold, 20-fold and 4-fold lower for imipenem, meropenem and ertapenem hydrolysis, respectively, as compared with OXA-48. These values are similar to those observed for OXA-244, with k_{cat}/K_m values 6-fold, 14-fold, and 10-fold lower for imipenem, meropenem and ertapenem hydrolysis, respectively, as compared with OXA-48.

These reduced catalytic efficiencies for carbapenems in OXA-933 were mainly due to a reduction in the turnover number. Interestingly, as observed for OXA-232, the k_{cat}/K_m of ertapenem for OXA-933 was 480-fold lower than that of imipenem, but only slightly affected as compared with the value for OXA-48 (1 versus $0.26 \text{ mM}^{-1} \text{ s}^{-1}$). Overall, the catalytic efficiencies of OXA-48-D159G and OXA-48-D159K are similar to those of OXA-933.

Determination of IC_{50} s showed that OXA-933 and OXA-48 are similarly inhibited by clavulanic acid (4.55 and $28.5 \text{ }\mu\text{M}$, respectively), and tazobactam (4.23 and $20 \text{ }\mu\text{M}$, respectively). The IC_{50} s of OXA-933 suggest that D159N is not involved in inhibition by neither tazobactam, nor clavulanic acid.

Molecular modelling. An *in-silico* study was performed to identify the structural determinants that could explain the experimentally determined differences between the hydrolytic profiles of OXA-933 and its variants in comparison with OXA-48. The three-dimensional structures of OXA-48, OXA-244 (i.e. OXA-48-R214G), OXA-933 (i.e. OXA-48-D159N), OXA-48-D159G and OXA-48-D159K were modelled, showing different conformations of key residues of the active site, and compared with the docking conformation of temocillin in the active site of OXA-48 (Figures 2 and S1). In all complexes, temocillin establishes hydrogen bonds with the backbone of Tyr211 and with the side chain of Thr209, and a strong ionic bridge with Arg250. In the OXA-48 structure, Arg214 interacts with temocillin through a strong ionic interaction and with Asp159 through a hydrogen bond. A hydrogen bond is also observed between Asp159 and Ser155 (Figure 2a). The mutation R214G in OXA-244 compared with OXA-48 induces a destabilization of the interaction with temocillin, which shows a 8-fold lower affinity (Figure 2b). In OXA-933, the OD1 atom of Asn159 is already engaged in the hydrogen bond with Ser155 and no hydrogen bond is possible between the ND2 atom of Asn159 and the side chain of Arg214. Therefore, Arg214 would change the conformation to make simultaneously a hydrogen bond with Thr213 and an ionic bridge with temocillin (Figure 2c). These interactions restore almost completely the affinity of the protein for temocillin. In the absence of possible interactions between Arg214 and the residue in position 159, the same conformational change is also observed for the OXA-48-D159G and OXA-48-D159K mutants (Figure 2d,e), with similar effects on the affinity for temocillin. All these results are in perfect agreement with the experimental data (Table 2).

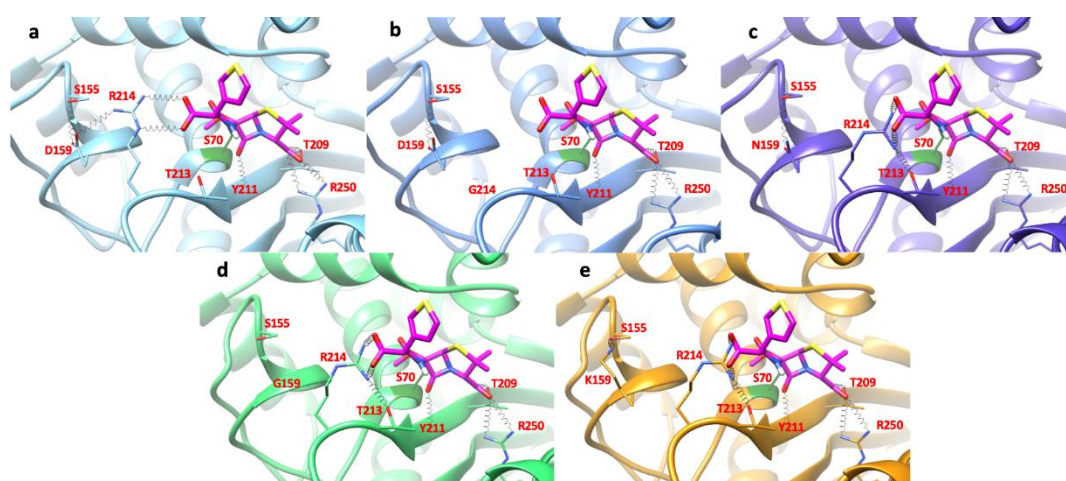


Figure 2: Docking conformation of temocillin (non-covalent binding) on OXA-48 (a), OXA-244 (b), OXA-933 (c), OXA-48-D159G (d) and OXA-48-D159K (e). The residue Ser70 is colored in green. The black strings represent hydrogen bonds

Discussion

Since the first description of an Enterobacterales producing OXA-48 (4) in 2004, a rapid dissemination has been observed, and OXA-48 producers became endemic in many countries and have been described on all continents (6). OXA-48 variants can be classified into 4 groups according to their hydrolysis profile: (i) those having similar hydrolytic activities as OXA-48 such as OXA-181, OXA-162, OXA 204 (7); (ii) those having lost their carbapenemase activity but gained an expanded-spectrum cephalosporin hydrolytic activity such as OXA-163 and OXA-405, (7,10,11); (iii) those capable of hydrolyzing carbapenems and expanded-spectrum cephalosporins equally well such as OXA-438, OXA-517 and OXA-793 (12-14), and finally (iv) those displaying a reduced activity especially for carbapenems and temocillin as compared to OXA-48 such OXA-232, OXA-244, OXA-484 (7,9). In the latter case, it has been shown that these differences in hydrolytic profile depend on the residue R214 of the β 5- β 6 loop, and has an impact on carbapenems and temocillin hydrolysis (9,15,16,18). In addition, crystallographic studies of OXA-48 and OXA-517 suggested that R214 stabilizes the shape of the active site and can interact with D159 through a salt bridge (13,15).

In 2019, the F-NRC received a *K pneumoniae* isolate 241B9 expressing a new variant of OXA-48 where a negatively charged aspartic acid is replaced by an uncharged asparagine (D159N). This clinical isolate displayed a decreased resistance towards carbapenems and temocillin, as compared to OXA-48, best evidenced with a Carba NP test used for CPE detection that was inconsistently positive, suggesting that the imipenem-hydrolysis was at the limit of detection of the assay. These observations were in favor of the hypothesis that the D159 plays also a major role in the hydrolytic profile of OXA-48 by establishing a salt bridge with R214 to stabilize the active site for carbapenem-hydrolysis (13). To validate this hypothesis, we generated mono-mutants of OXA-48 (D159A, D159G, D159K and D159W) and analyzed MICs for β -lactams and kinetic parameters for OXA-933, and for OXA-48-D159G and D159K. These mutations were chosen regarding their intrinsic properties: alanine and glycine as hydrophobic aliphatic small AA, tryptophan a bulky hydrophobic aromatic AA and lysine a polar positively charged AA. Thus, in comparison with OXA-48 we observed that substitution of the D159 led to an increase of susceptibility toward all the β -lactams. The D159W substitution has almost completely inactivated the β -lactamase (data not shown).

CarbaNP test revealing carbapenemase activity was evaluated on several *E. coli* TOP10 isolates

expressing OXA-48, OXA-244, OXA-933 and point mutant derivatives of position D159 from pTOPO plasmids. In these constructs, the OXA-48-like gene is cloned without native promoter and without Shine-Dalgarno sequence, thus allowing for reduced expression, even though from a high copy number plasmid, to see differences between the variants. Except for pTOPO-OXA-48 and TOP10-pTOPO OXA-48D159G, the Carba NP test remained repeatedly negative suggesting very low carbapenem-hydrolytic activity that is below the detection limit of the assay. All these OXA-48-variants were sufficiently expressed, as the lateral flow immunoassay NG-Test Carba5 yielded positive signals in less than 2 minutes for all OXA-48 clones, except for TOP10-pTOPO D159W, suggesting that replacement with this bulky AA has substantially modified the structure of the protein, at least the part that is recognized by one of the antibodies included in the test.

The clinical *K. pneumoniae* 24B1 strain, *E. coli* Top10 pOXA-933 and *E. coli* Top10 pTOPO OXA-933 had different MIC values, likely due to species specific expression and impermeability problems. MIC values showed same reduction for all the substrates tested as compared to OXA-48. These reductions were comparable to OXA-244 (Table 1). Similarly, the MICs of the various mutants were similar to OXA-933, confirming a loss of ability to hydrolyze carbapenems (Table 1). Even though temocillin MICs were much lower for OXA-933 and the generated mutants as compared to OXA-48, this result could not be confirmed through steady state kinetics determination. Similar discrepancies between MIC for temocillin and its hydrolysis have already been observed for other OXA-48-like variants (7,9,13).

Differences in catalytic efficiencies were due to changes in both K_m and k_{cat} . The steady-state kinetics of OXA-933, which differs by D159N from OXA-48, revealed that the OXA-933 active site can still accommodate carbapenem and temocillin substrates, but with decreased catalytic efficiencies as compared with OXA-48. Kinetic parameters of the OXA-48-D159G and D159K variants show overall decreased catalytic efficiencies (k_{cat}/K_m) for ampicillin, cephalotin, imipenem and meropenem, similar to OXA-933. The most significant difference was observed for meropenem, with a decrease of 15 to 20-fold. This result is a consequence of at least a 10-fold decrease of affinity. Regarding imipenem, a weak decrease of catalytic efficiency was noticed mostly due to a diminution of the turnover (k_{cat}). Thus, these results tend to show the importance of residue D159 for the hydrolysis of carbapenems. We have shown previously (9,18), that R214 could interact also with the substrate and D159. This interaction

could stabilize R214 in the right position and thus stabilize the small substrates as carbapenems in the active site. S155 would also be a residue of a particular interest since it directly interacts with D159 and could be the target for further studies. Overall, OXA-933 and OXA-244, even though differing by the AA changes (R214G for OXA-244), have similar catalytic efficiency values (Table 2), further supporting the importance of the salt bridge established between these two residues.

The structural analysis of the active sites of these mutants (Figure S1) and their interaction with temocillin (Figure 2) show important changes in the conformation and in the size of the active site induced by these mutations. In OXA-244, the active site is enlarged as a consequence of the mutation R214G and the flexibility of this region is likely to be increased in the absence of the salt bridge R214-D159. This mutation also destabilizes the interaction of temocillin, leading to a significant decrease of affinity. In OXA-933, although the mutation D159N looks minor at the first sight, it also breaks the salt bridge R214-D159, allowing the R214 to change the conformation and to interact with T213 through a hydrogen bond and with temocillin through an ionic bridge. The conformational change and interactions are also observed in the OXA-48-D159G and OXA-48-D159K mutants, leading to an important remodeling of the active site surface in this region and to the reinforcement of the interaction with temocillin. Overall, these changes in the conformation of the active site and in the interaction with temocillin are in agreement with the kinetic data determined experimentally (Table 3).

Most OXA-48-like variants are associated with different *Tn1999* variants inserted on IncL plasmids, but some such as OXA-181 and OXA-232 are associated with *ISEcp1*, *Tn2013* on ColE2, and IncX3 types of plasmids (6,25). The pOXA-933 natural plasmid of 7872-bp in size was closely related (5-24 nucleotide differences) to plasmids carrying OXA-48 from an *K. pneumoniae* clinical isolate TUM17816 from Vietnam (AP019554), an *E. coli* IPCEC31 isolate from Japan (AP026786), an *E. coli* 53 isolate from Switzerland (CP048364), and *K. pneumoniae* TUM17823 from Japan (AP019557), suggesting that this type of plasmid may have already spread on several continents and in different species. *IS1*-based composite transposons have the tendency to transpose at high frequency in Enterobacterales, and could thus promote even further the spread of OXA-48 type enzymes, even beyond Enterobacterales, in a similar manner to *Tn9* or *Tn1681*, two highly dispersed composite transposons carrying the *cat* gene, conferring chloramphenicol resistance and a heat stable toxin, respectively (26,27).

Conclusion

This study characterized a new variant of OXA-48, OXA-933 with a D159N amino acid change, an AA involved in salt bridges with R214 and S155 that stabilize the active site. Our work further supports the role of these salt bridges, and especially the role of residue D159 in the hydrolytic profile of OXA-48. The precise role of S155 another residue supposed to be involved in active site stabilization by interacting with D159 needs also to be explored. OXA-933 is another example of single AA derivative of OXA-48, where the AA change is located outside the β 5- β 6 loop, with yet significant changes in enzymatic activity (28). According to Dabos et al. OXA-933, even though with reduced activity towards carbapenems, has still enough carbapenemase activity to be considered as a true carbapenemase, especially when expressed in bacteria with impaired outer membrane permeability (29).

As this new variant has reduced hydrolytic activities, especially for carbapenems, its detection using hydrolysis tests, such as carba NP may be challenging, but the use of LFIA, such as NG-Test CARBA 5 may be an interesting tool to detect these OXA-48-like variants. Their detection from rectal swab samples may still be possible using chromogenic selective media unlike for what has been observed for OXA-244 (9, 30). Even though OXA-933 hydrolyses carbapenems only weakly, its transfer into a porin-deficient genetic background may yield high level of carbapenem-resistance (Table 4). Finally, OXA-933 is present on a novel composite transposon made of two *IS1* elements, Tn2208, inserted in a high copy number plasmid further illustrating the genetic variability associated with OXA-48 carbapenemases.

Materials and methods

Bacterial strains

Klebsiella pneumoniae 241B9 clinical isolate was identified using MALDI-TOF (MALDI Biotyper, Bruker Daltonics, Hamburg, Germany). *Escherichia coli* TOP10 (Invitrogen, Saint-Aubin, France) and *E. coli* BL21 (DE3) (Novagen, VWR International, Fontenay-sous-Bois, France) were used for cloning and over expression experiments, respectively. Azide-resistant *E. coli* J53 was used for conjugation assays (13).

Susceptibility testing and carbapenemase detection

Antimicrobial susceptibilities were determined by disk diffusion technique on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and Minimal inhibitory concentration (MIC) values were determined using the Etest technique (BioMérieux, Paris, France). Susceptibility results were interpreted according to the EUCAST breakpoints, updated in 2022 (<http://www.eucast.org>). Detection of a carbapenemase activity were carried out with the Carba NP test as previously described (19). Lateral flow immunoassays (FLIA) were carried out using NG-Test CARBA 5 (NG Biotech, Guipry, France) according to the manufacturer's instructions (20).

Whole genome sequencing

Total DNA was extracted from colonies using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, US) following the manufacturer's instructions. The DNA concentration and purity were controlled by a Qubit® 2.0 Fluorometer using the dsDNA HS and/or BR assay kit (Life technologies, Carlsbad, CA, US). The DNA library was prepared using the Nextera XT-v3 kit (Illumina, San Diego, CA, US) according to the manufacturer's instructions and then run on Next500 (Illumina) for generating paired-end 150-bp reads. De novo assembly was performed by CLC Genomics Workbench v 9.5.3 (Qiagen) after quality trimming ($Q_s \geq 20$) with word size 34. The acquired antimicrobial resistance genes were identified by uploading the assembled genomes to the Resfinder server v2.1 (<http://cge.cbs.dtu.dk/services/ResFinder-2.1>) (22). The Multi Locus Sequence Typing (MLST) and the identification of the different plasmids were also obtained by uploading the assembled genomes to the MLST 1.8 (21) and PlasmidFinder 1.3 (24) servers, respectively, available at

Plasmid characterization, and conjugation assay

Plasmid DNA of the clinical isolate *K. pneumoniae* 241B9 was extracted using the Kieser method (31). The Kieser extract was used to transform *E. coli* TOP10 strain by electroporation (GenePulser, Biorad, Marne-La-Coquette, France), and the electroporants were plated on a TSA plate containing 100 µg/ml ampicillin. The presence *bla*_{OXA-933} gene was sought by PCR as previously described (7,18). From the transformants harboring the *bla*_{OXA-933} gene, plasmid DNA extraction was done using the Kieser's method and subsequently analyzed on 0.7% agarose gel stained with ethidium bromide. Plasmids of ca. 154, 66, 48, and 7 kb of *E. coli* NCTC 50192 were used as plasmid size markers (13).

Quantitative filter mating-out assay was performed as previously described (13), and transconjugants were selected with 100 µg/ml ampicillin and 100 µg/ml azide.

Site directed mutagenesis

Site-directed mutagenesis was done using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Les Ulis, France) as recommended the manufacturer's protocol, and the pTOPO-*bla*_{OXA-48} plasmid as a template (18). Specific primers used for the different mutations, were designed using the QuikChange Primer Design software (Agilent Technologies): D159A (a: 5'- CGAAATTCGAATACCACCGGCGAGCCAGAACTGTCTACA-3' and b: 5'- TGTAGACAGTTTCTGGCTCGCCGGTGGTATTCGAATTTTCG-3'); D159K (a: 5'- GAAATTCGAATACCACCCTTGAGCCAGAACTGTCTA-3' and b: 5'- TAGACAGTTTCTGGCTCAAGGGTGGTATTCGAATTTTC-3'); D159W (a: 5'- GGCCGAAATTCGAATACCACCCCAGAGCCAGAACTGTCTACATT-3' and b: 5'- AATGTAGACAGTTTCTGGCTCTGGGGTGGTATTCGAATTTTCGGCC-3'); D159G (a: 5'- CGAAATTCGAATACCACCGCCGAGCCAGAACTGTCTACA-3' and b: 5'- TGTAGACAGTTTCTGGCTCGGCGGTGGTATTCGAATTTTCG-3'). Plasmids pOXA-48-D159G, pOXA-48-D159A, pOXA-48-D159K and pOXA-48-D159W were then electroporated into *E. coli* TOP10 and the presence of the mutation was confirmed by Sanger sequencing using an automated ABI Prism 3100 sequencer (Applied Biosystems, Les Ulis, France) as previously described (7).

PCR and cloning experiments

Total DNAs of *K. pneumoniae* 241B9 isolate extracted using the QIAamp DNA mini kit (Qiagen, Courtaboeuf, France) was used as templates for PCR amplification of *bla*_{OXA-933} gene using the Phusion High-Fidelity DNA Polymerase (ThermoFischer Scientific, Villebon-sur-Yvette, France). The amplicon obtained was then cloned into the pCR®-Blunt II-TOPO® plasmid (Invitrogen, Illkirch, France), downstream of the pLac promoter, in the same orientation, resulting in pOXA-933 as previously described (18). The *bla*_{OXA-933}, *bla*_{OXA-48-D159G}, and *bla*_{OXA-48-D159K} gene fragments amplified using primers INF-48 F (5'-AAGGAGATATACATATGGTAGCAAAGGAATGGCAAG-3'), INF-48 R (GGTGGTGGTGCTCGAAGGGAATAATTTTTTCCTGTTTGAG-3') corresponding to the mature β -lactamase were cloned into the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois, France) as previously described (18). The recombinant plasmid pET41-OXA-933, pET41-OXA-48D159G, and pET41-OXA-48D159K were then transformed into the chemically competent *E. coli* strain BL21 (DE3) (Novagen).

Recombinant plasmids were verified by Sanger sequencing using T7 promoter, M13 and T7 terminator specific primers (depending on the plasmid) and the automated ABI Prism 3100 sequencer (Applied Biosystems, Les Ulis, France). Nucleotide sequences were analyzed using software available at the National Center for Biotechnology Information website (<https://www.ncbi.nlm.nih.gov>).

β -Lactamase production and purification

Overnight cultures of *E. coli* BL21(DE3) harboring recombinant plasmid pET41b-OXA-933, pET41b-OXA-48-159G and pET41b-OXA-48-D159K were used to inoculate 2 liters of LB medium broth containing 50 mg / liter of kanamycin. Bacteria were grown at 37 °C until the culture reached an OD₆₀₀ of 0.6. Expression of OXA-933, OXA-48-D159K and OXA-48-D159G was induced overnight at 25 °C with 0.2 mM IPTG. Cultures were centrifuged at 6000 g for 15 min and the pellets were resuspended in 10 ml of a buffer composed of 20 mM phosphate buffer, 175 mM K₂SO₄, 40 mM imidazole, pH 7.4. Cells were then disrupted by sonication and two centrifugation steps were required to remove debris at 10 000 g and 48000 rpm for 1h each. Purification was done by affinity chromatography using NTA-Ni column (GE Healthcare, Freiburg, Germany). Protein purity was estimated by SDS-PAGE, pure fractions

were pooled and dialyzed against 100 mM sodium phosphate, 50 mM K₂SO₄ (pH 7.0) and concentrated using Vivaspin® 10 kDa columns (GE Healthcare, Freiburg, Germany). Protein concentration was determined by Bradford Protein assay (Bio-Rad) (13).

Steady-state kinetic parameters

Kinetic parameters of purified OXA-933, OXA-48-D159K and OXA-48-D159G were determined at 30°C in 100 mM sodium phosphate buffer (pH 7.0). The k_{cat} and K_{m} values were determined by analyzing hydrolysis of β -lactams under initial-rate conditions with an ULTROSPEC 2000 model UV spectrophotometer and Swift software (GE Healthcare). Steady-state kinetic parameters were calculated using the Eadie–Hoffstee linearization of the Michaelis–Menten equation, as previously described (7,13). The β -lactams were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Comparative activation of OXA-933 by CO₂ was conducted by monitoring the rate of hydrolysis of penicillins in the same buffer supplemented with 50 mM sodium hydrogen carbonate (NaHCO₃) as the source of CO₂ (7,9). OXA-48 kinetic parameters, used in the comparison, were previously determined by Docquier et al. (15).

Molecular modelling

UCSF Chimera package (32) was used for structural analysis and image plotting. The three-dimensional structures were generated by homology modeling using MODELLER version 10.3 (33) and the structure 6PQI (34) as template, or directly from sequence using AlphaFold (35), through a local implementation of ColabFold (36). Three-dimensional structures of the β -lactam ligands were generated using the Corina (version 3.60) program (Molecular Networks GmbH, Erlangen, Germany). Molecular docking calculations were performed using the Gold program (Cambridge Crystallographic Data Centre, Cambridge, UK) (37) and the GoldScore scoring function. The binding site, defined as a 20-Å-radius sphere, was centred on the OG oxygen atom of Ser70. All other parameters had default values.

Nucleotide sequence accession numbers

The nucleotide sequence of the *bla*_{OXA-933} gene has been submitted to the EMBL/Genbank nucleotide sequence database under the accession number MW073108. The nucleotide sequence of plasmid pOXA-933 has been submitted also, under the accession number

OQ915017. The Whole Genome Shotgun of *K. pneumoniae* 241B9 has been deposited at the DDBJ/ENA/GenBank under the accession code JARYTJ000000000.

Acknowledgments

This work was supported by the Assistance Publique – Hôpitaux de Paris (AP-HP), the University ParisSaclay, INSERM, and by grants from the French National Research Agency [ANR-19-AMRB-0004 and ANR-20-PAMR-0010]. LD, TN and BII are members of ESCMID Study Group for Antimicrobial Resistance Surveillance - ESGARS.

Conflicts of interest

None to disclose.

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Supplemental material

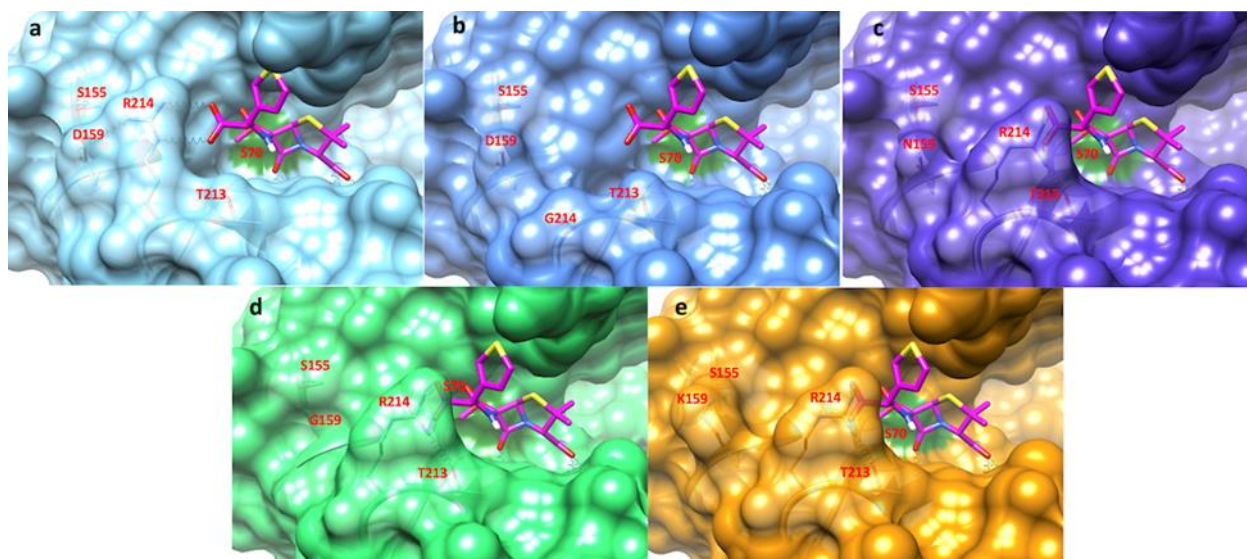


Figure S1: Docking conformation of temocillin (non-covalent binding) on OXA-48 (a), OXA-244 (b), OXA-933 (c), OXA-48-D159G (d) and OXA-48-D159K (e), in surface representation. The surface of residues in positions 155, 159, 213 and 214 is partially transparent to show the conformation of the corresponding residues, represented as sticks. The surface of Ser70 is colored in green. The black strings represent hydrogen bonds.

Molecular, genetic and biochemical characterization of OXA-484, a difficult to detect R214G variant of OXA-181

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Keywords: oxacillinase, carbapenemase, OXA-181, antibiotic resistance, beta-lactamase, OXA-48-like

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Abstract

Increasing number of variants of the carbapenem-hydrolyzing class D β -lactamase OXA-48 are identified in Enterobacterales worldwide. Among them, OXA-244 an R214G variant of OXA-48 is of particular interest, as this substitution results in reduced hydrolysis of carbapenems and temocillin, two compounds classically used in CPE screening media. Here, we have characterized an *E. coli* 172D10 and an *C. youngae* 175G8 isolates expressing OXA-484, an R214G variant of OXA-181.

Both isolates displayed reduced susceptibility to carbapenems but were lacking significant carbapenem-hydrolyzing activity as revealed by negative biochemical confirmatory test results. However, they were positive for OXA-48-like enzymes by immunochromatography and PCR assays. WGS revealed in both isolates the presence of an *bla*_{OXA-181-like} gene, that codes for OXA-484, an R214G variant of OXA-181. The *bla*_{OXA-484} gene was located on a 58,409-kb IncP1-like plasmid (pNOXA-484). The MIC values of *E. coli* TOP10 (pTOPO-OXA-484) revealed reduced susceptibilities to most substrates as compared to OXA-181, especially imipenem (MIC= 0.25 μ g/ml versus 0.75 μ g/ml for OXA-181) and temocillin (MIC= 16 μ g/ml versus 1028 μ g/ml for OXA-181). When pNOXA-484 was expressed in *E. coli* HB4 with impaired permeability, MIC values for carbapenems and Temocillin were above 32 μ g/ml. Catalytic efficiencies of OXA-484 were reduced as compared to OXA-181 for most β -lactams including imipenem (27.5-fold reduction) and temocillin (21,7-fold reduction), in a similar manner to OXA-244. Molecular modelling studies evidenced that R214 in OXA-181, establishes a salt bridges with D159 and with the R1 substituent's carboxylate group of temocillin. These interactions are not possible with G214 in OXA-484, explaining the reduced affinity of temocillin for this enzyme and the resulting changes in active site's water network that would explain the decrease in hydrolysis rate of carbapenems.

Overall, OXA-484 has weak imipenem-and temocillin hydrolytic activity, likely resulting in under detection using selective screening media containing these two antibiotics or by biochemical tests monitoring imipenem hydrolysis, thus resulting in silent spread.

Introduction

Clinically-relevant carbapenemases in Enterobacterales belong to class A (KPC-type), B (NDM, VIM, IMP) and D (OXA-48-like) (1). OXA-48, initially identified in Turkey, has since rapidly spread in the Mediterranean area, middle East, Europe, India and is now turning

into a major global threat (2). OXA-48 hydrolyzes penicillins including temocillin, narrow-spectrum cephalosporins, and also carbapenems at low rate, but spares expanded-spectrum cephalosporins (ESC) e.g. ceftazidime and cefepime (3,4). Along with the spread of OXA-48, several variants have been reported that differ from OXA-48 by amino-acid (AA) substitutions or deletions (<http://bldb.eu/BLDB.php?class=D#OXA>) mostly located in the β 5- β 6 loop (5). OXA-181 that differs from OXA-48 by 4 amino-acid substitutions (T103A, N110D, E169Q and S171A) is the second most prevalent OXA-48 variant and displays similar β -lactam hydrolyzing activity as compared to OXA-48 (2). Some variants of both enzymes, such as OXA-244 (R214G of OXA-48) or OXA-232 (R214S of OXA-181), have been described to confer lower MICs towards carbapenems and temocillin as compared to OXA-48/OXA-181-producers (6,7). Recently, OXA-484 presenting an R214G in an OXA-181 backbone was initially described in the UK in *K. pneumoniae*, and subsequently in *E. coli* isolates from Germany, Switzerland, South Africa, China and in an environmental sample from Ireland (8-14). In most of these cases the *bla*_{OXA-484} gene was carried on a 51kb IncX3 plasmid present in *E. coli* ST410 isolates (8-14).

Substitution at position R214 in the β 5- β 6 loop, are particularly interesting, as they significantly impair carbapenem- and temocillin hydrolyzing activity as compared to their parental enzymes (6,7). The position R214 plays a pivotal role in the hydrolysis of carbapenems and temocillin (7). As temocillin and carbapenems are present in several screening media used for the detection of CPEs, reduced hydrolysis of these molecules results in difficulties in detection of these variants (15,16). As a consequence of the difficulties of detection, R214 mutants in the β 5- β 6 loop are increasingly isolated worldwide (2,17-19).

Here we investigated the effect of R214G substitution in a OXA-181 backbone, compared it to OXA-244 and OXA-232. In addition, we analyzed the genetic environment of OXA-484 in different Enterobacterales isolates, from France, UK and Germany. Finally, using molecular modelling based on the X-ray structure of OXA-181 and OXA-232, we were able to propose an explanation to the reduced carbapenem and temocillin hydrolysis.

Results

Antimicrobial susceptibility testing and carbapenemase confirmation tests

E. coli 172D10 and *C. freundii* 175G8 isolates displayed reduced susceptibility to carbapenemase and were thus sent to the French National Reference Center for carbapenem resistant Enterobacterales in 2019 for further expertise. Bacterial identification was confirmed by

MALDI TOF. Biochemical tests based on imipenem hydrolysis (home made Carba NP [20]) gave repeatedly negative results, although the resistance phenotype was compatible with the production of an OXA-48-like carbapenemase especially since the immunochromatographic NG-Test Carba5 (NG Biotech, Guipry, France) assay gave positive results for OXA-48 in both isolates (**Table 1**). Finally, attempts to grow these isolates on ChromID® CARBA SMART (BioMérieux), failed on the CARBA side and on the OXA-48 side of the plate suggesting an OXA-244-like variant with lower carbapenem and temocillin hydrolytic activity (15).

Concomitantly, PCR experiments with primers specific for known carbapenemases genes confirmed the presence of a *bla*_{OXA-48-like} gene and sequencing of the product revealed the presence of *bla*_{OXA-484}, a *bla*_{OXA-181} gene variant showing a single nucleotide substitution, resulting in a single amino acid substitution, R214G (DBL numbering).

The *bla*_{OXA-484} gene was cloned into pTOPO plasmid, transformed into *E. coli* TOP10, and MIC values were compared to those of *E. coli* TOP10 harboring pTOPO-OXA-48, -OXA-244, -OXA-181, and -OXA-232. The effect of the R214G substitution in the two main OXA-48-like backbones (OXA-48 and OXA-181) on the hydrolysis of several β -lactams including imipenem and temocillin were similar. Reduced MICs for temocillin and imipenem were observed and were similar to those observed by Oueslati et al. on a S214G mutant of OXA-232 (Table 1). In addition, OXA-484 displayed similar MIC values to OXA-232, an R214S OXA-181 derivative. Overall, MIC values for ampicillin and cephalothin were not affected and MIC values of the mutants for the other carbapenems (meropenem and ertapenem) vary in the same way as for imipenem. MIC values of *E. coli* HB4 expressing the natural plasmid pN-OXA-484, revealed increased MICs (>32 μ g/ml for carbapenems) (**Table 1**) suggesting that these enzymes may yield carbapenem-resistance when expressed in a bacterium with impaired outer membrane permeability.

Figure 1: Kieser DNA extractions, of (A) parental *E. coli* 172D10, (B) *E. coli* 50192 containing four plasmids of 7-, 38-, 66- and 154-kb; (C) *E. coli* TOP10 pN-OXA-484 electroporant and (D) *E. coli* J53 pN-OXA-484 transconjugants.

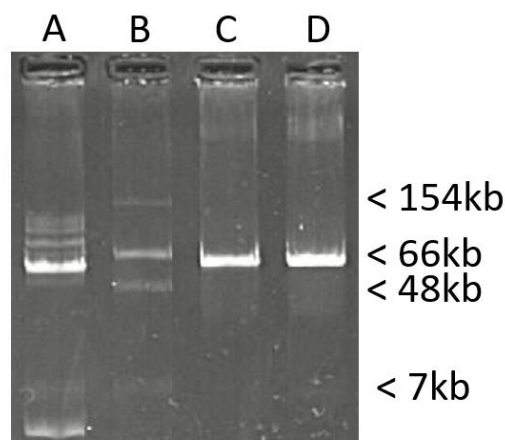


Table 5. Antimicrobial susceptibility results.

MIC (mg/L)										
	<i>E. coli</i>	<i>C. youngae</i>	<i>E. coli</i> TOP10				<i>E. coli</i> HB4			
β-lactam	172D10	173G7	pN-OXA-484	pTOPO- OXA-181	pTOPO- OXA-484	pTOPO- OXA-244	pTOPO- OXA-48	-	pN-OXA-484	-
Amoxicillin	>256	>256	>256	>256	96	>256	>256	2	>256	16
Temocillin	64	128	64	>256	12	32	>256	4	>256	24
Cephalotin	ND	ND	ND	16	8	16	16	1.5	128	8
Cefotaxime	ND	ND	ND	1	0.064	0.064	0.25	0.06	1.5	0.75
Ceftazidime	0.25	1	0.125	0.5	0.38	0.38	0.25	0.12	1	0.75
Ertapenem	1	1	0.5	0.19	0.004	0.012	0.094	0.003	>32	0.75
Imipenem	1	2	0.25	0.75	0.25	0.25	0.75	0.25	>32	0.125
Meropenem	0.06	0.125	0.125	0.125	0.19	0.047	0.094	0.016	>32	0.38
CarbaNP test	-	+/-	-	+	-	-	+	-	-	-
NG test Carba 5	OXA	OXA	OXA	OXA	OXA	OXA	OXA	-	OXA	-

Genomic features of OXA-484 producers.

The genome of *E. coli* 172D10 and *C. freundii* 173G7 were analyzed to decipher its resistome. Only contigs bigger than 500-bp were retained for further analysis. The genomes were 5,462,546 bp and 4,744,353 bp in size, respectively with a mean coverage of >130 X. The bacterial identification using WGS data was confirmed for *E. coli* 172D10, however *C. freundii* 173G7 turned out to be a *C. youngae*. The Multi locus sequence typing (MLST) of *E. coli* 172D10 and *C. youngae* 175G8 was determined using MLST 2.0 software and revealed a ST69 and an ST491, respectively (22,23). *E. coli* ST69 belongs to a dominant uropathogenic lineage frequently associated with AMR (24), while *C. youngae* ST491 is rarely described especially among CPEs (25).

Both genomes were analyzed by searching for acquired resistance genes and for point mutations involved in resistance. Two acquired β -lactamases, *bla*_{TEM-1}, and *bla*_{OXA-484} carbapenemase gene were identified in *E. coli* 172D10, while in *C. youngae* 175G8 ST491 only *bla*_{OXA-484} was present (**Table 2**). In addition, an *aad1* gene, conferring resistance to spectinomycin and streptomycin, two sulfonamide resistance genes, *sul1* and *sul2*, *Tet(A)*, *mdf(A)* and two dihydrofolate reductases, *dfrA14* and *dfrA17* genes were also present in *E. coli* 172D10, while in *C. youngae* ST491 *AadA1*, *Sul1* and three macrolide resistance genes were identified: *ere(A)*, *mdf(A)* and *mph(A)*.

No mutations were identified in topoisomerase genes leading to substitutions known to confer to high-level resistance to fluoroquinolones in Gram-negative rods (28).

Table 2: Heatmap displaying the detected plamid INC-types, resistance genes and MLST within Enterobacterales harboring a carbapenemase gene.

					Plasmids													β-lactamases					aminoglycosides							Fluoroquinolones			Others																			
Isolate		MLST ^d	Country ²	year	Size	IncP1	col156	Col8282	IncB/O/K/Z	IncR	IncFIB(K)	IncFIA	IncFIB	IncX3	IncY	IncN	IncX4	p0111	TEM-1	Shv-28	OXA-1	OXA-9	CTX-M-15	CMY-	OXA-484	Aad1	aadA5	aph(6)-Id	aac(6')-Ib-cr	aac(3')-IId	aac(3')-IIa	aac(6')-Ib3	aph(3'')-Ib	aac(6')-Ib-cr	qoxA	qoxB	qnrS1	sul1	Sul2	Tet(B)	Tet(A)	ere(A)	mdf(A)	mph(A)	dfpA ³	fosA	cmIA1	reference	Genbank			
E. coli	172D10	69	F	2019	58.5																																									-						
C. youngae	173G7	491	F	2019	58.5																		157																							14			-			
K. pneumoniae	32333	14	UK		ND																																										1			8		
K. pneumoniae	15179	14	UK		ND																																										1			8		
E. coli	260213	410	UK		ND																			42																								8				
E. coli	EC-JS316	410	G		51.5																																											17			9	CP058621

1: * indicates a novel ST highly related to ST26; 2: Country of origin F France, UK United kingdom, and G Germany; 3: Number indicates the allelique number. Boxes in dark grey represent the plasmid carrying blaOXA-484.

Genetic support and environment of *bla*_{OXA-484} gene

According to plasmid finder, seven different plasmid replication origins belonging to the incompatibility groups, IncB, IncN, IncP1-like, IncX4, col156, Col8282 and p0111 were identified in *E. coli* 172D10, and an IncP1-like in *C. youngae* 175G8 (**Table 2**). Plasmid reconstruction revealed that the *bla*_{OXA-484} gene was carried by the IncP1-type plasmid of ca. 58.4-kb in both isolates. This plasmid was different in size, and in nature from previous plasmids described to contain *bla*_{OXA-484} genes such as IncX3 plasmid identified in *E. coli* ST410 from Germany, UK and from Switzerland (Figure 2 and 3) (8-14).

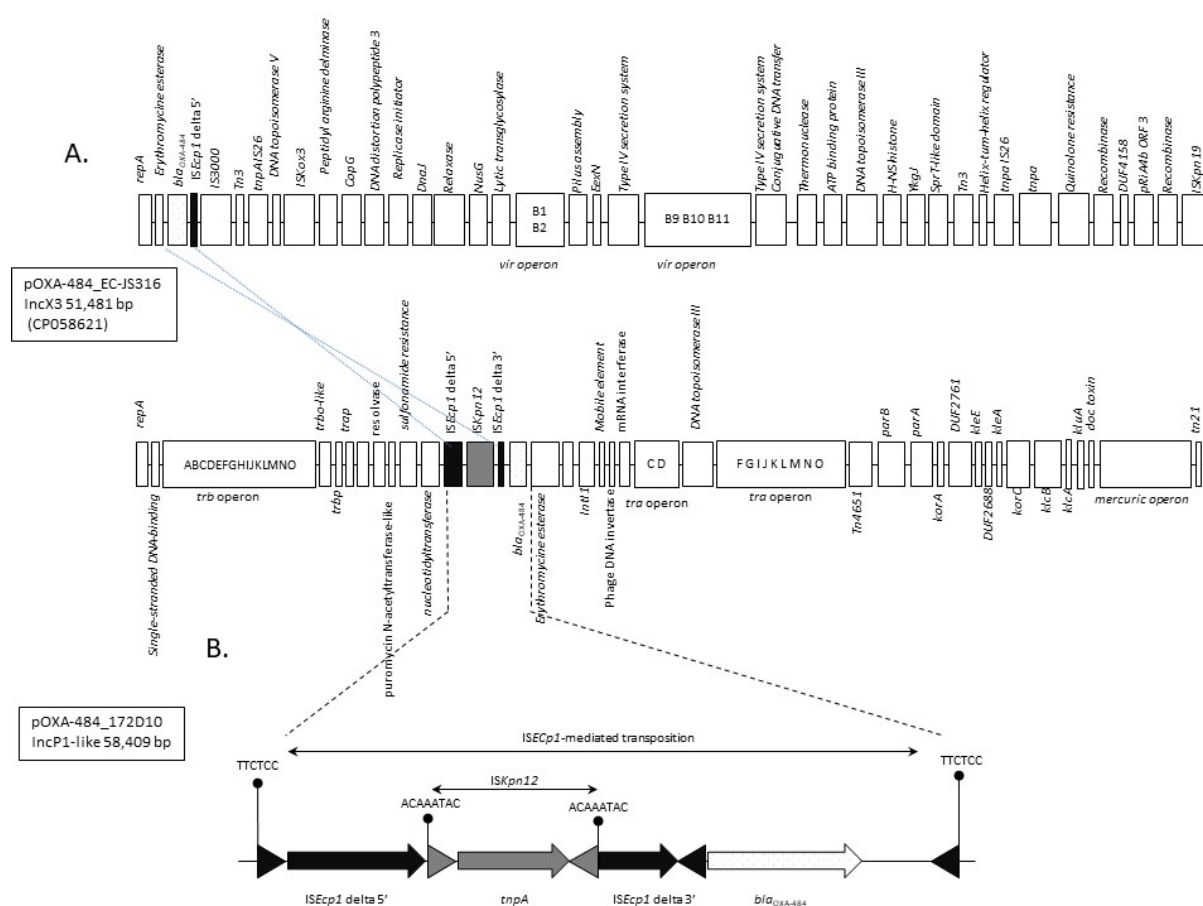


Figure 2: Schematic representation of (A) pOXA-484_EC-JS316 (IncX3 of 51,481 bp (CP058621) harbouring *bla*_{OXA-484} and *qnrS1* and pN-OXA484_172D10 (IncP1-like of 58.5kb) harbouring *bla*_{OXA-484}. Common structures are highlighted in dotted region lines (B) Schematic representation of ISEcp1 – *bla*_{OXA-484} transposon in pOXA-484_172D10. Genes are represented by arrows. Target site duplications are indicated and Inverted Repeat sequences are indicated by triangles. Coloured-dotted boxes indicate resistance genes : *bla*_{OXA-484} and *qnrS1*. ISEcp1 and ISKpn12 insertion sequences are represented by black and grey boxes, respectively.

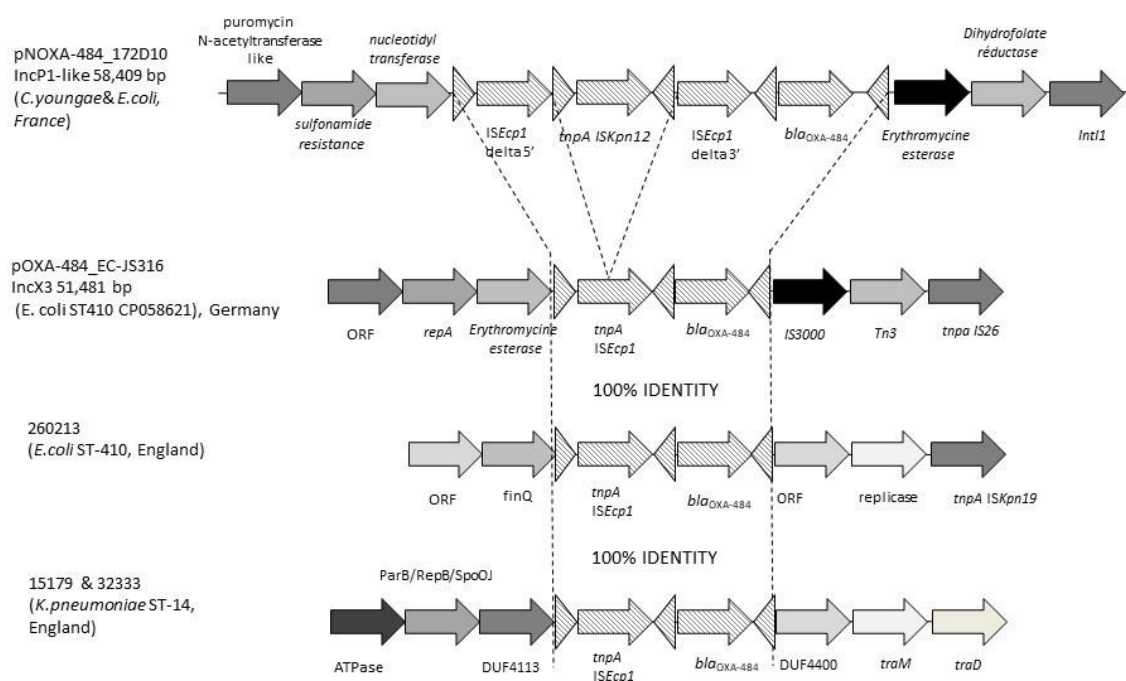


Figure 3: Structural features of the genetic environment of *bla*_{OXA-484} from pOXA-484_172D10 with pOXA-484_EC-JS316 (GenBank accession number CP058621 (9) and with three other strains from England (8). Common structures are indicated by dotted lines.

The only part that was shared with these plasmids is the *ISecp1*-based composite transposon carrying *bla*_{OXA-484} gene (Figure 2B). This structure inserted into the IncP1 backbone, as revealed by a target site duplication. In addition, in both isolates, the open reading frame of *ISecp1* is disrupted by the insertion of another insertion sequence, *ISKpn12*, unlike what is observed for the other plasmids (Figure 3).

Direct transfer of the β -lactam resistance marker into *E. coli* J53 by mating-out experiments revealed the transfer of the 58.4 kb plasmid in the recipient *E. coli* isolates (Figure 1) confirming the plasmidic origin and its self-transferable nature of plasmid pN-OXA-484—172D10. Similarly, electroporation of this plasmid into *E. coli* TOP10 yielded transformants resistant to β -lactams and with reduced susceptibility to carbapenems (Table 1).

Biochemical properties determination.

To further characterize the impact of the nature of the residue at position 214 on the hydrolytic profile, steady-state kinetic parameters of OXA-484 for several clinically-relevant substrates

(penicillins, cephalosporins and carbapenems) were determined and compared to those of OXA-48, OXA-244, OXA-181 and OXA-232 (5,7). In a previous work, Oueslati et al. had already investigated the role of S214G of OXA-232, which corresponds to OXA-484 on imipenem hydrolysis (7). To complete the hydrolytic profile, several additional substrates were investigated.

The k_{cat}/K_m of OXA-484 for the hydrolysis of penicillin G, ampicillin and cephalothin were similar to those of OXA-181 (**Table 3**) suggesting that the R214G substitution does alter the hydrolysis of these substrates. Interestingly, the hydrolysis of penicillin G is 30-fold lower with OXA-244. Oxyimino cephalosporins such as cefotaxime are weakly hydrolysed, but the k_{cat}/K_m could not be determined precisely, as the K_m value was >1000 mM to be determined experimentally. For ceftazidime, a bulkier oxyimino-cephalosporin, no hydrolysis was observed even with $1.92\ \mu\text{M}$ purified enzyme and up to $500\ \mu\text{M}$ substrate, as shown for OXA-48.^{4,17}

Compared to OXA-181, the OXA-484 has k_{cat}/K_m values 1.6-, 7.5-, and 27.5-fold lower for ertapenem, meropenem and imipenem hydrolysis, respectively (**Table 3**). The reduced catalytic efficiency for imipenem in OXA-484, are mainly due to a reduction in the turnover number. Interestingly, as observed for OXA-232, the k_{cat}/K_m for ertapenem of OXA-484 was 916-fold lower than that for imipenem, but was not affected as compared to the value for OXA-181 or OXA-48 (0.6 versus 2 and $1\ \text{s}^{-1}.\text{mM}^{-1}$, respectively).

Table 3. Steady-state kinetic parameters for hydrolysis of β -lactam substrates by OXA-48, OXA-181, OXA-232, OXA-484 and OXA-244.

Substrate	<i>K_m</i> (μ M)					<i>K_{cat}</i> (s ⁻¹)					<i>K_{cat}/K_m</i> (mM ⁻¹ s ⁻¹)				
	OXA-48	OXA-181	OXA-232	OXA-484	OXA-244	OXA-48	OXA-181	OXA-232	OXA-484	OXA-244	OXA-48	OXA-181	OXA-232	OXA-484	OXA-244
<i>Benzylpenicillin</i>	ND	90	60	33 \pm 1.9	450 \pm 37	ND	444	125	329 \pm 8.4	72 \pm 8	ND	5000	2100	9972 \pm 724	163 \pm 28
<i>Ampicillin</i>	400	170	220	653 \pm 72.2	657 \pm 163	955	218	132	394 \pm 20.6	373 \pm 158	2400	1300	600	604 \pm 43	549 \pm 133
<i>Temocillin</i>	45	60	60	178 \pm 25.1	364 \pm 23	0,3	0,3	0,03	0.04 \pm 0	0.11 \pm 0.02	6	5	0,5	0,23 \pm 0.03	0.31 \pm 0.05
<i>Cefalotin</i>	195	250	125	37 \pm 2.6	88 \pm 27	44	13	13	3 \pm 0.2	3.3 \pm 0.8	225	50	105	78 \pm 1.7	39 \pm 4
<i>Ceftazidime</i>	NH	NH	>1000	>1000	NH	NH	ND	>0,6	ND	NH	NH	ND	0,1	ND	NH
<i>Cefotaxime</i>	>900	>1000	>1000	>1000	>900	>9	>62	>6,5	ND	ND	10	13	6	ND	ND
<i>Ertapenem</i>	100	100	110	27 \pm 1.2	22.9 \pm 0.3	0,13	0,2	0,04	0.02 \pm 0	0.02 \pm 0.01	1	2	0,4	0,6 \pm 0.06	0.1 \pm 0.03
<i>Imipenem</i>	13	13	9	6 \pm 2.1	2.6 \pm 0.8	5	7,5	0,2	0.1 \pm 0.01	0.07 \pm 0.01	370	550	20	20 \pm 4.4	27 \pm 11
<i>Meropenem</i>	10	70	100	263 \pm 9.4	23 \pm 4	0,07	0,1	0,03	0.05 \pm 0.05	0.02 \pm 0.001	6	1,5	0,3	0,2 \pm 0.06	0.8 \pm 0.2

ND, not determined; NH, no detectable hydrolysis was observed with 1.92 μ M purified enzyme and up to 500 μ M substrate. Data are the means of three independent experiments. Data for OXA-48, OXA-181, OXA-232 and OXA-244 were from Docquier *et al.*(4) , Rima *et al.*(6) and Oueslati *et al.* (16) respectively

Regarding temocillin, K_m is 4-fold higher than OXA-181, showing that OXA-484 has a lower affinity for temocillin. Moreover, k_{cat} of temocillin in OXA-484 is 7.5-fold lower than OXA-181, thus resulting in a 21.7-fold lower catalytic efficiency for temocillin as compared to OXA-181.

Determination of IC_{50} showed that OXA-484, OXA-232, and OXA-181 are similarly inhibited by clavulanic acid (19 μM , 13.4 μM , 28.5 μM), tazobactam (1.09 μM , 0.75 μM and 20 μM) and NaCl ($0.113 \times 10^3 \mu M$, $97 \times 10^3 \mu M$, $35 \times 10^3 \mu M$), respectively.

Overall, the hydrolysis profiles were consistent with those of OXA-244. The catalytic efficiencies, k_{cat}/K_m s, were concordant with the MIC results, and confirm that the hydrolysis of imipenem depends on the nature of the residue in position 214, irrespective of the backbone of the enzyme.

Molecular modelling.

An *in silico* study was performed to identify the structural determinants that could explain the experimentally determined differences between the hydrolytic profiles of OXA-484 in comparison with OXA-181 and OXA-244. The OXA-181 and OXA-232 structures (PDB code 5OE0 and [5HFO](#)) were used as a starting point and the mutation S214G was modeled based on predicted low energy conformations. The resulting models didn't show any structural clashes (Data not shown).

A salt bridge is present in OXA-48 and in OXA-181 between D159 and R214, that is lost in OXA-484, as already observed in OXA-244. This mutation induces a significant change in the shape of the active site at its periphery (Figure 4). Moreover, we have shown previously that R214 in OXA-48 also establishes a favourable ionic interaction with the carboxylate group of the R1 substituent of temocillin, and that this interaction is lost in OXA-232, which has a serine residue in position 214 (7). Here we show, through molecular docking calculations, that this interaction is also absent in the complex of temocillin with OXA-484, which has a glycine in position 214 (Figure 4)

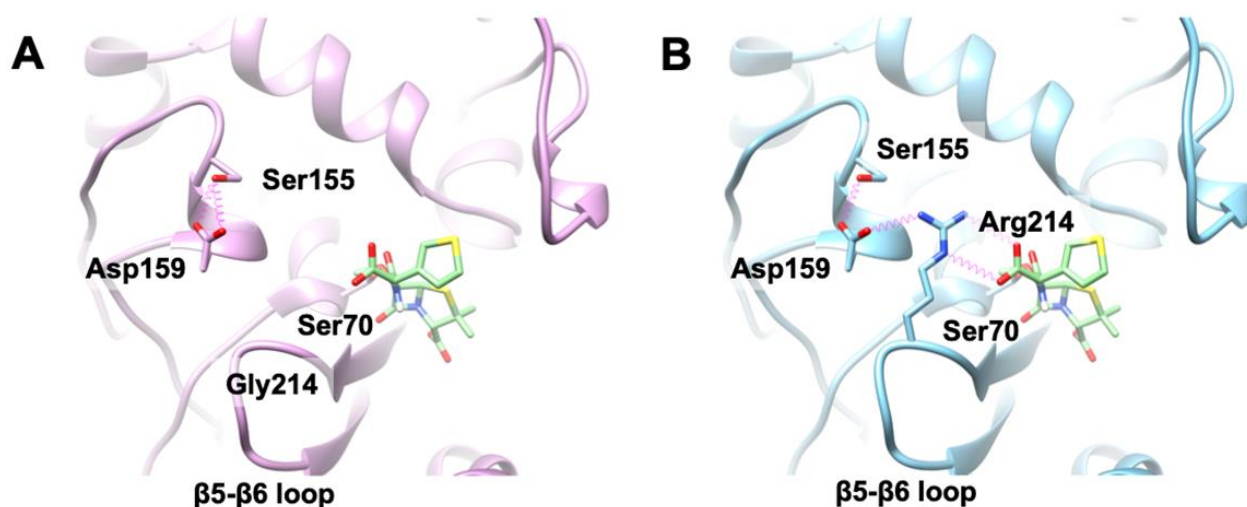


Figure 4: In silico model of OXA-484 (A) and crystal structure of OXA-181 (B, PDB code 5OE0 in cartoon representations, with the docking conformation of temocillin in stick representation. OXA-484, OXA-181 and temocillin are colored in pink, cyan and green, respectively. Hydrogen bonds are represented as springs colored in magenta.

Discussion

OXA-48-producing Enterobacterales are now endemic in many countries, such as Turkey, Middle East, North Africa, India and have widely spread across Europe (2). Since the first description of OXA-48, several variants have been described (1,2,5). These variants can be classified into 4 groups according to their hydrolysis profile. Most of them, including OXA-181 or OXA-162 (2), have an enzymatic activity similar to OXA-48 (26). The second group, represented by OXA-163, OXA-247, and OXA-405 (27), have no carbapenemase activity, but instead a marked hydrolytic activity against ESC, similarly to OXA-ESBLs (27). The third group, has carbapenemase and ESC hydrolytic activity, such as OXA-517 and OXA-438 (28,29). Finally, the fourth group represented by OXA-244 (6) and OXA-232 (7) exhibit an overall reduced activity towards all β -lactams including carbapenems as compared to OXA-48. The amino acid sequence comparison of OXA-48-variants suggested a link between the primary structure and the function of these enzymes. Indeed, all OXA-48-variants with an OXA-ESBLs phenotype (loss of carbapenem hydrolysis and the gain of activity towards ESC) have an amino-acid deletion in the β 5- β 6 loop (Figure 1), and those having both activity display a 2 amino acid deletion (28). This observation suggests that this loop plays a role in the substrate specificity. The

phenotypic and enzymatic study of OXA-232 (which differs from OXA-181 by only one substitution R214S) (7) underlined that the residue 214 is crucial for the carbapenem and temocillin hydrolysis. Similarly, phenotypic and enzymatic studies of OXA-244 (which differs from OXA-48 by only one substitution R214G) (6) confirmed these results. The impact of R214G substitution in an OXA-181 backbone was initially partially investigated by Oueslati et al., where enzymatic studies conducted on an in vitro generated mutant of OXA-232 (S214G) suggested that imipenem, and ertapenem hydrolysis could be impaired in a similar manner to OXA-244 (7). These results were further confirmed with specific activity measurements with OXA-484 (9). Here detailed enzymatic activity of OXA-484 variant with an R214G substitution in an OXA-181 backbone was analysed. `

From a treatment perspective, these enzymes, even though with reduced activity towards carbapenems, in a background of impaired outer membrane may lead to high level carbapenem resistance. Unlike ST410, that were all multidrug resistant, these ST69 were still susceptible to many classes of antibiotics.

Analysis of the 3D structure of OXA-48 and OXA-181 showed that R214 interacts with D159 *via* a salt bridge (5), which maintains the shape and the network of water molecules within the binding site. Our molecular modeling study revealed that with the G214 mutations, this interaction with D159 is lacking, which presumably increases the flexibility of this part of the binding site. Overall, we demonstrated that the AA position 214 in OXA-48-like β -lactamases is critical for the carbapenemase activity but also for the temocillin hydrolysis. This point is of utmost clinical importance and explains why detection of OXA-244, OXA-232 and now OXA-484-producers remains a challenge for clinical microbiology laboratories (15,16). Indeed, these isolates do not grow on ChromID Carba Smart (bioMérieux, Marcy L'Etoile, France), one of the most used medium for the screening of CPEs (15). The ChromID Carba Smart is a biplate containing on one side a carbapenem and on the other temocillin, two substrates that are only weakly hydrolyzed by R214G variants of OXA-48/181 (19).

The *bla*_{OXA-484} sequence was initially reported in 2017 in five *Klebsiella pneumoniae* isolates from the UK (8). In December 2019, it was described in an *E. coli* EC-JS316 strain belonging to ST 410 obtained from a rectal swab of a patient hospitalised in Germany (December 2019) due to acute dengue fever following a stay in India (9). *Bla*OXA-484 was carried on an IncX3 plasmid of 51.5kb. Recently, *bla*_{OXA-484}-ST410-*Ec* isolates originating from India were described in Switzerland to possess both an IncX3 *bla*_{OXA-484}-carrying plasmid and a core genome

identical to EC-JS316 (23 Δ SNVs) (13). This IncX3 plasmid is frequently described with *E. coli* ST-410 isolates harboring OXA-181 and OXA-484 either from isolates of patients but also from environmental source. It has been suggested that an IS26 composite transposon may be at the origin of the mobilization of *bla*_{OXA-484} from this IncX3 plasmid to an IncFII plasmid found in *Klebsiella* in Switzerland and China, and in *E. coli* ST1722 isolates from Switzerland. Here we evidenced a likely transfer of the transposon carrying OXA-484 to other plasmids, increasing the potential to spread to other *E. coli* isolates and species, such as *C. youngae*. Finally, the genes have also been described in patient isolates of *K. pneumoniae* from the UK but also in environmental *K. pneumoniae* isolates from South Africa present on IncF-type plasmids, but also in *K. varicola* from China, on an IncX3 plasmid (table2). In several cases a link with India has been evidenced (8-14), which is not surprising given the high prevalence of OXA-181 carried by a common IncX3 plasmid in this country (2). These data suggest that *bla*_{OXA-484} and *bla*_{OXA-181} may have evolved from a common ancestor and spread to different hosts, and now to different plasmids thanks to ISEcp1, an element proficient in transposition at high frequency without insertion site preference of *bla*_{CTX-M}-type ESBLs worldwide.

In conclusion, importation of these difficult to detect pathogens from endemic areas to low-prevalence nations is a major concern, even more so with difficult to detect mechanisms, as OXA-244 and OXA-484. This may lead to uncontrolled spread and the occurrence of outbreaks. Several countries have reported this gene, from all the continents suggesting active dispersion, likely as a result of underdetection and high mobility through transposition to various plasmid backbones. Therefore, infection prevention and control programmes must be rigorously maintained, to mitigate any future clinical impact due to these peculiar CPEs.

Materials and methods

Bacterial strains

E. coli 172D10 and *C. youngae* 175G8 were initially identified with matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MALDI, Biotyper CA system, Bruker Daltonics, Billerica, MA, USA). *Escherichia coli* TOP10 (Invitrogen, Saint-Aubin, France) and *E. coli* BL21 (DE3) (Novagen, VWR International, Fontenay-sous-Bois, France) were used for cloning experiments. Azide-resistant *E. coli* J53 was used for conjugation assays. *E. coli* 50192 were used plasmid size marker (30).

Antimicrobial agents, susceptibility testing, and microbiological techniques

Antimicrobial susceptibilities were determined by disk diffusion technique on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the EUCAST breakpoints, updated in 2023 (<http://www.eucast.org>). Minimal inhibitory concentration (MIC) values were determined using the Etest technique (BioMérieux, Paris, France) and broth microdilution (Sensititre, Thermofisher, Grenoble, France). *E. coli* 172D10 and *C. youngae* 175G8 were plated in different chromogenic medias: chromID® ESBL (BioMérieux, Paris, France) and chromID® CARBA SMART (BioMérieux, Paris, France). Antibiotics were purchased from Sigma (Saint-Quentin-Fallavier, France) except temocillin (Eumedica, Brussels, Belgium).

Carbapenemase activity was investigated using the Carba NP test, as previously described (20) and carbapenemases were thought using NG-Test Carba 5 (21)(NG Biotech, Rennes, France) as recommended by the manufacturer.

PCR, cloning experiments, and DNA sequencing

Whole-cell DNAs of *E. coli* 172D10 isolate was extracted using the QIAamp DNA mini kit (Qiagen, Courtaboeuf, France) and was used as template for PCR amplification of OXA-484 with the following primers: preOXA-48A (5'-TATATTGCATTAAGCAAGGG-3') and cloning OXA-48B (5'-AAAAGGATCCCTAGGGAATAATTTTTTCCTGTTTGAGCA-3'). The amplicons obtained were then cloned into the pCR®-Blunt II-TOPO® plasmid (Invitrogen, Illkirch, France), downstream of the pLac promoter, in the same orientation, resulting in pTOPO- OXA-484, that was subsequently electroporated into *E. coli* TOP10 cells; the electroporants were plated on a TSA plate containing kanamycin (50 ug/ml). The recombinant plasmids pTOPO-*bla*_{OXA-232}, pTOPO-*bla*_{OXA-48}, pTOPO-*bla*_{OXA-181}, pTOPO-*bla*_{OXA-244} were obtained from previous studies (6,7).

The *bla*_{OXA-484} gene fragment corresponding to the mature β -lactamase was cloned into the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois, France) using the PCR-generated fragment with primers INF-OXA-48Fw (5'-AAGGAGATATACAT-ATGGTAGCAAAGGAATGGCAAG-3') and INF-OXA-48Rv (5'-GGTGGTGGTGCTC-GAAGGGAATAATTTTTTCCTGTTTGAG-3') and the NEBuilder® HiFiDNA Assembly

Cloning Kit (New England BioLabs® Inc, United Kingdom), following the manufacturer's instructions. The recombinant plasmid pET41-OXA-484 was transformed into the chemically-competent *E. coli* strain BL21 (DE3) as previously described (6,7).

Recombinant plasmids were extracted using the Qiagen miniprep kit and both strands of the inserts were sequenced using M13 primers for the pCR®-Blunt II-TOPO® plasmid (Invitrogen, Illkirch, France), and T7 primers for pET41b(+) (Novagen, VWR International, Fontenay-sous-Bois, France), with an automatic sequencer (ABI Prism 3100; Applied Biosystems). The nucleotide sequences were analyzed using software available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

Whole genome sequencing (WGS)

Total DNA was extracted from colonies using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, US) following the manufacturer's instructions. The DNA concentration and purity were controlled by a Qubit® 2.0 Fluorometer using the dsDNA HS and/or BR assay kit (Life technologies, Carlsbad, CA, US). The DNA library was prepared using the Nextera XT-v3 kit (Illumina, San Diego, CA, US) according to the manufacturer's instructions and then run on Miseq (Illumina) for generating paired-end 300-bp reads. De novo assembly was performed by CLC Genomics Workbench v 12.0 (Qiagen, Hilden, Germany) after quality trimming ($Q_s \geq 20$) with word size 34. The acquired antimicrobial resistance genes were identified by uploading the assembled genomes to the Resfinder server v4.0 (<https://cge.food.dtu.dk/services/ResFinder/>)(31). The Multi Locus Sequence Typing (MLST) and the identification of the different plasmids were also obtained by uploading the assembled genomes to the MLST 2.0 (<https://cge.food.dtu.dk/services/MLST/>; 32) and PlasmidFinder 2.1 (<https://cge.food.dtu.dk/services/PlasmidFinder/>; 33) servers

Plasmid characterization and conjugation assays

Plasmid DNA of the clinical isolates *E. coli* 172D10 was extracted using the Kieser method (34). The Kieser extract from *E. coli* 172D10 isolate was used to transform *E. coli* TOP10 strain by electroporation. The electroporants were plated on a TSA plate containing 100 µg/ml ampicillin. Transformants were analyzed by PCR using the primers OXA-48A and OXA-48B (5'-TTGGTGGCATCGATTATCGG-3' and 5'-GCCATCACAAAAGAAGTGCTC-3', respectively). From the transformants harboring the *bla*_{OXA-484} gene, plasmid DNA extraction was

done using the Kieser's method and subsequently analyzed on 0.7% agarose gel stained with ethidium bromide. Plasmids of ca. 154, 66, 48, and 7 kb of *E. coli* NCTC 50192 were used as plasmid size markers (30).

Filter mating-out assay, between *E. coli* 172D10 and *E. coli* J53 as recipient was done as previously described (oxa-517, Dabos). Transconjugants were selected with 100 μ g/ ml ampicillin and 100 μ g/ ml azide.

β -Lactamase purification

An overnight culture of *E. coli* strain BL21 (DE3) harboring pET41b-OXA-484 was used to inoculate 2 L of LB broth containing 50 mg/L kanamycin. Expression and purification of OXA-484 was done as previously described (28). Protein concentration was determined by Bradford Protein assay (Bio-Rad, Marnes-La-Coquette, France) (28).

Steady-state kinetic parameters

Steady-state kinetic parameters of purified OXA-484 were determined at 30°C in 100 mM sodium phosphate buffer (pH 7.0). The k_{cat} and K_m values were determined by analyzing hydrolysis of β -lactams under initial-rate conditions with an ULTROSPEC 2000 model UV spectrophotometer (Amersham Pharmacia Biotech) using the Eadie–Hoffstee linearization of the Michaelis–Menten equation, as previously described (35). The β -lactams were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Comparative activation of OXA-484 by CO₂ was conducted by monitoring the rate of hydrolysis of penicillins in the same buffer supplemented with 50 mM sodium hydrogen carbonate (NaHCO₃) as the source of CO₂ (28).

Molecular modelling.

Molecular modelling was performed to evaluate the effects of the mutations on OXA-232 β -lactamase. Substitution of amino acid was performed by mutating the OXA-232 structure (PDB code 5HFO) *in silico* using the Dunbrack rotamer library (*swapaa* command) as a part of the UCSF Chimera software (36,37). The Dunbrack rotamer library predicts the conformation of the amino acid side-chain based on the global energy minimum of the protein. The identification of interatomic clashes based on VDW radii (38) was performed with UCSF Chimera software (36,37). Three-dimensional structures of the β -lactam ligands were generated using Corina 3.60

(Molecular Networks GmbH, Erlangen, Germany). Molecular docking calculations were performed using Gold (Cambridge Crystallographic Data Centre, Cambridge, UK) (39) and the GoldScore scoring function. The binding site, defined as a 20 Å radius sphere, was centered on the OG oxygen atom of Ser70. All other parameters had default values. The receptor-ligand complex images were produced using UCSF Chimera (36).

Nucleic acid sequences

To be submitted. Genbank accession numbers.

Acknowledgments

This work was supported by the Assistance Publique – Hôpitaux de Paris (AP-HP), the University Paris-Saclay, INSERM, and by grants from the French National Research Agency [ANR-19-AMRB-0004, ANR-20-PAMR-0010 and ANR-17-ASTR-0018]. LD, TN and BII are members of ESCMID Study Group for Antimicrobial Resistance Surveillance - ESGARS.

Transparency declarations

We have no competing interests to declare.

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Chapter III

“Novel antibiotic candidates”

AMR is a global health threat that is potentiated by the lack of novel antibiotics with novel targets. In addition, the development of antibiotics is long (usually 20 years) as compared to the sometimes rapid appearance of resistance (a few days). This is best exemplified with Daptomycin, discovered before 1990s, but Food and Drug administration (FDA) approved only in 2003.

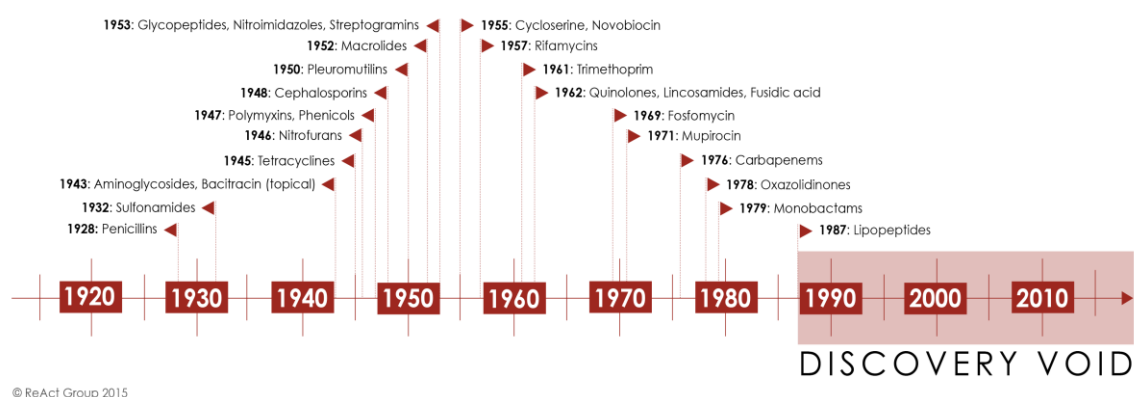


Figure 23: Timeline of the discovery of different antibiotic classes in clinical use. “The discovery void” refers to the period from 1987 until today, as the last antibiotic class that has been successfully introduced as treatment was discovered in 1987 (233).

According to WHO, the lack of novel antibiotics threatens global efforts to contain drug resistant infection. Several factors limit the development of new performant antimicrobials. The private investment in the development of such drugs is declining since the financial income is limited. It is time consuming and costly for the antibiotics to go through the different clinical trials and reach the market.

In addition, research and development for antibiotics is primarily driven by small- or medium-sized enterprises with large pharmaceutical companies continuing to exit the field .

For all these reasons, we are in desperate need of new antibiotics covering the priority list of pathogens set by the WHO, which includes carbapenem resistant *A. baumannii* and *P. aeruginosa* and carbapenem resistant / ESBL producing Enterobacterales.

Urgent and concerted investments in research and development by governments and the private sector are therefore needed to accelerate and expand the pipeline for antibiotics, especially those that can make an impact in low-resource settings, which are worst-affected by AMR.

We aim at characterizing the action of new classes of antibiotics from myxobacterial and actinobacterial strains in carbapenem resistant gram negative bacteria while potentiating their action by membrane-destabilizing antimicrobial peptides. This project is part of a French – German collaboration to optimize two novel antimicrobial families: Cystobactamids and chelocardins. Four derivatives of both compounds will be tested to escape resistance development over hundreds millions of years and which have been shown by one of us to be active against resistant clinical isolates. We focus on the development of new antibiotics inhibiting alternative bacterial targets and the understanding of their mechanism of action on a structural and biophysical level.

According to the WHO, many pathogens were listed as highly critical and were considered a priority to find antibiotics able to stop them due to the fact that they are becoming more and more resistant to almost all available therapeutic options.

The list comprises, carbapenem resistant *A. baumannii*, carbapenem resistant *P. aeruginosa*, carbapenem resistant Enterobacterales, and Enterobacterales resistant to third generation cephalosporins.

Cystobactamids and chelocardins are two promising candidates that could be released as two novel antibiotic families with broad activity against many pathogens including the ones listed by the WHO as critical. In the following, two derivatives of each family are tested on more than 200 bacterial isolates going from the most wild type bacteria to the most resistant ones to reveal the antibacterial activity of those compounds.

Several aspects will be studied:

- 1- Definition of activity spectrum and antimicrobial activity of the new compounds by testing combinations of new antibiotics on relevant clinical isolates and activity on MDR, and XDR Enterobacterales
- 2- Studying bacterial probability to resist the activity of compounds, definition of resistance frequency and compare resistant mutants genome to parental strain
- 3- Studying the effect of antibiotic exposure on bacterial response through RNA sequencing experiments



Article

In Vitro Activity of Two Novel Antimicrobial Compounds on MDR-Resistant Clinical Isolates

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Citation: Rima, M.; Pfennigwerth, N.; Cremanns, M.; Cirnski, K.; Oueslati, S.; Gatermann, S.G.; d'Amélio, N.; Herrmann, J.; Müller, R.; Naas, T. In Vitro Activity of Two Novel Antimicrobial Compounds on MDR-Resistant Clinical Isolates. *Antibiotics* **2023**, *12*, 1265. <https://doi.org/10.3390/antibiotics12081265>

Academic Editor: Alessia Carocci

Received: 29 June 2023

Revised: 24 July 2023

Accepted: 29 July 2023

Published: 31 July 2023



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Abstract: The development of novel antibiotics is mandatory to curb the growing antibiotic resistance problem resulting in difficult-to-treat bacterial infections. Here, we have determined the spectrum of activity of cystobactamids and chelocardins, two novel and promising classes of molecules with different modes of action. A panel of 297 clinically relevant Gram-negative and Gram-positive isolates with different antibiotic susceptibility profiles, going from wild type to multi- or even extremely drug resistant (MDR, XDR) and including carbapenem-resistant isolates, were tested using broth microdilution assays to determine the minimal inhibitory concentrations (MICs), MIC₅₀s and MIC₉₀s of two cystobactamids derivatives (CN-861-2 and CN-DM-861) and two chelocardin derivatives (CHD and CDCHD). Cystobactamids revealed potent activities on the majority of tested Enterobacteriales (MIC₅₀s ranging from 0.25 to 4 µg/mL), except for *Klebsiella pneumoniae* isolates (MIC₅₀s is 128 µg/mL). *Pseudomonas aeruginosa* and *Acinetobacter baumannii* showed slightly higher MIC₅₀s (4 µg/mL and 8 µg/mL, respectively) for cystobactamids. Chelocardins inhibited the growth of Enterobacteriales and *Stenotrophomonas maltophilia* at low to moderate MICs (0.25–16 µg/mL) and the chemically modified CDCHD was active at lower MICs. *A. baumannii* and *P. aeruginosa* were less susceptible to these molecules with MICs ranging from 0.5 to 32 µg/mL. These molecules show also interesting in vitro efficacies on clinically relevant Gram-positive bacteria with MICs of 0.125–8 µg/mL for cystobactamids and 0.5–8 µg/mL for chelocardins. Taken together, the cystobactamid CN-DM-861 and chelocardin CDCHD showed interesting antibiotic activities on MDR or XDR bacteria, without cross-resistance to clinically relevant antibiotics such as carbapenems, fluoroquinolones, and colistin.

Keywords: MDR; carbapenemase-producing Enterobacteriales CPE; antimicrobial; in vitro susceptibility testing

1. Introduction

Antimicrobial resistance (AMR) continues to increase, rendering the treatment of life-threatening infections with conventional antibiotics ineffective and rising the call to

search for new antibiotics able to overcome the spread of this dangerous threat [1]. The rapid spread of AMR can be attributed to the unnecessary use of antibiotics, improper administration, and over-the-counter availability in some countries, along with their miss- and overuse in veterinary and agriculture fields. Additionally, the lack of novel antibiotics, relying heavily on academic research laboratories and/or small companies, due to the “big” pharmaceutical companies dropping out of antibiotic research, is potentiating the existing crisis [2,3]. However, despite all these challenges, from 2017 until 2021, more than 40 antibiotics entered clinical phase I, II, and III trials. Unfortunately, due to high attrition rates, only a minority of these will be marketed [4]. Focus of research and development of new antibiotics is now shifting towards finding molecules with (i) lower ability to develop resistance; (ii) finding novel targets to avoid cross-resistance with existing antibiotics; (iii) finding molecules with selective toxicity not to harm the host; and (iv) avoiding efflux problems especially with molecules dedicated to Gram-negative bacteria [5].

Cystobactamids and chelocardins are two novel antibiotic families that exhibit promising antibacterial activities [6,7]. Cystobactamids constitute a novel class of antibiotics putatively acting by inhibiting bacterial type II topoisomerases [8]. They are non-ribosomally synthesized molecules derived naturally from myxobacteria whose derivatives are still under investigation [9,10]. According to recent studies, CN-861-2, a synthetic cystobactamid derivative, showed very potent antibacterial activity [9,10]. Structural modification led to the synthesis of another cystobactamid derivative, CN-DM-861 [10], with improved antibacterial activity. The second family of molecules investigated in this work are chelocardins, originating from *Amycolatopsis sulphurea* [11]. Chelocardins represent atypical broad-spectrum tetracyclines and have the advantage of acting on tetracycline-resistant isolates. Their exact mode of action is not fully understood, but it is believed that they act on bacterial membranes [12]. Several derivatives have been described to overcome the natural chelocardin resistance [13]. CDCHD, an amidochelocardin, is a promising compound derived from the natural chelocardin CHD, able to act on larger number of bacterial species including *Pseudomonas* spp. [13]. This study aimed to characterize the action of two novel compounds isolated from myxobacterial and actinobacterial strains on resistant bacteria, and especially Gram-negatives (ESKAPE pathogens) resistant to carbapenems and colistin, and carbapenemase-producing Enterobacterales CPE, of which some are listed by the WHO as high priority species for which new antibiotics are urgently needed. We have investigated the antimicrobial activities of two derivatives of cystobactamids (CN-861-2 and CN-DM-861) and of chelocardins (CHD, CDCHD) against multi- and even extremely drug-resistant (MDR and XDR) clinically relevant bacterial isolates.

2. Results

2.1. Activity on Reference Strains

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs), determined for reference strains, can be found in Table 1. Bacteria were more susceptible to CN-DM-861 and CDCHD, compared to CN-861-2 and CHD, respectively, thus confirming the importance of chemical modifications. Low MICs for the four compounds were found for *Escherichia coli* ATCC25922, whereas high MICs were found for *Klebsiella pneumoniae* reference isolates (MICs ranging from 0.5 µg/mL to 64 µg/mL for cystobactamids and 4 µg/mL to 32 µg/mL for chelocardins). All the other bacteria had MICs ≤1 µg/mL for CN-DM-861, but higher MICs for CDCHD chelocardins (≤4 µg/mL). Based on their MICs and MBCs, cystobactamids are bactericidal, while chelocardins act more bacteriostatic for most species tested.

2.2. Activity on Gram-Positive Isolates

Gram-positive isolates tested are indicated in Table 2. Most of the isolates are MDR and displayed low MICs when treated with cystobactamids (ranging from 0.125 µg/mL to 8 µg/mL) or chelocardins (ranging from 0.5 µg/mL to 8 µg/mL), except for *Nocardia* and

Corynebacterium sp., for which both cystobactamids and chelocardins display comparable MIC ranges, 8–128 µg/mL and 2–8 µg/mL (Table 2), respectively.

Table 1. MICs and MBCs for cystobactamids and chelocardins for reference strains.

Reference Strains	MIC (µg/mL)				MBC (µg/mL)			
	CN-861-2	CN-DM-861	CHD	CDCHD	CN-861-2	CN-DM-861	CHD	CDCHD
<i>Escherichia coli</i> ATCC25922	0.06	0.0019	0.5	0.25	0.125	0.015	2	2
<i>Klebsiella pneumoniae</i> ATCC15380	4	0.5	32	4	ND ^a	ND	ND	ND
<i>Klebsiella pneumoniae</i> ATCC700603	64	32	8	4	512	256	16	16
<i>Enterobacter cloacae</i> ATCC13047	8	0.125	2	1	ND	ND	ND	ND
<i>Staphylococcus aureus</i> ATCC25923	0.25	0.25	2	4	0.5	0.25	16	8
<i>Staphylococcus aureus</i> ATCC29213	0.125	0.5	2	2	0.5	1	256	4
<i>Staphylococcus aureus</i> NCTC12493	≤0.06	≤0.06	1	1	0.06	0.125	8	2
<i>Enterococcus faecalis</i> ATCC29212	0.25	0.25	2	1	1	1	512	128
<i>Pseudomonas aeruginosa</i> ATCC27853	0.5	0.25	8	4	1	2	512	32
<i>Acinetobacter baumannii</i> ATCC17978	2	1	8	2	ND	ND	ND	ND

^a ND: not determined.

Table 2. MICs of tested Gram-positive isolates.

Species	# of Tested Isolates	MICs (µg/mL)			
		CN-861	CN-DM-861	CHD	CDCHD
<i>Streptococcus pneumoniae</i>	2	0.25–2	0.5	0.5–4	2–4
<i>Enterococcus faecalis</i> ATCC29212	1	0.25	0.25	1	2
<i>Enterococcus faecium</i> vanA	4	1–4	1–8	4–8	2–4
<i>Enterococcus faecium</i> vanB	2	0.5	0.5–2	4–8	2–4
<i>Enterococcus faecium</i> Tet(M)	2	≤0.125–0.5	≤0.125–0.25	4–8	2–8
<i>Staphylococcus capitis</i>	1	0.25	0.125	2	1
<i>Staphylococcus aureus</i> ATCC25923	1	0.25	0.25	1	4
<i>Staphylococcus aureus</i> ATCC29213	1	0.25	0.5	2	2
<i>Staphylococcus aureus</i> NCTC12493	1	0.5	≤0.125	1	1
<i>Staphylococcus aureus</i> mecA	2	≤0.125	≤0.125	4	4
<i>Staphylococcus aureus</i> mecC	1	≤0.125	0.5	4	4
<i>Staphylococcus epidermidis</i> mecA	3	≤0.125	≤0.125	2–4	2–4
<i>Corynebacterium</i> sp.	1	8	8	1	0.5
<i>Nocardia asteroides</i>	3	64–>128	>128	4–8	2–4
<i>Nocardia farcinica</i>	1	>128	>128	8	4

2.3. Activity on Enterobacterales

The majority of Enterobacterales strains tested were MDR, as revealed by susceptibility testing against conventional antibiotics including carbapenems (Table 3). Eighty percent of these Enterobacterales expressed a carbapenemase belonging mostly to the big 5: *Klebsiella pneumoniae* carbapenemase KPC, oxacillinase OXA-48, New Delhi metallo β-lactamase NDM, Verona integron-encoded metallo β-lactamase VIM, imipenemase IMP, but also to minor carbapenemases (German imipenemase GIM, LMB, TMB, OXA-198, etc.). These isolates were either from the French and German National Reference Centers (NRC) for Antimicrobial Resistance (AMR), the clinical bacteriology laboratory of the Bicêtre Hospital or ATCC strains (Table 1).

Table 3. MIC50 and MIC90 of cystobactamids (CN-861-2, CN-DM-861) and chelocardin (CDCHD and CHD) in Enterobacteriales.

Species	n	MIC Range [MIC50] (µg/mL)				Percentage of Resistant Isolates (%)																	
		CN-861-2	CN-DM-861	CHD	CDCHD	CN-861-2	CN-DM-861	CDCHD	CHD	AMX	AMC	PRL	IMP	MER	ERT	CAZ	CTX	FEP	FF	CIP	LEV	TGC	CT
<i>E. coli</i> ^a	50	0.06–256 [0.25]	0.0019–256 [0.25]	0.25–32 [2]	0.25–16 [1]	2	2	16	8	100	100	100	68	81	87	100	96	100	25	ND	81	12.5	43
<i>K. pneumoniae</i> ^b	56	0.5–256 [128]	0.25–256 [128]	0.5–32 [8]	0.5–32 [2]	256	256	16	32	100	100	100	33	90	100	100	100	95	90	ND	90	81	46
<i>K. oxytoca</i> ^c	22	0.25–4 [0.25]	0.25–2 [0.25]	0.25–32 [0.5]	0.25–32 [1]	2	2	8	16	100	28	100	0	ND	0	28	30	23	ND ⁿ	7	7	ND	ND
<i>K. variicola</i> ^d	5	0.5–64 [8]	0.25 [0.25]	0.5–2 [1]	1–2 [1]	16	0.25	2	2	100	0	100	0	ND	0	0	0	0	ND	0	0	ND	ND
<i>Enterobacter</i> sp. ^e	28	0.25–64 [1]	0.25–1 [0.25]	1–16 [4]	0.5–32 [4]	64	2	16	16	100	100	100	72	72	90	100	100	90	72	ND	63	9	45
<i>C. freundii</i> ^f	20	0.25–1 [0.25]	0.25–1 [0.25]	1–16 [4]	0.5–4 [1]	1	1	4	8	100	100	100	87	87	100	100	100	100	6	ND	93	12.5	62.5
<i>M. morganii</i> ^g	7	2–64 [4]	0.5–1 [1]	4–16 [4]	2–32 [8]	64	1	32	16	100	100	100	100	100	100	100	100	100	ND	ND	25	100	
<i>Salmonella</i> sp. ^h	11	0.25–5 [0.5]	0.25–5 [0.25]	0.5–4 [2]	0.25–2 [1]	0.25	0.25	2	4	ND	40	0	0	0	0	ND	0	0	40	20	ND	ND	ND
<i>S. marcescens</i> ⁱ	12	1–128 [16]	0.25–32 [0.25]	0.25–4 [2]	0.5–32 [2]	128	32	32	4	100	100	100	80	80	100	100	100	100	40	ND	100	0	80
<i>S. flexneri</i> ^j	1 ^o	0.5	0.5	2	2				0	0	0	0	0	0	0	0	0	0	0	0	0	ND	ND
<i>K. aerogenes</i> ^k	4 ^o	0.5–8	ND	2–16	8–16				ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
<i>C. frakii</i> ^l	2	0.25–4	0.25–2	4–16	8				ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
<i>P. stuartii</i> ^m	1	128	128	4	4				ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
<i>P. mirabilis</i> ⁿ	4	8–32	8–32	1–4	4–16				ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0	0	100	0	100

Number of isolates with susceptibility testing results for conventional antibiotics: ^a, 16; ^b, 23; ^c, 14; ^d, 5; ^e, 11; ^f, 16; ^g, 4; ^h, 10; ⁱ, 5; ^j, 1; ^k, 0; ^l, 1; ^m, 4; ⁿ, ND: no data; ^o, for species with less than five tested isolates. MIC₅₀ and MIC₉₀ were not calculated; rather, MIC or a range of MIC values are presented. Antibiotic abbreviations: AMX: amoxicillin; AMC: co-amoxiclav; PRL: piperacillin; IMP: imipenem; MER: meropenem; ERT: eritapenem; CAZ: ceftazidime; CTX: cefotaxime; FEP: cefepime; FF: Fosfomycin; CIP: ciprofloxacin; LEV: levofloxacin; TGC: tigecycline; CT: colistin. ND: not determined.

MIC₅₀ for CN-861-2 and CN-DM-861 with *Klebsiella pneumoniae* isolates were very high (>128 µg/mL), while for CHD and CDCHD they were lower, 8 µg/mL and 2 µg/mL, respectively (Table 3). *Klebsiella oxytoca* (*n* = 22) and *Klebsiella variicola* (*n* = 5) were more susceptible to all compounds with MIC₅₀s ≤ 1 µg/mL except for *K. variicola* MIC₅₀ that displayed an MIC of 8 µg/mL for CN-861-2 (Table 3). MIC₅₀s for the 50 tested *E. coli* isolates were ≤ 2 µg/mL for the four molecules. *Salmonella* and *Shigella* displayed low MICs to cystobactamids and slightly higher values for chelocardins (Table 3). MIC₅₀s of *Enterobacter* and *Citrobacter* species were 1 µg/mL for cystobactamids, but slightly higher for chelocardins (4 µg/mL). Variable results were found for *Serratia marcescens* and *Proteus mirabilis*. One tested *Proteus stuarti* isolate showed much higher MICs for both compounds with values of 128 µg/mL for CN-861-2, 4 µg/mL for CHD, and 8 µg/mL for CDCHD. In general, lower MIC₅₀ values were found for the two chemically modified compounds CN-DM-861 and CDCHD as compared to their parental molecules.

2.4. Activity on Non-Fermenters

Most of the tested isolates were multidrug resistant and produced at least one β-lactamase, including carbapenemases and are challenging in terms of antimicrobial therapy. Regarding *Pseudomonas aeruginosa*, intermediate MIC values were found for cystobactamids, with MIC₅₀ of 4 µg/mL and 2 µg/mL, and MIC₉₀s of 8 µg/mL for both compounds (Table 4). For chelocardins, MIC₅₀s were high (>32 µg/mL) ranging from 16 µg/mL to 256 µg/mL. For *Acinetobacter baumannii*, variable MIC values were found for cystobactamids ranging from 0.25 µg/mL to 256 µg/mL with MIC₅₀ of 8 µg/mL and 4 µg/mL and MIC₉₀ of 128 µg/mL and 128 µg/mL for CN-861-2 and CN-DM-861, respectively (Table 4). For chelocardins, high MIC values were found, ranging from 4 µg/mL to 64 µg/mL. Concerning *Achromobacter xylosoxidans*, isolates have high MICs against cystobactamids (>128 µg/mL) and slightly lower values for chelocardins (16–32 µg/mL). *Stenotrophomonas maltophilia* had variable MICs, with values of MIC₅₀ of 4 µg/mL and 2 µg/mL and values of MIC₉₀ of 128 µg/mL and 32 µg/mL for CN-861-2 and CN-DM-861, respectively. It also showed relatively intermediate values for chelocardins ranging from 0.5 to 32 µg/mL. *Burkholderia cepacia* showed high MIC of 256 µg/mL for cystobactamids and intermediate MICs of 8 µg/mL and 4 µg/mL for CHD and CDCHD, respectively (Table 4).

Table 4. MIC₅₀ and MIC₉₀ of cystobactamids CN-861-2, CN-DM-861, and chelocardins CDCHD, and CHD on non-fermenters.

Species	<i>n</i>	MIC Range [MIC ₅₀] (µg/mL)				MIC ₉₀ (µg/mL)			
		CN-861-2	CN-DM-861	CHD	CDCHD	CN-861-2	CN-DM-861	CHD	CDCHD
<i>P. aeruginosa</i>	13 ^a	0.5–256 [4]	0.5–32 [2]	16–256 [64]	32–256 [32]	8	8	256	256
<i>A. baumannii</i>	22 ^a	0.5–256 [8]	0.25–256 [4]	16–64 [8]	2–64 [2]	128	128	32	64
<i>S. maltophilia</i>	7 ^a	0.5–256 [4]	0.5–64 [2]	0.5–32 [1]	0.5–4 [1]	128	32	16	4
<i>A. xylosoxidans</i>	4 ^b	128	128	16–32	4–16				
<i>B. cepacia</i>	2 ^b	256	128–256	8	4–8				

^a *n*: number of isolates tested; ^b for species with less than five tested isolates, MIC₅₀ and MIC₉₀ were not calculated; rather, a range of MIC values are presented.

2.5. Colistin-Resistant Isolates

Since colistin has become one of the last options for treating carbapenem-resistant Gram-negative bacterial infections, we aimed to assess if any cross-resistance with either cystobactamids or chelocardins could be found in colistin-resistant isolates (Table 5). For *K. pneumoniae*, MICs for cystobactamids were high, irrespective of the resistance to colistin (Tables 3 and 5), while for *E. coli*, MICs for cystobactamids remained low, irrespective of the colistin resistance mechanism.

Table 5. MICs for cystobactamids and chelocardins against colistin-resistant *K. pneumoniae* and *E. coli* strains.

Species	# of Isolates	Resistance Mechanism	MIC Range [MIC50] (µg/mL)				
			CN-861-2	CN-DM-861	CHD	CDCHD	Colistin
<i>K. pneumoniae</i>	5	<i>mgrB</i> mutation	256	256	0.5–8 [4]	4–32 [16]	8–128 [64]
<i>E. coli</i>	8	Plasmid-mediated resistance (<i>mcr-1</i>)	0.25–16 [0.25]	0.25–4 [0.25]	0.25–4 [0.5]	0.25–8 [8]	4–8 [4]
<i>E. coli</i>	11	Chromosomal resistance to colistin ^a	0.25–32 [0.25]	0.25–2 [0.25]	0.5–4 [1]	0.25–16 [0.5]	4–16 [4]

^a Mutations included frameshifts, *ISKpn7* insertions, and complete *MgrB* deletion.

As chelocardins are thought to also target the bacterial membrane [11], cross-resistance with colistin is a possibility. However, MICs for chelocardins remained low on colistin-resistant bacteria, thus ruling out any cross-resistance, and suggesting different mode of actions than that of colistin.

2.6. Fluoroquinolone-Resistant Isolates

Since cystobactamids are known to have the same target as fluoroquinolones, studying those compounds on fluoroquinolone resistant isolates allowed us to address possible cross resistance mechanism between these compounds. Seventeen fluoroquinolone-resistant Enterobacterales were tested, and the results are shown in Table 6. Except for *K. pneumoniae*, all the other tested isolates had low MICs to cystobactamids while exhibiting a high level of fluoroquinolone resistance. Our results suggest that cystobactamids may be active on fluoroquinolone resistant Enterobacterales, except for *K. pneumoniae*, which have high MIC for cystobactamids irrespective of additional resistance mechanism, suggesting a different mode of action for cystobactamids compared to fluoroquinolones.

Table 6. MICs of fluoroquinolone-resistant Enterobacterales tested for cystobactamids and chelocardins.

Species	Isolate #	β-lactam Resistance	MIC Range [MIC50] (µg/mL)					
			CN-861-2	CN-DM-861	CHD	CDCHD	CIP ^a	LEV ^b
<i>K. pneumoniae</i>	4	2 NDM, 1 CTX-M-15, 1 OXA-48	16–>128 [>128]	2–>128 [>128]	4–32 [32]	2–8 [2]	>4	>4
<i>K. oxytoca</i>	1	OXA-48 LIKE	<0.25	<0.25	8	2	>4	>4
<i>E. coli</i>	8	6 NDM, 2 CTX-M-15	<0.25	<0.25	1–8 [2]	0.5–2 [0.5]	>4	>4
<i>E. cloacae</i>	3	1 Hyper-AmpC, 1 VIM, and 1 OXA-48	<0.25	<0.25	1–16 [1]	0.5 [1]	>4	>4
<i>C. freundii</i>	2	OXA-48 LIKE	<0.25–0.5	<0.25–0.5	8–16	1	>4	>4
<i>C. brachii</i>	1	OXA-48 LIKE	<0.25	<0.25	4	2	>4	>4

^a ciprofloxacin; ^b levofloxacin.

3. Discussion

Despite enormous efforts in the last decade, AMR still represents one of the most challenging problems in healthcare systems worldwide. It is predicted that, unless significant measures are taken, AMR will cause 300 million deaths and a loss of 100 trillion dollars by 2050 [14]. Resistance acquisition and the mechanisms elaborated by bacteria to escape the activity of broadest antibiotics along with the absence of new antibiotics should not be ignored. Consequently, identification and characterization of novel antibiotics is of utmost importance. In this study, we aimed to characterize the action of two new classes of antibiotics from myxobacterial and actinobacterial strains regarding their potential to act on resistant bacteria, and especially Gram-negatives (ESKAPE pathogens) resistant to carbapenems and colistin, of which some are listed by the WHO as high priority species for which new antibiotics are urgently needed [15]. Especially carbapenem-resistant *A. baumannii*, carbapenem-resistant *P. aeruginosa*, and carbapenem-resistant Enterobacterales are of concern.

Cystobactamids and chelocardins are novel antibacterial compounds that showed promising activity on subsets of bacterial isolates, and thus represent potential drug candidates [6,9,10,16–18]. As no clinical breakpoints are available for these compounds, the different MIC values obtained using broth microdilution were classified into three categories: species with low MICs (≤ 2 $\mu\text{g/mL}$); (ii) species with moderate MICs (ranging from 2 to 16 $\mu\text{g/mL}$); and (iii) species with high MICs (above 16 $\mu\text{g/mL}$) (Table 7).

Table 7. Activity spectrum of cystobactamids (CN-861-2 and CN-DM-861) and chelocardins (CDCHD and CHD) on Gram-negative bacteria.

MICs	Cystobactamids		Chelocardins	
	CN-861-2	CN-DM-861	CDCHD	CHD
MIC ≤ 2 $\mu\text{g/mL}$	<i>K. oxytoca</i>	<i>K. oxytoca</i>	<i>K. oxytoca</i>	<i>K. oxytoca</i>
		<i>K. variicola</i>	<i>K. variicola</i>	
	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>
	<i>C. freundii</i>	<i>C. freundii</i>	<i>C. freundii</i>	
	<i>E. cloacae</i>	<i>E. cloacae</i>	<i>E. cloacae</i>	
	<i>Salmonella</i>	<i>Salmonella</i>	<i>Salmonella</i>	<i>Salmonella</i>
	<i>Shigella</i>	<i>Shigella</i>	<i>Shigella</i>	<i>Shigella</i>
		<i>M. morganii</i>		
			<i>S. maltophilia</i>	<i>S. maltophilia</i>
				<i>P. mirabilis</i>
2 $\mu\text{g/mL}$ < MIC ≤ 16 $\mu\text{g/mL}$				<i>P. stuarti</i>
				<i>C. freundii</i>
				<i>E. cloacae</i>
	<i>K. variicola</i>		<i>K. pneumoniae</i>	<i>K. pneumoniae</i>
	<i>M. morganii</i>		<i>M. morganii</i>	<i>M. morganii</i>
	<i>P. mirabilis</i>		<i>P. mirabilis</i>	
			<i>P. stuarti</i>	
	<i>P. aeruginosa</i>			
	<i>A. baumannii</i>	<i>A. baumannii</i>	<i>A. baumannii</i>	<i>A. baumannii</i>
	<i>S. maltophilia</i>		<i>N. asteroides</i>	
MIC > 16 $\mu\text{g/mL}$			<i>N. farcinica</i>	<i>N. farcinica</i>
			<i>B. cepacia</i>	<i>B. cepacia</i>
	<i>K. pneumoniae</i>	<i>K. pneumoniae</i>		
	<i>P. stuarti</i>			
			<i>P. aeruginosa</i>	<i>P. aeruginosa</i>
	<i>B. cepacia</i>			
	<i>N. asteroides</i>	<i>N. asteroides</i>		
	<i>N. farcinica</i>	<i>N. farcinica</i>		

Even though the mechanism of action is not completely elucidated, cystobactamids seem to act on bacterial gyrase thus inducing bacterial death by DNA synthesis inhibition [16,19,20]. Several derivatives have been chemically synthesized with improved activity, including the investigated CN-861-2 and CN-DM-861 [9], which were tested on a highly diverse panel of 297 bacterial isolates, mostly from clinical sources. The vast majority of Enterobacterales including *E. coli*, *K. oxytoca*, *K. variicola*, *C. freundii*, *C. braakii*, *E. cloacae*, *S. marcescens*, *Salmonella* sp., and *S. flexneri* had MICs ≤ 2 $\mu\text{g/mL}$ (Tables 3 and 6). *K. pneumoniae* seems to resist to the activity of cystobactamids, which could be due to the distinct capsular features of this species or due to intrinsic resistance mechanisms, as suggested for albicidin, an analogue of cystobactamids [20–22]. *Morganella morganii* and *Proteus* were among the bacteria having high MICs for cystobactamids. This could be due to the specificities of the gyrase, or to outer membrane permeability problems. Nevertheless,

finding derivatives of cystobactamids to bridge the species activity gap is important, as they are known to have reduced susceptibility to carbapenems and are naturally resistant to colistin. Moderate activity was shown for *P. aeruginosa* with MICs ranging from 0.5 to 32 µg/mL. *A. baumannii* seems to escape the action of cystobactamids despite the presence of a few strains having low to moderate MIC values (Table 4). Overall, the activity of cystobactamids on non-fermenting Gram-negative bacteria was not as potent. *S. maltophilia* showed diverse MIC values according to the tested strains, with MICs ranging from 0.5 to 256 µg/mL. Even though susceptibility results for clinically relevant antibiotics were available, no link between a given phenotype and higher MIC values of cystobactamids could be established. For *B. epacian*, both tested strains showed high MICs. As AMR in Gram-positive bacteria is also a growing concern, a few representatives of each species were included in the testing panel of isolates, among which *S. aureus*, *S. capitis*, and *E. faecalis* have low MICs to cystobactamids, unlike *S. pneumoniae*, *Corynebacterium* sp., *N. asteroides*, and *N. farcinica*. For all tested strains, CN-DM-861 showed better activity than CN-861-2, suggesting that it may be a promising lead that, however, still requires some optimization to include *K. pneumoniae*. As cystobactamids and fluoroquinolones have the same bacterial target, cross-resistance was something thought possible. Although many tested strains were resistant to fluoroquinolones, no obvious cross-resistance could be recorded. A recent study by Michalczyk E et al. showed that the binding of albicidin, an analog of cystobactamids, occurs at a different site than fluoroquinolones on the DNA gyrase, thus explaining the absence of cross-resistance [20]; a similar observation can be extrapolated to cystobactamids, although the exact binding site of cystobactamids to gyrase is not known. Thus, cystobactamids present an interesting alternative, especially for potential treatment of infections with fluoroquinolone-resistant bacterial isolates.

The second family of molecules investigated in this work are chelocardins. The activity of two chelocardin derivatives, the natural CHD and the amidochelocardin CDCHD, were tested on the same selection of MDR bacterial species. For Enterobacterales, including *K. pneumoniae*, moderate MIC values were found, with a slightly improved activity for CDCHD, suggesting that the chemical modification of CHD improved its activity. With *P. aeruginosa* strains, MICs for chelocardins were high and ranged from 32 to 256 µg/mL. The activity of these molecules on *A. baumannii* was similar, which is quite disappointing since the development of compounds that could inhibit those non-fermenters has become a necessity. Gram-positive strains showed moderate MICs that ranged from 1 µg/mL to 2 µg/mL of tested bacteria. Chelocardins are atypical tetracyclines due to their similar structures. They constitute another promising class of molecules, and no cross-resistance was found with other cyclines [17]. As the exact mode of action is still not fully understood and published studies indicate they target bacterial membranes, possible cross-resistance with colistin was also investigated [11] and refuted, suggesting a different mode of action than that of colistin.

K. pneumoniae, *P. aeruginosa*, and *E. coli* are all Gram-negative bacteria exhibiting similar types of virulence factors [23]. They can form biofilms [24–26], exposing different O antigens on LPS molecules [27] and producing siderophores [28], all characteristics that have been related to the development of resistance [29]. Due to their complexity, it is hard to interpret the observed MICs considering their structural differences.

4. Materials and Methods

4.1. The Tested Compounds

The cystobactamids CN-861-2, CN-DM-861, and chelocardins CHD and CDCHD were provided by the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS)—Helmholtz Center for Infection Research, Germany.

4.2. Bacterial Isolates

A total of 297 bacterial isolates were selected including both Gram-negative and Gram-positive isolates. These isolates corresponded to ATCC strains displaying wild-

type phenotypes, but also highly drug resistant isolates that are challenging in terms of treatment options.

Bacterial isolates were selected from the French National Reference Center for Antibiotic Resistance (F-NRC), from the bacteriology ward of the Bicêtre Hospital, France and from the German National Reference Center for Multidrug-resistant Gram-negative Bacteria, Bochum, Germany (G-NRC). There were a total of 223 Enterobacterales (*Escherichia coli* ($n = 50$), *Klebsiella pneumoniae* ($n = 56$), *Klebsiella oxytoca* ($n = 22$), *Klebsiella variicola* ($n = 5$), *Citrobacter freundii* ($n = 20$), *Citrobacter braakii* ($n = 2$), *Klebsiella aerogenes* ($n = 4$), *Enterobacter* sp. ($n = 28$), *Serratia marcescens* ($n = 12$), *Salmonella paratyphi* ($n = 5$), *Salmonella tiphymurium* ($n = 2$), *Salmonella choleraesuis* ($n = 3$), *Salmonella* sp. ($n = 1$), *Shigella flexneri* ($n = 1$), *Morganella morganii* ($n = 7$), *Providencia stuarti* ($n = 1$), *Proteus mirabilis* ($n = 4$)); 48 non-fermentative Gram-negative isolates (*Pseudomonas aeruginosa* ($n = 13$) and *Acinetobacter baumannii* ($n = 22$); *Stenotrophomonas maltophilia* ($n = 7$), *Alcaligenes xylosoxidans* ($n = 4$), and *Burkholderia cepacia* ($n = 2$)); and 26 Gram-positive bacteria (*Staphylococcus capitis* ($n = 1$), *Staphylococcus aureus* ($n = 6$), *Staphylococcus epidermidis* ($n = 3$), *Streptococcus pneumoniae* ($n = 2$), *Enterococcus faecalis* ($n = 1$), *Enterococcus faecium* ($n = 8$), *Corynebacterium* sp. ($n = 1$), *Nocardia asteroides* ($n = 3$), and *Nocardia farcinica* ($n = 1$)).

4.3. Bacterial Identification and Susceptibility Testing of Isolates

Bacteria were identified using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF MALDI Biotyper system, Bruker Daltonics GmbH & Co. KG, Bremen, Germany). Microbroth dilution assays were used to test the selected isolates using U-bottom plates according to EUCAST MIC testing protocol and results were determined according to EUCAST guidelines, with a final bacterial concentration of 10^{-5} CFU and a total volume of 100 μ L in each well [30]. A wide range of concentrations was tested, going from 0.0019 μ g/mL to 256 μ g/mL. As no breakpoints are available for these molecules, MICs were classified into three categories: low (MICs ≤ 2 μ g/mL), intermediate (4–16 μ g/mL), and high (>16 μ g/mL).

4.4. Minimum Bactericidal Activity

Minimal bactericidal concentrations MBCs were determined as recommended by EUCAST [30]. MIC plate wells with antibiotic concentrations equal to or greater than the MIC values were spread on Mueller Hinton agar to determine MBC. MBC was defined as the lowest antibiotic concentration that results in less than 0.01% surviving bacteria.

5. Conclusions

Our results indicate that cystobactamids and chelocardins have interesting in vitro activity on a wide range of multidrug-resistant bacteria (Gram-positive and Gram-negative) and that there is no cross-resistance with conventional antibiotics, especially with fluoroquinolones, colistin, and cyclines. Molecules derived from chemical analysis, such as CN-DM-861 and CDCHD, have improved efficacies on the tested panel of MDR pathogens.

Despite small variation among different strains, our data show that the resistance to both chelocardins and cystobactamids is highly dependent on the species, at least in the case of some of the most important ESKAPE bacteria such as *K. pneumoniae*, *P. aeruginosa*, and *E. coli*. For these bacteria, similar MIC values were found for each antibiotic class, independently of the tested isolates.

Our study on isolates resistant to antibiotics targeting the bacterial membrane (colistin) or the DNA gyrase (fluoroquinolones) was aimed at highlighting a possible cross-resistance, which would have suggested the main bacterial target of chelocardins and cystobactamids, respectively. However, even though cystobactamids have been suggested to interact with bacterial gyrase, no cross-resistance was observed with fluoroquinolones. Similarly, even though chelocardins are believed to interact with the bacterial membrane, no cross-resistance was observed with colistin. The reasons may be complex, as fluoroquinolones are structurally different from cystobactamids and may target different regions of DNA

gyrase, as observed for albicidin, a related class of molecules [23]. Chelocardins are also very different from colistin and their structural similarity to tetracycline suggests that they may also target the bacterial ribosome. Structural studies and further investigations (transcriptomics and sequencing of resistant strains) will be needed to obtain deeper insight into the molecular mechanisms accounting for the activity of these interesting classes of antibacterial molecules.

Author Contributions: Conceptualization, T.N., J.H., R.M., S.G.G., K.C. and N.d.; methodology, T.N. and N.P.; validation, T.N. and N.P.; formal analysis, M.R. and M.C.; investigation, M.R. and M.C.; resources, T.N. and N.P.; writing—original draft preparation, M.R. and T.N.; writing—review and editing, all authors; supervision, T.N., S.O. and N.P.; funding acquisition, T.N., J.H., R.M., S.G.G. and N.d. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by NATURAL-ARSENAL (ANR-19-AMRB-0004/01KI2125B). This work has been supported as part of France 2030 program “ANR-11-IDEX-0003”, and the OI HEALTHI of the Université Paris-Saclay.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Raw data are available upon request.

Acknowledgments: We would like to thank Anja Kaminski for excellent technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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Cystobactamids efficiently kill multi-drug resistant *Escherichia coli* and induce a novel QseBC-regulated resistance mechanism leading to LPS modifications

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Abstract

Due to steadily increasing antimicrobial resistance and the shortage of novel antimicrobials reaching the market, the need to evaluate and develop new antibacterial agents is higher than ever. In the present study, we undertook an extensive microbiological and mechanistic evaluation of a novel natural compound class, the cystobactamids, acting as potent inhibitors of bacterial gyrase with IC₅₀ values in the sub-μM range. We focused our research on *Escherichia coli*, an important nosocomial pathogen, and could demonstrate that cystobactamids efficiently kill multi-drug resistant (MDR) *E. coli* with high potency. In vitro studies revealed a bactericidal mechanism with a rapid onset of action. Cystobactamid-resistant *E. coli* mutants developed at a low frequency of resistance, 10^{-8} - 10^{-10} . Intriguingly, no mutations were found in *gyrA* or *parC* target genes and instead, all mutations were observed in QseBC, a virulence-regulating, quorum-sensing *E. coli* two-component system and resistance to cystobactamids did not induce a fitness cost in the *E. coli* mutants. RT-qPCR showed that exposing wildtype *E. coli* to cystobactamid did not result in a significant change in *qseBC* and *pmrAB* transcript levels. However, transcriptome analyses of resistant mutants revealed that the two-component systems QseBC and PmrAB are strongly interconnected and together they initiate a cascade of events leading to lipopolysaccharide (LPS) modifications. In case of cystobactamids, we observed lipid A modifications connected to the *arn* operon: addition of either one or two 4-amino-4-deoxy-L-arabinose units. Encouragingly, no cross-resistance with other antibiotic classes was observed, including fluoroquinolones and polymyxins (colistin).

The full manuscript cannot be provided due to it's confidentiality, RNAseq experiments performed on cystobactamid resistant mutants revealed interesting outcome. Part of the results can be found in the following pages.

Results

Transcriptomics reveals upregulation of genes responsible for LPS modification and downregulation of virulence genes. Deletion of *qseC* was reported to result in abnormal bacterial morphology, i.e. causing bacterial cells to be longer and wider [56]. Thus, we investigated whether similar morphological changes can be observed for cystobactamid-resistant *E. coli* mutants carrying SNPs in *qseB* and *qseC*. Scanning electron microscopy did not reveal any obvious changes of the bacterial surface (Fig S2). However, cells of resistant mutant #4, carrying a *qseC* P251S mutation, were significantly shorter, wider, and had a larger diameter than wildtype cells. No statistically significant changes in cell dimensions were detected for cells of resistant mutant #3 carrying the *qseB* A80V mutation (Fig 8).

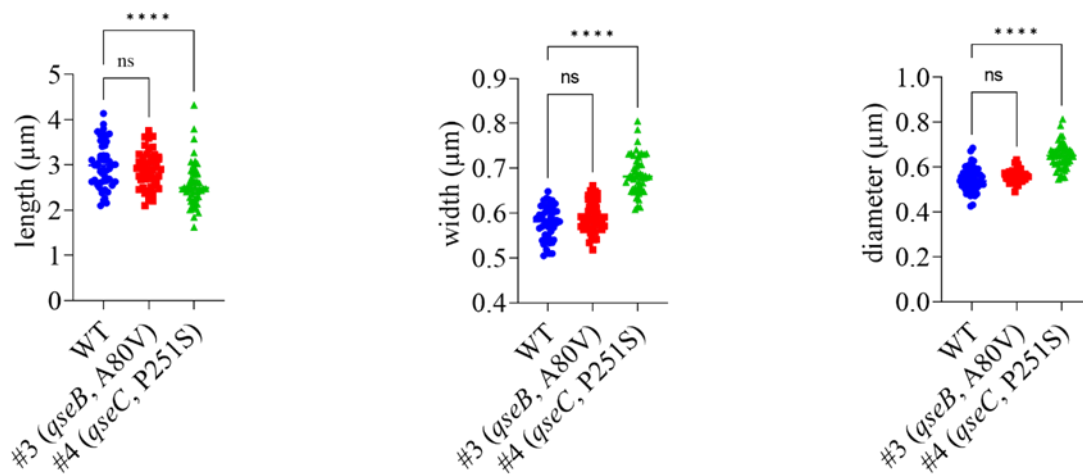


Figure 8. Cell dimensions assessment on selected cystobactamid CN-DM-861 resistant mutants of *E. coli* ATCC-25922 $\Delta tolC$ and wildtype. Length (left), width (middle), and (right) diameter. Ordinary one-way ANOVA was used to analyze the results. ns, not significant; ****, $p < 0.001$.

The results from RT-qPCR experiments were ineffective in providing hints on *qseBC* interactions with *pmrAB* and the *mar* operon, both already linked to antibiotic resistance in the literature. Additionally, changes in transcript levels of *pmrB* and *marR* in the cystobactamid-resistant *E. coli* mutant #3 (*qseB*, A80V) and #4 (*qseC*, P251S) were not associated with cross-resistance to other antibiotic classes. Thus, the exact mechanism of resistance of cystobactamids in *E. coli* remained unclear, and we thought to study the global transcriptome changes of selected *E. coli* mutants. The transcriptomes of several *E. coli* mutants that were obtained by selecting for resistance against cystobactamids [#1 (*qseC*, P251S), #2 (*qseC*, 259In), #6 (*qseC*, 441Fs), #7

(qseC, V257E)] and the qseC P251A mutant that was generated in a targeted approach were compared to wildtype *E. coli*. In summary, the mutants had 35 upregulated and 58 downregulated genes in common (Fig 9), and the full list of significantly up- or downregulated genes and operons can be found Table S12. Most prominently, we found the following genes or operons significantly upregulated: *arn* and *qse* operons responsible for synthesis and translocation of 4-amino-4-deoxy-L-arabinose (L-Ara4N) onto lipid A [57] and quorum-sensing, respectively [51]; *waaH* (implicated in incorporation of glucuronic acid to the LPS [58]), *ygiW* (implicated in stress response, co-transcribed with *qseBC* [59]), *ygiV* (gyrI-like small molecule binding domain protein, described in literature to confer resistance against albicidin in *Salmonella Typhimurium* and *E. coli* [60]), *eptA* and *cptA* (connected to the addition of phosphoethanolamine (pEtN) to lipid A and the core region of LPS, respectively [61]), *ugd* (involved in formation of L-Ara4N), and *ygiS* (implicated in stress response [62]). Despite the upregulation of genes involved in LPS modifications, transmission electron microscopy did not reveal obvious morphological changes of the outer or plasma membrane (Fig S3). In addition, several genes encoding hypothetical proteins were also found to be significantly upregulated.

The most significantly downregulated genes or operons included the *flg*, *fli* and *flh* operons, responsible for flagellar assembly [63], and the genes *motB* (flagellar assembly [63]), *yhjB* (putative response regulator [64]), and *yrbL* (Pho-regulatory network protein [65]). Based on the described functions of the upregulated genes, we concluded that cystobactamid resistance is likely caused by specific LPS modifications in a manner similar to colistin resistance despite the lack of cross-resistance between these two antibiotic classes. The downregulation of genes related to motility and virulence in *E. coli*, is fully in accordance with literature-described QseBC functions [66-68] and our findings from in vitro motility and biofilm assessments.

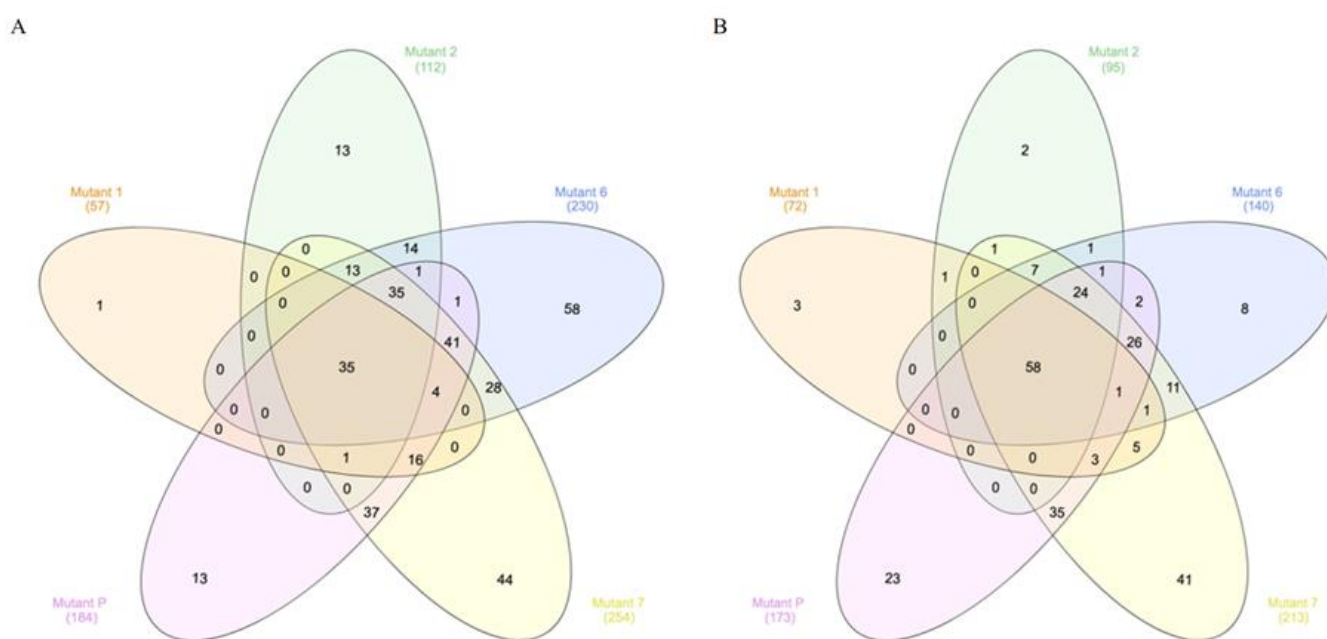


Figure 9: Venn diagram of upregulated and downregulated genes. (A) Upregulated genes, FC > 2. (B) Downregulated genes, FC < 0.5. Adjusted p-value < 0.05. mutant 1: qseC (P251S); mutant 2: qseC (259In); mutant 6: qseC (441Fs); mutant 7: qsec (V257E); mutant P: qseC (P251A). Mutants 1, 2, 6, and 7 were obtained during spontaneous resistance development, mutant P was achieved using seamless mutagenesis. In, insertion; Fs, frameshift.

Materials and methods

In vitro resistance development.

Strain glycerol stock was streaked out on a CASO agar plate and incubated overnight in a static incubator at 37 °C. Following day, a single colony was picked up and used to inoculate fresh Mueller-Hinton broth (MHB). Liquid culture was incubated at 37 °C at 180 rpm for approximately 16-18 hours. OD600 of overnight culture was determined and used to calculate the volume of culture needed to plate 5×10^9 CFU/plate. Bacterial load was confluent spread over the surface of the agar plate until it was completely soaked in. Plates were incubated in a static incubator for 24 hours at 37 °C. Frequency of resistance was determined at four times and eight times the MIC value. Total viable count was determined by serial dilutions of the overnight culture and plating it on non-selective CASO agar plates. Frequency of resistance was determined as ratio between the number of bacteria growing on selective plates and the number of bacteria in the inoculum. Obtained resistant clones were assessed to determine the MIC shift. RNA isolation and RT-qPCR.

Overnight cultures were diluted 1:100 in fresh medium and incubated at 37°C and 180 rpm until they reached OD600 0.5. Next, cultures that were not induced were directly subjected to RNA isolation. Induction was performed at OD600 0.5 and cultures were left on the shaker until they reached OD600 0.8-0.9. Exposure to CN-DM-861 was done at 0.5x MIC values. RNA isolation and cDNA synthesis was performed as described before [86]. Purity and concentration of RNA samples was determined using a Nanodrop spectrometer. Peqstar 96Q (Peqlab, Erlangen, Germany) cyclor was used to assess gene expression levels. cDNA was diluted 1:10 and primer pairs in S15 Table were used. qPCR was ran in 10 µL reactions using GoTaq Master Mix (Promega, Madison, WI, USA) in triplicates. Comparative threshold cycle ($\Delta\Delta C_t$) method [118] was used to determine relative mRNA quantity with *ifhB* normalization.

Transcriptomics.

RNA isolation and purification:

Overnight cultures of *E. coli* were diluted 100-fold in fresh MHB and allowed to grow to an OD600 of 0.5. 2 ml of each culture were taken and mixed with RNA protect according to the manufacturer's recommendations. Cells were immediately pelleted by a 10 min centrifugation at 5000 g. Biological triplicates were performed. RNA was extracted using GeneJet RNA extraction kit. DNase treatment was performed with the dsDNase kit according to the manufacturer's protocol. RNA concentration was measured using Qubit RNA BR assay kit, and RNA quality was evaluated using the Agilent 2100 Bioanalyzer RNA 6000 Nano Kit. RIN > 9 were obtained for all the RNA samples.

RNA library and sequencing:

A total of 250 ng of total RNA for each sample was used for the preparation of cDNA libraries. Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus was used according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The quality of the cDNA was validated using the Agilent 2100 Bioanalyzer DNA1000 kit (Agilent Technologies) and quantity was determined with the Qubit dsDNA BR Assay (ThermoFisher Scientific). Equimolar pool of RNA-Seq libraries was made to a final molarity of 2 nM, and 1.4 pM were loaded in the sequencer. Sequencing of the libraries was performed with an Illumina NextSeq 500 in single-read mode with 75 cycles, with 1% of PhiX intern control sequences.

RNASeq data analysis and differential expression analysis:

Raw sequencing data were demultiplexed and quality control was done using the Aozan tool [119]. An average of $23.4 \text{ M} \pm 4.8 \text{ M}$ reads per sample was obtained, with a Q30 of 92.6%.

PhiX sequences account for 1.85% of total reads, close to what was expected. Ribodepletion efficiency was assessed with FastQ Screen and was below 2% on average [120]. Reads generated from strand-specific RNA-seq experiments were aligned to the genome of *E. coli* ATCC-25922 genome (NZ_CP009072.1) by using the software Bowtie (version 0.12.7) [121]. Reads that mapped in more than four different positions on the genome were discarded i. e. reads corresponding to rRNA. RNA-seq data were analysed as described [122] by using Rsamtools (version 1.26.2), GenomicAlignments (version 1.10.1), GenomicFeatures (version 1.26.4) and DESeq2 (version 1.14.1) and SARTools [123] in R 3.3.1. Only adjusted p-values were used and were obtained using the Benjamini-Hochberg correction for false discovery rate [124]. Read count data were visually assessed using the Artemis genome viewer [125]. Venn diagrams were drawn using InteractiVenn [126].

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Response to Cystobactamid and Chelocardin Exposure in *Escherichia coli* ATCC25922 and mutant selection

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Keywords: Antibiotics, Cystobactamid, Chelocardin, RNAseq, resistance

Abstract (298)

In the light of the rising antimicrobial resistance (AMR) problem rendering conventional antibiotics inefficacious, novel compounds are desperately needed to overcome AMR. Despite the lack of discovery of novel classes of antibiotics, a few are going through clinical trials and are being tested as future antibiotic candidates with promising antibacterial activity. Cystobactamids and chelocardins are two promising novel molecules with potent activity on multidrug resistant (MDR) isolates. Here we have investigated the propensity of these molecules to select in vitro resistance to assess the frequency of resistance to those compounds as well as the genes that could be at the origin of resistance acquisition through comparative genomics. In addition, the study of the transcriptomic landscape following exposure to cystobactamids and chelocardins, of *E. coli* ATCC25922 was also investigated to identify regulatory events that may lead to resistance.

Cystobactamid resistant *E. coli* isolates were obtained at a frequency of 0.27×10^{-8} . Whole genome sequencing of these mutants and comparison to their parental strain revealed a high number of SNPs (117 SNPs as average), some of which are located in universal regulatory elements, such as , QseC, in colistin resistance genes, Arn Operon and pmrB, and in iron uptake operon, SitABCD.

Exposure of *E. coli* ATCC25922 for 10 min to 10x its MIC for both compounds and subsequent RNAseq experiments revealed increased expression of DNA repair, SOS response and iron metabolism after cystobactamid exposure, while genes implicated in processes like protein synthesis, TCA cycle, biotin synthesis, and motility were affected by chelocardin exposure. Both approaches revealed similar genes either mutated or upregulated, giving further hints about the potential targets of these molecules. Nevertheless, especially RNA seq experiments, remain preliminary and need further investigations as transcriptomic bacterial response is not so easy to control and limit to one condition.

Introduction

Even before the first use of penicillin to treat human infection, bacteria resistant to this molecule were described (1). The rapid emergence of resistant bacteria after clinical use, has led Alexander Fleming to warn that miss- and over-use will inevitably lead to antimicrobial resistance (AMR) (2). After more than 80 years of extensive use, AMR has become a serious global health threat affecting patients' lives by limiting the therapeutic options to treat

infections caused by multidrug resistant (MDR) bacteria (3, 4). This problem is potentiated by horizontal transmission of mobile elements carrying most of the genes able to confer antibiotic resistance (5).

Unfortunately, in parallel to the spread of AMR, a slowdown of development and commercialization of novel antibiotics with improved efficacy against MDR bacteria was witnessed, a consequence of high financial cost of new antibiotics R&D projects, short return of investment periods, and the appearance of bacterial resistance (6, 7). Interestingly, the number of immune-oncology agents in development is estimated at 4000 compounds, while, only 30 to 40 new antibiotic candidates are in the clinical trial phases of development (7, 8). In recent years, health authorities including WHO have realized how dramatic the situation of AMR has become providing even a list of pathogens for which urgently novel molecules are needed: carbapenem resistant *Acinetobacter baumannii* (CR-AB), carbapenem resistant *Pseudomonas aeruginosa* (CR-PA) and MDR Enterobacterales (8, 9). Many novel antibiotics are either active on Gram-positives (novel glycopeptides), are derivatives of existing molecules (cefiderocol) or are a combination of an existing β -lactam and an β -lactamase inhibitor, such as ceftazidime/avibactam, imipenem/relebactam, and meropenem/vaborbactam. These antibiotics have shown their usefulness as they allowed to treat infections that were considered a therapeutic dead end, but resistance has rapidly emerged (234). The resistance appearance is linked to selection of point mutations in specific genetic backgrounds that display already reduced susceptibility to the β -lactam antibiotics (ex PBP3 insertion in *E coli*). Thus, novel molecules with novel targets are desperately needed to overcome this problem. Cystobactamids and Chelocardins constitute two novel families with promising antimicrobial activities (13).

Cystobactamids, are novel myxobacteria derived compounds, they were isolated from *Cystobacter* and are found to act by inhibiting topoisomerases (14). From a structural point of view, those natural products are based on para-aminobenzoic acid (PABA) and seem to have a broad activity spectrum especially on gram-negative bacteria (15, 16). Since its discovery, researchers tried to find enhanced versions of the first natural cystobactamid product CN-861-2, through biosynthetic engineering, and the modifications lead to the discovery of CN-DM-861, having broader antimicrobial activity (17). Cystobactamids are characterized by their activity against gram negative pathogens including *P. aeruginosa*, fluoroquinolone resistant Enterobacterales and pathogens of the ESKAPE complex. As cystobactamids and fluoroquinolones have the same bacterial target, cross resistance was something feared, but no

obvious cross resistance could be recorded (13). A recent study by Michalczyk E et al. has shown that the binding of albicidin, an analog of cystobactamids occurs at a different site than fluoroquinolones on the DNA gyrase, thus explaining the absence of cross resistance (18), similar observation can be extrapolated to cystobactamids, although the exact binding site of cystobactamids to gyrase is not known. Thus, cystobactamids present an interesting alternative especially for potential treatment of infections with fluoroquinolone resistant bacterial isolates. Chelocardins (CHD), discovered in 1970s are atypical tetracyclines, due to their similar structures (19). They are naturally produced by *Amycolatopsis sulphurea*, and showed interesting activity against ESKAPE complex pathogens and no resistance was observed in *K. pneumoniae* against CHD, the natural chelocardin's derivative. Despite the high structural identity between CHD and tetracyclines, they are able to overcome the efflux and ribosomal resistance mechanisms to cyclines (20). As the exact mode of action is still not fully understood and published studies indicate they target bacterial membranes, possible cross resistance with colistin was also investigated and refuted suggesting a different mode of action than that of colistin.

Here we have used two complementary approaches to gain further insights in the mode of actions of these compounds. We thus investigated the effect of exposure of the novel compounds on *E. coli* ATCC25922, by studying its transcriptional response. In addition, we investigated the frequency at which a bacteria may become resistant, and through what gene mutation/inactivation.

Materials and methods

1- Compounds

The cystobactamids CN-861-2, CN-DM-861, and chelocardins CHD and CDCHD were provided by the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) – Helmholtz Center for Infection Research, Germany

2- Bacterial strains and MIC determination

A clinical *E. coli* isolate (O89E4) as well as an *E. coli* ATCC25922. The MICs of both strains against cystobactamids and chelocardins were determined by broth microdilution assays as previously described using U bottom plates according to EUCAST MIC testing protocol (13).

3- Mutants selection procedure and Frequency of resistance

For each parental strain, one single colony was subcultured over-night in Brain heart infusion media (BHI) at 37 °C . Hundred µl of each culture was grown on TSA plates supplemented with 1, 2 and 4X the MIC of cystobactamids. Plates were incubated over night at 37 °C, and on the subsequent day, single colonies were used to determine their MICs of cystobactamids and chelocardins . The frequency of resistance was calculated by dividing the number of colonies obtained on TSA supplemented with cystobactamids by the total number of colonies obtained on the same plates without cystobactamids supplementation.

4- Next generation sequencing and data analysis

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). Sequences were analyzed by CLC genomics and different mutants compared with the basic variant detection tool.

5- RNA isolation and purification:

Overnight cultures of *E. coli* ATCC25922 were diluted 100-fold in fresh BHI and allowed to grow to an OD600 of 0.5. For each antibiotic, two 2 ml of each culture were prepared, among which one contained CN-DM-861, and CDCHD were added at concentrations ten times greater than the MIC of *E. coli* ATCC25922 (0.019 µg/ml for CN-DM-861 and 2.5 for CDCHD µg/ml).. Ten min later, 2 ml of each culture were taken and mixed with RNA protect (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. Cells were immediately pelleted by a 10min centrifugation at 5,000 g. Biological triplicates were performed. RNA was extracted using GeneJet RNA extraction kit (ThermoScientific, ThermoFisher Scientific, Villebon sur Yvette. France). DNase treatment was performed with the dsDNase kit (ThermoScientific) according to the manufacturer's protocol. RNA concentration was measured using Qubit RNA BR assay kit (Invitrogen. ThermoFisher Scientific), and RNA quality was evaluated using the Agilent 2100 Bioanalyzer RNA 6000

Nano Kit (Agilent Technologies, Santa Clara, CA). RIN >9 were obtained for all the RNA samples. Verification of complete removal of any contaminating DNA was performed via PCR amplification of recA housekeeping gene. A total of 2.5 µg of total RNA was treated with the Ribo-Zero rRNA Removal kit according to the manufacturer's instructions (Illumina, CA, USA) to remove 16S and 23S rRNAs. rRNA removal efficiency was then analyzed via the Bioanalyzer RNA 6000 Nano Kit. Ribodepleted RNA concentration was measured using Qubit™ RNA HS assay kit (Invitrogen).

6- RNA Library preparation and sequencing

cDNA libraries were prepared using the NEBNext R Ultra Directional RNA Library Prep Kit for Illumina protocol (New England Biolabs, MA, USA). The quality of the cDNA was validated using the Agilent 2100 Bioanalyzer DNA1000 kit (Agilent Technologies) and quantity was determined with the Qubit dsDNA BR Assay (ThermoFisher Scientific). Sequencing of the library was performed with an Illumina HiSeq 2500 in single-read mode with 50 cycles.

7- RNA-Seq Data Analysis and Differential Expression Analysis

Reads generated from strand-specific RNA-seq experiments were aligned to the genome of *E. coli* ATCC25922 (CP009072) by using the software Bowtie (version 0.12.7) (21). Reads that mapped in more than four different positions on the genome were discarded i.e., reads corresponding to rRNA. RNAseq data were analyzed as described (10, 22) by using Rsamtools (version 1.26.2), GenomicAlignments (version 1.10.1), GenomicFeatures (version 1.26.4) and DESeq2 (version 1.14.1) and SARTools (11) in R 3.3.1. Only adjusted p-values were used and were obtained using the Benjamini–Hochberg correction for false discovery rate (12). Read count data were visually assessed using the Artemis genome viewer (23). The coverage (expressed as the average density of reads over 500 nucleotide fragments) was computed on each strand of pBICa sequence and visualized by using a heatmap representation (IGV software) (24). The gene expression values were quantified in terms of reads per million (RPKM) defined as the total number of reads mapping to the feature divided by feature length (in kbp) normalized by the total number of reads (in millions) (25). The significantly up- and downregulated genes were analyzed for GO term enrichment separately with PANTHER ([http:// pantherdb.org/webservices/go/overrep.jsp](http://pantherdb.org/webservices/go/overrep.jsp); (26) using batch upload, and the

significantly enriched terms further explored on the REVIGO web application (<http://revigo.irb.hr/>) to identify and visualize relationships among the GO terms (27). The default settings were used on REVIGO with a list of results of medium size, and *E. coli* GOterms database used as reference. The functions of genes and gene regulons were annotated using the following web databases: EcoCyc ([http://biocyc.org/ ECOLI/organism-summary](http://biocyc.org/ECOLI/organism-summary); (28) and RegulonDB v8.2 (<http://regulondb.ccg.unam.mx/index.jsp>). Regulation for genes in the SoxRS, OxyR, IscR and Fur regulons were identified as annotated in RegulonDB v8.2 (29).

8- RT-qPCR

In order to confirm the results obtained after *E.coli* ATCC25922 exposition to CN-DM-861 and to CDCHD, two upregulated genes and two downregulated genes were selected for *E. coli* exposed to CN-DM-861, whereas one upregulated gene and two downregulated genes were selected for *E. coli* exposed to CDCHD. Expression levels were analyzed by quantitative RT-PCR to validate the RNASeq data. The primers used are listed in Table S1 in Supplementary data. An input of 100 ng of total RNA, with no contaminating DNA was used. One-step RT-qPCR was performed using One-step RT-PCR kit (Qiagen) according to the manufacturer's protocol, and the reactions were carried out in 96-well plates with CFX96 Real-time PCR System (22)(BioRad, USA). All qRT-PCRs included an initial denaturation step of 30 s at 95°C, 35 cycles of denaturation (95°C/1s), annealing (52°C/5s), and extension (72°C/7s). Expression of each gene was normalized using *rpoB*, *idnT* and *yiaJ* genes as previously described (30). Expression of these genes was not influenced by the presence of imipenem according to the RNA-Seq data. All reactions were carried out in triplicates. The relative abundance of gene transcripts among the imipenem treated group was calculated using the 2^{-11Ct} method (31).

9- Sequence accession numbers

Submission to NCBI in progress

Results

Mutants selection and Frequency of resistance

In order to understand the mutational rate of cystobactamids, the frequency of resistance was calculated. After an overnight culture of *E. coli* ATCC25922 on mediums supplemented with x2 MIC concentrations of cystobactamids, an average of 33 clones were obtained after culture

on CN-861-2 and 21 clones were obtained after culture with CN-DM-861. This number divided by 12×10^9 , which represents the number of bacteria obtained without antibiotics gives a final frequency of 0.27×10^{-8} for CN-861-2 and 0.17×10^{-8} for CN-DM-861. Growing *E. coli* ATCC25922 on mediums containing higher antibiotic concentrations revealed a lower value for resistance frequency, which ranged from 0.14×10^{-8} and 0.28×10^{-8} for CN-861-2 and remained 0 for CN-DM-861 (Table 1).

Table 6: Frequency of resistance to cystobactamids calculated by dividing the number of obtained colonies by the total number of bacteria obtained on mediums without antibiotic.

	Number of obtained colonies					
	CN-861-2	CN-DM-861	CN-861 X4	CN-DM-861	CN-861 X8	CN-DM-861 X8
	X2	X2		X4		
#1	31	26	25	0	15	0
#2	28	12	49	0	22	0
#3	40	27	30	0	16	0
Average of triplicates	33	21	34	0	17	0
Frequency of resistance	0.27×10^{-8}	0.17×10^{-8}	0.28×10^{-8}	-	0.14×10^{-8}	-

X represents the dilutions above the MICs

Concerning chelocardins, frequency of resistance was calculated after bacterial growth on media supplemented with CDCHD at MIC x2, x5 and x8. The average number of colonies obtained after three trials was 6, 3 and 1 respectively resulting in resistance frequencies ranging from 0.0027×10^{-8} to 0.058×10^{-8} (Table 2).

Table 7:: Frequency of resistance to chelocardins calculated by dividing the number of obtained colonies by the total number of bacteria obtained on mediums without antibiotic.

	Number of colonies (N)		
	CDCHD X2	CDCHD X5	CDCHD X8
#1	5	3	0
#2	10	2	0
#3	6	3	1
Average of triplicates	7	2.6	0.33
Frequency of resistance	0.058×10^{-8}	0.021×10^{-8}	0.0027×10^{-8}

1-Comparison of mutants to parental strains

a- Microbroth dilution assays and number of SNPs

Microbroth dilution assays revealed significant shifts towards higher MICs in all obtained mutants. New MIC results are shown in Table 2. After WGS and data analysis, the number of SNPs found in each mutant was determined by comparing the genomes of all mutants to the parental strains through CLC genomics workbench (version 12.0) and Basic variant detection tool. Interestingly, the number of SNPs was found to be relatively high ranging from 16 to 211 SNPs (Table 3).

Table 3: MIC shifts and number of SNPs in mutants compared to their parental isolate

Isolate	MIC to Cystobactamids (µg/ml)		Number of SNPs
	CN-861-2	CN-DM-861	
O89E4 parental strain	0.5	0.5	
O89E5	32	16	95
O89E6	16	8	100
O89E7	>128	>128	104
O89E8	>128	>128	107
O94D10 parental strain	0.06	0.0019	
O94E1	4	4	28
O94E2	4	0.25	16
O94E3	2	0.125	20
O94E4	1	0.125	34
O94E5	2	0.25	43
O94E6	0.25	0.06	55
O94E7	0.5	0.06	113
O94E8	0.125	0.015	209
O94E9	0.5	0.06	205
O94E10	0.25	0.06	202
O94F1	0.25	0.06	206
O94F2	0.25	0.06	94

O94F3	0.25	0.06	211
O94F4	0.25	0.06	205

b- Comparative genomics

For further understanding of resistance mechanisms, altered genes of each mutant were identified. Genes are presented in the table below (Table 4).

Table 4: Mutated genes found in the various mutants. Shading in the table means that the gene was found to be mutated

	<i>SitA</i>	<i>SitB</i>	<i>SitC</i>	<i>SitD</i>	<i>dprM</i>	<i>pmrB</i>	<i>YihH</i>	<i>Transposase InsG for insertion sequence</i>	<i>Polyketide synthase modules and related</i>	<i>Putative large exoprotein involved in heme</i>	<i>UPF0380 proteins YafZ and homologs</i>	<i>Phage tail fiber, tail fiber assembly protein</i>	<i>Phage tail fiber Mup49</i>	<i>Type 1 fimbriae regulatory protein FimB</i>	<i>S-adenosylmethionine synthetase</i>	<i>Undecaprenyl phosphate-aminocarbinose</i>	<i>UDP-N-acetylmuramoyl-dipeptide--2,6-dia-</i>	<i>Antirestriction protein klcA</i>	<i>Large exoproteins involved in heme utili-</i>	<i>LysR family transcriptional regulator HdfR</i>	<i>Sensory histidine kinase QseC</i>
<i>O89E5</i>																					
<i>O89E6</i>																					
<i>O89E7</i>																					
<i>O89E8</i>																					
<i>O94E1</i>																					
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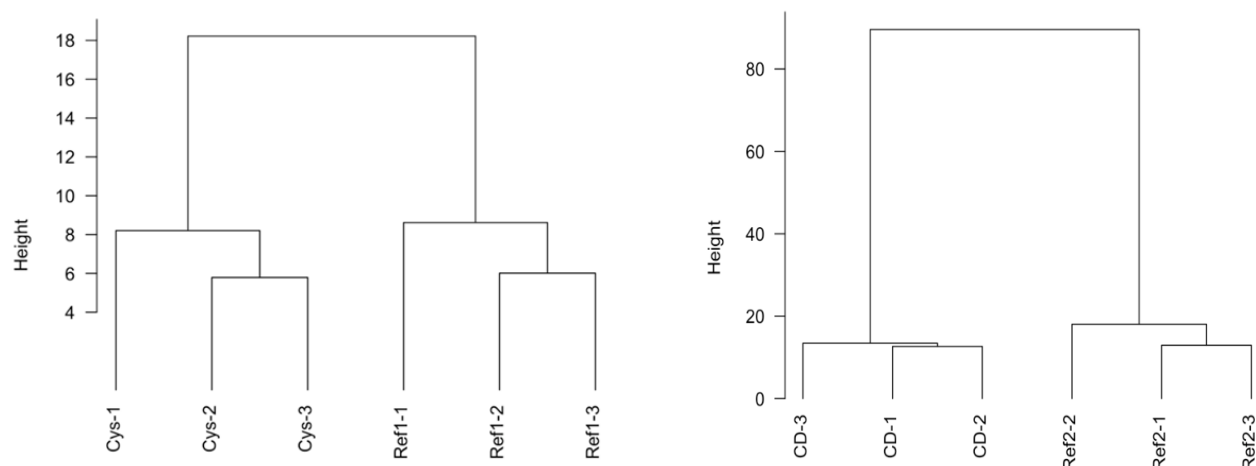


Figure 1: Cluster dendrograms. Sample clustering based on normalized data (A) cystobactamid triplicates clusterization ; (B) chelocardin triplicates clusterization – Euclidean distance – Ward criterion hclust. Cys 1, Cys 2 and Cys 3 represents the three rounds of *E. coli* exposure to CN-DM-861; CD 1, CD 2, CD 3 represents the three rounds of exposure to CDCHD and REF 1, REF 2, REF 3 represents the reference for each trial

2.b.-Differentially expressed genes after CN-DM-861 exposure

2.b.1.Up regulated genes after CN-DM-861 exposure

After exposure to 10x the MIC of CN-DM-861, 18 genes were up-regulated ($FC > 2$), and had significant p-values (Table 1).

Table 8: Genes implicated in DNA repair and SOS response up-regulated following exposure to CN-DM-861.

Protein	Fold Change	p-value
DNA repair protein RecN	4.548	3.62E-282
DNA polymerase IV	2.874	1.50E-59
cell division inhibitor sulA	2.8	1.19E-92
protein yebF	2.707	2.32E-79
yebG family protein	2.703	1.77E-74
protein umuD	2.616	5.10E-20
Repressor LexA	2.55	3.78E-142
MATE efflux family protein	2.54	4.29E-60
inner membrane protein ydjM	2.483	5.51E-36
acetyltransferase family protein	2.467	2.91E-14
protein RecA	2.412	4.95E-133
DNA-damage-inducible protein I	2.401	9.31E-71
alpha/beta hydrolase fold family protein	2.337	8.46E-23
colicin-E1	2.137	1.67E-08

Proteins encoded by overexpressed genes are overall implicated in DNA damage response, where some of them induce DNA damaged repair (DNA repair protein RecN, FC 4.548 ; inner

membrane protein YdjM FC 2.483 ; protein RecA FC 2.412 ; protein UmuD, FC 2.616), in DNA synthesis (DNA polymerase IV FC 2.874), and in SOS response (cell division inhibitor SulA FC 2.8 ; repressor LexA FC 2.55).

2.b.2 Down regulated genes after CN-DM-861 exposure

Following exposure to CN-DM-861, 14 genes were found to be downregulated, FC < 0.5 (Table 2).

Table 9: Downregulated genes after exposure to CN-DM-861

Protein	Fold Change	p-value
isochorismate synthase entC	0.331	4.55E-15
siderophore-iron reductase FhuF	0.363	4.72E-31
ferric enterobactin transport system permease protein fepD	0.384	9.48E-17
enterochelin esterase	0.39	1.03E-10
ferrichrome-iron receptor	0.395	1.35E-15
enterobactin exporter EntS	0.4	6.93E-13
ferrous iron transport protein A	0.42	0.000259
ferric enterobactin transport system permease protein fepG	0.427	3.12E-09
glycerol-3-phosphate dehydrogenase2C anaerobic2C A subunit	0.461	0.000194
bacterioferritin	0.486	6.65E-13
iron(3+)-hydroxamate import ATP-binding protein FhuC	0.491	1.20E-15
ribonucleoside-diphosphate reductase2C alpha subunit	0.493	3.42E-09
hemin uptake hemP family protein	0.493	1.05E-07
4Fe-4S dicluster domain protein	0.494	0.001184

Proteins encoded by down-regulated genes are overall implicated in iron metabolism. Isochorismate synthase EntC involved in the biosynthesis of the siderophore enterobactin (FC 0.331); siderophore iron reductase FhuF, involved in the reduction of ferric iron in cytoplasmic ferrioxamine B (FC 0.363); ferric enterobactin transport system permease protein FepD and FepG, which are part of the ABC transporter complex FepBDGC involved in ferric enterobactin uptake (FC 0.384, and 0.427); enterochelin esterase having a role in iron ion transport (FC 0.39); ferrichrom iron transport, also responsible for iron uptake (FC 0.395); enterobactin exporter EntS, which underexpression increases intracellular arabinose concentrations (FC 0.4); Ferrous iron transport protein A also implicated in iron transport (FC 0.42); iron(3+)-hydroxamate import ATP-binding protein FhuC, which is part of the ABC transporter complex FhuBGCD involved in iron3+-hydroxamate import (FC 0.491); and 4Fe-4S dicluster domain

protein implicated in iron binding (FC 0.494).

2.c. Differentially expressed genes after CDCHD exposure

2.c.1. Up regulated genes after CDCHD exposure

Following exposure to CDCHD, 171 genes were upregulated with $FC > 2$. These genes can be classified in several categories.

1- Overexpression of genes involved in protein synthesis.

Proteins encoded by upregulated genes of this category are listed in Table 3: rRNA ligases responsible for the catalysis and the formation of phosphodiester bonds between the 5'-phosphate and 3'-hydroxyl termini of RNA (FC 48.1 and 41); AAA domain protein involved in protein degradation, membrane fusion, DNA replication, microtubule dynamics, intracellular transport, transcriptional activation, protein refolding, disassembly of protein complexes and protein aggregates (FC 16.7); and Ribosome binding factor A, Translation initiation factor IF-2 and Peptide chain release factor H (FC of 2.8, 2.3 and 2.3 respectively) (Table 3).

Table 3: Genes responsible for protein synthesis, upregulated after exposure to CDCHD

Protein	Fold change	p-value
rRNA ligase	48.1	1.71E-59
rRNA ligase	41	1.96E-135
AAA domain protein	16.7	6.57E-183
Ribosome binding factor A	2.8	1.13E-175
Translation initiation factor IF-2	2.3	1.23E-162
Peptide chain release factor H	2.3	3.60E-26

1- Overexpression of genes involved in efflux

E. coli upregulates efflux and more specifically the ABC efflux system, suggesting that the efflux mechanism could be a mechanism by which *E. coli* resists the action of CDCHD.

Table 10: Efflux genes upregulated in *E. coli* after exposure to CDCHD

Proteins	Fold change	p-value
Phosphate ABC transporter 2C phosphate binding protein PstS	2.8	2.18E-102
asmA family protein	2.7	2.08E-145
ABC transporter periplasmic binding protein ytfQ	2.477	4.27E-12
Phosphate ABC transporter 2C permease protein PstC	2.294	1.42E-37

Glutathione regulated potassium efflux system protein KefB	2.273	2.59E-31
Efflux transporter 2C RND family 2C MFP subunit	2.093	1.20E-20

Genes encoding several efflux families were affected. The phosphate ABC transporter 2C phosphate binding protein PstS and PstC, which is part of the ABC-type transporter complex pstSACB involved in phosphate import (enhanced under phosphate starvation) was 2.8 times upregulated. Other transporters of the ABC family were up-regulated, such as ABC transporter periplasmic binding protein YtfQ (FC 2.477), part of the ABC transporter complex YtfQRT-YjfF involved in galactofuranose transport; OmpF family was also affected through the upregulation of AsmA family protein (FC 2.7), AsmA mutations were isolated as extragenic suppressors of an OmpF assembly mutant may have a role in LPS biogenesis; and RND family efflux transporter 2C MFP subunit was slightly upregulated (FC 2.093).

2- Genes implicated in iron metabolism

Table 11: Genes implicated in Iron metabolism, overexpressed following exposure to CDCHD

Protein	Fold change	P-value
Formate hydrogenlyase subunit 7	4.6	1.43E-72
4 Fe-4S dicluster domain protein	4.3	1.88E-48
formate hydrogenlyase regulatory protein hycA	2.355	0.000133
CsiD protein	2.1	3.23E-05

Several genes involved in iron metabolism were altered, out of these, formate hydrogenlyase subunit 7 and 4 Fe-4S dicluster domain protein both responsible for iron sulfur cluster binding showed FC values of 4.6 and 4.3 respectively. In addition, CsiD protein, implicated in iron ion binding and oxidoreductase activity was also upregulated FC 2.1.

3- Genes implicated in TCA cycle

TCA cycle was largely affected after CDCHD exposure (Table 6).

Table 12: Up regulated genes implicated in TCA cycle, after CDCHD exposure

Protein	Fold change	P-value
thioredoxin	4.2	6.81E-146
glutaredoxin2C GrxA family	4.048	2.98E-57
glycerol-3-phosphate dehydrogenase2C anaerobic2C C subunit	2.798	1.65E-07
dimethylsulfoxide reductase2C chain B	2.789	5.67E-28
alkyl hydroperoxide reductase subunit F	2.742	1.51E-154
succinate dehydrogenase2C hydrophobic membrane anchor protein	2.546	1.40E-32
DMSO reductase anchor subunit family protein	2.538	2.27E-32
trimethylamine-N-oxide reductase c-type cytochrome TorC	2.363	0.003922
anaerobic dimethyl sulfoxide reductase2C A subunit2C DmsA/YnfE family protein	2.33	0.000659
succinate dehydrogenase2C cytochrome b556 subunit	2.107	8.31E-31
acetyl-CoA C-acyltransferase family protein	2.058	1.66E-16
NAD-binding of NADP-dependent 3-hydroxyisobutyrate dehydrogenase family protein	2.057	2.26E-06
electron transfer flavodomain protein	2.044	0.000325
fructose-like permease IIC component 1	2.037	0.015976
22C4-dienoyl-CoA reductase [NADPH]	2.031	5.12E-06
UDP-glucose/GDP-mannose dehydrogenase family2C NAD binding domain protein	2.021	0.017992
glutaminase A	2.014	1.59E-07
L-tartrate/succinate antiporter	2.007	0.002863
xylose isomerase-like TIM barrel family protein	2.11	1.12E-19

Many genes involved in defense against oxidative stress were upregulated. Out of these genes thioredoxin and glutaredoxin 2C GrxA family genes showed FCs of 4.2 and 4.048 respectively. While other are involved in anaerobic respiration such as glycerol-3-phosphate dehydrogenase2C anaerobic2C C subunit (FC 2.798), DMSO reductase anchor subunit family protein (FC 2.538).

4- Genes implicated in *E. coli* motility

Table 13: Bacterial motility genes upregulated following CDCHD exposure

Protein	Fold change	P-value
motility protein B	2.148	2.59E-84
flagellar motor stator protein MotA	2.124	4.82E-80

E. coli motility seems also to be affected after exposure to CDCHD, where motility protein B and flagellar motor stator protein MotA, were upregulated with FCs of 2.148 and 2.124, respectively.

6- Other upregulated genes

Many other genes were upregulated, each one having a distinct function (Table 8).

Table 14: Other upregulated genes

Protein	Fold change	P-value
WYL domain protein	4.954	5.13E-07
yejG-like family protein	4.936	3.93E-192
bacterial regulatory s2C gntR family protein	4.289	6.32E-56
bacterial regulatory helix-turn-helix2C lysR family protein	3.916	2.06E-56
transcriptional repressor BetI	3.779	3.29E-99
formate hydrogenlyase maturation HycH family protein	3.673	3.54E-37
Cold shock protein CspA	3.49	4.43E-103
Cold shock protein CspG	3.336	2.21E-85
Multidrug resistance protein D	3.119	8.74E-154
Obg family GTPase CgtA	2.691	1.46E-162
Regulatory protein soxS	2.55	6.02E-49
hydrogenase assembly chaperone HypC/HupF	2.541	1.71E-13
tRNA-splicing ligase RtcB family protein	2.524	1.12E-67
tRNA pseudouridine(55) synthase	2.422	3.83E-145
ompA family protein	2.405	0.001827
formate hydrogenlyase regulatory protein hycA	2.355	0.000133

type II secretion system (T2SS)2C M family protein	2.205	2.36E-07
Universal stress protein G	2.12	1.89E-56
chaperone protein fimC	2.059	0.019121
hydrogenase-2 operon protein hybE	2.038	5.28E-24
hydrogenase expression/formation protein	2.037	1.20E-22

2.c.3. Down regulated genes after CDCHD exposure

Following the exposure to CDCHD, 228 genes were downregulated with FCs <0.5. Overall, those genes could be classified in four categories.

1- Genes implicated in iron metabolism

Table 15: Genes of iron metabolism downregulated after CDCHD exposure

Protein	Fold change	P-value
enterochelin esterase	0.105	1.67E-12
22C3-dihydroxybenzoyl adenylate synthase	0.151	7.39E-14
isochorismate synthase entC	0.151	2.47E-10
iron(3+)-hydroxamate-binding protein fhuD	0.174	3.38E-101
iron(3+)-hydroxamate import system permease protein fhuB	0.175	8.57E-116
enterobactin synthase component F	0.181	6.12E-14
ferrienterobactin receptor	0.191	1.08E-08
iron(3+)-hydroxamate import ATP-binding protein FhuC	0.205	4.18E-23
fecR family protein	0.208	4.03E-11
Isochorismatase	0.212	3.55E-12
siderophore-iron reductase FhuF	0.219	5.10E-13
catecholate siderophore receptor fhu	0.25	1.15E-06
fe(3+) dicitrate transport system permease protein fecD	0.261	6.23E-63
enterobactin exporter EntS	0.263	6.59E-08
ferrienterobactin-binding periplasmic protein	0.317	5.98E-14
iucA / IucC family protein	0.31	1.77E-13
iucA / IucC family protein	0.314	2.66E-07
ferrienterobactin-binding periplasmic protein	0.317	5.98E-14
L-lysine 6-monooxygenase	0.338	7.69E-48
ferric enterobactin transport system permease protein fepG	0.346	4.40E-06
hemin transport protein HmuS	0.351	4.56E-11
protein tonB	0.368	5.86E-17
ferric enterobactin transport system permease protein fepD	0.368	2.95E-05
hemin uptake hemP family protein	0.383	1.73E-05

FeS assembly scaffold SufA	0.386	1.22E-06
Ferrichrome iron receptor	0.4	6.36E-11
putative ferrichrome-binding protein	0.411	3.11E-23
putative heme utilization radical SAM enzyme HutW	0.424	4.26E-09
ferrous iron transport protein A	0.443	2.45E-19
2Fe-2S iron-sulfur cluster binding domain protein	0.448	1.39E-55
2OG-Fe(II) oxygenase superfamily protein	0.475	5.39E-06
FeS assembly protein SufB	0.477	1.36E-10
ferrous iron transport protein C	0.485	1.35E-09
ferric enterobactin (Enterochelin) transport	0.489	1.61E-47
bacterioferritin-associated ferredoxin	0.501	2.75E-05
outer membrane heme receptor ShuA	0.448	0.002097
alpha-hemolysin translocation ATP-binding protein HlyB	0.461	7.96E-44
bacterioferritin-associated ferredoxin	0.501	2.75E-05
22C3-dihydro-22C3-dihydroxybenzoate dehydrogenase	0.295	3.77E-19

2- Genes implicated in efflux and transport

Genes encoding efflux components seem to be largely affected by CDCHD (Table 9)

Table 16: Efflux affected by CDCHD exposure

Protein	Fold change	P-value
peptide transport system ATP-binding protein SapF	0.232	4.66E-94
ABC transporter family protein	0.243	6.35E-89
ABC transporter family protein	0.251	4.47E-11
PTS system trehalose-specific EIIBC component	0.268	8.92E-82
inner membrane transport permease ybhR	0.294	3.07E-31
ABC 3 transport family protein	0.309	8.86E-66
inner membrane ABC transporter ATP-binding protein YddA	0.352	4.06E-08
AcrB/AcrD/AcrF family protein	0.397	7.68E-35
putative threonine efflux protein	0.429	2.80E-32
ABC 3 transport family protein	0.443	4.61E-13
carboxylate/amino acid/amine transporter family protein	0.448	1.78E-41
outer membrane porin protein ompD	0.455	5.21E-80
TRAP transporter solute receptor2C DctP family protein	0.454	0.014404
putative N-acetylneuraminic acid outer membrane channel protein		
NanC	0.464	0.000132
binding--dependent transport system inner membrane component		
family protein	0.469	1.52E-16

ABC 3 transport family protein	0.482	6.82E-12
binding--dependent transport system inner membrane component		
family protein	0.485	2.73E-18
amino ABC transporter2C permease2C 3-TM region2C		
His/Glu/Gln/Arg/opine family domain protein	0.487	2.47E-32
MATE efflux family protein	0.49	4.00E-31
outer membrane lipocarrier LolA family protein	0.494	1.47E-21
thiamine ABC transporter2C ATP-binding protein	0.494	4.72E-18
ABC transporter family protein	0.498	0.000302
cyanate transporter family protein	0.328	2.98E-30
Inner membrane transport permease ybhS	0.328	2.09E-29
MMPL family protein	0.392	2.17E-60
outer membrane porin protein ompD	0.406	6.00E-100
nickel import ATP-binding protein NikE	0.253	5.48E-74
arginine transport ATP-binding protein ArtP	0.469	3.24E-45
type II secretion system (T2SS)2C K family protein	0.438	0.012118

3- Adhesion and motility

Many genes responsible for making the flagella were significantly downregulated (Table 11). This includes FlgI, FlgJ, FlgC, FlgQ with FC values ranging from 0.379 to 0.474. Genes having crucial function in adhesion are also affected, we cite fimG, fim F, SfaH, with FC values of 0.266, 0.278, and 0.374 respectively.

Table 17: Genes involved in adhesion and motility downregulated after CDCHD exposure

Protein	Fold change	P-value
protein fimG	0.266	1.77E-22
protein fimF	0.278	2.46E-17
flagellar hook-length control FliK family protein	0.292	6.29E-150
flagellar biosynthetic protein FliR	0.307	9.78E-37
fimbrial family protein	0.318	0.000288
fimbrial family protein	0.339	5.37E-21
S-fimbrial protein subunit SfaH	0.374	1.66E-88
flagellar rod assembly protein/muramidase FlgJ	0.379	7.05E-103
flagellar P-ring family protein	0.401	4.05E-110
flagellar L-ring family protein	0.402	3.00E-91
PRS fimbrial major pilin protein	0.446	3.77E-100

flagellar export protein FliJ	0.407	1.28E-33
flagellar transcriptional regulator FlhC	0.413	7.62E-79
flagellar transcriptional activator family protein	0.426	1.15E-48
flagellar biosynthetic protein FliQ	0.474	8.12E-19

4- TCA cycle and electron transport

Eventhough some genes implicated in TCA cycle were upregulated (Table 6), few were found to be downregulated (Table 12) meaning that the whole metabolism was affected.

Table 18: Genes involved in TCA cycle metabolites downregulated after CDCHD exposure

Protein	Fold change	P-value
succinate-CoA ligase2C alpha subunit	0.341	8.38E-90
succinate-CoA ligase2C beta subunit	0.359	2.40E-88
dihydrolipoyllysine-residue succinyltransferase2C E2 component of oxoglutarate dehydrogenase complex	0.375	1.49E-94
cytochrome d ubiquinol oxidase subunit 1	0.394	3.66E-77

5- Biotin synthesis

The synthesis of biotin which is a covalently attached enzyme cofactor required for intermediary bacterial metabolism change after CDCHD exposure (Table 10).

Table 19: Genes implicated in Biotin synthesis downregulated after CDCHD exposure

Protein	Fold change	P-value
biotin synthase	0.285	1.89E-107
malonyl-acyl carrier protein O-methyltransferase BioC	0.32	8.45E-49
adenosylmethionine-8-amino-7-oxononanoate transaminase	0.336	4.23E-78
dethiobiotin synthase	0.362	4.42E-38

Discussion

Mutations

Despite the growing number of studies performed on Cystobactamids, few studies are looking for the mechanisms of resistance towards these novel compounds. For that reason, we aimed to elucidate the mechanism of resistance to Cystobactamids by inducing resistant mutants and comparing the genomes of obtained mutants to their parental isolate. For this, a clinical *E. coli* isolate and an *E. coli* ATCC25922 were used to induce mutants by growth and selection on various CN-861-2 and CN-DM-861 concentrations. After mutants selection, frequency of

resistance was calculated. It was found to be 0.27×10^{-8} for CN-861-2 and 0.17×10^{-8} for CN-DM-861. Those values are relatively normal compared to the frequency of error caused by DNA polymerase, which is estimated at 10^{-9} or 10^{-8} .

At a second time, obtained mutants were collected and their MICs towards both derivatives of Cystobactamids were determined through microbroth dilution assays. Significant differences in MIC values were found for all mutants, whereas the shifts in MICs were diverse. Moreover, *pmrB*, is mutated in one mutant. This gene encoded a part of a two component system, the *pmrAB*, found to have a major role in resistance to colistin through LPS modification (32-34). These findings, indicate that maybe, this mutant is resistant to colistin, which is not the case where no cross resistance between cystobactamids and colistin was found through broth microdilution assays.

In comparison to the parental isolates, number of SNPs was quite important suggesting a mutagenic feature of Cystobactamids, which recalls the possibility of those compounds to be toxic. However, the existing published studies made on mouse in vivo models, ensures that those compounds are safe (35). After further investigation and comparison to their parental isolates, mutants had various alterations in several genes. For instance, the operon *SitABCD*, seemed to be altered in the first batch of mutants, suggesting an involvement of its substrates, mainly iron and manganese in *E. coli*, in the mechanism of resistance to Cystobactamids (36). The adenine methyl transferase, encoding gene *DpnM* was also found to be mutated in the first batch of selected clones. Interestingly, this enzyme works on DNA single strands which recalls the mode of action of Cystobactamids having topoisomerases IIA as target (37). The mutated *YibH*, consists also of a transmembrane protein, involved in many cellular processes including transport and cellular response to damage, which may be related to the large number of SNPs observed in the Cystobactamid resistant mutants.

A variety of genes were mutated in the isolates generated out of *E. coli* ATCC25922. Some genes were commonly mutated in all mutants, whereas others were more specific. Transposase *InsG* insertion sequence element *IS4* gene was mutated in 12 mutants. This gene encodes a protein that binds to DNA, and has a transposase activity is responsible for transposition function (38). Polyketide synthase modules and related proteins, which were also altered, have also a transferase function (39). Putative large exoprotein involved in heme utilization or adhesion of *ShlA/HecA/FhaA* family were mutated in 7 mutants, this family consists of a group of proteins that are secreted by Gram-negatives. According to Uniprot, they

are involved in many functions including adhesion, invasion and colonization of host cells. The heme utilization recalls the implication of iron in the process. No relevant information was published concerning the function of UPF0380 proteins YafZ and homologs, which are mutated also. More importantly, many genes involved in adhesion and motility were mutated, such as Phage tail fiber, tail fiber assembly protein Tfa, Phage tail fiber Mup49, and Type 1 fimbriae regulatory protein FimB. Those genes are generally under the control of a two component system, the QseBC, which is in its turn altered in some mutants. This two component system consists of a sensor kinase, the QseC, and a response regulator, the QseB. This system is thought to regulate genes involved in motility, adhesion, and other biological processes (40). Another interesting mutated gene, is Undecaprenyl phosphate-aminoarabinose flippase subunit ArnF, which is part of the Arn operon, responsible for LPS modifications. Mutations in genes belonging to Arn operon could result in resistance to colistin by altering the LPS and thus modifying the membrane charge. This suggests that maybe, a similar resistance mechanism could confer resistance to cystobactamids. UDP-N-acetylmuramoyl-dipeptide-2,6-diaminopimelate ligase involved in the peptidoglycan synthesis was found to be mutated, indicating that maybe an alteration in this process was also noted. Antirestriction protein klcA, mutated in 6 cystobactamid resistant mutants, could be implicated in carbapenem resistance dissemination (41). LysR-type transcriptional regulators (LTTRs) regulate various genes, including virulence genes, metabolism genes, quorum sensing and motility, suggesting that the mutations found in lysR family transcriptional regulator HdfR, could be related to the alteration of QseBC two system component.

For the moment, no clear explanation of cystobactamid resistance mechanism could be elaborated, further studies are needed to find a solid hypothesis for this phenomenon, but our findings suggests the implication of many genes in this mechanism including some genes responsible for colistin resistance.

RNAseq

In order to have a deep understanding of the modes of action or mechanisms by which bacteria evade to cystobactamids and chelocardins, we aimed to study the bacterial response to exposure to CN-DM-861 and CDCHD. Conditions of exposure were chosen in a way to put stress on bacteria just to have an overview how it will react following the exposure. *E. coli* ATCC25922, chosen for its well-studied genome, were exposed to concentrations 10x the MICs for both compounds for 10 min to induce a highly stressful condition and RNA was extracted

directly following the exposure.

Cystobactamid exposure effect

Eighteen genes were upregulated, and fourteen were downregulated after exposure to CN-DM-861, the most potent cystobactamid derivative. Overexpressed genes were mainly entailed in DNA repair function and SOS response meaning that CN-DM-861 induced DNA repair which goes along with the fact that cystobactamids are topoisomerase inhibitors. The SOS response set by the bacteria, is a proof that bacteria is trying to increase its fitness especially if it is trying to survive a lethal dose of antibiotic (42), so here clearly *E. coli* was trying to overcome the effect of high CN-DM-861 dose by starting this response.

Downregulated genes are mainly involved in iron homeostasis, which may have a role in antimicrobial resistance. This indicate that bacteria may have altered iron transport and thus highlights the importance to test the MICs of cystobactamid resistant mutants for cefiderocol (43), the siderophore cephalosporin, to find any potential cross-resistance between both antibiotics.

Chelocardin exposure effect

After exposure to CDCHD, *E. coli* showed an up-regulation of 171 genes and a downregulation of 228 genes. Among the overexpressed genes, we largely found genes involved in protein synthesis, which recalls the activity of tetracyclines. In fact, the structural homology between tetracyclines and chelocardins (44), explains the effect of CDCHD on ribosomal proteins such as rRNA ligase, or ribosome binding factor A which were significantly upregulated (FC 48.1 ; FC 2.8). For instance, the putative target of chelocardins comprise the ribosome and they are believed to inhibit protein synthesis, although they act in a dual mechanism by inhibiting protein synthesis and by inducing a bacterial membrane depolarization (19). Our findings come in favor with the published studies about the mode of action of chelocardins, which shows an effect of those compounds on protein synthesis inhibition. In addition, genes encoding efflux proteins were significantly altered suggesting that *E. coli* may overregulate these genes to extrude the unwanted compound outside and thus avoid it's devastating effects. Maybe efflux is the mechanism by which *E. coli* resist the action of chelocardins. Moreover, genes responsible for iron homeostasis were altered, some of them were upregulated (4) while others were clearly downregulated (39). Many studies have elucidated the involvement of iron homeostasis in antibiotic resistance (18, 45). Maybe iron-induced breathing promotes resistance to CDCHD

The overexpression of genes encoding proteins regulating TCA cycle could be the result of stress caused by CDCHD exposure. However, it could also be the way *E. coli* is trying to resist CDCHD. The metabolites in the TCA cycle, were found to promote resistance in some species towards certain antibiotics as resistance of *Edwardsiella tarda* to chloramphenicol (46). From another point of view, TCA cycle alteration could be the consequence of stressful conditions the bacterial cell went through. For example, researchers found that in some cases a dysfunctional TCA cycle enhances the fitness of bacteria when exposed to antibiotics, like *Staphylococcus epidermidis* when exposed to β -lactams. So we assume, it's either Krebs cycle that may trigger resistance against CDCHD or an alteration of the Krebs cycle might be necessary to withstand the stress of exposure to this same compound (47).

Out of the other up-regulated genes, several ones are of a particular interest where *gntR* for example is 4.289 times upregulated and *GntR* family are transcription factors responsible for the regulation of some antibiotics by controlling the expression of permeases which in our case are also altered following CDCHD exposure (48).

Concerning down regulated genes, the first list comprises permeases as *fhuB*, *fecD*, *fepG*, *fepD*, which are implicated in iron transport recalling the importance of iron homeostasis in antibiotic resistance. Add to this, inner membrane transport permease *ybhR*, which is part of ABC YbhFSR transporter complex that may be involved in the efflux of many antibiotics suggesting that resistance to CDCHD might be linked to modifications in *AcrAB* expression (49). Many publications shed the light on the role of *AcrAB-TolC* family in the efflux and tetracyclines, likewise, many components of this family were altered after CDCHD exposure (49, 50). The huge number of altered genes may indicate that *E. coli* was in a highly stressful situation that affected many pathways. All the discussed points remain hypothetical until further investigations, especially that chelocardin's mode of action is not fully understood.

Conclusions

All our findings enable us to set hypotheses for the understanding of cystobactamids and chelocardins effect on *E. coli* ATCC25922. However, bacterial response remain blurred due to the fact that transcriptomes could rapidly fluctuate and it's not easy to control all the conditions affecting it. CN-DM-861, has clearly induced DNA repair and SOS response in *E. coli*, while CDCHD affected ribosome binding proteins involved in protein synthesis recalling tetracycline's mode of action. Note that even efflux is affected after CDCHD exposure suggesting a potential role in resistance mechanism.

Acknowledgements

We would like to thank Axel Philip for excellent technical assistance.

Funding

This research was funded by NATURAL-ARSENAL (ANR-19-AMRB-0004/01KI2125B). This work has been supported as part of France 2030 program “ANR-11-IDEX-0003”, and the OI HEALTHI of the Université Paris-Saclay

Conflicts of interest

The authors declare no conflict of interest.

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Discussion

Our thesis project went from epidemiological observations, to better understanding the emergence of some OXA-48 variants, to finding novel therapies active on these MDR bacteria. Thus in a first set of experiments we tried to address from a molecular point of view the epidemiological situation of carbapenem resistance in Lebanon, and more precisely in three hospitals in the northern part of the country. We focused on the three bacteria, Enterobacterales, *Pseudomonas* and *Acinetobacter* isolates, considered as critical for the discovery of novel therapies according to WHO (235). Based on our findings, the prevalence of carbapenem resistance in north Lebanon, follows an increasing trend over the years. Among the circulating enzymes, OXA-48-like and NDM were the most prevalent one. This result was quite surprising as in many neighboring countries high incidence of KPC-producers have been described (236). We reported for the first time in Lebanon OXA-162, and OXA-48 variant and NDM-19, both displaying increased imipenem-hydrolysis as compared to their parental enzyme. Interestingly the most prevalent enzyme was the plasmid encoded OXA-48, but over the years, NDM is increasing its prevalence. In addition, we showed, on a limited number of isolates that OXA-23 is responsible of carbapenem-resistance in *baumannii* isolates, while in *P. aeruginosa* non enzymatic carbapenem-resistance is observed, as in many European countries, where OprD mutations are the main mechanism for carbapenem-resistance (237).

Factors standing behind this alarming situation in Lebanon are various, (i) the possibility of obtaining antibiotics without a prescription, (ii) the high prevalence of inappropriate antibiotics prescription, regardless of the specialty of the doctor, (iii) the uncontrolled use of antibiotics in the animal sector, add to this, (vi) the availability of antibiotics for online purchase, which increases the circulation of medicines and decreases the quality of care. Likewise, there

is a poor level of awareness to the general population of the AMR situation and the responsibilities of the society in this problem. In addition, the detection carbapenemase-producing Gram-negatives either in carriage or in infections is not efficient in most hospitals, thus preventing implementation of infections control strategies to reduce transmission and circulation in the hospital settings. Moreover, the massive immigration of Syrian refugees and their placement in camps with poor sanitation conditions in Northern Lebanon have worsened the situation. This factor was already shown to spread resistance in the region. For instance, the presence of carbapenem resistant *A. baumannii* CRAB by production of NDM-1 type enzymes, was first reported in Lebanon by Rafei et al. in Syrian injured patients hospitalized in Tripoli, northern Lebanon (238). Our findings along with previously published studies confirmed that enzymatic resistance mechanisms remain the most prevalent (223).

Our results advocate for serious actions, such as a reinforced surveillance system along with respect of standard infection control measures. Rapid and proper diagnosis of CPE carriers and their isolation should limit their dissemination. Moreover, the government should take several actions including the prohibition of over the counter sales of antibiotics, and the organization of awareness campaigns to ensure the compliance of patients to their prescribed therapeutic regimen. All these actions will help in the limitation of AMR problem.

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 especially with an economic collapse. 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 (225). 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 (239, 240). 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. As an important Lebanese community is living abroad, especially in France, our results are also important for hospitals accepting frequently transferred patients from Lebanese hospitals or patients have visited relatives in Lebanon.

OXA-48-like enzymes remains the most prevalent carbapenemase among our collection of isolates. However, a diversification of variants has been observed, especially with the spread of OXA-244 variant. The reason of this emergence, are still not fully understood, but it is thought that difficulties in detection, leads to underdetection, and thus to silent spread. Indeed bacteria expressing OXA-48 variants with modified R214 AA display increased susceptibility to carbapenem and temocillin (Emeraud). During this thesis we investigated biochemically and genetically three OXA-48/OXA-181 variants with R214 changes: OXA-244, OXA-484 and OXA-933, as well as several laboratory generated OXA-48 mutants.

The increasing prevalence of OXA-48 is facilitated by their horizontal transfer mediated by an IncL plasmid (187). Along with its dissemination evolution of the carbapenemase has been witnessed, and characterized by the accumulation of mutations likely in response to environmental stress and thus resulting in the appearance of novel OXA-48 variants with modified hydrolytic profiles and stability. OXA-48 enzymes are commonly found in the Middle East, North Africa, India, and Europe (130). Thus the OXA-48 variants that have lost carbapenem hydrolysis activity and gained activity against extended-spectrum cephalosporins, had amino acid deletions in the β 5- β 6 loop, suggesting its major role in substrate specificity. Similarly, OXA-232, which differ from OXA-181 by a single substitutions at position 214 (R214S), emphasized the importance of residue 214 in carbapenem and temocillin hydrolysis (178). Here we confirmed the key role of R214 in the hydrolysis of OXA-244 and OXA-484. Our work demonstrates that changes at position 214 in β 5 β 6 loop has a major impact on the conformation of the active site and therefore on the hydrolytic profile of the enzyme. In the first description of the 3D structure of OXA-48 by Docquier et al. a salt bridge between R214 and D159 could stabilize the active site and allow temocillin to position ideally for efficient hydrolysis (241). R214 delimits the active site cavity of OXA-48 and may be in part responsible for the closed conformation of the β 5- β 6 loop by forming a salt bridge with D159 in the omega loop. The β 5- β 6 loop and the omega loop may also influence the formation of a network of water molecules probably having an impact on both the rate at which a water molecule may enter the active site and its most stable position once inside. The MIC for imipenem for the R214S mutants (178) suggests that maintaining the R214-D159 salt bridge is important for the hydrolysis of imipenem, allowing the best conformation to facilitate imipenem hydrolysis and keep R214 and both loops in a conformation that maintains a good water molecule network. We focused on

R214 natural variants as they are increasingly isolated worldwide (183). Even though these variants show reduced hydrolytic activity of carbapenems and temocillin. In addition, the *bla*_{OXA-244} gene is usually encoded on bacterial chromosome in *E. coli* ST38, the main bacteria spreading OXA-244, thus resulting in even lower levels of enzyme production as compared to plasmid locations (1 copy versus 10–20 copies) (183). R214 is believed to interact with D159, resulting in the stabilization of the active site and therefore contributes to the substrate specificity of the enzyme. We were very fortunate during our thesis that an D159N variant was evidenced at the French National reference center, allowing to study the precise role of D159.

We were able to show that the substitution of residue 214 results in a significant decrease in carbapenemase activity where *k_{cat}/K_m* of imipenem, and ertapenem showed significant decrease of 10-folds as compared to OXA-48, and that of meropenem was decreased by 8 folds. Decreases in hydrolysis was mainly due to a decrease in both *K_m* and *k_{cat}*.

The salt bridge established between residues 214 and 159 delimits at the active site a hydrophilic pocket. Thus water molecules would lodge in this cavity located near the serine 70 (S70), promoting the catalysis step. We showed that, the loss of R214-D159 interaction no longer allows to have water molecules to proximity to the active site, which therefore decreases hydrolytic activity.

OXA-933, a novel natural OXA-48 variant with a D159N replacement was subsequently characterized. As for OXA-244, OXA-933, displayed reduced carbapenem and temocillin hydrolysis. The activity was still compatible with a carbapenemase as its *K_{cat}/K_m* was above 0.27 mM⁻¹ s⁻¹, the cut-off set by Dabos *et al.* for carbapenemase definition (242). Site directed mutagenesis was undertaken to further characterize the impact of AA substitutions at position 159. the following enzymes were constructed: D159G, with glycine having no side chain; D159A with alanine having a methyl side chain; D159W with tryptophane having a

bulky hydrophobic side chain and; D159K with lysine having a positively charged side chain. CarbaNP test results showed that when D159 is replaced by Asp (N), Lys (K), Ala (A) or Trp (W) no detectable activity was evidenced. With D159G, detectable activity was evidenced. Using immunochromatographic assay, we could show that showed positive OXA presence for TOP10-pTOPO OXA-933 and the generated mutants except for TOP10-pTOPO all the mutants could still be detected, except for D159W, that probably underwent drastic structural changes. W residue, being very bulky likely interferes with the binding of one of the antibodies to the enzyme or through sterical changes modifies dramatically the overall structure of the enzyme. . While all the D159 mutants conferred lower MICs and hydrolytic activities to carbapenems then OXA-48, with temocillin, MICs were lower, but these changes were not confirmed through steady state kinetics determination. . Reduction in the catalytic efficiency in imipenem, meropenem and ertapenem was due to a slight increase in K_m in OXA-933 and D159G, but for D159K k_{cat} reduction of imipenem responsible for the change in the hydrolysis ratio. In order to confirm our results, other AA replacements need to be undertaken, especially with a glutamic acid, to see the role of the charges of the AA.

The third part of this thesis was dedicated to the study of alternatives to overcome the scarcity of treatment options and thus reducing mortality, morbidity and economical burden. We have characterized, in the frame of a European project the action of two new classes of antibiotics from myxobacterial and actinobacterial strains hoping they will act on resistant bacteria precisely, bacteria that belongs to ESKAPE complex and those listed by WHO as high priority species for which new antibiotics are urgently needed , (carbapenem resistant *Acinetobacter baumannii*, carbapenem resistant *Pseudomonas aeruginosa*, carbapenem resistant, and ESBL-producing Enterobacterales). Cystobactamids and chelocardins have shown promising

results in few published studies (210, 243, 244). Cystobactamids act on bacterial gyrases resulting in bacterial death by DNA synthesis inhibition (243). The evaluation of cystobactamid derivatives on a large panel of Enterobacterales showed that these compounds are active against *E. coli*, *K. oxytoca*, *K. variicola*, *C. freundii*, *C. brakii*, *E. cloacae*, *S. marcescens*, *Salmonella* sp. and *S. flexneri*. *K. pneumoniae* seems to escape the activity of cystobactamids, this may be due to the intrinsic characteristics of *K. pneumoniae* especially the fact that this species has some distinct capsular features (245). This should be further explored maybe by testing *K. pneumoniae*, with agents able to inhibit the capsular expression and see if any shifts in MICs could be observed. Further chelical improvements should be done so that more species (e.g. *P. aeruginosa* and *A. baumannii*) be covered. Overall, cystobactamids activity on non-fermenting gram-negative bacteria was not as potent as expected. Although many tested strains were resistant to fluoroquinolones and colistin, no obvious link of cross resistance with cystobactamids was established regardless that fluoroquinilones and cystobactamids have somehow similar bacterial targets, but cross resistance between the two families was excluded, which renders cystobactamids active on fluoroquinolone resistant isolates and thus encourages further studies on these compounds. Finally, this class of molecules showed also excellent activity against Gram positives.

Two chelocardins derivatives were tested, among which the natural CHD and the amidochelocardin CDCHD (245). revealing relatively good activity against *K. oxytoca*, *K. variicola*, *E. coli*, *C. freundii*, *C. brakii*, *S. marcescens*, *M. morganii*, *Salmonella* sp. and *S. flexneri*. Results with *P. aeruginosa* and *A. baumannii*, did not come in favor of chelocardins. Gram positive strains were not as susceptible to chelocardins as they are to cystobactamids.

Despite the growing number of studies performed on Cystobactamids, few studies were investigating the mechanisms of resistance. By defining the frequency at which bacteria may

become resistant, we also aimed at identifying the locus leading to that resistance, and thus fine tuning the mode of action of this class of molecules. Mutation frequencies of 0.27×10^{-8} for CN-861-2 and 0.17×10^{-8} for CN-DM-861 were found and are compatible with DNA polymerase generated errors (10^{-9} or 10^{-8}). The mutants had increased MICs to these molecules, but presented large numbers of SNPs, as compared to the parental strain, suggesting a mutagenic feature of Cystobactamids, and likely toxicity. However, in a mouse in vivo model, these compounds didn't show any toxicity (246). In depth analysis of mutated alleles, revealed genes involved in iron and manganese uptake such as the SitABCD operon. The adenine methyl transferase, encoding gene DpnM was also found to be mutated in the first batch of selected clones. Interestingly, this enzyme works on DNA single strands which recalls the mode of action of Cystobactamids having topoisomerases IIA as target. The mutated YibH, consists also of a transmembrane protein, involved in many cellular processes including transport and cellular response to damage, which may be related to the large number of SNPs observed in the Cystobactamid resistant mutants. Putative large exoprotein involved in heme utilization or adhesion of ShlA/HecA/FhaA family were mutated in 7 mutants. According to Uniprot, they are involved in many functions including adhesion, invasion and colonization of host cells. The heme utilization recalls the implication of iron in the process. More importantly, many genes involved in adhesion and motility were mutated, such as Phage tail fiber, tail fiber assembly protein Tfa, Phage tail fiber Mup49, and Type 1 fimbriae regulatory protein FimB. Those genes are generally under the control of a two component system, the QseBC, which is in its turn altered in some mutants. This two component system consists of a sensor kinase, the QseC, and a response regulator, the QseB. This system is thought to regulate genes involved in motility, adhesion, and other biological processes. Another interesting mutated gene, is Undecaprenyl phosphate-aminoarabinose flippase subunit ArnF, which is part of the Arn operon, responsible

for LPS modifications. Mutations in genes belonging to Arn operon could result in resistance to colistin by altering the LPS and thus changing the membrane charge. This suggests that maybe, a similar resistance mechanism could confer resistance to cystobactamids. UDP-N-acetylmuramoyl-dipeptide-2,6-diaminopimelate ligase involved in the peptidoglycan synthesis was found to be mutated, indicating that maybe an alteration in this process was also noted. Antirestriction protein klcA, mutated in 6 cystobactamid resistant mutants, could be implicated in carbapenem resistance dissemination. LysR-type transcriptional regulators (LTTRs) regulate various genes, including virulence genes, metabolism genes, quorum sensing and motility, suggesting that the mutations found in lysR family transcriptional regulator HdfR, could be related to the alteration of QseBC two system component.

For the moment, no clear explanation of cystobactamid resistance mechanism could be elaborated, further studies are needed to find a solid hypothesis for this phenomenon, but our findings suggests the implication of many genes in this mechanism including some genes involved in colistin resistance. As cystobactamids might DNA damage inducing antibiotics, we wanted to see using RNAseq experiments the changes in the transcriptional landscape of *E. coli* ATCC25922 exposed for a short time to cystobactamids and chelocardins.

Exposure to cystobactamids caused significant upregulation of genes encoding DNA cellular response to damage, confirming our previous observations. Concerning the downregulated genes, they were mostly implicated in iron metabolism and transport, which made us question the activity of cefiderocol in cystobactamid resistant mutants since this antibiotic is a siderophore cephalosporinase that takes advantage of iron transport system to penetrate the bacterial cell and act on it. If iron transport is affected by cystobactamids, then the MICs towards cefiderocol should show shifts towards higher values. This was confirmed for nine mutants out of 18.(Data not shown). For chelocardins exposure, the expression of many genes were

altered. Despite the studies showing that chelocardins act on bacterial membranes (198), our findings reinforces the outcome of few studies talking about their activity on ribosomes (REF) since they show a large structural similarity to tetracyclines, and showed many upregulated genes that are implicated in protein synthesis. Efflux is also affected by exposure to chelocardins especially components of ABC family. After chelocardin exposure, many processes were affected, this includes genes of iron metabolism, TCA cycle, motility proteins. RNA sequencing was also confirmed by RT-qPCR for few genes

Conclusion

In conclusion, during this thesis we contributed to (i) the understanding of the epidemiological situation of carbapenem resistance in Lebanon, (ii) the unraveling the role of R214 and D159 in the carbapenemase activity of OXA-48 type, (iii) the assessment of the spectrum of activity of two novel antibacterial compounds, and (iv) the mode of action of these molecules. The AMR situation in Lebanon is very alarming with difficult to treat bacterial infections with only very limited treatment options, such as tigecycline, or colistin, as all the newer molecules are not yet available. It is expected that the situation is going to degrade even more unless actions are taken to control the spread but giving the dramatic economic crisis the country is going through, and the numerous refugee's camps established in Lebanon, where the refugees are living in very precarious conditions (over-crowded camps, no access to quality controlled drinking water and no sanitation devices), it is very unlikely that these actions may occur. For sure, nationwide epidemiological studies based on WGS are required to monitor the ongoing epidemiological situation and to trace dissemination routes. The isolation of more *E. coli* isolates expressing carbapenemases is scary, as this indicates that these enzymes are now present in the community and influx into the hospital through admitted patients, rather than through nosocomial spreading. This has been clearly shown in our epidemiological studies, with the different *E. coli* isolates being unrelated.

We have witnessed the emergence of OXA-48 variants with impaired carbapenem and temocillin hydrolysis in Lebanon, France and in fact worldwide (178, 225, 247). While we have now clues on how these AA changes alter the hydrolysis of these substrates, we still don't know what could have triggered the selection of these variants. In 16 out of the 22 OXA-48 variants with changes in the $\beta 5$ - $\beta 6$ loop, the R214 is changed to S (n=3), G (n=3), N (n=1), D (n=3), K (n=1), E (n=2), and G (n=3) (141). In 5 out of the 6 remaining, R214 is deleted and only in one

case, in OXA-438, the R214 is still present, but two amino acids 215-216 have been deleted (193). These results clearly indicate that OXA-48 R214 variants, even though with reduced hydrolytic activity are constantly selected. As the observed conformation of the loop in OXA-48 is promoted by a salt bridge of R214 with the Ω -loop D159 residue, which itself is stabilized by S155 (178, 241) future investigations are needed to explore this interaction in more detail. Finally, the fitness cost of these different variants need to be evaluated to understand their emergence.

As carbapenemase producers are resistant to most commercially available antibiotics, novel molecules with novel targets to avoid cross resistance with older molecules are urgently needed. We have explored two novel antibiotics that display very interesting spectrum of activity, for most Gram positive and Gram negative bacteria, except *K. pneumoniae*. Future chemical modifications are needed to extend the spectrum of activity to include *K. pneumoniae*, as this is a major nosocomial pathogen.

The growing AMR issue has harsh effects on mortality rates and a large economic cost. In addition to all the important studies that help in AMR limitation, serious actions must be taken: Robust surveillance systems must be established on national and global levels to monitor the emergence of novel resistance mechanism, way before they become endemic, and too late to intervene efficiently. Awareness campaigns should be widespread to ensure everyone uses antibiotics only when needed to avoid unnecessary use of these compounds, which leads to AMR. While it is going to be difficult to control community spread, we have to keep our hospitals safe places for everyone, thus globally, screening and isolation of carrier patients should be implemented. But in many LMIC, health issues are secondary, and AMR is not even considered as a problem.

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Article 11 – In progress

Structural analysis and mechanism of action of Cathelicidin-BF, an antimicrobial peptide active towards resistant clinical isolates

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Keywords: antimicrobial peptide, drug resistance, molecular dynamics, solution NMR, solid-state NMR, minimal inhibitory concentration

Abstract

Cathelicidin-BF (CatBF) is an antimicrobial peptide found in many vertebrates with an exceptional portfolio of antimicrobial, antiviral, antifungal and anticancer activities. Contrary to many AMPs, it showed a good pharmacological profile with a half-life of at least one hour in serum and it has proven effective against bacterial infections in mice. With the aim of investigating its capabilities in the context of resistant nosocomial infections, we have tested its activity against more than 50 resistant clinical isolates and we investigated the molecular mechanisms by which CatBF interacts with its bacterial target by using phospholipid membranes. By NMR spectroscopy and MD simulations we found how CatBF selectively recognises bacterial over mammalian phospholipid model bilayers. Its N-terminal portion forms a stable helix onto the target membrane and penetrates it with its axis almost parallel to the surface, while lysine and arginine residues form salt bridges with phosphate groups of phospholipids with a preference for negatively charged ones. At higher concentrations, CAT-BF causes membrane deformation as a possible prelude to its rupture and the consequent bacterial death.

1. Introduction

Multidrug resistance is responsible for tens of thousands of deaths every year (1, 2). Resistant microbes cause many nosocomial infections in health system users (2–6). They usually colonize multiple surfaces such as catheters, implants, stents, contact lenses or ventilators, among others (1, 2). Their disinfection is not a trivial task. Several methodologies are currently being used although they are not always effective in case of extremely resistant pathogens. Many of those bacteria are able to form spores or biofilms which are hard to tackle (1, 2).

Antimicrobial peptides (AMPs), often referred to as host defense peptides (HDPs), are produced by most organisms and constitute a part of their innate immune system (7, 8). They are usually short, positively charged and constituted by a high proportion of hydrophobic residues that allow them to selectively target membranes of pathogens (9, 10), which usually expose negatively charged lipids to the outer leaflet (11, 12). AMPs are known for their broad spectrum of microbicidal activity and their mode of action is usually less prone to the development of resistances (13–16) because of their high killing rate (higher than common

antibiotics) which do not allow microbial replication (17–19). Furthermore, remodeling of the membrane to impair their action may require more effort compared to antibiotic targeting a single molecular target (16).

AMPs are particularly interesting in the fight and in the prevention of nosocomial infections as they can be incorporated into coatings used in the fabrication of implants or catheters (1, 2, 6, 20) but also metallic implants (1, 2, 20, 21). In addition, their immobilization onto surfaces can often bypass some of their weakness, such as their short half-life caused by the action of proteases (22, 23).

Cathelicidins are a family of AMPs produced by many vertebrates. They are released by proteolytic cleavage from their inactive precursors upon leukocyte activation (usually neutrophils and macrophages) (9, 10, 24, 25). They display bactericidal actions, but also antiviral, antifungal or anticancer. Additionally, they play important immunomodulatory roles and regulate inflammation processes. Their name is due to the presence of “cathelin”, a relatively well conserved domain, at their N-termini. The homology is higher in the precursor form, while mature forms show different amino acid sequences, structures and lengths (9) especially at the level of the C-terminus (9, 24, 25). Cathelicidins, as other AMPs, usually exert their action by damaging membranes, but they can modulate the immune response targeting receptors (9, 10, 26) or act as a cell penetrating peptide (27–31) which exert their intracellular actions by transpassing the external membrane rather than damaging it (32–39).

Cathelicidin-BF is a peptide isolated from the venom of the snake *Bungarus fasciatus* (40) with antibacterial (40–43), antifungal (40, 44, 45), anticancer (27–29, 46) and immunomodulatory (47–53) properties.

It has been successfully used against *Staphylococcus aureus*, *Propionibacterium acnes*, *Salmonella typhi*, *Shigella dysenteriae*, and *Escherichia coli* (54–56). Even if CatBF degrades quickly in simulated intestinal fluid, it displayed a half-life of at least one hour in both serum and simulated gastric fluid and it has proven effective against *Pseudomonas aeruginosa* and *Staphylococcus aureus* in murine models (30, 57, 58). In this work, we tested its activity against relevant resistant clinical isolates and investigated its mechanism of action at molecular level by means of NMR and MD simulations.

2. Materials and Methods

2.1. Peptide synthesis

The peptide CatBF (KFFRKLKKS VKKRAKEFFKKPRVIGVSIPF) was synthesized following the standard Fmoc-synthesis protocol on a Millipore 9050 automatic peptide synthesizer. The peptides were purified by reverse phase HPLC (Gilson, Villiers-le-bel, France) using a preparative C18 column (Luna, C-18-100 Å-5 mm, Phenomenex, Le Pecq, France) and an acetonitrile/water gradient (Figure S1). Their identity and purity (>90%) were checked by MALDI mass spectrometry (MALDI-TOF Autoflex, Bruker Daltonics, Bremen, Germany) (Figure S2).

2.2. Susceptibility testing

To test antimicrobial susceptibilities to the CatBF peptide on various bacteria isolates, broth microdilution using cation-adjusted Muller Hinton broth (CA-MHB) as recommended by EUCAST was used. Roughly an inoculate of McFarland 0.5 was obtained with fresh colonies grown on Mueller-Hinton agar plate. Within 15 min the adjusted bacterial suspension was diluted in CA-MHB to reach approximately 10×10^5 CFU/mL in a final volume of 50 µL. To this, 50 µL of the diluted CatBF were added, to reach a final concentration of 5×10^5 CFU/mL of bacteria in each well.

Stock solution of CatBF was prepared at 9.8 mg/ml, and sequentially diluted in Water. 50 µl of bacterial suspension was added to each dilution of CatBF in order to test a range of final concentrations between 128 µg/mL to 0.125 µg/mL. One row of each well plate served as a growth control without antibiotics. A sterility control was also performed. Microdilution plates were incubated at 37°C for 20 h and subsequently analyzed by the naked eye. Each MIC determination was done in triplicate.

The bacterial isolates used were pre-characterised multidrug-resistant clinical isolates from the strain collection of the German National Reference Centre for Multidrug-resistant Gram-negative Bacteria (Bochum, Germany) and (Paris, France). Pre-characterisation was performed as previously described (59). Testing was performed according to ISO 20776-1.

2.3. Liquid-state NMR spectroscopy and sample preparation

1. 2.3.1. Titration of CatBF with DPC micelles

50 mM phosphate buffer with 10% of D₂O and 0.1 mM deuterated sodium 3-(trimethylsilyl)propionate-d₄ (TSP-d₄) (used as internal reference) was used to solubilise CatBF in a final volume of 500 µl. The final concentration was 0.8 mM. Small amounts of a 1 M stock solution of dodecylphosphocholine-d₃₈ (DPC-d₃₈) were added gradually up to a final concentration of 60 mM. All the experiments were run at a temperature of 310K.

2. 2.3.2. Paramagnetic NMR experiments

The paramagnetic probe 16-doxyl stearic acid (16-DSA) was dissolved in deuterated methanol and was gradually added to the samples of CatBF with DPC micelles until the paramagnetic effect was observed as an increase of the linewidth of at least one peak, (usually for a ratio 16-DSA/DPC of 1:100). The percentage reduction of intensity of H/C cross peaks in ¹H, ¹³C-HMQC spectra was used to assess paramagnetic contributions. Errors were evaluated using the propagation formula in ratios $A = \frac{B}{C} : \Delta A = A \left(\frac{\Delta B}{B} + \frac{\Delta C}{C} \right)$, where B is the cross-peak intensity without 16-DSA, C is the cross-peak intensity with 16-DSA, and ΔB and ΔC are the spectral noise. Tests were carried out at 310 K.

2.3.3. Titration of CatBF with isotropic bicelles

CatBF was solubilized in the same buffer as the one used for titration with DPC micelles (see section 2.3.1). The final concentration was 0.8 mM. Small amounts (in the order of microliters) of a stock solution of bicelles were gradually added until reaching a final concentration of 60 mM. Isotropic bicelles were obtained by mixing in chloroform 1,2-

dihexanoyl-sn-glycero-3-phosphocholine (DHPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG) at molar ratios 66.7/25.0/8.3. After the mixing, chloroform was evaporated under a nitrogen stream and overnight lyophilisation. Experiments were run at a temperature of 310 K.

2.3.4. NMR acquisition and processing

A 500 MHz Bruker spectrometer equipped with a 5 mm Broadband Inverse (BBI) probe was used to perform all the liquid-state NMR experiments. ^1H , ^{13}C -HMQC, ^1H , ^1H -TOCSY (mixing time of 60 ms), and ^1H , ^1H -NOESY (mixing time of 200 ms) were used to assign backbone resonances. Spectra were analyzed by TopSpin 4 (Bruker BioSpin) and NMRFAM-SPARKY (60). Secondary structure prediction by examining the chemical shift deviations (61–63) was done comparing with reference random coil values obtained by POTENCI web server (<https://st-protein02.chem.au.dk/potenci/>) (64) at 310K.

2.4. Solid-state NMR spectroscopy and sample preparation

Samples for solid-state NMR spectroscopy were prepared by co-dissolving POPE:POPG 3:1 (molar ratio) or *E.coli* total lipid extract with appropriate amount of CatBF peptide in methanol:chloroform (1:1 by volume). Solution was carefully applied onto 25 ultrathin cover glasses (8 x 18 mm; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), dried under vacuum overnight and hydrated at 93%r.h. at 37°C for 2 days. Thereafter glass plates were stacked on top of each other, sealed and proceeded to NMR measurements.

Proton-decoupled ^{31}P solid-state NMR spectra were acquired at 121.575 MHz using a Bruker Avance wide-bore 300 solid-state NMR spectrometer equipped with a commercial double-resonance flat-coil probe (Bruker, Rheinstetten, Germany) [52, (Adélaïde et al. 2023)]. A Hahn-echo pulse sequence [53] was used with a $\pi/2$ pulse of 5 μs , a spectral width of 100 kHz, an echo delay of 100 μs , an acquisition time of 10.2 ms, and a recycle delay of 3 s. An exponential line broadening of 50 Hz was applied before the Fourier transformation. External 85% H_3PO_4 at 0 ppm was used for calibration. The temperature was set to 37°C.

The proton-decoupled ^{15}N cross-polarization spectra of static aligned samples were

acquired at 30.43 MHz using a Bruker Avance wide bore 300 NMR spectrometer. An adiabatic CP pulse sequence was used with a spectral width, acquisition time, CP contact time, and recycle delay times of 25 kHz, 10.2 ms, 0.4 ms, and 3 s, respectively [51,54]. The ^1H $\pi/2$ pulse and spinal-64 heteronuclear decoupling field strengths B_1 corresponded to a nutation frequency of 31 kHz. Then, 40 k scans were accumulated, and the spectra were zero-filled to 4 k points. An exponential line broadening of 100 Hz was applied before the Fourier transformation. Spectra were externally referenced to $^{15}\text{NH}_4\text{Cl}$ at 39.3 ppm [55]. Samples were cooled with a stream of air at a temperature of 37°C.

Next, ^2H solid-state NMR spectra were acquired using a quadrupole pulse-echo sequence [56] with a recycle delay of 0.3 s, an echo time of 100 μs , a dwell time of 0.5 μs , and a $\pi/2$ pulse of 5 μs . Before the Fourier transformation of the free induction decay, an exponential apodization with a line broadening of 300 Hz was applied. Spectra were externally referenced to $^2\text{H}_2\text{O}$ (0 Hz). The temperature was set to 37°C.

2.5. Molecular dynamics simulations

Lipid bilayer systems were generated using the CHARMM-GUI web-server (65) and GROMACS software (65) was employed to run the simulations under the CHARMM36m forcefield (66). Bilayers contained a total of 128 lipids and the initial structure of the peptide was obtained by I-Tasser (67). Peptide molecules were placed over the upper leaflet at a distance $>10\text{\AA}$. Lysine and arginine side chains were protonated. Water molecules were described by the TIP3P model (68) and a 50- \AA thick water layer was added above and below the membrane. Ions were added to obtain a concentration of 150 mM of NaCl, CaCl_2 or MgCl_2 where appropriate.

Particle mesh Ewald (PME) method was used for long-range electrostatic interactions (69, 70) and periodic boundary conditions were applied for all the simulations. All bonds were constrained by the LINear Constraint Solver (LINCS) algorithm (71), allowing a 2 fs integration step. Every system was energy-minimized with a 5000-step steepest-descent method. Berendsen barostat (72) was used during the equilibration while Parrinello-Rahman barostat (73, 74) was employed to maintain pressure (1 bar) semi-isotropically with a time constant of 5 ps and a compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$. The temperature was maintained at 310.15 K using the Nose-Hoover thermostat (75, 76) with a time constant of 1 ps. Simulations were run

for 500 ns (except when a different duration is specified in the manuscript) after the standard CHARMM-GUI minimization and equilibration steps (77). Minimization, equilibration and production runs were repeated to have two repetitions of membranes in absence of peptide and three in their presence. Convergence was assessed by the analysis of polar contacts and RMSD.

MD trajectories were analyzed by GROMACS tools and Fatslim (78). Graphs and images were created with GNUplot (79) and PyMol (80). MOLMOL (81) and VMD (82) were used for visualization.

3. Results and discussion

3.1. MIC tests

We were especially interested in evaluating the performance of CatBF against resistant bacterial strains involved in nosocomial infections. To this end, more than 80 strains were studied covering some of the most challenging species such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacteriales* (Table 1).

Interestingly, the MIC values obtained in the cation adjusted medium (containing Ca^{2+} and Mg^{2+}) were significantly lower than in the absence of these metal ions. Indeed, some cationic amphipathic AMPs are able to disrupt the outer membrane of Gram negative bacteria by displacing Ca^{2+} and Mg^{2+} ions which bridge lipopolysaccharide LPS molecules (83–87).

In order to further understand the mechanism of action we studied the structural properties of CAT-BF with models of bacterial membranes

3.2. NMR studies of CatBF interacting with model membranes

3.2.1. CatBF is largely unstructured in solution

CatBF (KFFRKLKKS VKKRAKEFFKKPRVIGVSIPF) is an antimicrobial peptide whose sequence is compatible with the formation of a rather amphipathic alpha helix (Figure 1A). Nonetheless, structure prediction by I-TASSER web server (67) predicts an alpha helical structure on only two thirds of its sequence (up to proline 21) (Figure 1B), where residues 21–30 make van der Waals contacts with the apolar face of the helix formed by residues 1–20, thus protecting it from the exposition to the polar solvent.

The amide region of the ^1H NMR spectrum of CatBF presents significant overlap, which is rather compatible with a random coil, in agreement with previous studies (40). The complete assignment of backbone atoms (HN, $\text{H}\alpha$, $\text{C}\alpha$) was achieved by ^1H , ^1H -TOCSY, ^1H , ^1H -NOESY and ^1H , ^{13}C -HMQC (Table S1) and used to detect the presence of elements of secondary structure along the sequence (Figure 1C,D). Deviations from random coil values (as calculated by the POTENCI web server (64)) are well below the threshold values expected for the presence of alpha helices or beta strands (0.1 ppm for ^1H and 0.7 ppm for ^{13}C) (88), thus revealing that CatBF is indeed largely unstructured, at least at the concentrations used in our experiments. CD spectra (Figure S3) confirm our findings being characterized by a large minimum at about 200 nm.

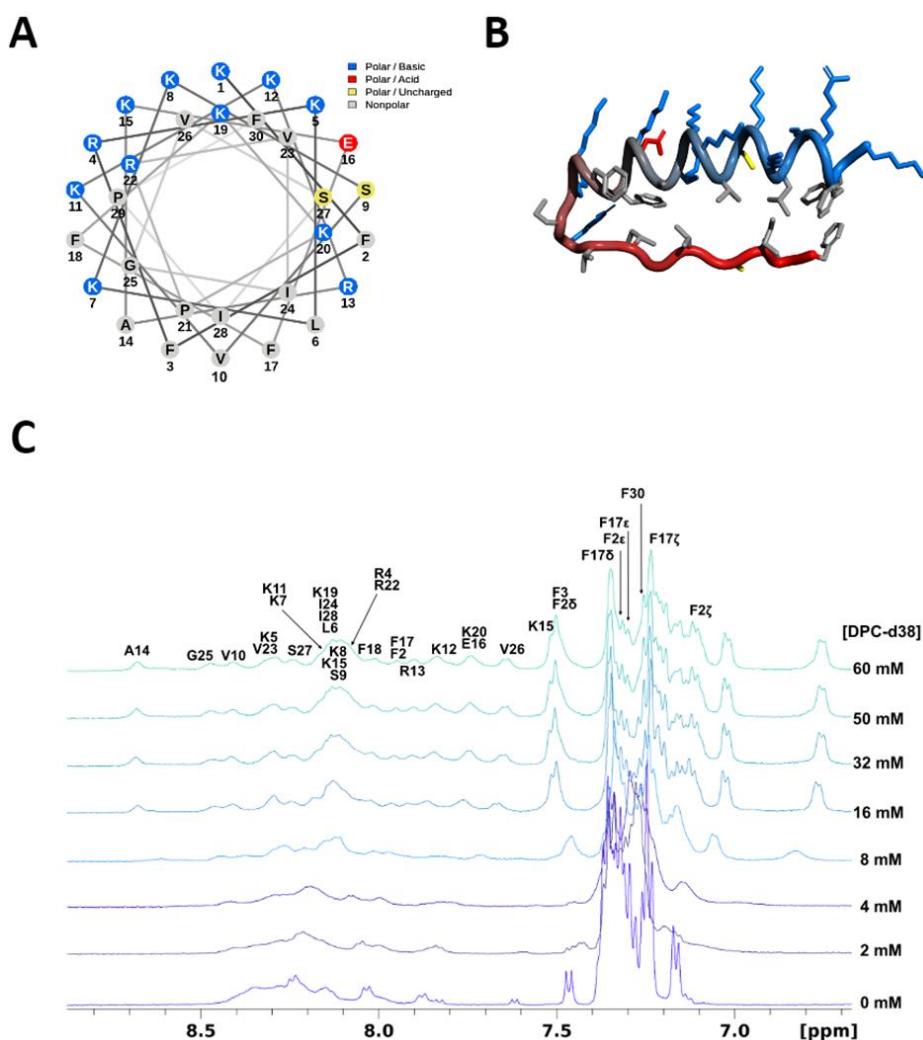


Figure 1. (A) Helical wheel for CatBF peptide (created by Netwheels (93)) and (B) structure prediction by I-TASSER web server (67). (C) 500 MHz ^1H NMR spectra (amide and aromatic region) of CatBF in the presence of increasing concentrations of DPC-d₃₈.

3.2.2. The N-terminal part of CatBF forms a stable alpha helix in membrane models

In order to study the interaction with membranes, we first used a very simplified model constituted by DPC micelles. Such systems allow the study of peptides and proteins in solution because of their small size, fast rotational tumbling and consequent relatively thin NMR lines (89). While DPC cannot form bilayers, the choline headgroup reproduces the surface of biological membranes and the apolar chains form an hydrophobic core while not relying on detergents such as SDS. The NMR spectrum of CatBF changes significantly in the presence of DPC micelles (Figure 2A).

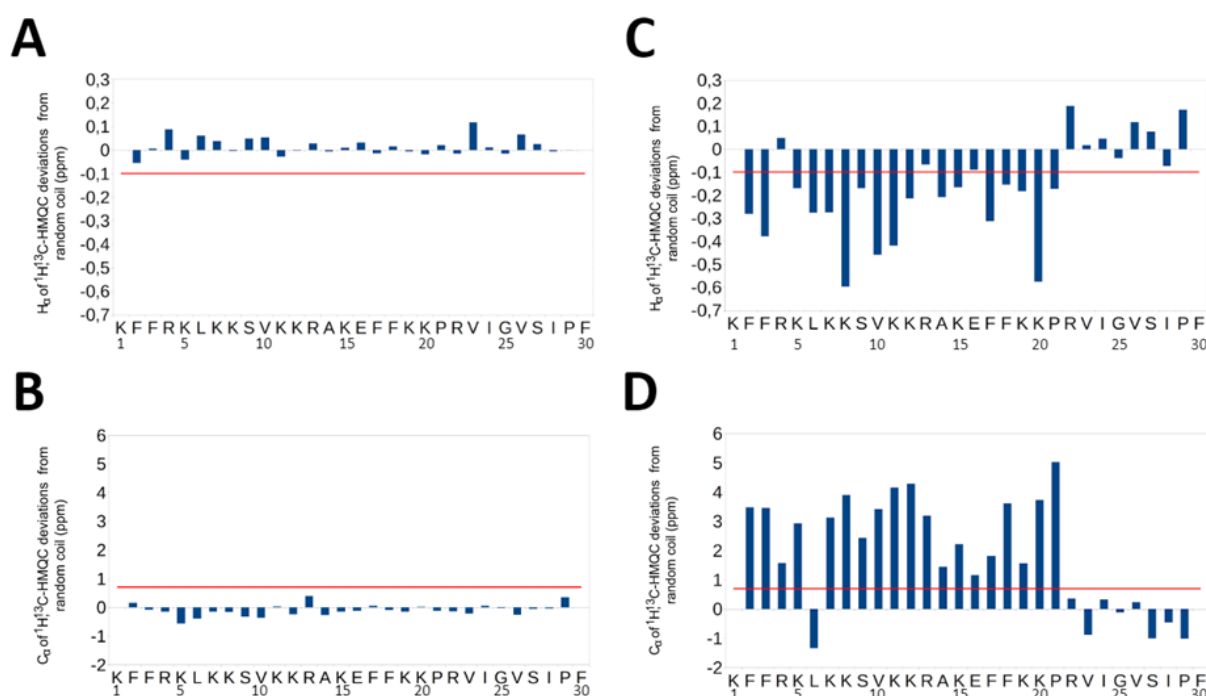


Figure 2. (A,B) H_α (A,C) and C_α (B,D) chemical shift deviations from random coil values estimated by POTENCI (<https://st-protein02.chem.au.dk/potenci/>) (64) of CAT-BF in the absence (A,B) and in the presence of DPC micelles (C,D).

The spectrum totally changes at peptide:lipid ratio 1:16, the sudden changes being indicative of slow exchange on the NMR time scale, typical of a sub-micromolar dissociation constant. Backbone atoms (HN, H_α, C_α) of the bound form of CatBF were assigned by ¹H, ¹H-TOCSY, ¹H, ¹H-NOESY and ¹H, ¹³C-HMQC (Table S1, Figure S4). The analysis of chemical shift deviations from random coil values clearly reveals that the peptide gets structured in the presence of micelles, the negative and positive deviations of respectively H_α and C_α being

indicative of alpha helical conformation up to lysine 20. The C-terminal tail (residues 21-30), starting by proline (which is notably an alpha helix disruptor) seem unstructured and highly flexible as suggested by the strong intensity of its backbone cross-peaks in 2D spectra (Figure S4). These data are in very good agreement with what was observed in SDS micelles and TFE/H₂O mixtures (40).

Paramagnetic probes can be used to confirm the alpha helical structure of the peptide and determine the orientation of the helix within the membrane model. To this aim, a paramagnetic center can be placed at the center of DPC micelles by addition of 16-DSA (16-doxyl Stearic Acid), a stearic acid derivative bearing a spin label at the very end of its acyl chain. By inserting itself into DPC micelles, 16-DSA affects the intensity of NMR signals of atoms in the proximity of the spin label placed in the micelle core. The effect is inversely proportional to the sixth power of their distance from the paramagnetic center (90–92). Extensive overlap did not allow a complete determination of paramagnetic effects induced by 16-DSA on the backbone H α /HN cross peaks in ¹H,¹H-NOESY spectra. In ¹H,¹³C-HMQC H α /C α peaks are less overlapped but often very weak, even in the absence of the paramagnetic species. Nonetheless, we can clearly observe complete disappearance of H α /C α peak of A14, H δ /C δ peak of L6 in the methyl region and aromatic peaks of F3 and F17 (Figure S5 and S6). Aromatic signals of F2 and F18 are also affected but to a lesser extent in the presence of 16-DSA. When observing the helical wheel of figure 1A, these effects are totally consistent with the orientation of the helix facing the center of the micelles by its apolar chains while lysines and arginines are pointing outwards. Indeed, despite the severe overlap among the α , β , γ , δ and ϵ of the 9 lysines and α , β , γ , δ of arginine (each within their groups) it is clear that these groups of signals are affected by the 16-DSA in the order $\alpha > \beta > \gamma > \delta > \epsilon$, as expected for a chain pointing far away from the micelle core. Furthermore, the region of the methyls of Valines (two out of which are in the unstructured C-terminal tail) and Isoleucine are not particularly affected, indicating that the C-terminal unstructured tail remains outside the micelle.

Paramagnetic relaxation enhancements have provided a rather well defined picture of the structure of CatBF inserted in DPC micelles. Isotropic bicelles can be used to better model its target membranes by allowing the introduction of headgroups typically found in bacteria such as PG. For this reason, we titrated a solution of CatBF with DHPC/DMPC/DMPG isotropic bicelles (Figure S7). Also in this case, the ¹H spectrum changes drastically when the

concentration of bicelles is increased, with new peaks appearing while those of the free form gradually disappear as expected for a slow exchange on the NMR time scale. The larger size of the system resulted in broadening of $\text{Ca/H}\alpha$ peaks beyond detection in ^1H , ^{13}C -HMQC spectra which prevented the assignment. However, the spectrum obtained in the presence of bicelles resembles that of Figure 2B, suggesting a similar structure as that defined in micelles. Indeed, structuring onto the membrane is testified by a clear increase of the peak dispersion in the amide region while peaks belonging to aromatic and methyl protons shift upfield, probably due to the insertion of their moieties inside the apolar core of the bilayer.

3.2.3. Most CatBF peptides lies with their helical axis parallel to the membrane surface while a second pool appears dynamically oriented at higher concentrations

The high curvature of micelles and the small surface of bicelles, together with the constraints in the nature and relative amounts of lipid head groups for both systems, limit their capability of reproducing biological membranes. Liposomes are vesicles that can reproduce better the curvature of a cell and its phospholipid composition can be freely varied to represent different biological unicellular organisms. Unfortunately, their relatively large size (from 20 nm to tens of μm) results in extremely large NMR linewidth due to short transverse relaxation rate caused by their slow tumbling. In these cases, important information can be obtained by magic angle spinning solid state NMR (MAS-ssNMR), in which the fast rotation of the sample at the magic angle of 54.74° with respect to the static magnetic field is able to quench dipolar interactions giving rise to relaxation. Alternatively, the large linewidth resulting from the observation of many different molecular orientations, each displaying different chemical shifts, can be significantly reduced in oriented systems, where all molecules maintain the same angle with respect to the magnetic field. In this work we have used oriented membranes to study the interaction of CatBF with bilayers composed of POPG and POPE, and even *E.coli* total lipid extract. In oriented systems the chemical shift is a function of the angle between an interatomic vector and the external magnetic field. Since in alpha helical structure amide HN are nearly parallel to the helix axis we have decided to use ^{15}N chemical shift to determine the orientation of the alpha helix formed by CatBF in biomembranes. For the sake of robustness, we have selectively labeled two probes relatively distant on the helical structure determined by liquid

state NMR: V10 and F17. Figure 3B shows the ^{15}N spectrum of CatBF ^{15}N label on F17 amide nitrogen. The good alignment of the membrane is evidenced by the major ^{31}P peak intensity at 28 ppm originating from the phosphate group of macroscopically aligned phospholipids in ^{31}P NMR spectrum (Figure 3A). The observed ^{15}N chemical shift of 65 ± 4.5 ppm is compatible with multiple orientations of the HN vector and more information is required to better define the tilt and pitch angle of the helix. For this reason, we used ^2H labeling of the methyl protons of A14. In the case of ^2H spectra, the quadrupolar coupling is also a function of the orientation of the methyl group with respect to the external magnetic field. The quadrupolar coupling of CatBF ^2H labeled at the level of A14 is 20 ± 3 KHz (Figure 3C). For better mimicking bacteria membrane we have used *E.coli* total lipid extract for glass plates oriented sample with CatBF peptide ^{15}N -labeled at V10 residue (Figure 3D). The ^{15}N chemical shift spectrum of V10 amide nitrogen in this more natural membrane showing the peak at 70 ± 4.5 ppm (Figure 3E) similar to the one observed in POPE:POPG 3:1 membrane (data not shown). It provides additional information for peptide topology determination (Figure 3F,G).

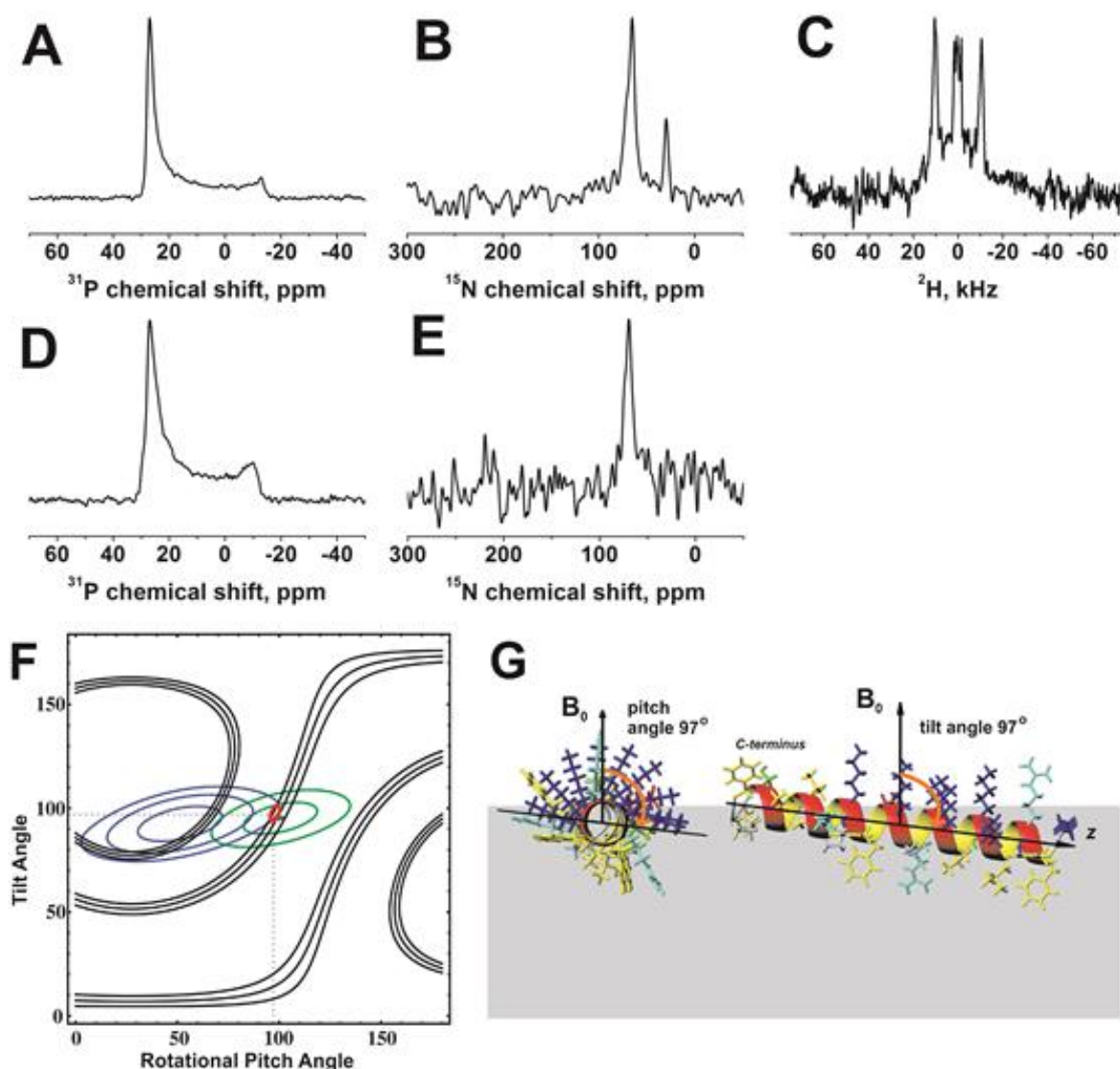


Figure 3. (A) Proton-decoupled ^{31}P (A,D), ^{15}N (B,E), and ^2H (C) spectra of 1 mol% CatBF in POPE:POPG 3:1 (A-C) or 2 mol% (D,E) CatBF in *E. coli* total lipid extract at 310 K, carrying ^{15}N label on Phe17 (B) or Val10 (E) and ^2H label on the methyl group of Ala14 (C). (F) Restriction analysis and corresponding peptide alignments relative to the membrane normal (G). The restrictions for the tilt and pitch angle of the ideal helix formed by CatBF are shown in blue for ^{15}N -Val10 (70.0 ± 4.5 ppm), green for ^{15}N -Phe17 (65.0 ± 4.5 ppm) and black ^2H -Ala14 (20.0 ± 3.0 kHz) (panel G). The restraints were obtained assuming rocking (18° Gaussian distribution) and wobbling (10°) motions of an ideal helix ($\phi = -65^\circ$, $\psi = -45^\circ$). In panel G, leucines, isoleucines, phenylalanines and valines are shown in yellow, alanine and proline in gray, lysines in blue, glutamines in magenta, and arginines and serine in cyan. One possible solution is found for pitch and tilt angles, corresponding to the values highlighted by a red circle in panel F.

Each of the three parameters (two ^{15}N chemical shifts and one ^2H quadrupolar coupling) obtained by oriented NMR spectra are compatible with multiple combinations of the pitch and tilt angle which perfectly define the orientation of the helix in POPE/POPG bilayers. However,

when the possible solutions are compared (Figure 3F) only one combination of pitch and tilt angle satisfies the three experimental values. Such combinations are shown in Figure 3G, where the helix axis lies almost parallel to the membrane surface and lysine residues point upward. Remarkably, this orientation is perfectly compatible with the one found in micelles and defined by paramagnetic effects (see section 3.2.2) and its biological significance is supported by the fact that the same ^{15}N chemical shift values are obtained in bilayers formed by *E.coli* total lipid extracts.

3.3. Molecular dynamic simulations of CatBF interacting with mammalian and bacterial membrane models

Molecular dynamics simulations have been widely used to get insight into the membrane-destabilizing action of several AMPs. In this study, we have explored the interaction of CatBF with membrane models mimicking bacterial and mammalian cells (11), the latter used for ascertaining potential cytotoxicity. Following previous studies (94–97), we have used a simple POPC bilayer as a mimic of a mammalian cell outer leaflet. Indeed, POPC exposes the phosphatidylcholine moiety which is the most common headgroup found in mammalian bilayers. On the contrary, bacterial membranes expose phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and possibly cardiolipin (CL). For our study we used molar ratios close to those found in many gram-negative bacteria (11), namely 70/30 for POPE/POPG and 67/27/6 for POPE/POPG/CL. Besides its antibacterial activity (40–43), CatBF displays also antifungal (40, 44) and anticancer (27–29, 46) properties, whose study must take into account the exposition of PS (cancer cells and fungi) and PE (fungi) on the target membrane. For all these activities, membranes formed by pure components (PE, PG, CL and PS) were also examined to ascertain the contribution of each lipid to the interaction with CatBF.

The activity of AMPs can be impaired by several factors such as temperature, pH and buffer. As most of the media used to determine their activity contain Na^+ , Mg^{2+} and Ca^{2+} , we have performed simulations with each of these ions separately. Magnesium is an essential factor for the action of many antibiotics such as tetracycline (98–100), but can impair the action of peptides targeting the cell membrane (101). For some peptides, the equilibrium between self-

aggregation and the interaction with a membrane target may be modulated by Calcium (102–107). Ca^{2+} binds to the phosphate groups of the bilayer, thus shielding its negative charge and reducing the affinity for positively charged residues of the peptide. Sometimes, calcium can form salt bridges connecting charged glutamate or aspartate residues of the peptide to the phosphate groups of the membrane, enhancing the peptide interaction (107, 108).

3. 3.3.1 CatBF poorly interacts with mammalian model membranes

Along MD simulations CatBF rarely interacts with POPC membranes. Polar (salt bridges and H-binds) and van der Waals contacts were estimated by the radial distribution function (68) at the relevant distances (Figures... contacts and Figure van der Waals). In the case of CatBF, few superficial contacts are established, indicating a poor and infrequent interaction. Other parameters support this view. The electron density profile along the bilayer normal provides a clear view of the membrane thickness and the localization of phosphate groups during the simulation. Such profiles clearly show the absence of alterations (including lipid interdigitation) induced by the presence of CatBF (Figure electron density). Furthermore, in the presence of important interactions, the membrane surface tends to invaginate resulting in an increase and decrease in the surface area per lipid of the internal and external leaflet of the bilayer, respectively. This parameter remains constant for the two leaflets (Figure area per lipid) even when the peptide concentration is increased by introducing 6 peptides in the simulations.

3.3.2. CatBF strongly interacts and penetrates in bacterial model membranes

Contrarily to what observed in the presence of POPC membranes, CatBF promptly interacts with phospholipids typically found in bacterial membranes (PG, PE and CL) and their mixtures (POPE/POPG and POPE/POPG/CL). This is demonstrated by the significant increase in both polar (Figures S14-S18) and apolar contacts (Figures S19-S21). It is interesting to point out that in all systems CAF-BF clearly prefers the interaction with POPG, as we had hypothesized (Figure 4G). Preference for negatively charged PG and CL is clear when analyzing the area per lipid of pure lipid bilayers, which reflect the deformation of membrane induced by the strong interaction with the peptide (Figure S24). Occurrence of polar contacts

in simulations with pure PE, PG and CL bilayers or their mixtures POPE/POPG/CL show that this preference follows the order of the global charge: CL > PG > PE (Figures S14, S15, S16 and S18). Another interesting feature highlighted by polar contacts is that the peptide tends to approach the membrane with its N-terminus, in agreement with the tilt angle found by solid state NMR (Figure 3H).

While polar contacts are informative about the first stages of the interaction, van der Waals contacts tend to inform on the final stages, when the peptide is in close contact with the hydrophobic tails of the lipids that constitute the model membrane (109, 110). Our solid state NMR experiments have highlighted an important perturbation of the order parameter of the palmitoyl chains in POPG/POPE mixtures used to model bacterial membranes. Such parameters can be calculated from MD simulations and directly compared to our experimental data (Figure 4B,E), provided the timescale of the phenomena are comparable. In 500ns simulations we can observe the first stages of the interaction. As already observed for other highly charged peptides (97, 109–114), CatBF initially increases the order parameter of both pure bacterial phospholipids and their mixtures while it decreases it once it manages to get deeper in the membrane core (Figures S9 and S10).

One limitation of all-atom molecular dynamics simulation over other kinds of enhanced sampling methods is that, while they allow to decipher the mechanism of action of the peptides at an atomic level, they cannot easily explore long time scales (115). Because of that, we decided to run long simulations (4 μ s) for both PC and PE/PG mixture. Simulations were repeated with six molecules of CatBF to simulate high concentrations.

While CatBF continues to avoid the interaction with PC bilayers, in PE/PG models of 4 μ s we can observe a partial penetration of the peptide, highlighted by a clear fluidification of the membrane (lower values of the order parameter, see Figure 5B but also S25, S26, S28 and S30). Also van der Waals contacts (Figure S21) testify the vicinity of the peptide to lipid acyl chains, especially in the presence of Ca²⁺ (Figure S28) but much less in the presence of Mg²⁺ (Figure S32). At variance with our experiments in micelles, van der Waals contacts are compatible with the internalization of the unstructured C-terminal tail (residues 22–30, see Figure S21). This discrepancy might be due to the intrinsic difference between micelles and liposomes. In any case it should be noted that C-terminal truncated versions of CAF-BF maintain their antibacterial activity.

The electron density profile (Figure S12) is very informative, showing significant lipid interdigitation (higher density in the membrane core) and the separation of the maxima related to phosphate groups into two doublets, indicative of membrane deformation.

Once in close contact with the bilayer, CatBF tends to lose its alpha helical structure (Figure 5C and S23) which is otherwise well conserved in all 500 ns simulations (Figure S22). This is an interesting point, as it might provide a molecular explanation of the dynamically oriented pool of peptides which has to be hypothesized to reproduce experimental solid state NMR spectra in oriented bilayers at high CatBF concentrations (see figure 3F and S8).

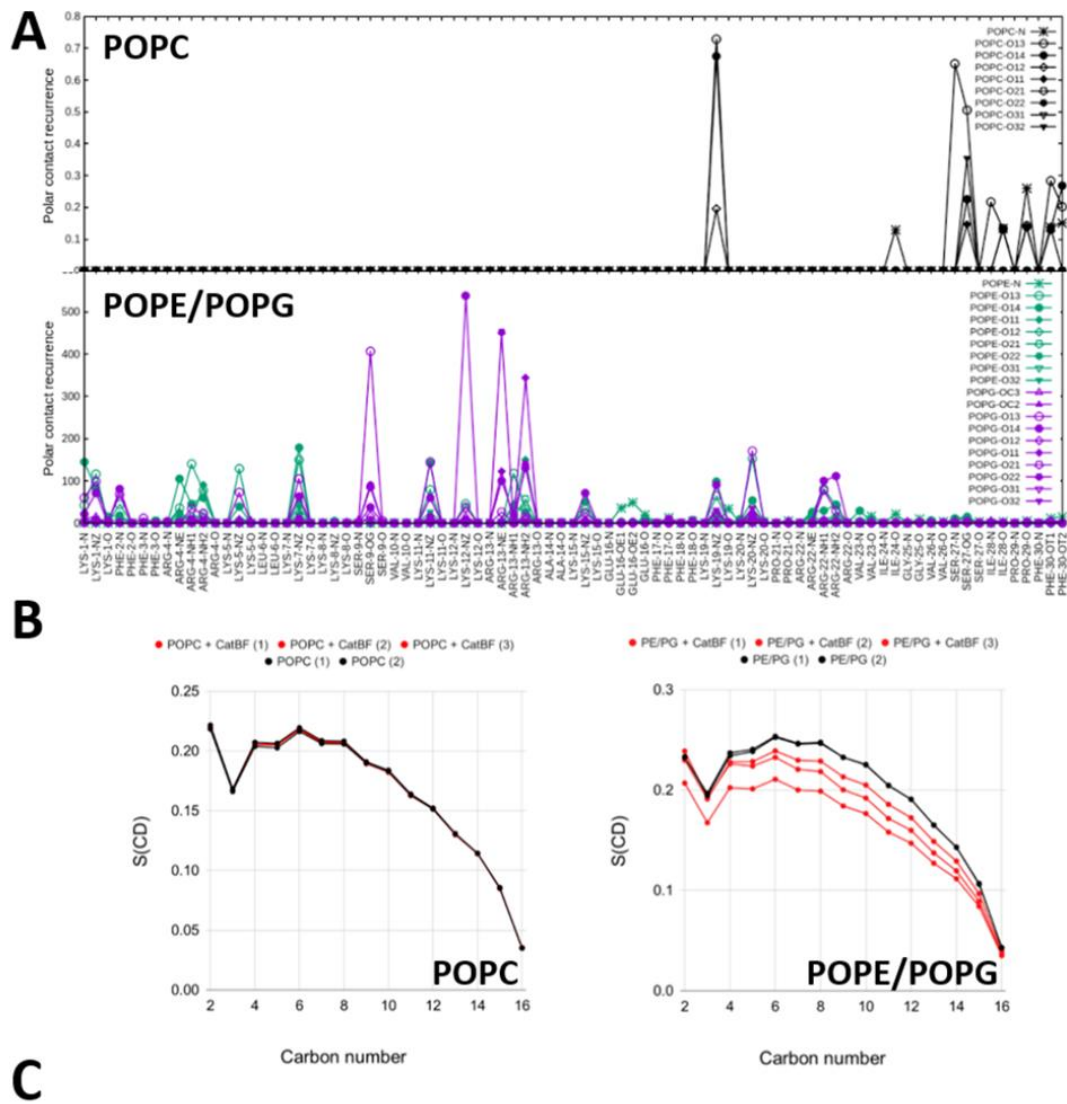


Figure 5. (A) Recurrence of polar atom contacts (H-bonds and salt bridges) between CatBF peptide and POPC (top) and POPE/POPG (bottom) bilayers calculated along MD simulation trajectories. (B) Palmitoyl chain order parameters of POPC and POPE (in POPE/POPG bilayers) calculated by 4 μ s MD simulation in the presence (red) and in the absence (black) of six CatBF peptides. (C) MD snapshot of CatBF interacting with POPE/POPG bilayers. Color

code: phosphorus atom: yellow, POPC black (body) and light gray (choline group), POPE dark green (body), turquoise (headgroup), light green (amine of the headgroup); POPG dark violet (body), violet (headgroup), light violet (hydroxyls of the headgroup). For clarity, only functional groups of headgroups are shown (spheres) in the upper leaflet.

3.4.3 Effect on CatBF on phosphatidylserine membranes

CatBF has been reported to play further biological activities as immunomodulator, anticancer and antifungal (against *Candida* species) agent (27–29, 45, 46). In all these functions, phosphatidylserine is an essential lipid because it is found exposed on cell membranes (11, 12, 116–119). Because of this, we decided to explore the interaction of CatBF with POPS membranes by MD. When looking into membrane dynamics (Figure order) we can observe similar trends as those observed for POPG and POPE. Polar and apolar contacts are Very similar to those obtained for POPG, probably because of the negative charge of both phospholipids. In models containing multiple peptides we observe membrane rigidification (higher values of the order parameter (Figure order 6pep) and an important membrane invagination (Figure area-per-lipid), as previously observed for other PS-targeting anticancer peptides (97, 110, 114).

These preliminary data will constitute the basis for further future investigations.

Conclusions

In this work we have tested the activity of CatBF towards clinical isolates of resistant bacteria giving rise to nosocomial infections. We found that CatBF displays low MIC values (sub- μM concentrations) against most isolates, independently of the bacterial species and of their mechanism of resistance (expression of carbapenemases, porin loss, expression of efflux pumps). At low concentrations of Ca^{2+} and Mg^{2+} ions, MIC values drop to about 70 nM for some species such as *E.coli*. In gram-negative bacteria these ions reinforce the bacterial membrane by bridging negatively charged LPS molecules, thus compensating the inter-lipid repulsive charge. Cationic AMPs are known to act on the outer membrane by displacing Ca^{2+} and Mg^{2+} ions exploiting their same positive charge.

In order to get insight into the mechanism of action of cationic AMPs at the level of the inner membrane (or the outer membrane of gram-positive bacteria) we investigated the interaction of CatBF with phospholipid bilayers exposing head groups typically found on their surface: PG and PE. We found that CatBF strongly interacts with these lipids and especially with PG. At low concentrations, the unstructured peptide assumes an alpha helical structure onto the membrane which orients with its axis almost parallel to the surface and penetrates the bilayers by its N-terminus. At higher concentrations, the membrane surface is deformed and consequently a new pool of dynamic peptides change the initial orientation while the internalized peptide establishes new sets of intermolecular polar and apolar contacts partially disrupting its helical structure.

Competing interest The authors declare that they have no competing interests.

Funding This work and the PhD fellowship of Morgane Adélaïde were funded by the ANR-BMBF NATURAL-ARSENAL project (ANR-19-AMRB-0004).

Author contributions Conceptualization, C.S., B.B. and N.D'A.; methodology, E.S., F.R-M., B.B. and N'DA.; software, F.R-M.; validation, F.R-M., C.S., B.B. and N.D'A.; formal analysis, M.A., E.S., F.R-M. and N.D'A.; investigation, M.A., E.S., F.R-M. and C.A. ; resources, C.S., B.B. and N.D'A.; data curation, M.A., E.S., F.R-M. and C.A.; writing—original draft preparation, M.A., F.R-M., N.D'A.; writing—review and editing, M.A., E.S., F.R-M., C.A., C.S., B.B., N.D'A.; visualization, M.A., E.S., F.R-M. and N.D'A.; supervision, C.S., B.B. and N.D'A.; project administration, C.S., B.B. and N.D'A.; funding acquisition, C.S., B.B. and N.D'A. All authors have read and agreed to the published version of the manuscript.

Acknowledgements We would like to thank the Matrics platform at the University Picardie Jules Verne for providing computing resources. We would also like to thank Anja Kaminski for excellent technical assistance and Dominique Cailleu for his help on setting up NMR experiments.

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