CHARLES UNIVERSITY
Faculty of Medicine in Pilsen
Department of Microbiology

DISSERTATION THESIS

Development and validation of methods for rapid detection of β-lactamases using MALDI-TOF mass spectrometry for the early detection of infectious disease agents and their antibiotic resistance

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

In Pilsen, 21.7.2020

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Veronika Pašková

Abstract

The discovery of antibiotics and their subsequent clinical use has had a tremendous and beneficial impact on human health. β-lactam antibiotics are considered the most widely used therapeutic class of antibacterials prescribed in human and veterinary clinical practices due to their excellent safety profile and broad antimicrobial spectrum. β-lactams have undergone continuous development since their introduction in order to improve properties such as potency, spectrum of activity, pharmacokinetic and safety profiles and to counter the emergence of resistance. Resistance can occur by multiple mechanisms, including, notably, the production of β-lactamases and modification of β-lactam receptors - penicillin-binding proteins.

The resistance of bacterial pathogens to common antimicrobial therapies and the emergence of multidrug-resistant bacteria are increasing at an alarming rate in Czechia. Understanding the mechanisms of resistance in clinical isolates is critical to the design of novel therapeutics and the improvement of detection techniques. Insight into the genetic basis of resistance can also reveal drug design strategies for curtailing the spread of resistance and combatting multidrug-resistant organisms.

This dissertation thesis concerns different thematic investigations, beginning with the evolution of β-lactam antibiotics to a background of the biochemistry of β-lactam antibiotic resistance, and continuing with publications focused on a) the development and validation of assays for rapid detection of carbapenemase activity, b) the validation of a commercial automatic program for the detection of MRSA strains, and c) the molecular-epidemiological characterization of carbapenemase positive Gram-negative isolates detected in hospitals in the Czech Republic.

The first study compares the efficiency of imipenem and meropenem hydrolysis assay for the detection of carbapenemase-producing *Enterobacterales* and *Pseudomonas aeruginosa* by MALDI-TOF mass spectrometer. Validation supported the high sensitivity and specificity of both assays to both strains. However, the study showed that the addition of special compounds is necessary for higher sensitivity of detection of carbapenemases.

The second study describes the first case of IMI-2-producing *Enterobacter asburiae* identified in the Czech Republic in 2016. The isolate was obtained from a patient without previous hospitalization and travel history. The strain lacked an obvious source of origin, suggesting a silent spread via unknown pathways.

The third study characterizes NDM carbapenemases isolated from *Enterobacterales* during an outbreak in 2016. Until then, the occurrence of NDM carbapenemases was rare. The results of the plasmid analysis showed that bla_{NDM} genes were located on $\mathrm{IncX_3}$ plasmids. These are the main factors contributing to the dissemination of NDM-like enzymes in the Czech Republic. Moreover, two distinct NDM-producing strains have been found in two different patients, suggesting the occurrence of horizontal gene transfer. These findings indicate that NDM-like producers pose an important public threat, mainly due to the rapid horizontal transfer of IncX3 bla_{NDM}-carrying plasmids.

The next study reports a case of VIM-1 producing *Enterobacter cloacae* isolate and description of the novel VIM-1-encoding plasmid. Until then the frequency of *Enterobacterales* with VIM carbapenemases has been rare with just a few sporadic cases detected in Czechia, although VIM carbapenemase has occurred frequently in Europe. Plasmid analysis suggests that this ColE1-type plasmid could be developed by obtaining a Tn*1721*-like transposon carrying the integron In*110* encoding VIM-1. This finding highlights the important role of mobile genetic elements in the spread of resistance determinants such as $bla_{\mathrm{VIM\text{-}1}}$.

The last study focuses on validation of a commercial automatic program on MALDI-TOF MS-based identification of MRSA. The assay showed 90% sensitivity and 100% specificity; however, repeatability and reproducibility were determined to be poor. Based on our data, the method is not suitable for routine use.

Abstrakt

Objev antibiotik a jejich následné klinické použití mělo obrovský prospěšný dopad na lidské zdraví. β-laktamová antibotika jsou považována za nejpoužívanější terapeutickou třídu antibakteriálních přípravků předepisovaných v humánní a veterinární klinické praxi kvůli jejich vynikajícímu bezpečnostnímu profilu a širokému antimikrobiálnímu spektru. β-laktamy procházejí od svého objevení neustálým vývojem, ke zlepšení vlastností, jako je účinnost, spektrum aktivity, farmakokinetické a bezpečnostní profily, a k zabránění vzniku rezistence. K rezistenci může dojít prostřednictvím mnoha mechanismů, zejména produkcí β-laktamáz a modifikací β-laktamových receptorů - penicilinových vazebných proteinů.

Odolnost bakteriálních patogenů vůči běžným antimikrobiálním terapiím a vznik bakterií rezistentních vůči více lékům v Česku znepokojivě roste. Pochopení mechanismů rezistence v klinických izolátech je zásadní pro návrh nových antibiotik a pro zlepšení detekčních technik. Nahlédnutí do genetického základu rezistence může také odhalit možnosti navrhování léčiv pro omezení šíření rezistence a boji proti organismům odolným vůči více lékům.

Tato disertační práce se zabývá různými tematickými výzkumy, počínaje vývojem β-laktamových antibiotik až po biochemii rezistence β-laktamových antibiotik, následně pokračuje publikacemi zaměřených na a) vývoj a validaci testů pro rychlou detekci aktivity karbapenemázy, b) validaci komerčního automatického programu pro detekci kmenů MRSA a c) molekulárně-epidemiologickými charakterizacemi gramnegativních karbapenemáza-pozitvních izolátů detekovaných v nemocnicích v České republice.

První studie porovnává účinnost testu hydrolýzy imipenemu a meropenemu pro detekci *Enterobacterales* a *Pseudomonas aeruginosa* produkujících karbapenemázy pomocí hmotnostního spektrometru MALDI-TOF. Validace potvrdila vysokou senzitivitu a specificitu obou testů na oba kmeny. Studie však ukázala, že pro vyšší senzitivitu detekce karbapenemáz je nutné přidání speciálních sloučenin.

Druhá studie popisuje první případ *Enterobacter asburiae* produkující IMI-2 identifikovaný v České republice v roce 2016. Izolát byl získán od pacienta bez předchozí hospitalizace a cestovní historie. Kmen postrádal zdroj původu, což naznačuje tiché šíření rezistence neznámými cestami.

Třetí studie charakterizuje NDM karbapenemázy izolované z *Enterobacterales* během epidemické epizody v roce 2016. Do té doby byl výskyt karbapenemáz typu NDM vzácný. Výsledky analýzy plazmidů ukázaly, že geny bla_{NDM} byly lokalizovány na plasmidech IncX3. Tyto plasmidy jsou hlavními faktory přispívajícími k šíření NDM enzymů v České republice. Kromě toho byly u dvou různých pacientů nalezeny dva odlišné kmeny produkující NDM, což naznačuje výskyt horizontálního přenosu genů. Tato zjištění naznačují, že producenti NDM karbapenemázy představují významnou obecnou hrozbu, zejména kvůli rychlému horizontálnímu přenosu pomocí IncX3 plazmidů.

Následující studie uvádí případ izolátu *Enterobacter cloacae* produkujícího VIM-1 a popis nového plazmidu kódujícího VIM-1. Do té doby byla četnost *Enterobacterales* s VIM karbapenemázou vzácná. Dosud bylo v České republice zjištěno jen několik sporadických případů, zatímco v Evropě se tento typ karbapenemázy poměrně často vyskytuje. Plazmidová analýza naznačuje, že tento plasmid typu ColE1 by mohl být vyvinut získáním transpozonu podobného *Tn*1721 nesoucího integron *In*110 kódující VIM-1. Toto zjištění podtrhuje důležitou roli mobilních genetických prvků v šíření těchto determinantů rezistence.

Poslední studie se zaměřuje na validaci komerčního automatického programu pro identifikaci MRSA pomocí MALDI-TOF hmotnostního spektrometru. Test ukázal 90% citlivost a 100% specificitu; opakovatelnost a reprodukovatelnost však byly vyhodnoceny jako nedostatečné. Na základě našich zjištění není tato metoda vhodná pro běžné použití v rutinní praxi.

List of abbreviations

6-APA	6-amino penicillanic acid
7-ACA	7 aminocephalosporanic acid
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
D-Ala	D-alanine
DAP	Diaminopimelic acid
DBO	diazabicyclooctane
DDST	double-disk synergy test
D-Glu	D-glutamic acid
DHP-I	dehydropeptidase I
EDTA	ethylene diamine tetraacetic acid
ESBL	Extended spectrum β-lactamases
ESBLs	extended spectrum β lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EWE	Electron-withdrawing effect
GES	Guiana extended-spectrum carbapenemase
Glu	Glutamic acid
Gly	Glycine
His	Histidine
IEF	isolectric focusing
Ile	Isoleucine
IMI	imipenem-hydrolyzing β-lactamase
IMP	imipenemase enzyme
Inc	plasmid incompatibility
KPC	*Klebsiella pneumonie* carbapenemase
L-Ala	L-alanine
L-Lys	L-lysine
LPS	lipopolysaccharide
Lys	Lysine
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MBL	metallo-β-lactamases
MBLs	metallo β lactamases
MDR	multidrug resistant
MIC	minimum inhibitory concentration
MLST	multilocus sequencing typing
MRSA	Methicillin-resistant *S. aureus*
MSSA	methicillin susceptible *S. aureus*
MβL	metallo- β-lactamase
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic
NDM	New-Delhi MBL
NMC-A	non metallo-carbapenemase A

OXA	oxacillinase
PBP	Penicillin-binding protein
PBRT	PCR-base replicon typing method
PFGE	pulsed-field gel electrophoresis
SARs	Structure-activity relationships
SCC*mec*	staphylococcal cassette chromosome
Ser	Serine
SME	*Serratia marcescens* enzyme
SβL	serine-β-lactamase
Thr	Threonine
Tyr	Tyrosine
Val	Valine
VIM	Verona integron-encoded MBL
VRE	vancomycin-resistant Enterococcus

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1. Introduction

β-lactam antibiotics are a broad class of bioactive compounds including penicillins, cephalosporins, β-lactamase inhibitors, monobactams and carbapenems. Their molecular structure contains a four-membered β-lactam ring, which is key to their potency against bacteria, and additional substituents, such as fused rings, confer variability in their spectrum of activity. Different substituents can also impart new pharmacokinetic properties and alter the susceptibility of the compound to acid and enzymatic hydrolysis.[1] Monobactams, as their name suggests, are monocylic, and are structurally the most simple class of β-lactams. The core structure of penicillins and carbapenems is bicyclic. Both have fused five-membered rings, which in the case of the former is a thiazolidine group, whereas the latter contains a carbon atom instead of a heteroatom at position 1 and an unsaturated bond between carbons 2 and 3. Certain β-lactamase inhibitors also contain a β-lactam ring fused to a five-membered ring. These compounds have minor antibacterial activity of their own but are used in conjunction with antibiotics. Cephalosporins, by contrast, display a six-membered dihydrothiazine ring (Figure 1).[2]

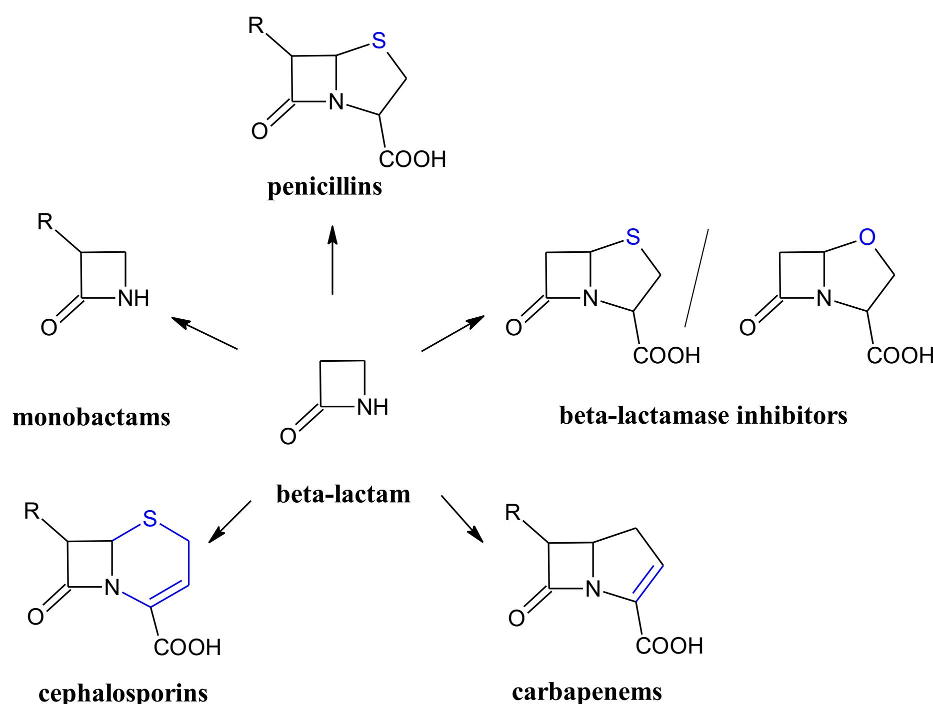

Figure 1 ***Principal structures of beta-lactam antibiotics***

Previous research has indicated that the precursors of β-lactam antiobiotics are amino acids. For example, the biosynthesis of penicillins and cephalosporins begins with *L*-isomers of cysteine and valine (Figure 2).[3]

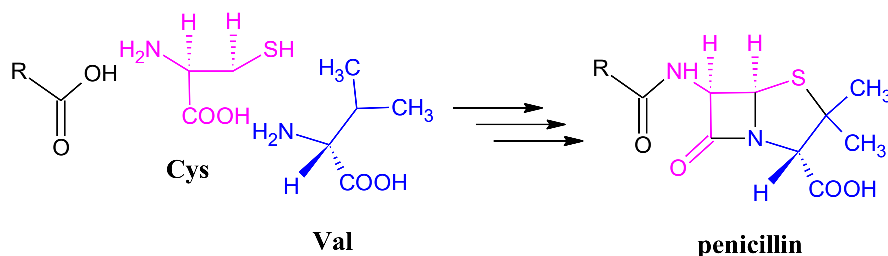

Figure 2 ***The biosynthetic precursors of penicillin***

β-lactam antibiotics inhibit the growth of sensitive bacteria by inactivating enzymes involved in the third stage of cell wall synthesis. Bacterial cell walls consist of a cross-linked peptidoglycan structure that lends osmotic and thermal stability to the organism. It incorporates two sugar derivatives, *N*-acetylglucosamine (NAG) and *N*-acetylmuramic (NAM) acid, and a peptide chain formed from L-alanine (L-Ala), L-lysine (L-Lys), unique *D*-isomers such as D-alanine (D-Ala) and D-glutamic acid (D-Glu) and the meso amino acid, diaminopimelic acid (DAP). The three-dimensional structure of peptidoglycan is formed in Gram-negative bacteria by a peptide bond between the amino group of DAP from the first peptide chain and the carboxyl group of terminal D-Ala^4 from the second peptide chain (Figure 3). In Gram-positive bacteria, the cross-link occurs through a short interbridge, which varies from species to species. For example, *Staphylococcus aureus* contains an interbridge consisting of five glycine (Gly) residues bonded to the terminal D-Ala^4 peptidoglycan chain (Figure 3). Gram-positive species have a cell wall composed of 20 – 30 peptidoglycan strands, whereas Gram-negative species have a complex outer surface that consists of lipid and polysaccharide layers interspersed with proteins that provide selective channels through which molecules are able to pass.

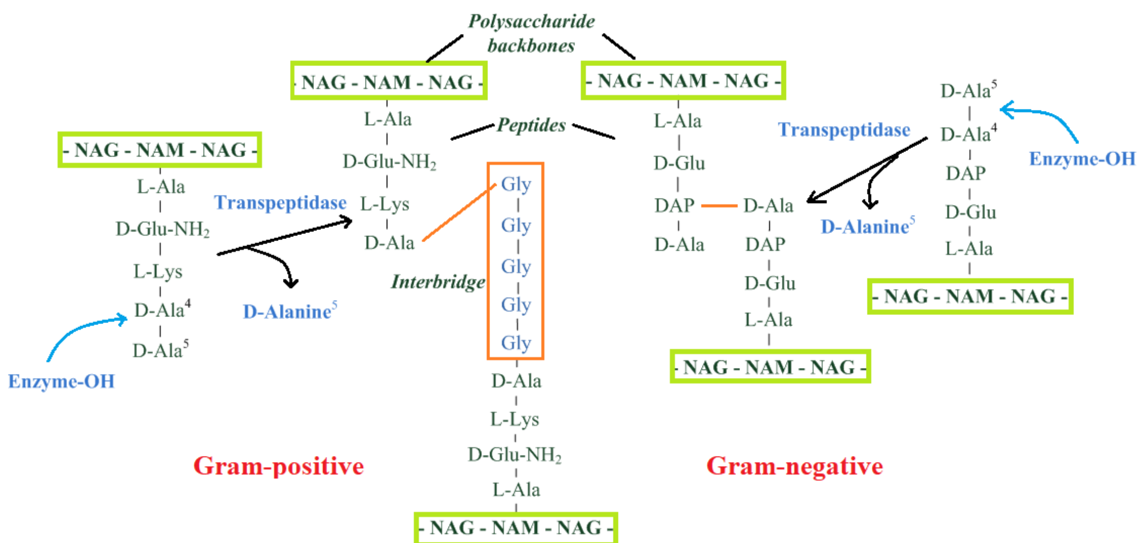

Figure 3 ***Peptidoglycan structure of bacterial cell walls and cross-linking in Gram-positive and Gram-negative bacteria***

More than 30 enzymes are involved in the biosynthesis of the cell wall. Among them, the transpeptidase enzyme, more commonly referred to as penicillin-binding protein (PBP), is responsible for the final cross-linking reaction. Under normal conditions, PBP catalyzes the formation of peptidoglycan cross-links in a two-step reaction.[4] In the first step, the catalytic serotonin (Ser) residue of the enzyme acts as a nucleophile to split the peptide bond between two terminal D-Ala^4-D-Ala^5 units in the peptide chain, leading to the release of the final D-Ala^5 and forming a new ester bond with the carbonyl group of D-Ala^4 (*Chyba! Nenalezen zdroj odkazů.*).[5] This step is the same for Gram-positive and Gram-negative bacteria, hile the second phase of cross-linking differs based on the chemical structure of peptidoglycan. In Gram-positive bacteria the pentaglycyl moiety of the peptide chain enters the active site of the acyl-enzyme and forms an ester bond with the carbonyl group of D-Ala^4, displacing the Ser residue in the process (Figure 3,Figure 4). Gram-negative bacteria lack the pentaglycyl moiety and alternatively, a peptide bond is formed between D-Ala^4 and DAP residues of the uniting peptide chains (Figure 3).[6]

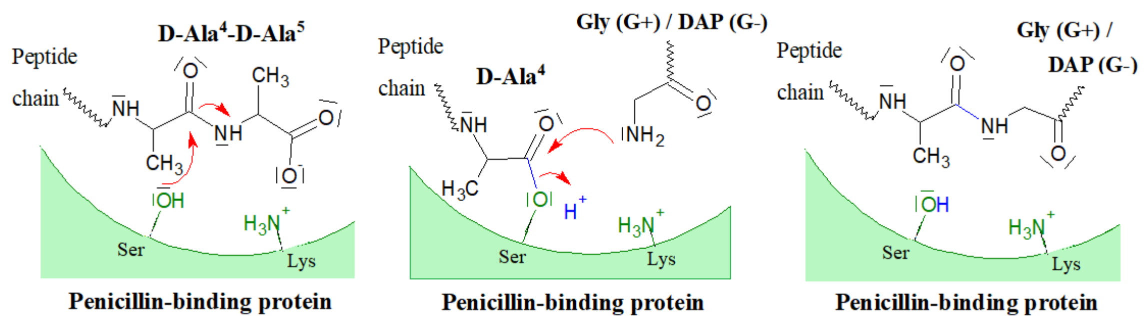

Figure 4 *Mechanism of penicillin-binding protein and its cross-linking*

β-lactam antibiotics irreversibly bind to the Ser residue of PBP, thus inhibiting the correct functioning of the enzyme. They behave as a mimic for the peptide sequence D-Ala^4-D-Ala^5 (Figure 5), but demonstrate greater affinity for the active site (Figure 6).

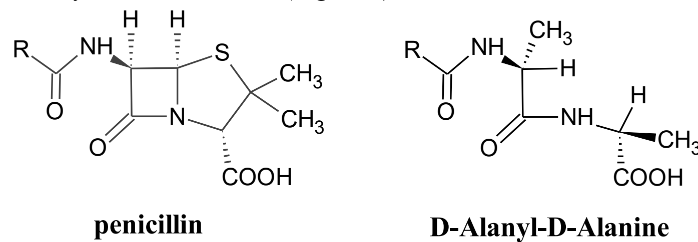

Figure 5 *Comparison of penicillin and D-Alanyl-D-Alanine*

Similar to the first step of the reaction shown in Figure 4, the β-lactam ring of penicillin undergoes a nucleophilic attack by the catalytic Ser residue. This results in a peptide bond as well as an ionic bond between –COO$^-$ and a neighboring lysine (Lys) residue, and prevents other molecules from approaching or displacing it (Figure 6).[7] Due to the inactivation of PBP, the peptidoglycan framework cannot be extended, becomes weak, and the bacterial cell bursts due to an osmotic imbalance.

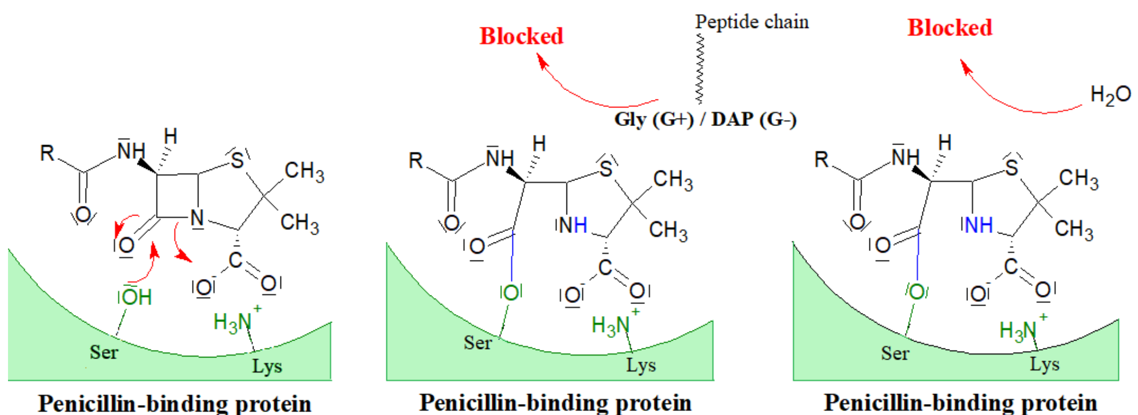

Figure 6 *Inhibition of penicillin-binding protein by a molecule of penicillin*

β-lactam remain some of the safest known antibiotics. From the mechanism of their action, it follows that they have good selectivity for prokaryotic cells (eukaryotic cells do not contain peptidoglycan). Therefore, these drugs are generally preferred for their low toxicity as well as their rapid bactericidal effect,

broad therapeutic range, minimal drug interactions, good pharmacokinetic properties, and relatively low cost.[8, 9] To illustrate how popular their use has become, Bush and Bradford have reported that β-lactam agents are currently the most used class of antibiotics for infectious disease in the United States (Figure 7).[10]

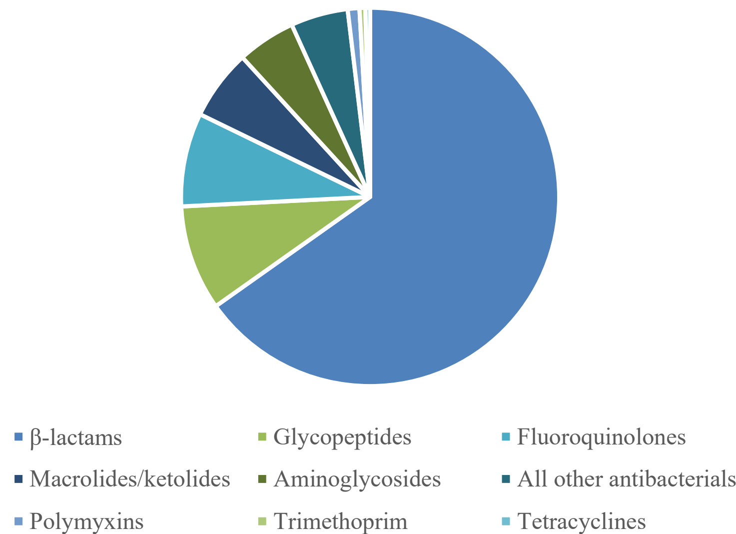

Figure 7 **Proportion of prescritions in the USA for injectable antibiotics by class for years 2004-2014:** *β-lactams 65,2%; glycopeptides 9%; fluorochinolones 8%; macrolides/ketolides 6%; aminoglycosides 5%; polymyxins 1%; trimethoprim 0,5%; tetracyclines 0,4%; all other antibacterials 4,9% (Adapted from Bush)*[10]

However, β-lactam antibiotics do carry the risk of adverse events. The side effects include an allergic reaction with an incidence of 1 % - 10 %, which can be expressed in various degrees of seriousness, from drug-induced fever and contact dermatitis to anaphylactic shock. Cases involving cross-reactivity of first- and second-generation cephalosporins with penicillin have also been reported. The newer generations of cephalosporins suffer less from this drawback, due to the decreased immunogenicity associated with bulky side chains in the molecule. Other side effects are rare and impact hematological, neurological and renal systems.[11]

Alteration of PBP is another mechanism of resistance that is witnessed especially in problematic Gram-positive bacteria such as *Staphylococcus* and *Streptococcus* species. The other two mechanisms prevent the approach of antibiotics to the enzyme active site by forced efflux or diminished permeability of the cell wall.[12] They are observed in smaller measure, but often occur at the same time with the propagation of β-lactamases or alteration of PBPs.[13]

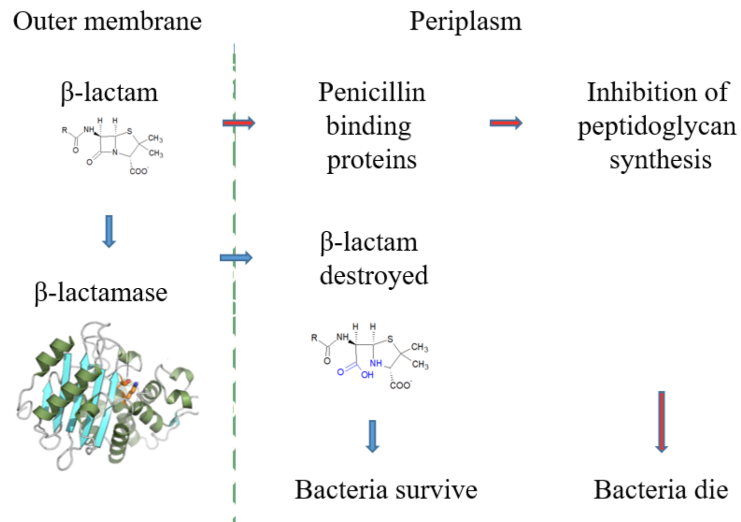

Figure 8 Interaction of β-lactam antibiotics with Gram-negative bacteria.

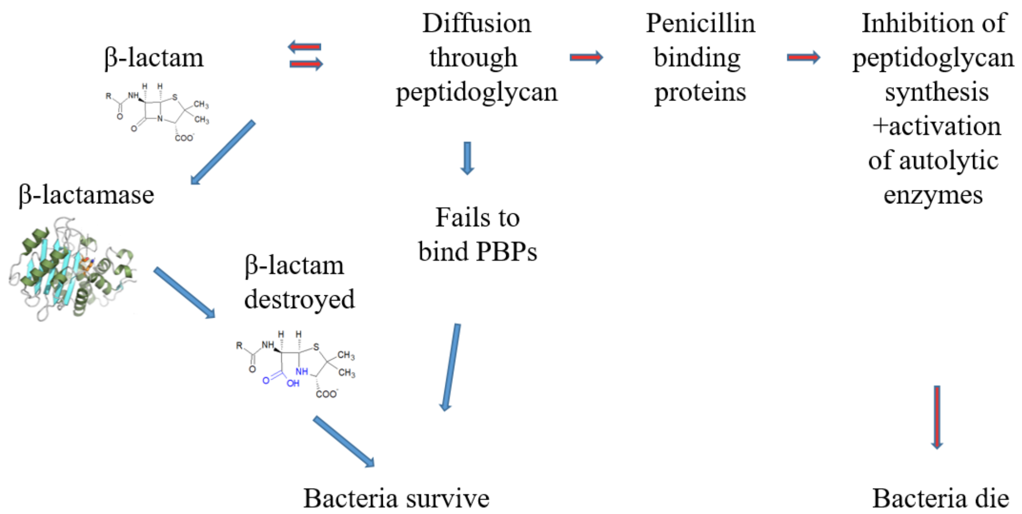

Figure 9 Interaction of β-lactam antibiotics with Gram-positive bacteria.

2. β-lactam antibiotics

2.1 Penicillins

In 1928, Alexander Fleming found a bacterial culture that had been left uncovered in a laboratory for several weeks and had become infected with fungus. Around the area of the fungal colony, he observed that the bacterial colonies were dying. He concluded that an antibacterial agent was being produced by the fungus, which he later identified as a relatively rare species of *Penicillium (Penicillium notatum)*. The temperature of the room was also found to play a major role since each of the two microorganisms preferred different thermal conditions for growth. At first, it had to be warm for bacterial growth to occur and then colder, both for the fungus to spread and for the bacterial colony to remain static. Fleming was unsuccessful at isolating and purifying the active substance, penicillin, even after several years of attempts.[14] However, a decade after his observation, production of this compound was achieved by Florey and Chain through a combination of freeze-drying and chromatography techniques.[15] Penicillin revolutionized the treatment of infectious diseases when it came into general use after World War II even though the molecular structure was unknown.[16] In 1945, the structure was established by X-ray crystallographic analysis conducted by Dorothy Hodgkin, which allowed reaserchers to contemplate derivatives.[17] In the 1940s and 1950s, the deep fermentation procedure of biosynthesis of penicillin allowed a large-scale production of the bioactive compound.[18] And the first antibiotics, namely penicillin G and V, were obtained by this procedure, while the newer generations of penicillins were prepared by a semisynthetic pathway from 6-amino penicillanic acid (6-APA) in the 1960s.[19]

The antimicrobial spectrum of elementary penicillins is narrow and includes only Gram-positive microbes that cause streptococcal, syphilitic, and meningococcal infections. Within a few years of the introduction of penicillin into clinical practice a specific type of β-lactamase, – penicillinase, was observed in Gram-positive bacteria. Penicillinase-producing *Staphylococcus aureus* started to disseminate in hospitals, which led to a worldwide pandemic of nosocomial penicillin-resistant staphylococcal infections. This initiated the development of penicillinase-resistant antibiotics leading, most notably, to the release of methicillin to the market. However, roughly a year after its introduction, a new type of resistance in *S. aureus* became apparent. These strains, now called methicillin-resistant *S. aureus* (MRSA), express a modified form of PBP showing low affinity for methicillins. Thus, methicillin is no longer manufactred because it is replaced by more stable such as oxacillin and cloxacillin.[9, 20]

Broad-spectrum penicillins (e.g., ampicillin, amoxicillin, carbenicillin, and ticarcillin) are less active against Gram-positive and anaerobic organisms than penicillin G, but are effective against infections caused by Gram-negative rods. Similar to penicillin, the rapid rise of resistance to ampicillin in the early 1960s occurred in Gram-negative bacteria expressing a plasmid mediated TEM β-lactamase.[21] Their greater efficacy against Gram-negative organisms is observed when they are combined with β-lactamase inhibitors such as sulbactam (for ampicillin), clavulanic acid (for amoxicillin and ticarcillin) and tazobactam (for piperacillin).[2] The ureidopenicillins mezlocillin and piperacillin have a greater activity against *Pseudomonas aeruginosa*.[8]

The pharmacokinetics of penicillins are characterized by good diffusion into bodily fluids and tissues, but they do not penetrate as well into living cells and do not dissolve in lipids. High levels can be found in the central nervous system, especially during inflammation of the meninges. They are actively excreted by the kidneys into urine, which can exhibit penicillin concentrations several orders of magnitude higher than in serum.[22, 23]

Pharmacodynamically, penicillins belong to a group of antibiotics with a concentration-independent effect. The preferred treatment dosing maintains the effective concentration above minimum inhibitory concentration (MIC) for susceptible pathogens during at least 40 % of the dosing interval.[8]

The term penicillin generally refers to compounds that contain the β-lactam structure and possess antibacterial activity, just like the first described antibiotic, benzylpenicillin. Their further classification is determined by their unique substituents, which alter the drug's spectrum of activity, mediated by the affinity

of the compound to PBP, its susceptibility to β-lactamases, its ability to cross the cell membrane, and the rate at which it is pumped out of the cell of Gram-negative organisms. Pharmaceutical companies have responded to the frequent emergence of antibiotic-resistant pathogens by developing drugs with increased stability[24, 25] guided by teachings from the study of structure-activity relationships (SARs; Figure 10).

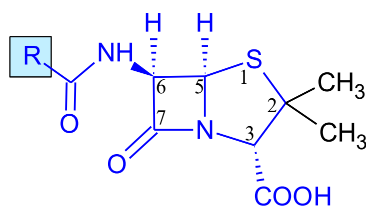

Figure 10 Structure-activity relationships of penicillins

- The strained β-lactam ring and its fusion with a second thiazolidine ring is required for binding to PBP and the associated antibacterial activity. However, the bicyclic system suffers large angle and torsional strains, making it sensitive to low pH environments, which facilitate ring-opening reactions.
- The sulphur at position 1 is common but not essential to its activity.
- The carboxylate group at position 3 is usually coordinated to a sodium or potassium cation, creating a salt. The affinity for the PBP active site is mediated by the binding of the carboxylate ion to the positively charged nitrogen of a Lys residue, and the creation of an ester bond between the oxygen of carboxyl group at position 8 and a Ser residue -OH.
- The carbonyl group at position 7 is highly susceptible to nucleophiles because the amide bond is not stabilized by resonance with the lactam nitrogen atom. Resonance would lead to prohibitive ring strain, resulting from a cyclic sp^2 nitrogen and the attendant preference for 120° degree angles between its substituents (Figure 11).[9]

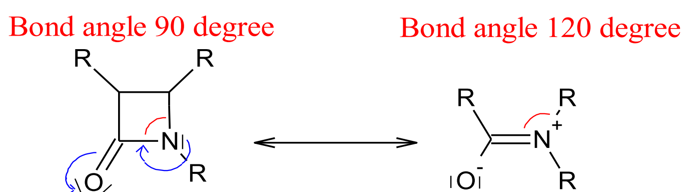

Figure 11 Comparison of β-lactam carbonyl group and tertiary amide group

- An electron-withdrawing R group in the acylamino side chain at position 6 is critical (Figure 12) to the pH stability of the compound. It draws electrons away from the carbonyl oxygen atom, which reduces its ability to act as a nucleophile, and this decreases the tendency of the compound to degrade in the presence of biliary acids. Drugs such as penicillin V, with an electron-withdrawing phenoxymethyl R group can therefore be administered orally.

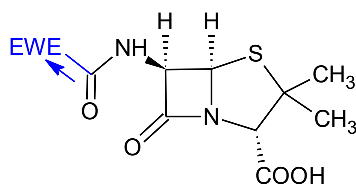

Figure 12 Electron-withdrawing effect

- Bulky R groups act as a steric shield, blocking the approach of penicillin to the active site of β-lactamase enzymes (Figure 13). However, the bulky group should not be so large to prevent penicillin-PBP binding. This teaching lead to the development of methicillin.

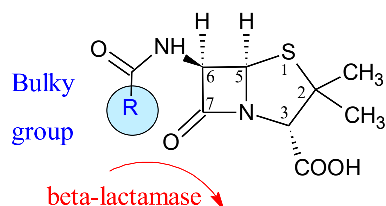

Figure 13 The use of steric shield blocking

- The cis-conformation of the bicyclic ring with respect to the acylamino side chain is essential for its antibacterial effect (Figure 13).
- SAR studies have shown that preservation of the antibiotic activity of penicillin requires that any structural modifications be limited to the acylamino side chain at position 6.

2.1.1 First generation of penicillins

The first generation of penicillins is also referred as the natural penicillins and they are effective against most of non-β-lactamase producing Gram-positive cocci with a very limited number of Gram-negative bacteria. To this group belongs penicillin G and its modified successor penicillin V. Penicillin G has to be administered parenterally due to its susceptibility to biliary acids. By contrast, penicillin V can be taken orally because it contains an electronegative acyl R group with a strong electron-withdrawing effect (EWE), leading to greater stability under low pH conditions encountered in the stomach. Both antibiotics are susceptible to penicillinases; however, this problem was overcome by the introduction of bulky groups on the side chain at position 6. Due to the presence of a bulky dimethoxybenzyl R group, methicillin was the first semi-synthetic penicillin of this generation that was effective against β-lactamase-producing *Staphylococcus aureus*. Methicillin was useful against *S. aureus* infections until a mutation of PBP took place, leading to isolates subsequently referred to as methicillin-resistant *S. aureus* (MRSA). Because dimethoxybenzyl does not have a strong electron-withdrawing effect, methicillin is acid-sensitive and has to be injected. Oxacillin is an acid-stable corollary to methicillin that displays an isoxazolyl ring in the acylamino side chain, resulting in tolerance not only to biliary acids but also penicillinases (Figure 14). With the addition of halogen atoms to the benzene ring, as in cloxacillin, flucloxacillin, and dicloxacillin, the absorption is augmented due to greater affinity to plasma proteins.

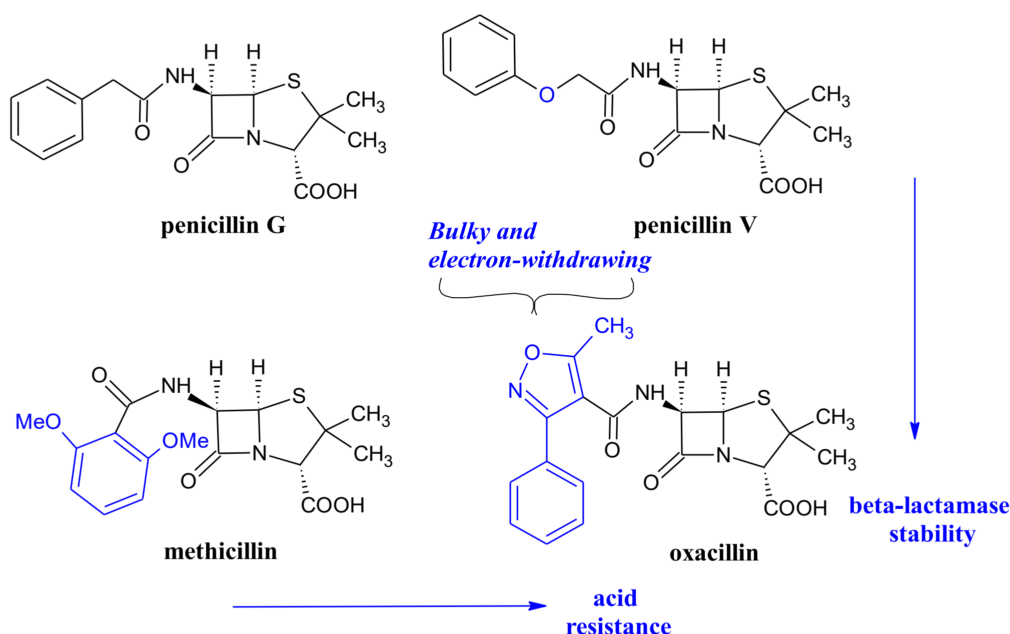

Figure 14 Structure-activity relationship of the first generation of penicillins

Penicillin G is active against non-β-lactamase-producing Gram-positive bacteria (strains of streptococci, *Listeria monocytogenes*) and Gram-negative cocci such as *Neisseria.* The antibiotic is effective for streptococcal, pneumococcal, gonococcal, and meningococcal infections. Penicillin V is recommended for infection causing tonsillitis, otitis, and rheumatic fever.[9] Methicillin used to be administered for infections caused by penicillin-resistant *S. aureus*, but is no longer preferred clinically due to the dissemination of MRSA and of its side effects including interstitial nephritis and kidney failure. Likewise, oxacillin and related β-lactams, have an increased stability towards β-lactamase enzymes but are only useful against methicillin susceptible *S. aureus* infections (MSSA).[26]

2.1.2 Second generation of penicillins – the aminopenicillins

The second generation of penicillins retained the important chemical groups of the first generation while providing improved properties through modifications in the side chain at position 6. This group of β-lactam derivatives is useful against Gram-negative bacteria, in contrast to the selectivity of first generation penicillins towards Gram-positive bacteria. This effect was achieved through an increased attention to the optimization of hydrophilic and hydrophobic properties (Figure 15**).** Other improvements ushered by the second generation of penicillins are summarized below:

- Variations of hydrophilic groups (e.g., NH_2, OH, COOH) on the side chain at position 6 of the β-lactam ring have a small effect on Gram-positive activity in the case of penicillin T or lead to a dramatic decrease in activity in the case of penicillin N. By comparison, these substituents lead to a major increase in activity against Gram-negative bacteria (Figure 16).
- Hydrophobic groups (e.g., benzyl groups in penicillin G) on the side chain at position 6 have a selective influence on the activity against Gram-positive bacteria.
- Introduction of an amino group to the molecule, as in ampicillin and amoxicillin, leads to enhanced stability under acidic conditions. Unfortunately, it also results in their relatively poor absorbance through the cell walls of the gastrointestinal tract, leading to diarrhea as a side effect. The decreased absorption through the gut wall is caused by ionization of both the amino and carboxyl group, but a prodrug strategy can be used to prevent

ionization of the latter (position 3). For example the carboxyl group can be protected by acyloxymethyl esters to improve its oral absorption. Such masking is exhibited in antibiotics such as pivampicillin, talampicillin, and bacamipicillin (Figure 15).[27]

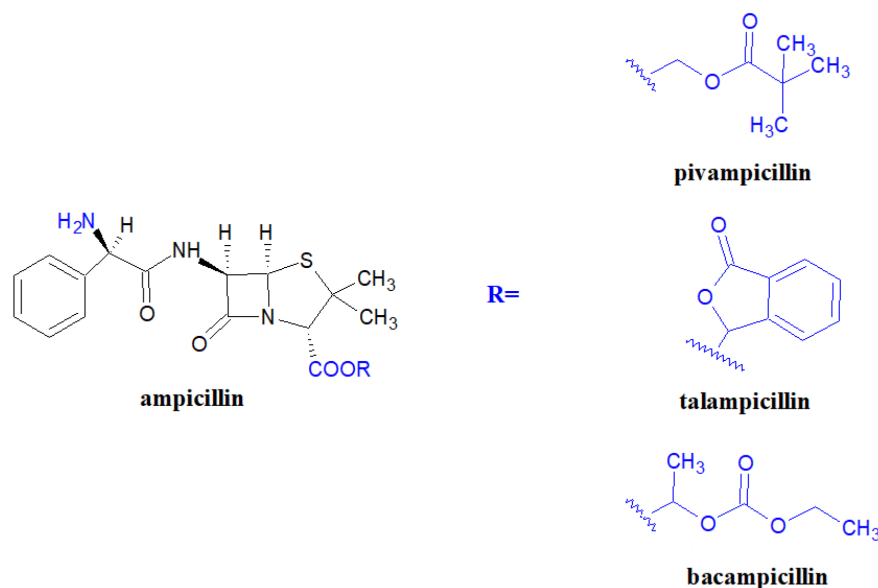

Figure 15 ***Prodrugs of ampicillin***

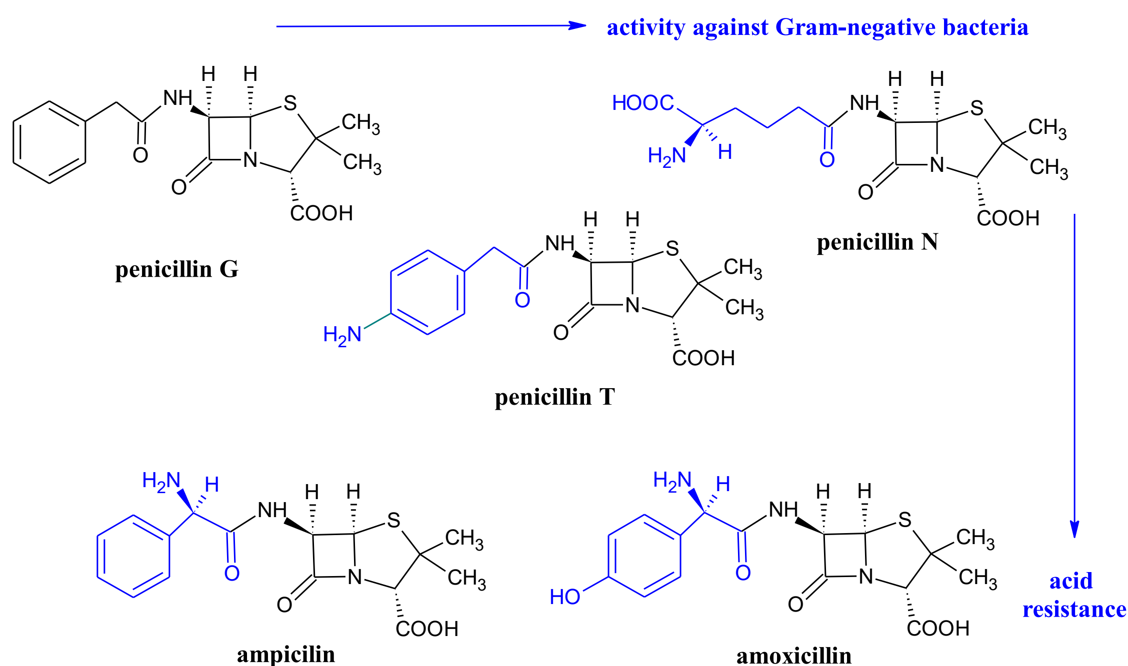

Figure 16 ***Structure-activity relationship of the second generation of penicillins***

Aminopenicillins have a similar spectrum of activity to penicillin G, but are more active against Gram-negative and anaerobic bacteria.[28] *Staphylococcus* and some species of *Enterobacterales* (*Klebsiella, Enterobacter, Serratia)* are intrinsically resistant to aminopenicillins as a result of β-lactamase production. Ampicillin has been used in the treatment of the respiratory tract infections such as otitis media, sinusitis, and bronchitis. Amoxicillin is currently used to treat respiratory and urinary tract infections.[9] Although ampicillin and amoxicillin are acid resistant due to electron-withdrawing side chains of the acylamino

group, they do not contain steric shields and are sensitive to β-lactamases (Figure 16). An increase of their spectrum of activity against β-lactamase-producing bacteria is achieved when they are administered with β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam.[9] These inhibitors irreversibly bind to the catalytic site of β-lactamase enzymes and prevent the hydrolytic degradation of penicillins (Figure 17). Their combination offers increased antimicrobial activity to β-lactamase-producing Gram-positive and certain *Enterobacterales* strains.[28] β-lactamase inhibitors belong to a subgroup of β-lactam antibiotics and will be discussed further in this thesis.

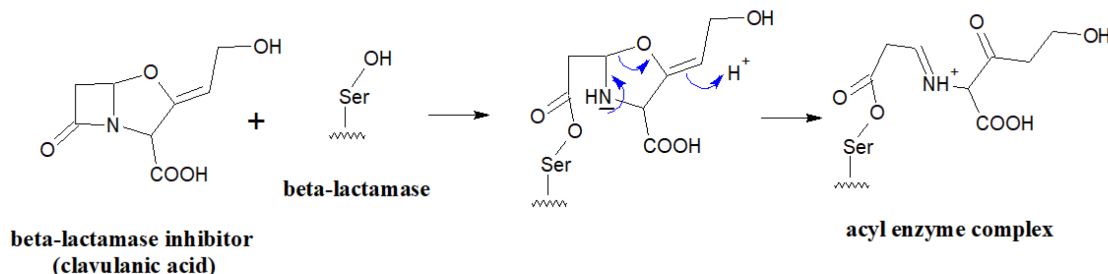

Figure 17 ***Irreversible binding of β-lactamase inhibitor to β-lactamase***[5]

2.1.3 Third generation of penicillins – the carboxypenicillins

Aminopenicillins not only extended the antimicrobial spectrum but also served as a prototype for the development of carboxypenicillins with an even wider spectrum of activity, which were deeply needed due to the emergence of resistant Gram-negative bacteria. The first carboxypenicillin, carbenicillin, was developed by replacing the amino group of ampicillin with a hydrophilic carboxyl group, giving rise to the archetypal name. The carboxyl group on the side chain at position 6 extends the range of activity.

In comparison with ampicillin, carbenicillin is active against a broader range of Gram-negative bacteria and was particularly effective against penicillin-resistant strains. However, carbenicillin has lower activity and thus requires higher doses. The production of carbenicillin was succeeded by ticarcillin, which has further activity against Gram-negative bacilli including *Pseudomonas aeruginosa* and does not have as many adverse effects as carbenicillin (Figure 18). Nowadays, ticarcillin is administered with β-lactamase inhibitor such as clavulanic acid to provide greater coverage against β-lactamase-producing *S. aureus*, *H. influenza*, bacteroides, and ticarcillin-resistant strains of *Enterobacterales*. On the other hand, their combination is not effective against *P. aeruginosa* expressing β-lactamases or MRSA strains.[9, 22, 29, 30]

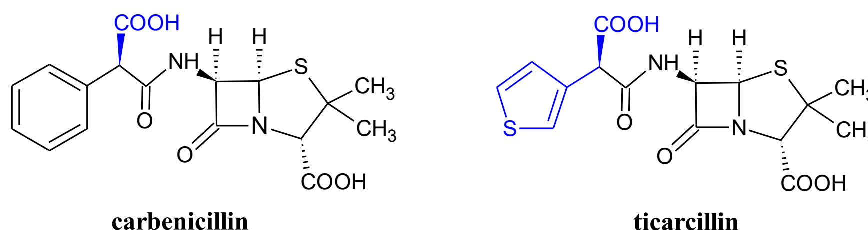

Figure 18 ***Structure of the third generation of penicillins***

2.1.4 Fourth generation of penicillins – ureidopenicillins

Ureidopenicillins are characterized by a very broad antibacterial spectrum comprising both Gram-positive and Gram-negative bacilli, especially *in vitro*. They show activity against streptococci, enterococci, *Enterobacterales*, *Pseudomonas*, and *Bacteroides fragilis,*[31] and they have more potency and a wider spectrum of activity in comparison with carbenicillin and ticarcillin.[32] They have an enhanced

anti-pseudomonal activity, due to good penetration through cell walls and greater affinity for PBPs, and they also evince fewer side effects than carboxypenicillins.

- Incorporation of polar groups on the side chain of the acyl amino group enhances penetration into Gram-negative bacteria while providing decreased susceptibility to β-lactamases-producing Gram-negative bacteria (Figure 19).

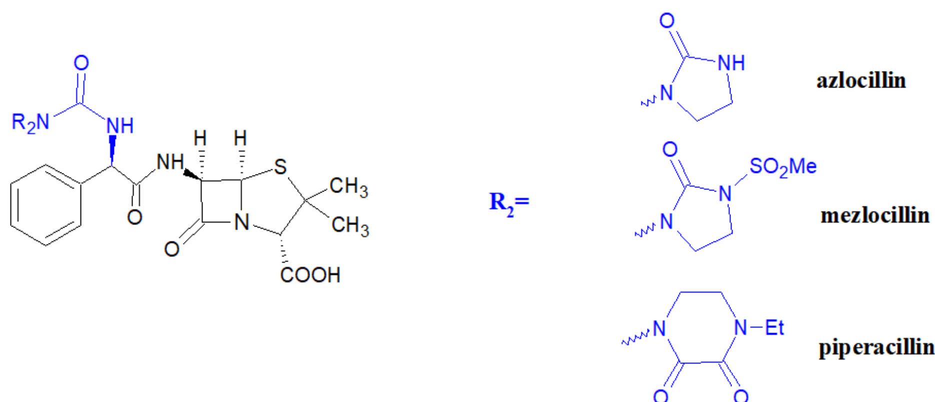

Figure 19 ***Structure-activity relationship of the fourth generation of penicillins***

Ureidopenicillins include the anti-*Pseudomonas* agent azlocillin, which is a more effective antibiotic against this species than carbenicillin from the previous generation. Mezlocillin has a higher affinity to PBPs and can more easily cross the outer membrane of Gram-negative bacteria. Piperacillin has similar activity against Gram-positive species compared to ampicillin and demonstrates twice higher activity than azlocillin and four times higher than mezlocillin and ticarcillin against *P. aeruginosa*.[33] Piperacillin is administered in combination with the β-lactamase inhibitor tazobactam to widen its spectrum of activity to bacteria that produce β-lactamases in staphylococci and a wide range of anaerobic and certain aerobic Gram-negative bacteria.

2.2 Cephalosporins

Cephalosporins are the second major group of β-lactam antibiotics. Their first known example, cephalosporin C (Figure 20), was discovered by Giuseppe Brotzu in 1940, who noted that the water in sewage is periodically cleared of microorganisms. He believed that organisms in the water were producing antibacterial substances, which he eventually confirmed. From his collected samples, a fungus called *Cephalosporium acremonium* (today known as *Acremonium chrysogenum*) was isolated, and after several years, in 1948, investigators at Oxford University purified cephalosporin C from a crude extract.[34] Its molecular structure was determined by X-ray crystallography in 1961.[35] Cephalosporin C can be enzymatically hydrolyzed or fermented to give 7-aminocephalosporanic acid (7-ACA) for a synthesis of later cephalosporins.[36]

Cephalosporins are similar to penicillins in that their core structure is bicyclic and contains a β-lactam ring. Furthermore, both groups of antibiotics have the same mechanism of action and are derived from the same biosynthetic precursors, cysteine and valine. By contrast, cephalosporins have a six-membered dihydrothiazine ring, which confers a relatively weak antibacterial activity in comparison with penicillins, although they are effective against both Gram-negative and Gram-positive species as well as anaerobes.[9, 37] Compare to penicillins, cephalosporins are highly active against bacteria producing broad-spectrum β-lactamases.

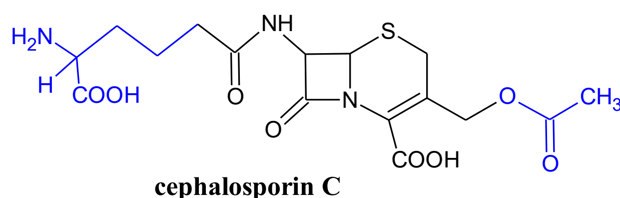

Figure 20 Cephalosporin C

Cephalosporins are divided into five generations according to their antimicrobial activity. Generally, with each consecutive generation, greater activity against Gram-negative species was achieved, accompanied by decreased activity against Gram-positive organisms.[37] The third generation cephalosporins are recommended as the drug of choice against meningitis-causing as they are active against penicillin-resistant *Haemophilus influenzae*.[38] The fourth generation have increased stability from degradation by chromosome or plasmid mediated β-lactamases produced by *Enterobacterales* and *P. aeruginosa.*[16, 39] Unfortunately, these bacteria have rapidly developed resistance to most cephalosporins.[39] The fifth generation cephalosporins were also developed with a focus on combatting resistant strains of bacteria. In particular, ceftobirole medocaril and ceftaroline fosamil are effective against methicillin-resistant *Staphylococcus aureus* (MRSA).[40] Ceftolozane in combination with tazobactam shows notable antipseudomonal activity and is effective against enteric bacteria producing extended-spectrum β-lactamases (ESBLs).[41]

The pharmacokinetics of cephalosporins is dependent on the physiochemical properties of the individual molecules. Generally, this class of compounds is described as having relatively good penetration through biological fluids but poor absorption from the gastrointestinal tract. Specifically, their inactivation by gastric acid prevents their passage through the mucosal barrier of the small intestine. The biological half-life of elimination of cephalosporins is also short: approximately 0.5 – 3 hours with the exception of ceftriaxone, which has a prolonged half-life of 8 hours.[8] However, modification of the cephalosporin core at position 3 can help to improve pharmacokinetic properties.[42]

The pharmacodynamics of cephalosporins is similar to penicillins, having an effect that is independent of concentration. The preferred treatment dosing maintains the effective concentration above minimum inhibitory concentration (MIC) for susceptible pathogens during at least 50 % of the dosing interval.[8]

Side effect of cephalosporins are neutropenia, nephrotoxicity and neurotoxicity only in large doses.[43] Cephalosporins are reported with a lesser incidence of allergic reactions than penicillin, even though a wide variety of these type of β-lactams is increasingly prescribed.[44]

Various chemical modifications to 7-ACA have led to the discovery of antibiotics with improved activity and pharmacokinetic properties. Individual changes of the molecule are described below by SARs (Figure 21).

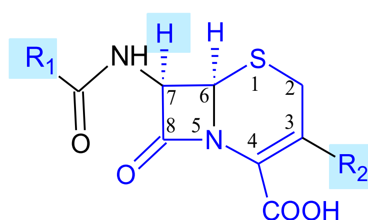

Figure 21 Structure of cephalosporins with positions for possible modification of cephalosporins (shadings)

- The β-lactam and fused dihydrothiazine rings are required for binding to PBP to effect the corresponding antibacterial activity.

- A sulphur atom at position 1 is common but not essential to its activity.
- The substituent at position 3 has an impact on chemical, metabolic and acid stability; however, variations at this position have a minimal effect on antibacterial activity. Addition of heterocycles allows protein binding and affects the half-life of the drug. The addition of a pyrrolidinium group to this position has an anti-pseudomonal effect.
- An unsaturated bond is present between positions 3 and 4 of the dihydrothiazine ring.
- In the active site of PBP, the carboxylate ion at position 4 of the cephalosporin core binds to positively charged nitrogen of a lysine (Lys) residue and the carboxyl group at position 8 reacts to form an ester bond with a Ser residue.
- Substituents at position 7 can be varied to influence the spectrum of activity via PBP affinity and β-lactamase resistance.
- The cephalosporin structure contains two chiral centers at positions 6 and 7, and their cis transformation is essential to antibacterial activity.

These findings led to the development of a range of cephalosporin derivatives and to their recognition as an important group of antibacterial agents. Beneficial chemical alterations are possible through modifications involving position-3 and -7 substituents, which allows greater freedom to tailor the molecule to achieve target properties than is possible for penicillin

2.2.1 First generation of cephalosporins

The first generation of cephalosporins includes antibiotics such as cephalothin, cephaloridine, cefalexin, and cefazolin. Generally, they have lower but broader activity comparable to penicillins. Most of them have to be administered by injection because of their poor absorption through the gut wall.[9] They can be used against staphylococci, streptococci, and anaerobic cocci except *Bacteroides fragilis.*[45] They are able to permeate the outer membrane of Gram-negative bacteria such as *E. coli, K. pneumoniae,* and *P. mirabilis*.[46] The first generation was successfully prescribed until resistance was observed from Gram-negative bacilli that produce β-lactamases.[47] Nowadays the first-generation is primarily used for the treatment of mild to moderate skin infections caused by methicillin-susceptible *S. aureus* (MSSA).[48]

Cephalothin was the first commonly used cephalosporin. Its acetyloxymethylene group at position 3 is important in the mechanism of PBP inhibition due to the ability of the acetoxy moiety to act as a good leaving group in reactions at the active site. However, it is readily hydrolyzed by esterase enzymes leading to a shortened period of activity. Cephaloridine displays a pyridiniummethylene ring at position 3, which is metabolically more stable against esterases while pyridinium is also a good leaving group for facilitating the inhibition mechanism. Cephaloridine, a zwitterion, is soluble in water and has to be administered by injection because of its poor absorption through the gut wall. Cefalexin is noteworthy because its methyl substituent at position 3 makes oral absorption possible, even though it does not improve activity. Activity comparable to that of cephaloridine and cephalothin can be restored by the addition of a hydrophilic amino group at the 7-acylamino side chain. Cefazolin has a prolonged biological half-life compared to other agents and is the preferred drug for surgical prophylaxis.[9] None of these antibiotics penetrate to the central nervous system, and are not first-choice drugs for any infections.[49] The first-generation cephalosporins contain diverse chemical substituents to achieve optimal pharmacokinetics. Their SARs are shown in Figure 21.

- Substitutions at the α-carbon of the C-7 side chain can introduce groups such as thienyl and tetrazolyl rings to increase activity against staphylococci, streptococci, *E. coli*, *K. pneumoniae* and *P. mirabilis.*
- Addition of hydrophilic groups (e.g., NH_2, OH) at the α-carbon of the C-7 side chain enhances Gram-negative activity. Such a change is observed in the case of cefalexin.
- An increase in lipophilicity of the C-7 side chain leads to decreased activity towards Gram-negative organisms *(all bullets from Neu)*[46]

- Molecules with an acetoxymethylene group at the 3C side chain have a relatively short half-life (e.g., cephalothin).[50]
- The pyridiniummethylene group at the C-3 side chain provided cephaloridine with metabolic stability, low serum binding, water solubility and excellent serum levels (e.g., cephaloridine), but greater nephrotoxicity at high concentrations.[51]
- Heterocyclic thioyl structures at the 3C side chain lead to higher levels of the drug in serum and a prolonged half-life (e.g., cefazolin).[51]

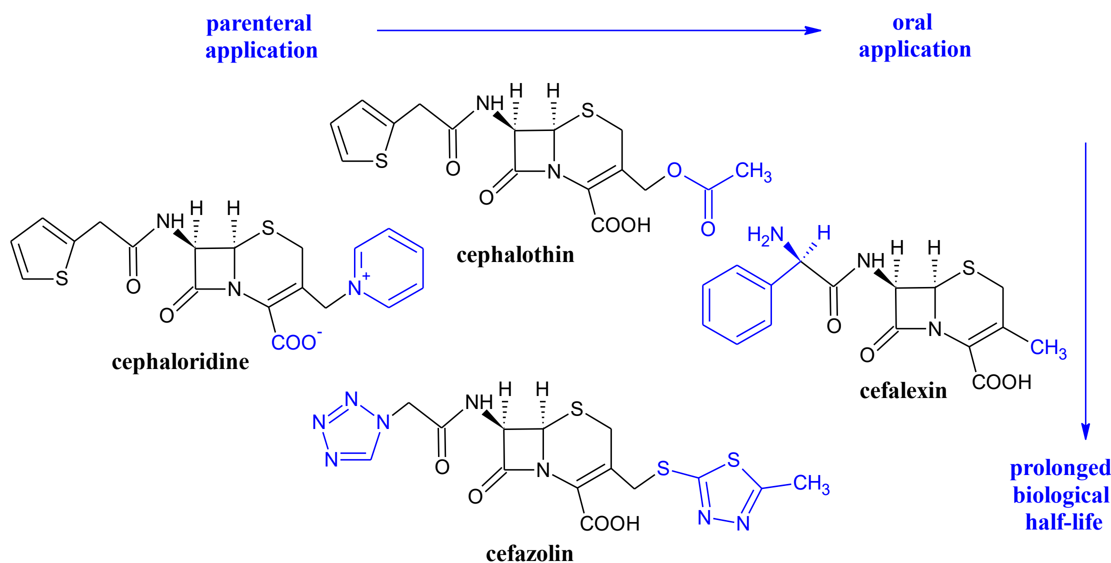

Figure 22 Structure-activity relationship of the first-generation cephalosporins

2.2.2 Second generation of cephalosporins

The second generation has practically the same effect on Gram-positive bacteria as cephalosporins of the first generation but increased activity against Gram-negative organisms. This generation is composed of two groups: cephamycins and oxyimino-cephalosporins.

2.2.2.1 Cephamycins

Cephamycins are sometimes classified as a separate group of β-lactams because their parent compound, cephamycin C, was isolated from *Streptomyces clavuligerus*, which also produces clavulanic acid belonging to a group of β-lactam inhibitors as well as a number of other metabolites.[52] Cefoxitin was synthesized from cephamycin C and exhibits a broader spectrum of activity than first-generation cephalosporins (Figure 23). It also possesses a high degree of resistance to β-lactamases due to the presence of a methoxy group in the 7-α-position of the β-lactam ring.

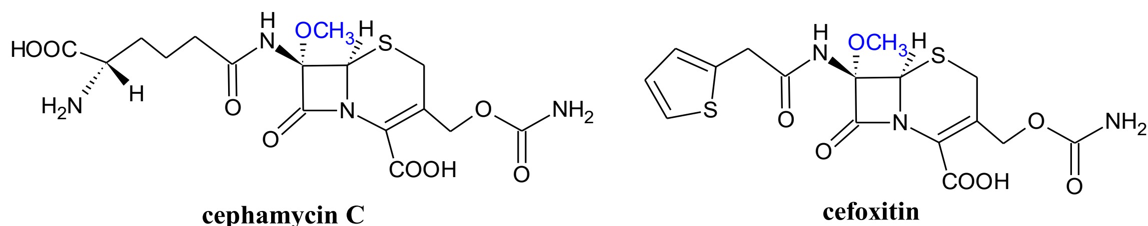

Figure 23 Structures of second generation of cephalosporins, the cephamycins

2.2.2.2 Oxyimino-cephalosporins

Oxyimino-cephalosporins contain an iminomethoxy substituent at the α-position of the 7-β-acyl side chain, which significantly improves pharmacological properties (Figure 24). The implementation of this group in cephalosporins has been a major advance, particularly in β-lactam research and is incorporated in all subsequent generations of this antibiotic class.

- The α-iminomethoxy substituent showed high levels of β-lactamase stability. A *syn*-position of the oxyimino group with the neighboring hydrogen increased antibacterial activity and resistance to various β-lactamases.[53] Interestingly, there is no interference between the oxyimino group and the β-lactam ring.[50]
- Replacement of phenyl and thienyl groups with a furyl group imparts increased antimicrobial activity.[46]

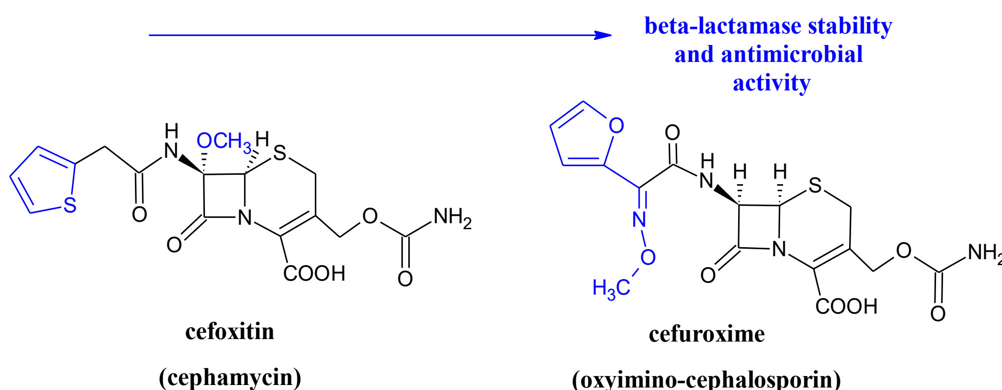

Figure 24 ***Comparison of cefoxitin and cefuroxime***

The first useful oxyimino-cephalosporin was cefuroxime with a wide spectrum of activity against bacterial species including Gram-negative rods (*Klebsiella* and *Proteus* spp.) with the exception of *P. aeruginosa.*[54] Clinical use is particularly prevalent for otitis, sinusitis and epiglottitis infections.[8]

2.2.3 Third generation of cephalosporins

The third-generation cephalosporins represent a very important development in the evolution of β-lactam antimicrobial agents. These compounds contain the aforementioned iminomethoxy substituent in addition to an aminothiazolyl group, which increases stability against β-lactamases and significantly increases potency in comparison to previous generations of cephalosporins.[50] These groups lead to a greater affinity for PBP and enhance the penetration of the antibiotic through the outer membrane of Gram-negative bacteria.[9] However, the aminothiazolyl side chain does not improve activity against Gram-positive organisms and anaerobic species. The third generation of cephalosporins is extensive and includes, most notably, ceftazidime, cefotaxime, and ceftriaxone with different substituents at position 3 to vary the pharmacokinetic properties (Figure 25).

- The 2-aminothiazolyl group at the 7-side chain has a high affinity for PBP in Gram-negative bacteria (mainly PBP1a and PBP3).[55] Replacement of the amino group of the thiazolyl ring with –H, –OH, or $–NHCH_3$ groups resulted in decreased activity against *S. aureus*, *Enterobacterales*, and *P. aeruginosa*.[56]
- The acidic and heterocyclic thiomethylene group at the C-3 position results in prolonged high levels in blood and binds to serum proteins (e.g., ceftriaxone).

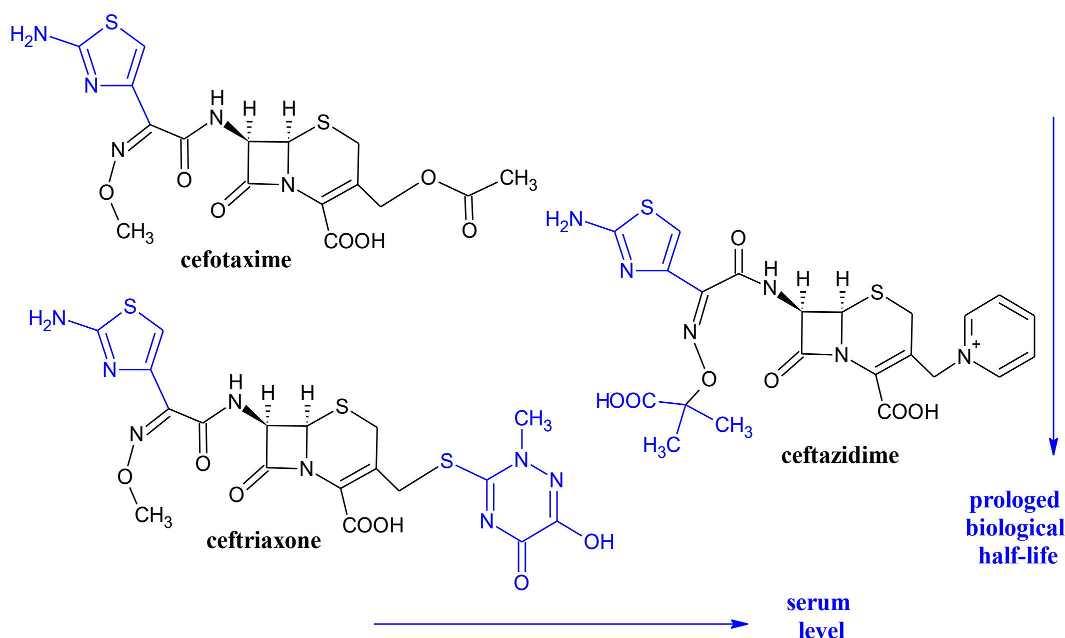

Figure 25 Structure-activity relationship of third generation cephalosporins

Third-generation cephalosporins have excellent activity against Gram-negative bacteria, but vary in their activity against Gram-positive cocci. Most of them are active against MSSA except ceftazidime which is weakly active with a decreased affinity to PBP in *S. aureus*.[54] This generation of antibiotics is very useful in the management of hospital-acquired Gram-negative bacteremia. Their major advantage is their enhanced activity against Gram-negative rods. They are able to cross the blood-brain barrier and distribute in the spinal fluid to treat meningitis.[45] They are also active against *P. aeruginosa*, although their effectiveness varies, with ceftazidime being one of the most prescribed third-generation cephalosporin for this purpose.

2.2.4 Fourth generation of cephalosporins

The fourth generation of cephalosporins contains oxyimino and aminothiazolyl groups, which were introduced in the previous generations. The only difference in the fourth-generation examples is the inclusion of positively charged nitrogen heterocycles at the 3C methylene position, making the drugs zwitterionic. This enhances the ability of the compounds to penetrate the outer membrane of Gram-negative bacteria with a high penetration to PBP and low affinity for a variety of β-lactamases.[9] Cefepime and cefpirome are the only molecules of this generation with similar *in vitro* spectra of antimicrobial activity. However, some differences are observed. Their nitrogen substituents at position 3C have significant effects on the biologic and pharmacokinetic properties of the compound. Cefpirome contains a cyclopentapyridinium group which has excellent activity against *S. aureus*. Cefepime contains a methylpyrroidinium group with lower antistaphylococcal activity, but it is two- to fourfold more active than the other aminothiazolyl cephalosporins against *Enterobacterales* (Figure 26).[46]

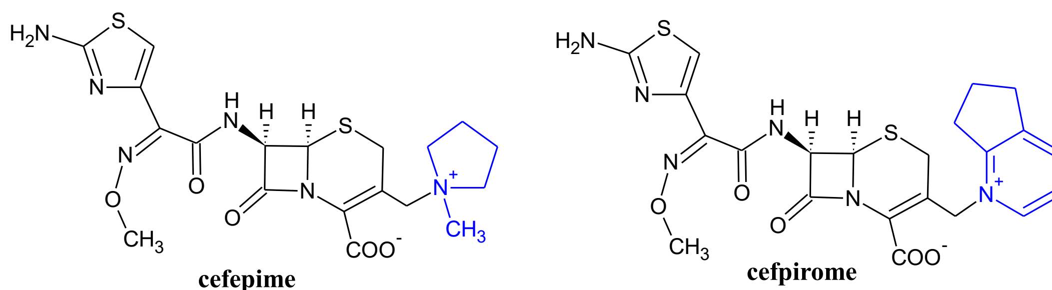

Figure 26 Structures of fourth-generation of cephalosporins

Both of them have enhanced activity against *Enterobacter* and *Citrobacter* species that are resistant to the third generation of cephalosporins. Cefepime has similar activity against *P. aeruginosa* but is more active against streptococci and MSSA in comparison with ceftazidime (3rd generation cephalosporin).[45] The molecules from the fourth generation of cephalosporins are the most active cephalosporins against *Enterobacterales*.

2.2.5 Fifth generation of cephalosporins

Ceftaroline fosamil and ceftobiprole medocaril are recently approved fifth-generation cephalosporins that are active against serious Gram-negative and Gram-positive bacterial infections, including methicillin-resistant *Staphylococcus aureus* (MRSA) carrying additional PBP2a (Figure 27). This modified PBP reacts with the early β-lactam antibiotics 10- to 1000-fold slower than native PBP even in the presence of high concentrations of the drugs.[57, 58] Ceftaroline has documented potent activity against vancomycin-resistant MRSA strains, multidrug-resistant *Streptococcus pneumoniae* strains, as well as common Gram-negative organisms.[59, 60] Ceftobiprole has also been reported to possess activity against methicillin-resistant staphylococci, *Enterococcus faecalis,* and penicillin-resistant pneumococci, while preserving the anti-Gram-negative activity of third- and fourth-generation cephalosporins.[37] The effectiveness of these antibiotics against MRSA appears to be a combination of factors:

- Their 3C side chains bind effectively to the active site of PBP2a due to the 1,3-thiazolyl ring, a permanently positive charge on the methylpyridinium ring of ceftaroline, and the strongly basic pyrrolidonyl group of ceftobiprole, which will be protonated at physiological pH.[61] The data showed that increased lipophilicity of the drugs improved activity against MRSA, whereas the introduction of acidic functionalities decreased the activity considerably.[62]
- The 1,2,4-thiadiazolyl ring in the 7C side chain facilitated Gram-negative membrane penetration and led to a high affinity for PBP.[63]
- The presence of the phosphonate group in ceftaroline fosamil significantly increased water solubility of the prodrug followed by rapid conversion in plasma into the bioactive agent.[63] The same effect was achieved with the prodrug ceftobiprole medocaril.[64]

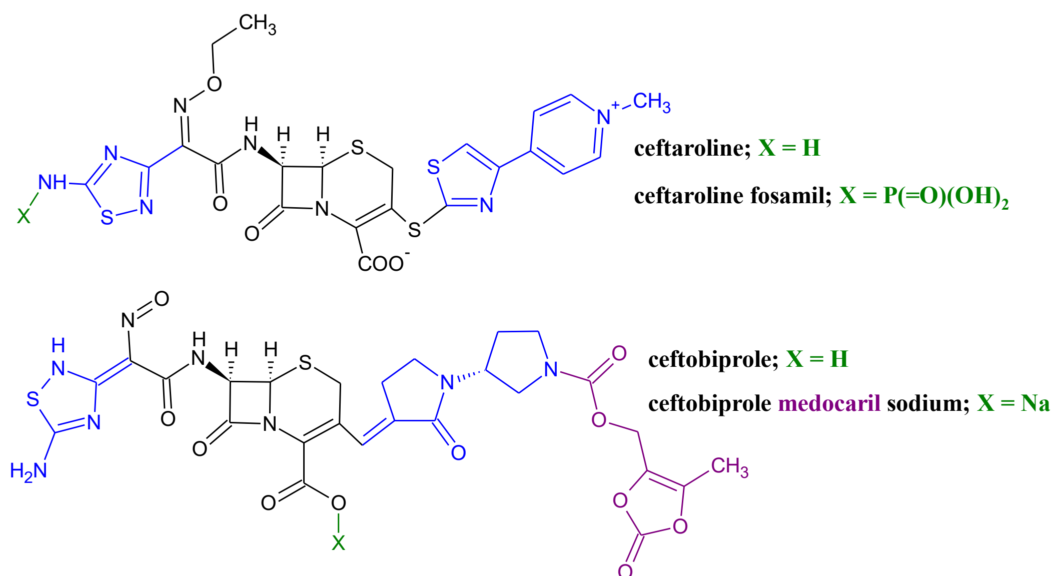

Figure 27 SAR of ceftarolin and ceftobiprole

Ceftolozane has high activity against *Pseudomonas* and *Enterobacterales* species, including *E. coli* and *Klebsiella* species (Figure 28). Ceftolozane is used in combination with the β-lactamase inhibitor tazobactam with an attempt to address multidrug-resistant Gram-negative bacteria such as *Enterobacterales* with extended spectrum β-lactamases (ESBLs) and some AmpC β-lactamases. However, it is not active against *Enterobacterales* producing carbapenemases.[65] Unlike ceftaroline and ceftobiprole is not effective for infections caused by *Staphylococcus aureus*.[66]

- The substituted pyrazolium ring on a side chain at position C-3 confers potent antipseudomonal activity[41] with high affinity to a broader inhibition profile toward the essential PBPs of *P. aeruginosa*.[67]

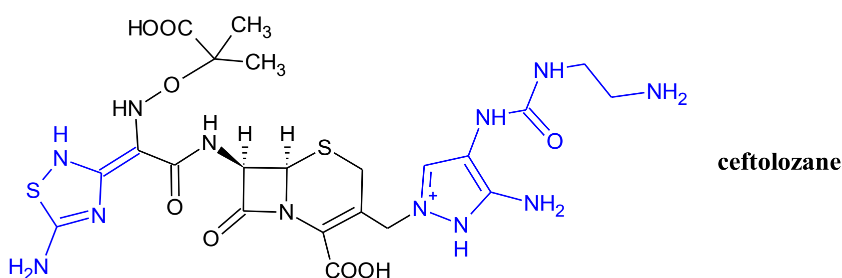

Figure 28 Structure of ceftolozane

2.2.6 Siderophore cephalosporins

Siderophore cephalosporins are one of the most promising groups of antibiotics to emerge recently and, of all available antibiotics, they possess the broadest spectrum against Gram-negative bacteria. They have shown activity against class A, B, C and D β-lactamase producers such as *A. baumannii*, *P. aeruginosa*, and *S. maltophilia.* Cefiderocol, for example, approved for medical use in the European Union in April 2020. FETCROJA®[68] (cefiderocol), has been shown to be potent *in vitro* against a broad range of Gram-negative bacteria. The chemical structure contains a catechol moiety on the 3-position side chain (Figure 29) that enables Fe^{3+} ions to be actively transported into bacteria via the ferric iron transporter system with subsequent destruction of cell wall synthesis.[69] Cefiderocol’s effects are attributed not only to

efficient uptake into bacterial targets via the active siderophore systems but also to the high stability of the compound to carbapenemase hydrolysis.

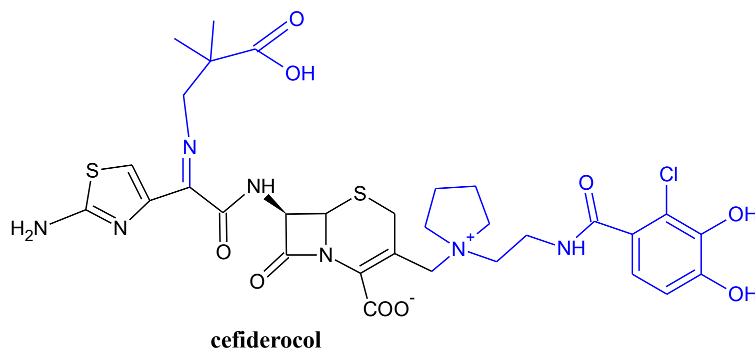

Figure 29 Structure of cefiderocol

2.3 Monobactams

Monobactams are a monocyclic β-lactam antibiotics that are found in various soil bacteria such as *Acinetobacter*, *Chromobacterium*, *Flexibacter*, and *Gluconobacter*.[70] The first marketed monobactam, aztreonam, arose around at the same time as the fourth generation of cephalosporins in the 1980s. Not surprisingly, aztreonam and subsequent derivatives incorporate the same substituents that were found to lend favorable activity to cephalosporins, namely oxyimino and aminothiazol moieties. In contrast to antibiotics with a bicyclic core structure, they contain a sulfonate group at the 1N position, which activates the β-lactam ring towards the acetylation of PBP (Figure 30).[71]

- The aminothiazolyl side chain supports to the activity against Gram-negative bacteria.[72]
- The iminopropyl carbonyl group on the side chain enhances antibacterial activity and provides stability against β-lactamases found in *P. aeruginosa*.[72]

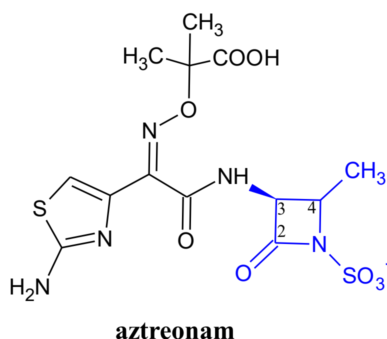

Figure 30 Structure of aztreonam

Aztreonam has a spectrum of activity limited to aerobic Gram-negative bacilli; it is not able to bind to PBP in Gram-positive bacteria or anaerobic organisms.[72] Aztreonam is widely distributed in body fluids and tissues, and concentrations exceeding the minimal inhibitory concentrations of important Gram-negative pathogens are attained in those compartments in which infections are most common. This drug is resistant to the most common chromosomal- and plasmid-mediated β-lactamases, i.e., TEM-1, TEM-2, SHV-1, as well as AmpC- and metallo-β-lactamases encoded by *Enterobacterales* and certain non-fermenting bacilli such as *Pseudomonas* and *Acinetobacter*.[37,73] Due to its high resistance to metallo-β-lactamases (MBLs), aztreonam can be used in unique applications, such as combination therapy

with a serine β-lactamase inhibitor to treat infections caused by multi-β-lactamase-producing bacteria.[74] Several other monobactams have been developed and briefly marketed, such as carumonam and the orally-absorbed tigemonan. They have not reached popularity in part because aztreonam, their predecessor, was not itself a marketing success. The most recent example, the monobactam BAL-30072, did not make it to market even though it was found to have improved activity due to the introduction of a siderophoric 3-hydropyridone substituent (Figure 31). In general, siderophores are secreted by microogranisms, exhibit high affinity for chelating iron, and have the ability to transport iron across a cell membrane. The 3-hydropyridone group competes with the homologous motif in bacteria whose primary function is nutrient acquisition.[75]

- The 3-hydropyridone moiety enhances activity against non-fermentating bacteria producing KPC-2 serine carbapenemase.[76]

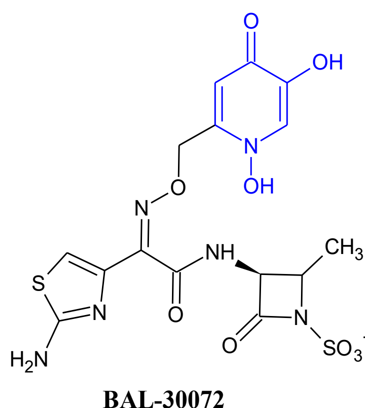

Figure 31 Structure of BAL-30072

BAL-30072 possessed an advantageous spectrum within the Gram-negative family based on in vitro and in vivo evidence but its development never reached Phase I because of its insufficient effect.

2.4 β-lactamase inhibitors

The protection of antibiotics by co-administration with enzyme inhibitors is an alternative strategy to the use of enzyme-stable drugs for overcoming resistance. The first β-lactamase inhibitor, clavulanic acid, was isolated from *Streptomyces clavuligerus* in 1976 and showed weak antibiotic activity but high resistance to most β-lactamases. As a result of their inefficacy when administered alone, they are combined with β-lactam antibiotics, particularly penicillins. Co-administration allows practitioners to decrease the dose level of the β-lactam and increases its spectrum of activity. For example, in combination with clavulanic acid, the minimum inhibitory concentration of amoxicillin against *S. aureus*, *E. coli*, *K. pneumoniae* and *Proteus mirabilis* was significantly reduced.[77] The mechanisms of β-lactamase inhibition include (1) irreversibly binding to the β-lactamase active site ("mechanism-based" inhibition), (2) reversible formation of an enzyme-substrate analog complex, and (3) formation of an enzyme-substrate analog complex, which upon hydrolysis yields an inactive derivative of the starting inhibitor (for example, the reaction of clavulanic acid with TEM-2 β-lactamase).[37]

The effectiveness of mechanism-based inhibitors varies across β-lactamase classes, with class A being the most susceptible, probably due to differences in the active site structure.[5] Inhibitors have mixed results when applied to specific enzymes within this class. For example, SHV-1 is more resistant to inactivation by sulbactam than TEM-1, while more susceptible to inactivation by clavulanate.[78] The structure of clavulanic acid is unique because the β-lactam ring is fused to an oxazolidine ring (oxygen atom at position 4), while sulbactam and tazobactam, which also belong to this group, are penicillinate sulfones containing an R_2SO_2 group at position 4. Sulbactam has a broader spectrum of activity against

β-lactamases than clavulanic acid, but it is not as potent. Tazobactam has both a broader spectrum of activity and greater potency than clavulanic acid.[9] The essential structural requirements for β-lactamase inhibition, in the case of clavulanic acid (Figure 32), are the presence of:

- a strained β-lactam ring
- an enol ether group
- *Z* geometry of the enol double bond (activity is reduced but not eliminated if *E* geometry is present)
- (*R*)-stereochemistry at C-2 and C-5
- a carboxyl group at position C-2
- without substitution at position C-6

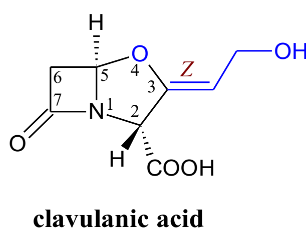

Figure 32 Structure of the β-lactamase inhibitor clavulanic acid

Clavulanic acid has potent inhibitory activity against the broad-spectrum β-lactamase from *K. pneumoniae* (e.g. SHV-1), as well as most class A β-lactamases, including ESBLs[79] and to a lesser extent, serine carbapenemases.[73] It acts synergistically with penicillin and cephalosporins against β-lactamase-producing enteric bacteria whereas in combination with ticarcillin, and taken parenterally, the same antibiotics are effective against nosocomial infections caused by *P. aeruginosa.*[80] Amoxicillin/clavulanate can be administered orally and is used in pediatric populations.[81]

Sulbactam and tazobactam (Figure 33) have a similar range of activity compared to clavulanic acid. Although effective against class A β-lactamases, all three inhibitors are generally less potent against class B, C, and D enzymes.[5] Sulbactam offers direct antimicrobial activity against *Acinetobacter* species and co-administration with ampicillin is prescribed for the treatment of various types of infections such as upper and lower respiratory tract infection, renal and urinary tract infection, intraperitoneal infection, genital infection, skin and soft tissue infection.[82] whereas tazobactam, in combination with piperacillin, has been used to treat nosocomial infections including those caused by *P. aeruginosa*.[83]

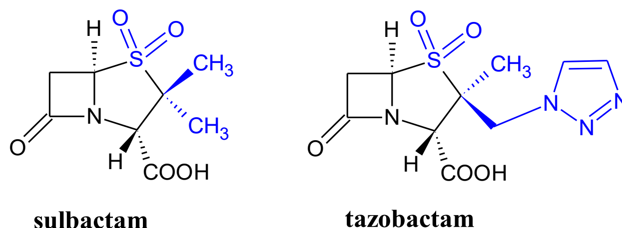

Figure 33 Structures of sulbactam and tazobactam

β-lactamase inhibitors with a diazabicyclooctane (DBO) core structure instead of a β-lactam ring have also been developed.[84] Avibactam was one of the first members of this group discovered to have a broader spectrum of activity than clavulanic acid and the penicillinate sulfones. Unlike these previous drugs, avibactam is a reversible inhibitor for most of the enzymes it is active against, including class A penicillinases, ESBLs, serine carbapenemases such as KPC-2, class C cephalosporinases and some class D

oxacillinases as well.[85, 86] MBLs and a few β-lactamases of class A such as TEM-30 and SHV-10 are not inhibited by avibactam.[84,87] The latter has been approved for therapeutic use in combination with ceftazidime while formulations of ceftaroline-avibactam and aztreonam-avibactam are currently under development.[88-90]

Other DBOs are currently being explored (Figure 34). Relebactam and nacubactam have been tested in combination with a carbapenem, imipenem, and exhibit a similar spectrum of activity to avibactam. However, relebactam is less effective against class D β-lactamases such as OXA-48,[91] whereas nacubactam has an additional benefit of intrinsic antibacterial activity against enteric bacteria.[92] Nacubatam combinations with meropenem or cefepime can overcome most metallo-β-lactamase-mediated resistence.[93]

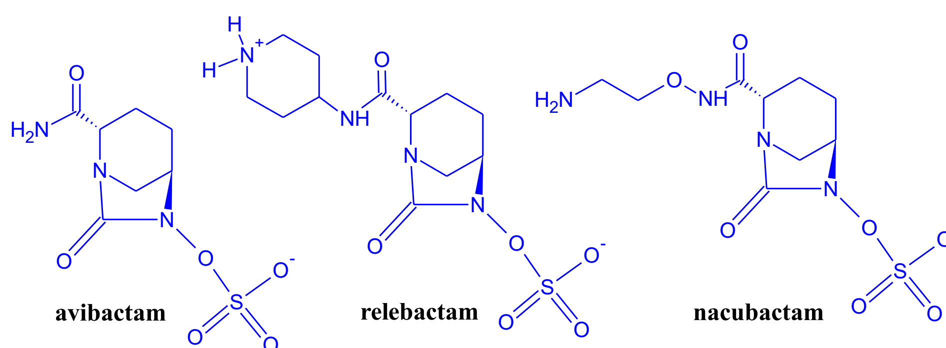

Figure 34 Structures of diazabicyclooctanes

Another family of β-lactamase inhibitors, known for many years, display boron heterocycles and are effective inhibitors of serine β-lactamases.[94] Compound RPX7009 - vaborbactam is currently under development and being used in combination with meropenem, another carbapenem, to target Gram-negative bacteria producing serine carbapenemases (Figure 35).[95]

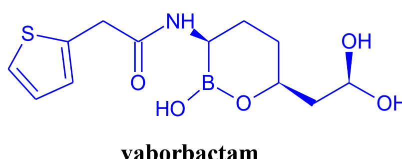

Figure 35 Structure of vaborbactam

2.5 Carbapenems

Carbapenems possess the broadest spectrum of antibacterial activity of any of the known groups of β-lactam antibiotics. They are often considered a last resort for patients infected by highly antimicrobial-resistant bacteria. Unfortunately, the rise of multidrug resistant (MDR) strains seriously threatens this class of antibiotics as well.[96]

The development of the first carbapenem antibiotics was preceded by the discovery of olivanic acid, which is naturally produced by the Gram-positive bacteria *Streptomyces clavuligerus.* Olivanic acid (Figure 36) differs to penicillins by:

- the presence of an unsaturated bond between C-2 and C-3
- the replacement of sulfone with a methylene in position 1 of the fused ring
- the stereochemistry of C-6 (opposite from the stereochemistry of the penicillin C-6)
- increased potency (in comparison with cephalosporins as well as penicillins) when there is *trans* geometry at the C-5 – C-6 bond[25]

- a hydroxyethyl group instead of an acylamino chain, which plays a major role in β-lactamase resistance.[9]

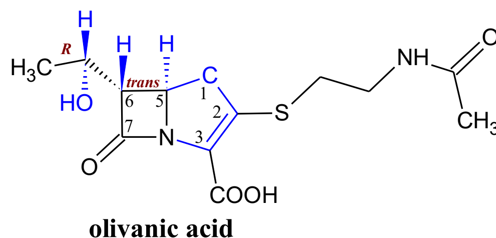

Figure 36 Structure of olivanic acid

Olivanic acid is no longer being pursued as a treatment due to low penetration into the bacterial cell as well as chemical instability; however, it showed impressive β-lactamase inhibitor activity, in some cases exceeding the potency of clavulanic acid by 1000 times.[9, 25]

Several notable compounds similar to olivanic acid have been reported, including thienamycin (Figure 37). A natural product of *Streptomyces cattleya,* thienamycin was identified as a promising β-lactamase inhibitor in 1976, subsequent to the discovery of olivanic acid and in the same year as the discovery of clavulanic acid.[97] Thienamycin is considered the first and model compound for the carbapenem group.[25] The chiral center on the side chain at position C-6 has a different absolute configuration (*S*-isomer) than olivanic acid (*R*-isomer) (Figure 37), which increases its activity and serves as a key attribute of carbapenems.

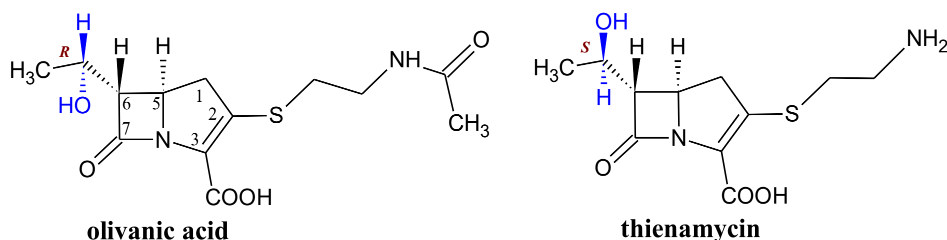

Figure 37 Structures of olivanic acid and thienamycin

Nonetheless, thienamycin was unstable in aqueous solutions, sensitive to mildly basic pH, and reactive with nucleophiles.[98] In addition, the primary amino group in the C-2 side chain of one molecule can react with a second molecule of thienamycin to form an inactive dimer.[3] These drawbacks led to the development of a chemically more stable derivative, imipenem. Imipenem contains the *N*-formimidoyl group, which is less sensitive to base hydrolysis (Figure 38).

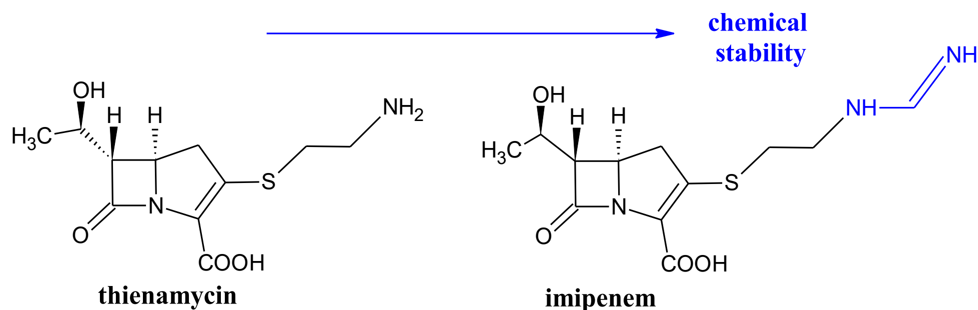

Figure 38 Comparison of thienamycin and imipenem

This drug was the first carbapenem used for the therapy of complex microbial infections caused by Gram-positive, Gram-negative, non-fermenting and anaerobic bacteria. It demonstrated high stability against common β-lactamases with high affinity for PBPs.[99] However, imipenem is susceptible to the renal metalloenzyme – dehydropeptidase I (DHP-I),[100] thus it´s administration with a DHP-I inhibitor such as cilastatin is necessary.[101] Co-administration of an inhibitor with a carbapenem can be avoided by the addition of a methyl group to the C-1 position, as in the structure of meropenem.[102] It has also been suggested that the methyl substituent in position 1 produces general activity against Gram-negative bacteria, but the effects on antipseudomonal activity instead depend on the side chain at position 2.[103]

The presence of a basic C-2 side chain is indispensable for improving permeability through the outer membrane of *P. aeruginosa*, but antipseudomonal activity was not directly correlated with the strength of the basicity. Other improvements were obtained by modification of the C-2 chain with various *N*-heterocyclic amines having broader antimicrobial influence.[103] The spectrum and activity of carbapenems are dependent on many physical and chemical factors such as lipophilicity and the charge at physiological pH. Generally, lipophilic carbapenems have good activity against Gram-positive bacteria, whereas hydrophilic carbapenems have been shown to penetrate through the water-filled porins of Gram-negative organisms.[3] Carbapenems are notable for their stability to most β-lactamases,[99] with the exceptions of carbapenemase subtypes found primarily in Gram-negative bacteria.[104]

To summarize, the structure-activity relationships of the carbapenems are complex, as they derive from an equilibrium that is difficult to achieve between chemical stability, resistance against hydrolysis by DHP I, and antibacterial activity. Following is a description of the important SARs[25, 105]:

- Methyl substitution at position C-1 increases stability against hydrolysis by DHP-I but reduces the antibacterial activity, which then derives from the chain at C-2.
- The side chain at C-2 has an influence on chemical stability, tendency to be hydrolyzed by DHP-I, and antimicrobial activity.
- The basic substituents with either free or protonated amino groups enhanced activity against Gram-negative bacteria, including *P. aeruginosa.*
- Hydrophobic or aromatic substituents on the side chain at C-2 increased the activity against Gram-positive bacteria.
- A hydroxyethyl chain at C-6 yields optimal antibacterial activity.
- The zwitterionic molecules exhibit good activity against *P. aeruginosa* and the DHP-I enzyme.

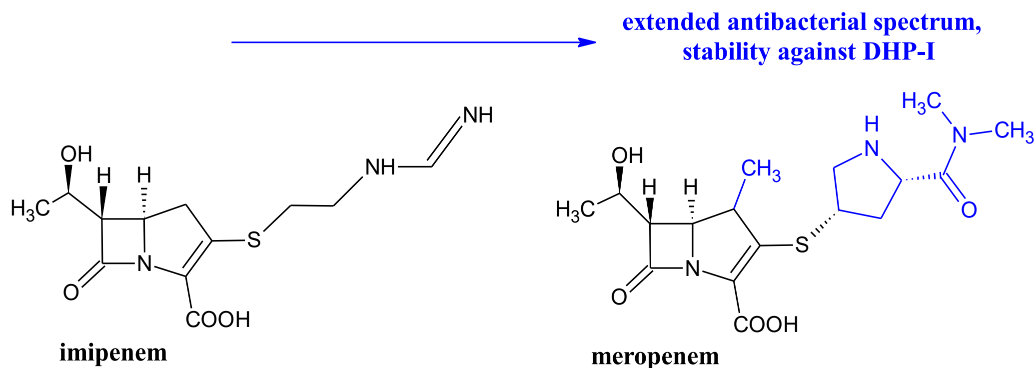

Figure 39 Comparison of imipenem and meropenem

In view of these SARs, more stable carbapenems were developed with a broader spectrum such as meropenem (Figure 39), ertapenem, and doripenem (Figure 40). These carbapenems are chemically more stable and have a longer half-life than imipenem. In general, imipenem and doripenem are potent antibiotics against Gram-positive bacteria,[106-109] while meropenem, ertapenem are more effective against Gram-negative bacteria.[106, 110] In addition, meropenem and doripenem retain greater activity against isolates of *P. aeruginosa* lacking the outer membrane porin protein OprD than imipenem,[111] but ertapenem is completely inactive.[112] By contrast, doripenem and imipenem were more active than meropenem against *Acinetobacter baumannii.*[25]

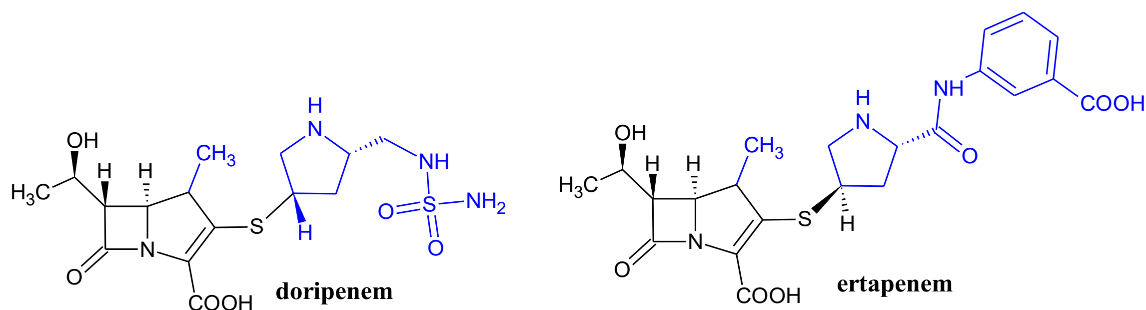

Figure 40 Structures of doripenem and ertapenem

Meropenem has unique applications and is the only carbapenem approved for use in meningitis because of its excellent penetration into the meninges.[113] When combined with clavulanic acid, it is also potent enough to kill multi-drug resistant *Mycobacterium tuberculosis*.[114] Doripenem has the additional benefit of being the least susceptible carbapenem to degradation by carbapenemases.[108]

The novel carbapenems strongly resemble the fifth generation of cephalosporins in structure and were focused on multi-drug resistant isolates. Carbapenem ME-1036 has a similar C-2 side chain compared to ceftaroline, containing a pyridinium ring, and shows the same advantageous affinity to PBP2a of MRSA.[115] By contrast, razupenem with a lipophilic thiazole group in the side chain at C-2 has extended antimicrobial activity across Gram-positive bacteria including MRSA and vancomycin-resistant *Enterococcus faecium* (VRE). However, the development of razupenem has been discontinued,[116] and there is no evidence that ME-1036 is progressing in clinical evaluations (Figure 41).

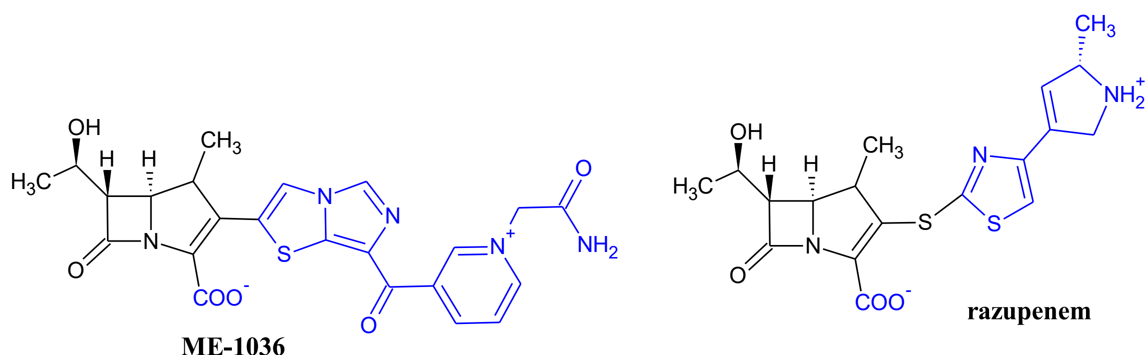

Figure 41 Structures of ME-1036 and razupenem

Carbapenems are often combined with other antimicrobial drugs for life-threatening infections. This combination is practiced for several reasons. It generally extends the antibacterial spectrum and provides additive or synergistic actions. Most importantly, however, combination therapy is used to avoid the emergence of resistant species, since the chance of developing resistance is lower for two co-administered drugs than for a single drug.[117]

3. β-lactam antimicrobial resistance

Antibiotics have revolutionized the treatment of infectious diseases worldwide. Unfortunately, once an antibiotic is widely used, resistant bacterial strains invariably emerge and become predominant. The serendipitous observation of Alexander Fleming in 1928 is considered one of the most significant milestones in the timeline of antibiotic discovery, but in 1940, several years before the introduction of penicillin to the market, a bacterial β-lactamase, penicillinase, which destroys the antibiotic, was identified. Although resistance is not usually discovered before the drug has been sold commercially, the general trend has been confirmed for other antibiotics, as shown in Figure 42.[118]

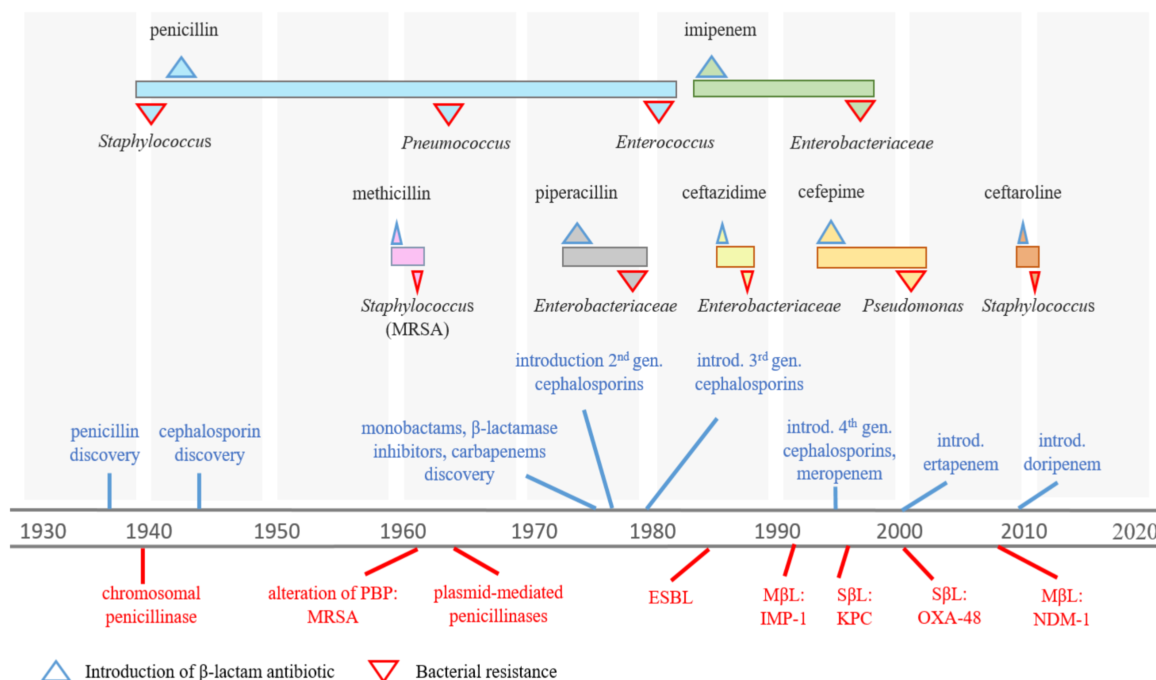

Figure 42 Timeline of notable β-lactam antibiotics: their discovery, clinical introduction and the appearance of antimicrobial drug resistance[119-122]

Interestingly, the identification of penicillinase before the clinical use of penicillin can now be appreciated from recent findings that a large number of resistance genes (r genes) are components of natural microbial populations.[123] This compensatory mechanism for survival can be difficult to overcome and prevents the discovery of otherwise effective antibiotics. However, in the majority of cases, acquired antibiotic resistance is promoted by the misuse and extended use of antibiotics in human and veterinary medicine as well as in agriculture. The resulting infections from resistant strains lead to greater mortality, morbidity, and a higher cost of treatment. Additionally, the growing inability to prevent infections with antimicrobial prophylaxis will increase the risk of complex medical procedures such as surgery, chemotherapy, and transplantation.

The mechanisms underlying bacterial resistance to β-lactam antibiotics are varied and often depend on a primary resistance mechanism assisted in important ways by secondary ones. For each group of β-lactam antibiotics, the primary mechanism of resistance is generally different for Gram-positive bacteria compared with Gram-negative species.

Usually, Gram-positive bacteria utilize a modification of the penicillin binding protein (PBP) active site that allows them to retain their normal physiological activity with decreased sensitivity to antibiotics. This is in contrast to the erection of effective permeability barriers combined with enzymatic degradation as a minor pathway, which is most often characteristic of resistance in Gram-negative bacteria (Figure 43**)**.[119, 124] The alteration of PBPs was first observed in *Staphylococcus aureus* isolates within a few years of the introduction of methicillin, a semi-synthetic penicillinase-resistant penicillin. Introduction of methicillin marked the onset of a second wave of resistance in *S. aureus*, well known as methicillin-resistant *S. aureus* (MRSA).

β-lactam resistance in Gram-negative bacteria can arise through three possible mechanisms: (1) alteration of PBPs, just as with Gram-positive bacteria, as well as (2) production of β-lactamases and (3) limited access of the antibiotics to target PBPs. β-lactamases are capable of inactivating β-lactam antibiotics by hydrolysis of the amide bond in the four-membered ring. Limited access to PBPs is made possible because they are located in the periplasmic space of Gram-negative bacteria, thus before β-lactam antibiotics can reach their sites of action, they have to pass over the bacterial outer membrane. Any modulation that restricts entry adds an additional challenge, such as porin loss or efflux pumps specific to

β-lactam antibiotics. These alterations will confer resistance to antibiotics with the potential for cross resistance with other antimicrobial agents if they are sharing the same channel (Figure 43**)**.

In Gram-negative bacteria the most prevalent resistance mechanism is the production of β-lactamases while alterations to PBP are fundamental for Gram-positive bacteria.[125]

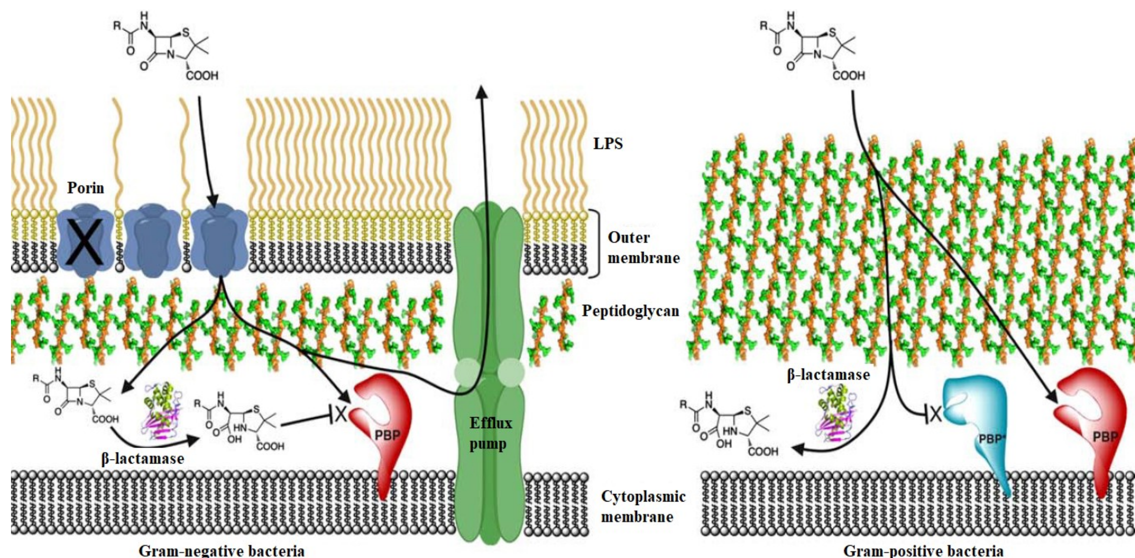

Figure 43 ***A schematic representation of β-lactam resistance mechanism exploited by Gram-negative and Gram-positive bacteria. (Adapted from Llarul)[24]***

As can be observed in Figure 42, an accelerated expansion and diversity of antimicrobial resistance became increasingly evident over the past 50 years, correlated with the increased development of antimicrobial compounds capable of overcoming or bypassing these resistance mechanisms. Unfortunately, over the past 3 decades, new classes of antimicrobial compounds have failed to emerge, which results in a problem compounded by the relentless increase in new and modified mechanisms of antimicrobial resistance in disease-causing microbes. This evolution is especially evident when considering the β-lactam class of antibiotics, which has become less effective due to a ten-fold increase in inactivating bacterial enzymes between 1990 and 2010.[126] Understanding the mechanism of resistance in clinical isolates is therefore important and thorough knowledge of the mechanism could enable the design of novel therapeutics.

3.1 Background to the genetics of antimicrobial resistance

As of today, numerous genetic loci associated with antibiotic resistance have been identified. Bacterial resistance can be intrinsic or acquired or both. Many bacterial species, both commensals and pathogens, are naturally resistant to certain classes of antimicrobial agents. The insensitivity is termed intrinsic resistance, the occurrence of which limits and complicates drug selection for treatment, due to the lack of targets, its poor affinity to the antibiotics or natural production of enzymes inactivating antibiotics. For example, intrinsic resistance to cephalosporins can be found in some species of enterococci that contain a D-Alanine-D-Serine terminus in peptidoglycan instead of a D-Alanine-D-Alanine terminus.[127]

Intrinsic resistance can increase the risk for development of acquired resistance in other bacterial species. Acquired resistance is the result of either the mutation of existing genes in different chromosomal loci or the incorporation of extraneous DNA encoding a novel gene. The latter is the result of horizontal gene transfer, where antibiotic-resistant genes are carried on mobile genetic elements such as transposons, integrons, and plasmids (Figure 44) that behave as vectors for inter- or intraspecies transfer.[128]

Acquired resistance is demonstrated by Gram-negative organisms when the uptake of β-lactam drugs is reduced by selectively alteration of cell membrane porin channels. Reduction of outer membrane permeability in this manner prevents the β-lactams from reaching their target.

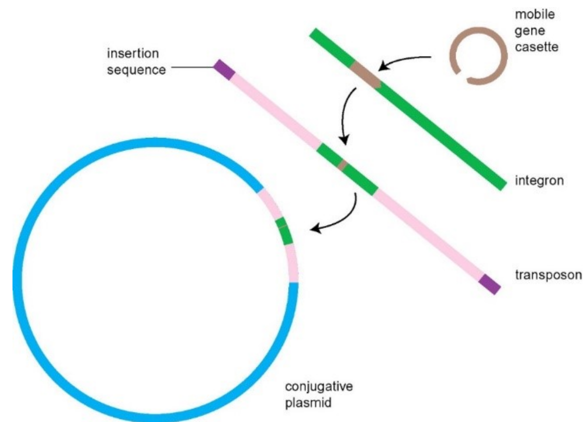

Figure 44 Mobile genetic elements. (Adapted from Nordmann)[128]

Horizontal transfer of a new gene from one bacterium to another accomplished via processes such as conjugation, transformation, and transduction illustrated in Figure 45 below. These mechanisms allow for the spread of plasmid-mediated β-lactamases genes, for instance, which are a great concern, contributing to the remarkable expansion of resistance throughout the microbial world.[129]

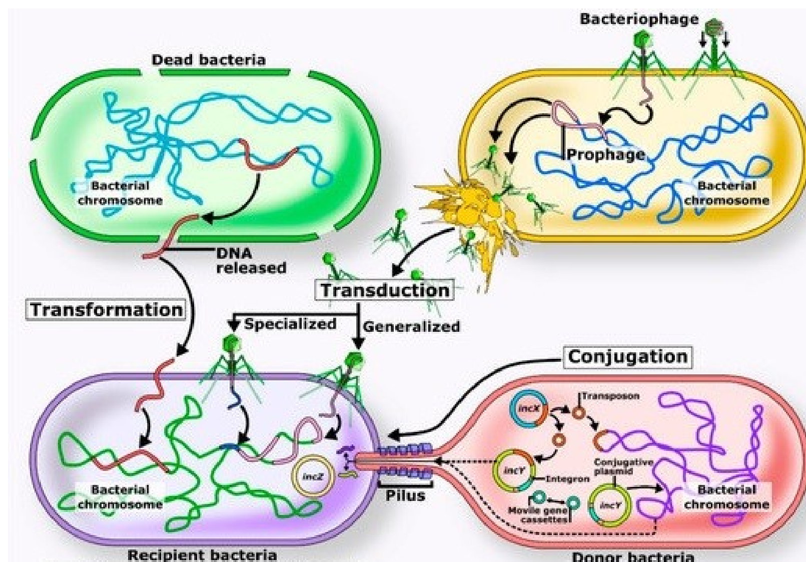

Figure 45 Horizontal gene transfer. (Adapted from Bello-Lopéz)[130]

The genes encoding β-lactamases can be located on the bacterial chromosome, or plasmids. They are commonly found on mobile elements such as transposons or gene cassettes of integrons.[131] Integrons encoding resistence gene cassettes are increasingly found in resistant bacteria. The genetic environment of the β-lactamase (*bla*) gene dictates whether the β-lactamases are produced in a constitutive or inducible manner.

Franco et al.[132] detail how metallo-β-lactamases (MBLs) were first found encoded in opportunistic and environmentally pathogenic bacteria including *Stenotrophomonas maltophilia*, *Bacillus cereus*, and *Aeromonas species*. However, in the 1990s chromosomal MBLs were detected in most carbapenem-resistant *Pseudomonas aeruginosa* and subsequently *Acinetobacter* species in clinical

specimens. Moreover, MBL genetic materials have been found on mobile elements in the *Enterobacterales* family.[133, 134]

3.2 Background to the biochemistry of antibiotic resistance

Only four general mechanisms, independently or in combination, are thought to explain resistance to antibiotics in all known bacterial species: (1) - (3) modifications to the cell wall including decreased permeability of the outer membrane, the presence of active efflux pumps, and the alteration of PBPs, as well as (4) the production of enzymes modifying or degrading antibiotics (like β-lactamases).

An amazing variety of antibiotic resistance mechanisms can be observed in a single strain of bacteria. Which of these mechanisms predominate depends on the nature of the antibiotic, its target site, the bacterial species, and whether it is caused by chromosomal mutation or mediated by horizontal transfer gene. It is noteworthy that the synergy achieved through the combination of these diverse mechanisms plays an important role in determining the final expression of resistance.

The mechanism that has been investigated the most thoroughly is the production of β-lactamases, which is also the most prominent mechanism of resistance in Gram-negative bacteria. Therefore, β-lactamases will be discussed in greater detail than the other mechanisms associated with cell wall modifications.

3.2.1 Modification of the cell wall of bacteria

All bacteria possess a cell envelope with a complex multi-layered structure that serves to protect these organisms from their external environment. Based on the unique characteristics of the cell wall, bacteria are divided into Gram-positive and Gram-negative species. In general, a Gram-positive cell wall has a simpler chemical composition than the cell wall in Gram-negative bacteria. Both contain inner membranes (IM) and peptidoglycans while Gram-negative bacteria possess an additional outer membrane (OM) consisting of a phospholipid bilayer. Between the two membranes lies the periplasm, where resistance enzymes are localized among other entities. The OM contains proteins such as porins and efflux pumps and exhibits lipopolysaccharides (LPS) on its exterior.[135] LPS bilayers are more rigid than typical phospholipid membranes and consist of polysaccharides (O-antigen), core oligosaccharides, and Lipid A molecules. It provides protection from potentially harmful compounds in the extracellular space. Porins help hydrophilic molecules pass through the OM. They are water-filled channels composed of 16 β-strand proteins. They provide an electrostatic field that plays an important role in selectivity for the size and charge of the entering molecules. Efflux pumps are transporter proteins, which actively force out a variety of compounds from the intercellular space of bacteria (Figure 43).[136] The cell envelope of Gram-negative bacteria is less permeable to many antibiotics compared with Gram-positive bacteria due to the additional barrier provided by the OM. Therefore, Gram-negative species are intrinsically resistant to a greater number of antibiotics.

3.2.2 Decreased permeability of the outer membrane

Molecules can penetrate the OM of Gram-negative bacteria in the following ways: diffusion through the lipid layer, diffusion through porins, or by self-promoted uptake[137] (Figure 43). Generally, hydrophobic antibiotics (macrolides) use a lipid-mediated pathway and hydrophilic antibiotics (β-lactams) diffuse through porins. Modifications of the protein (porin) or lipid (LPS) structures lead to the emergence of antibiotic resistance in bacterial species.[138]

Porins affect many classes of antibiotic and vary in specificity and mechanism.[139, 140] Some of them are drug-specific but most are multidrug transporters able to accomodate a wide spectrum of structurally unrelated antibiotics. Porin loss or alteration can promote antibiotic resistance, including multidrug resistance (MDR).[141, 142] Porin deficiency is a contributing factor of β-lactam resistance in *E. coli*, *K. pneumoniae, P. aeruginosa*, *A. baumanii, Enterobacter spp.,* and others.[13] The *P. aeruginosa* porin OprD is a substrate-specific protein that is involved in the diffusion of carbapenems, especially imipenem,

into the cell.[143] OprD-mediated resistance occurs as a result of decreased expression of the *oprD* gene and/or loss-of-function mutations that disrupt carbapenem diffusion.[144]

A detailed discussion about bacterial resistance to antibiotics via the lipid-mediated pathway is beyond the scope of this work.

3.2.3 Efflux pumps

Efflux pumps are membrane proteins able to export antibiotics that have penetrated within the bacterial cell. They are active transporters requiring a source of chemical energy to perform their function, and they can be found in both Gram-negative bacteria as well as Gram-positive bacteria[145] (Figure 43). They may be specific to one substrate or capable of transporting a range of structurally dissimilar compounds, thus they may also be associated with multidrug resistence (MDR). Some bacteria carry multiple chromosomal genes that encode MDR efflux pumps, but some of them have been mobilized onto plasmids that can transfer between bacteria. The transporters are classified into five major families based on their amino acid sequence and their energy source for transport substrates: MF (major facilitator), MATE (multidrug and toxic efflux), RND (resistance-nodulation-division), SMR (small multidrug resistance) and ABC (ATP binding cassette).[146] Mutations of the regulatory elements that control the production of efflux pumps can lead to an increase in antibiotic resistance. For example, the mutation of the *mexAB* system in *P. aeruginosa* leads to overexpression of the MexAB efflux pump that confers increased resistance to β-lactam antibiotics.[140, 147-149] Another well-studied example includes the multidrug efflux pump AcrB in *E. coli*, which contains two distinct binding pockets to accommodate substrates of different properties and sizes. This characteristic explains how the pumps are able to transport and provide resistance to a broad spectrum of antibiotics.[150-152]

3.2.4 Alteration of penicillin binding protein

As was mentioned in the first chapter, penicillin-binding proteins (PBPs) are involved in the final synthesis of peptidoglycan. Inhibition of PBPs by β-lactam antibiotics leads to destruction of the cell wall of bacteria via lysis. An increased production of the normal target enzyme can provide resistance to such antibiotics,[153] as can modifications to the structure of the enzyme. In cases where PBPs have been modified, the β-lactams may be unable to bind properly to the target site (Figure 46). This adaptation occurs by the acquisition of a gene encoding an analogous enzyme that has a much lower affinity for the antibiotic. One of the most important examples of a target modification is the case of MRSA, where the modification of PBPs in *Staphylococcus aureus* resulted in a low affinity to methicillin and other β-lactam antibiotics. This type of resistance is mediated by the acquisition of mobile genetic elements called the staphylococcal cassette chromosome *mec* (SCC*mec*) incorporating resistance genes *mecA* and *mecC,* which encode the β-lactam insensitive PBP2a and PBP2c, respectively. The altered PBPs (PBP2a and PBP2c) are also involved in the assembly of the peptidoglycan structure of the bacterial cell wall.[154, 155]

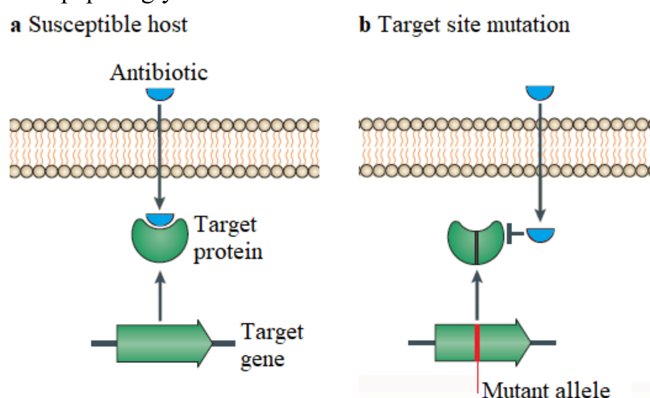

Figure 46 ***Target site changes. a)*** *A susceptible host in which an antibiotic is able to bind tightly to its specific target and exert an inhibitory effect.* ***b)*** *Mutation of the target site results in a functional*

target with reduced affinity for the antibiotic, which does not bind efficiently, and therefore has a reduced or negligible effect. (Adapted from Blair)[156]

3.2.5 Antibiotic inactivation

This mechanism includes the production of enzymes that modify or degrade the antibiotis. They are excreted in bacterial periplasm and inactivate the antibiotics before they reach their target. The most common enzymes for destroying β-lactam antibiotics are β-lactamases. These are globular proteins that contain characteristic secondary structures as well as other structural features. Mechanistically, these enzymes have a strong similarity to PBPs, in particular, a high affinity for binding to β-lactams. The mechanism of action of β-lactamases is hydrolytic cleavage of the amide bond of the β-lactam ring, which may deactivate the antibiotic before it reaches its target. The approach of the β-lactam agent is guided by relatively long range electrostatic attractions between the carboxylate of the β-lactam and the cationic side chain of an active site amino acid in β-lactamase. A proximal residue catalytically hydrolyzes the β-lactam ring, forming an acyl-enzyme complex, which is followed by further degradation (Figure 47).

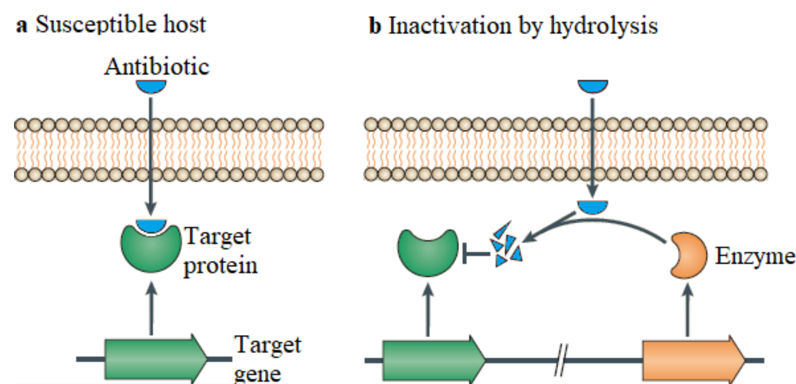

Figure 47 **Direct interactions with antibiotics.** ***a)*** *A susceptible host with a target that is efficiently inhibited by an antibiotic.* ***b)*** *Acquisition and production of an enzyme that destroys the antibiotic, prevents binding to the target, and confers resistence. (Adapted from Blair)*[156]

β-lactamases are produced by both Gram-positive and Gram-negative bacteria and more than 2000 unique β-lactamases have been reported.[157] They are codified by two main classification systems. The first one was proposed by Richard Ambler in 1980[158] and the second by Karen Bush et al. in 1988.[159] The Ambler classification divides the β-lactamases into four groups: A, B, C, and D, based on their amino acid sequences. The classes A, C, and D, are the active-site serine β-lactamases (SβL) while class B contains the metallo-β-lactamases (MβL) containing a bivalent metal ion(s), usually Zn^{2+}, for their proper activity (Figure 48).[160]

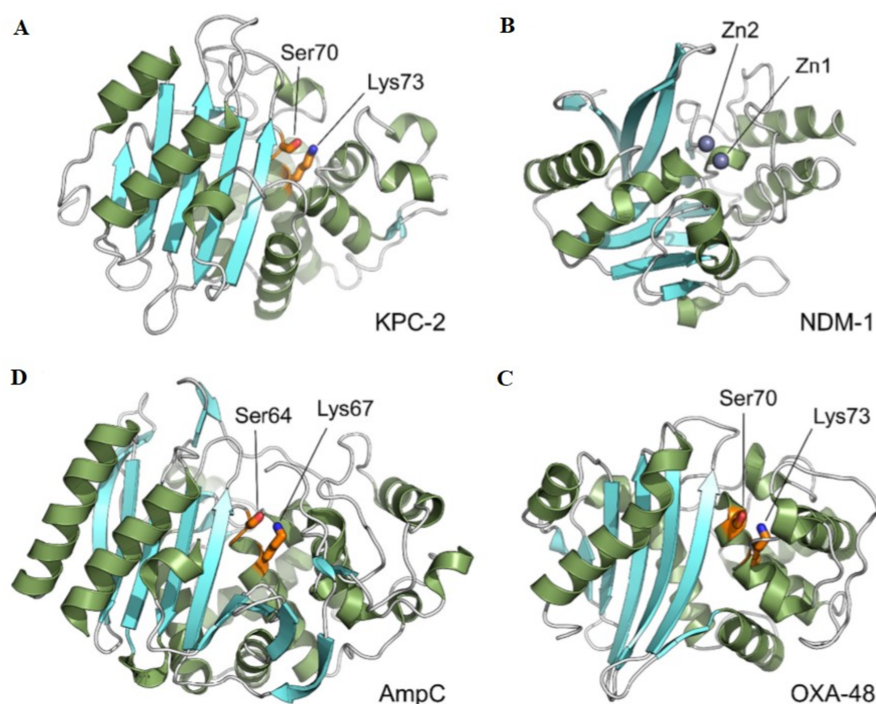

Figure 48 Crystal structures of β-lactamases from A, B, C, and D class. (Adapted from Tooke)[161]

Both mechanisms work on the same principle of opening the β-lactam ring and thereby inactivating the antibiotic (Figure 49).

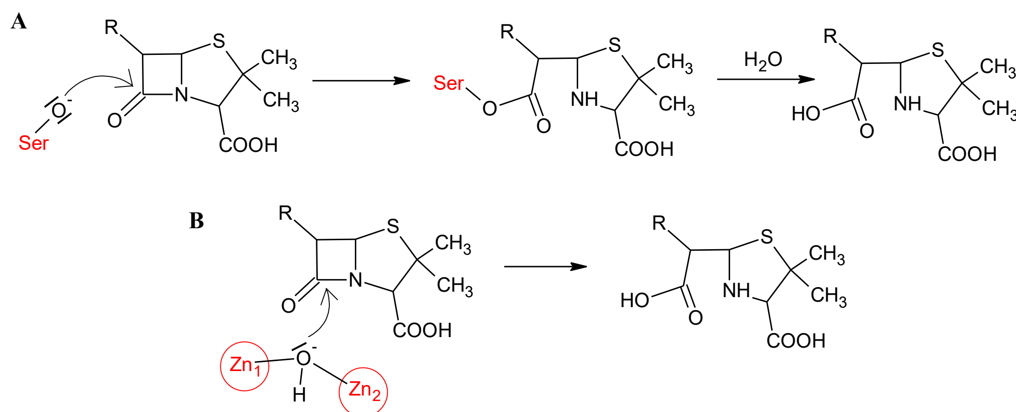

Figure 49 General mechanism of β-lactamase-mediated hydrolysis of penicillin.
***A)** Serine-β-lactamases utilize an active site serine as nucleophile. **B)** Metallo-β-lactamases utilize two zinc ions and a solvent molecule as nucleophile.*

The Bush system is used to assign a clinically useful description to a family of enzymes. It divides enzymes into functional groups numbered 1 to 3, and subgroups *a* to *f*, which are designated according to substrate spectrum, inhibitor profiles, molecular masses, and isoelectric points. In 2010, this system was updated with the addition of peptide sequences to the existing list of attributes describing new β-lactamases.[162, 163] In Table 1 are shown both classifications which organize the considerable differences between β-lactamases. In addition, they provide information for understanding the substrate/inhibition profiles of certain clinically relevant β-lactamases.

Table 1 Representative β-lactamases labeled according to Ambler and Bush classifications.[162]

Ambler class	Bush group	Penicillinase activity	Cephalosporinase activity	ESBL activity	Carbapenemase activity	Monobactamase activity	Examples of enzyme
A	*2b*	Y	Y	N	N	N	TEM-1, SHV-1
	2be	Y	Y	Y	N	Y	CTX-M-15
	2br	Y	Y	N	N	N	TEM-30
	2f	Y	Y	Y	Y	Y	KPC-2
B	*3a*	Y	Y	Y	Y	N	VIM, IMP, NDM
	3b	Y	N	N	Y	N	CphA
C	*1*	N	Y	N	N	N	AmpC
	1e	N	Y	Y	N	N	CMY-37
D	*2d*	Y	N	N	N	N	OXA-10
	2de	Y	Y	V	N	N	OXA-11
	2df	Y	N	Y	Y	N	OXA-48

Y = yes; N =no; V = variable

Table 2 Prevalent mechanisms of resistance among pathogens with extended-drug resistance (XDR). (Adapted from Karaiskos)[306]

Classification	Mechanism	Common bacterial species	Examples	Substrate
β-lactamase Ambler class A	Extended-spectrum or ESBLs	*Enterobacterales, Pseudomonas aeruginosa, Acinetobacter spp., Kluyvera spp.*	SHV-like, CTX-like, KLUG-like	Penicillins, cephalosporins (except cephamycins), aztreonam frequently co-transferred with VIM
β-lactamase Ambler class A	Serine carbapenemases Acquisition of a mobile genetic element	*Klebsiella spp.*	KPC-like, IMI-like	Penicillins, cephalosporins, aztreonam, carbapenems
β-lactamase Ambler class B	Metallo-β-lactamases, carbapenemases Acquisition of a mobile genetic element	*Stenotrophomonas maltophilia, P. aeruginosa, Bacteroides fragilis, Acinetobacter baumannii*	VIM-like, IMP-like, NDM-like, GIM, SPM, SIM	Penicillins, cephalosporins, and carbapenems. Monobactam are stable
β-lactamase Ambler class C	Extended-spectrum, cephalosporinases, Mainly chromosomal	*Enterobacter spp., Klebsiella spp., Proteus spp., Citrobacter spp., E. coli*	AmpC, P99, ACT-like, CMY-like, MIR-like	-
β-lactamase Ambler class D	Carbapenemases	*A. baumanii, P. aeruginosa, E. coli*	OXA-like (OXA-51, OXA-23)	Penicillin, aztreonam, carbapenems
Porin mutations (Loss of outer membrane permeability)	Chromosomal mutation	*P. aeruginosa, A. baumannii*	OprD CarO	Imipenem
Efflux pumps	Chromosomal mutations Different antimicrobial classes	*P. aeruginosa*	MexAB-OprM	Ticarcillin, aztreonam, cefepime, meropenem, quinolones

β-lactam antibiotics such as penicillins, cephalosporins, monobactams, and carbapenems are hydrolyzed by a diverse range of β-lactamases. The expansion of antibiotic classes, including derivatives with improved properties, has been shadowed by the emergence of bacterial enzymes that have an altered spectrum of activity. The early β-lactamases active against the first generation of β-lactams were followed by extended spectrum β-lactamases (ESBLs) that are able to destroy oxyimino-cephalosporins (the first, second, third and fourth generation of cephalosporins) and monobactam (aztreonam),[156, 164] although they are inhibited by clavulanic acid or tazobactam.[165] The most challenging β-lactamases are carbapenemases, which are able to hydrolyze not only oxyimino-cephalosporins and cephamycins but also carbapenems, agents of last resort for many complicated bacterial infections.[25, 73] These enzymes are produced by Gram-negative bacteria such as *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* and underpin the emergence of resistance to all types of β-lactam antibiotics, which has a serious impact on the treatment of severe infections, particularly in hospital patients.[166-168]

Each of Ambler class will be discussed separately, the overview is going to be focused on structure and mechanism of action primarily in Gram-negative bacteria. Then followed by briefly epidemiology and clinical relevance, a logical order since it is often subtle structural changes leading to distinct substrate profiles that bring certain β-lactamase variants to our attention.

3.2.5.1 Ambler class A β-lactamases

Class A β-lactamases are the most numerous and, mechanistically, the most thoroughly characterized serine β-lactamase class. Historically, these β-lactamases were described as “penicillinases” for their ability to catalyze penicillin hydrolysis. This class of enzymes is often plasmid-encoded, but can also be located on the bacterial chromosome or within integrons.[169] In general, class A β-lactamases are susceptile to inactivation by the clinically available β-lactamase inhibitors. Many class A β-lactamases have substrate profiles that include expanded-spectrum cephalosporins, and are known as extended-spectrum β-lactamases (ESBLs). However, few enzymes of this class show carbapenem-hydrolyzing activity. They have been recognized for decades for their prevalence in clinically important species from Gram-positive to Gram-negative bacteria including troublesome species such as *S. aureus* and *E. coli*.

3.2.5.1.1 Structural Components and Catalytic Mechanism

A great diversity of amino acid sequences was observed between the different clusters of class A β-lactamases. However, their structures are generally alike and bear important similarities in the features surrounding the active site.[170, 171] They consist of an α domain and an α/β domain. The alpha domain is largely α-helical. By contrast, the alpha/beta domain consists of a five-stranded β-sheet flanked by α-helices (Figure 50).

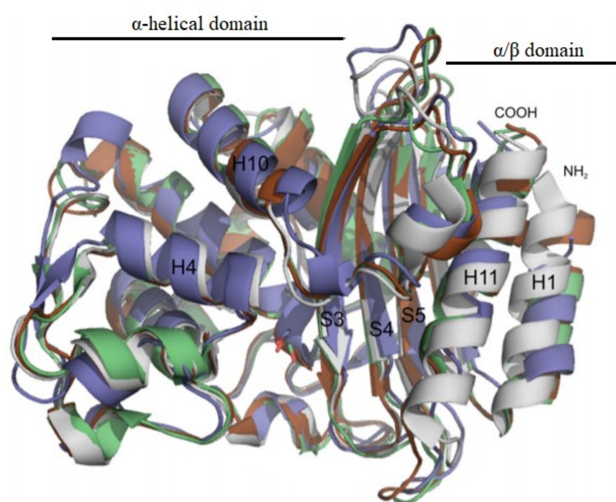

Figure 50 Superposition of four subclasses of A β-lactamases: TEM-1 (gray), KPC-2 (green), PC1 (blue), and OXY-1 (brown.). (Adapted from Philippon)[172]

These two domains form a cleft that harbors the active site, including, in TEM β-lactamase, for example, the residues Ser70, Ser130, Glu166, Lys73 and Lys234. Each of these residues is required for complete SBL-mediated β-lactam hydrolysis, which begins with acylation of the nucleophile Ser70 and is driven by coordination of the other invariant active site residues. Lys234 coordinates to the substrate carboxylate group while the backbone amides of residues Ser70 participate in hydrogen bonding interactions with the β-lactam carbonyl.[173]

The hydrolytic reaction include 2 step reactions- acylation and deacylation. For the acylation step have been proposed two detailed mechanisms. Both reactions begins with a nucleophile attack by the active site Serine OH on the carbonyl group of the β-lactam ring in the presence of base. In the first mechanism, Glu166 acts as a base to deprotonate Ser70 via a bridging water molecule.[174] The second mechanism proposes that Lys73 performs the role of base in the activation of Ser70.[175] This results in high-energy tetrahedral intermediate which is dependent upon N-protonation and Ser130 is widely accepted as the proton donor (Figure 51). The ring strain of this intermediate is relieved through the breaking of the lactam ring leading to the formation of a covalent acyl-enzyme complex, an *O*-linked Ser70 residue ester. It is also possible that both mechanisms are valid, which may help explain why these enzymes can hydrolyze a diversity of substrates.[176]

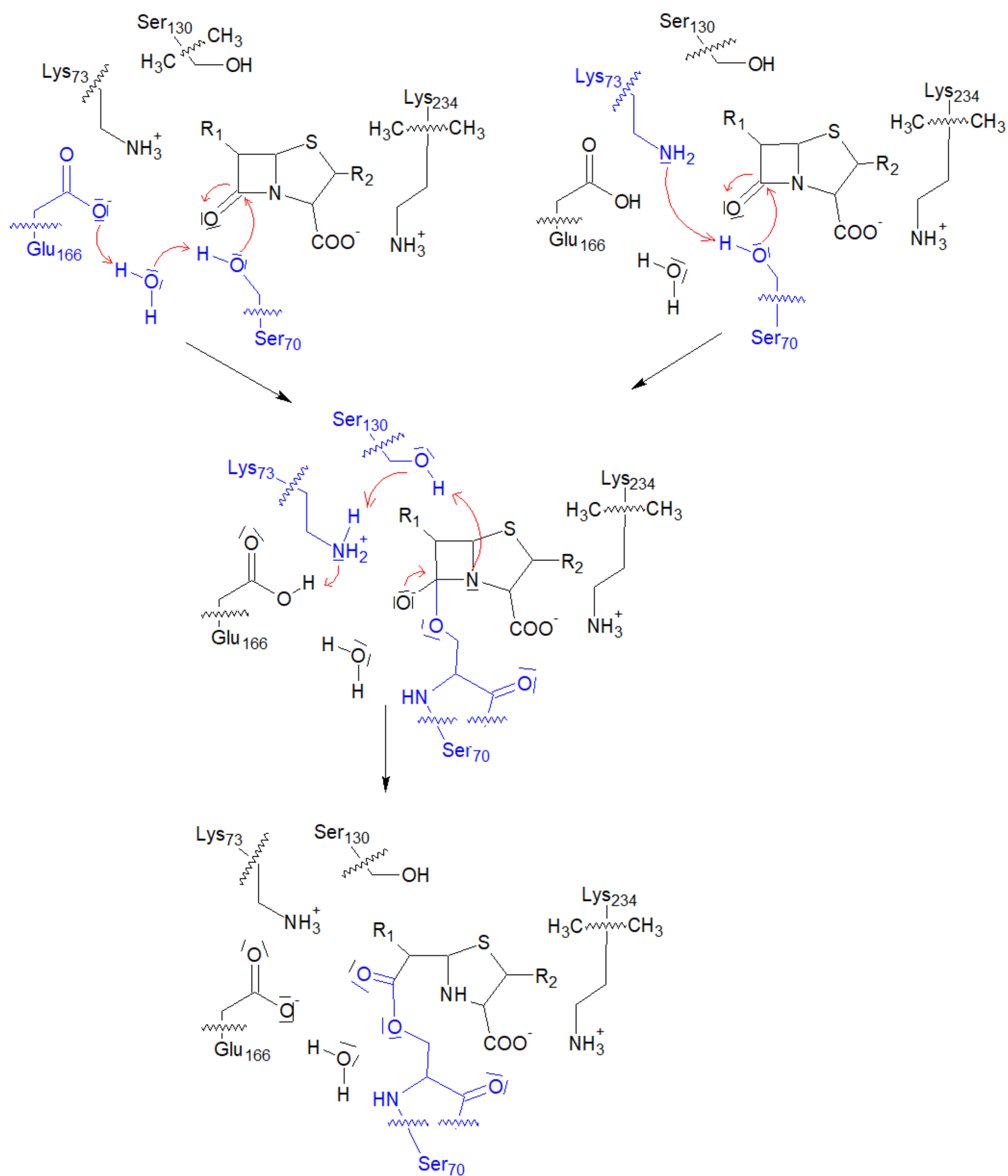

Figure 51 ***Acylation of class A SBL.***[176]

Deacylation occurs with Glu166 again acting as base to deprotonate water, which then attacks the acyl-enzyme intermediate. Collapse of the tetrahedral intermediate likely comes about with Ser130 shuttling the proton from Lys73 to the Ser70 leaving group (Figure 52).[177]

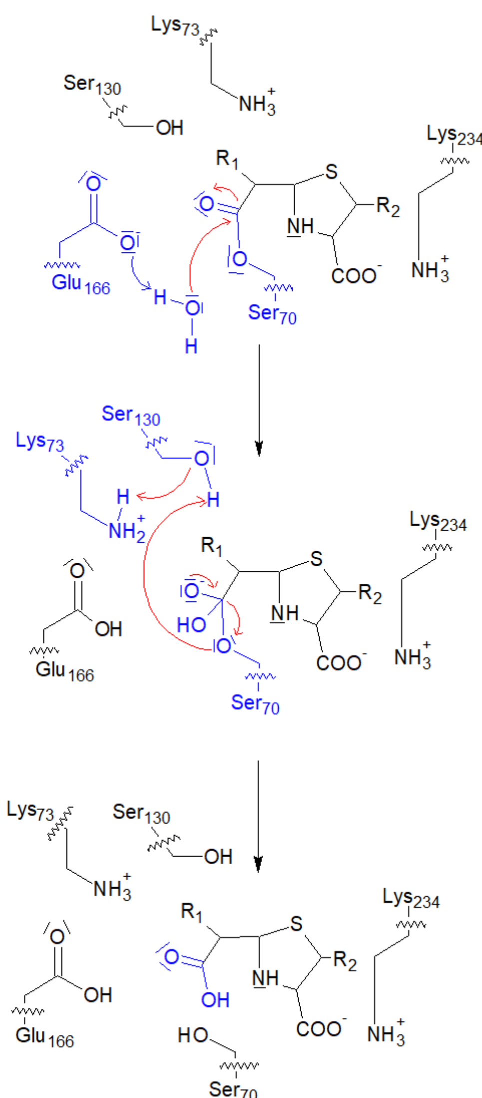

Figure 52 ***Deacylation of class A SBL.[178]***

3.2.5.1.2 Clinically important class A β-lactamases

In Gram-negative bacteria the two most important enzymes were found to be TEM-1 and SHV-1. TEM-1 was isolated from and named after a Greek patient named Temoniera. It is the most common β-lactamase, first identified in *E. coli* in 1963.[179] TEM-1 is commonly plasmid-encoded but, in some cases, has been observed to transfer chromosomally via mobile elements such as transposons.[180] TEM enzymes are closely related to SHV β-lactamases (contain a sulf-hydryl variable active site), sharing 68% sequence homology. The active site of SHV-1 is approximately 0.7 to 1.2 Å wider than that of TEM-1, having important differences in the positioning of critical active-site residues.[181] TEM and SHV β-lactamases are mostly found in *E. coli* and *Klebsiella* spp. After the introduction of expanded-spectrum cephalosporins (oxyimino-cephalosporins), stable against TEM-1 and SHV-1, extended-spectrum β-lactamases (ESBLs) have been observed.[182]

ESBLs are derived from TEM-1, TEM-2 or SHV-1 by mutations that alter the amino acid configuration around the active site of these β-lactamases. They confer bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems). However, they are inhibited by β-lactamase inhibitors such as clavulanic acid.[183] ESBLs are of molecular class A with the exception of the OXA-type enzyme, which belongs to class D of the Ambler

classification. The Bush-Jacoby-Medeiros system devides the ESBLs into groups labeled *2be* (CTX-M type) as well as *2d* (OXA-type), which shares most of the fundamental properties of group *2be* (Table 1).[184]

The *2be* group is derived from group *2b* β-lactamases (TEM-1, TEM-2, and SHV-1) where *e* of the *2be* denotes that the enzymes have an extended spectrum. CTX-M enzymes belong to the *2be* group, their name reflecting the potent hydrolytic activity against cefotaxime; in addition, some CTX-M type ESBLs can hydrolyze ceftazidime.[183, 185] Tzouvelekis[186] reported that CTX-M type β-lactamases hydrolyze cefepime with high efficiency and their MICs are higher than observed in bacteria producing other ESBL types.[187] The aztreonam MICs are variable and tazobactam has a better activity than clavulanic acid against CTX-M enzymes.[188] The CTX-M enzymes have become a global problem due to their ability to hydrolyze a broad range of β-lactam antibiotics with rapid evolution and spread under antibiotic pressure.[189]

The other class A enzymes garnering attention are the serine carbapenemases, enzymes able to hydrolyze not only penicillins, cephalosporins, and monobactams but additionally carbapenems. The major class A carbapenemases include five major families of plasmid encoded enzymes: KPC and GES, as well as chromosomally encoded enzymes NMC, IMI, and SME.[96]

The first member of the KPC (*Klebsiella pneumoniae* carbapenemase) family was discovered in North Carolina in 1996.[190] The KPC family rapidly spread along the east coast of the United States and in a few years, reports of its occurrence began to appear worldwide. In 2005, the *K. pneumoniae* strain producing KPC-2 was isolated from a patient who returned from New York after hospitalization in France.[191] Infections with pathogens expressing KPC are difficult to treat because many of them express plasmids of various sizes carrying bla_{KPC} and additional genes conferring resistance to fluoroquinolones and aminoglycosides, thus limiting the antibiotics available for treatment. Although bla_{KPC} has been mapped to a chromosomal location in some cases,[192] the KPC carbapenemases have the greatest potential for spread due to the enzyme's gene location on plasmids, especially since they are most frequently found in *K. pneumoniae*, a bacteria notorious for its ability to accumulate and transfer resistance. KPC-type enzymes pose significant threats due to their rapid international spread and multidrug resistance associated with high mortality rates.[193, 194]

The GES-type (Guiana extended-spectrum) carbapenemases are acquired β-lactamases recovered from *Enterobacterales*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. The GES family differs from other serine A carbapenemases by only two amino acid substitutions with cysteine resiudes at Ambler position 69 and 238. GES enzymes were first classified as ESBLs for their broad hydrolysis spectrum that included penicillins and extended-spectrum cephalosporins. Their hydrolysis spectrum was expanded in 2001 to include imipenem after the report of GES-2 in a *P. aeruginosa* isolate.[195]

NMC-A (non metallo-carbapenemase A) is a chromosomal carbapenemase originally isolated from *Enterobacter cloacae* in France. Currently, reports of this particular β-lactamase are still rare. NMC-A is highly similar to IMI-I with 97% amino acid identity and both are related to SME-1 with 70% amino acid identity.[196] Likewise, IMI-1 (imipenem-hydrolyzing β-lactamase) was initially recovered from the chromosome of an *E. cloacae* in the southwestern part of USA.[197] A variant of IMI-1 and IMI-2 has been identified on plasmids isolated from *E. asburiae* found in United States rivers and from *E. cloacae* isolate obtained in China.[198, 199] IMI carbapenemase-producing *E. asburiae* was isolated from a Czech patient in 2016 with no history of travelling abroad or previous hospitalization.[200] SME-1 (*Serratia marcescens* enzyme) carbapenemase was first detected in England from two *S. marcescens* isolates that were collected in 1982.[196, 201] Infrequently, SME-2 and SME-3 have since been isolated in North America and Switzerland.[202]

3.2.5.2 Ambler class B β-lactamases

Class B β-lactamases, or metallo-β-lactamases (MBLs), require zinc ions to catalyze the hydrolysis of β-lactam antibiotics such as penicillins, cephalosporins, and carbapenems with the exception of monobactams.[203] They are not affected by mechanism-based inhibitors such as clavulanic acid, sulbactam, or tazobactam but are inactivated by metal chelators such as EDTA (ethylene diamine tetraacetic acid).[169] A variety of MBL genes have been discovered on chromosomes of opportunistic and environmental bacteria such as CcrA from *Bacteroides fragilis,*[204] L1 from *Stenotrophomonas maltophilia,*[205] and CAU-1 from *Caulobacter crescentus*.[206] On the other hand, horizontally acquired MBL genes, for example,

bla$_{IMP}$,[207] *bla*$_{VIM}$,[208] *bla*$_{NDM}$,[209] and *bla*$_{SPM}$[210] have mainly been found on the plasmids of members of the family *Enterobacterales, Pseudomonas* spp., and *Acinetobacter* spp.

3.2.5.2.1 Structural Components and Catalytic Mechanism

MBLs are divided into three subclasses (B1, B2, and B3) based on their amino sequence, homology, and metal requirement. The subclass B1 β-lactamases have Zn1 coordinated to three His residues (His116, His118, and His196) and Zn2 with Asp120, Cys221, and His263 residues, whereas the subclass B3-lactamases have two zinc ions with similar binding affinity. The subclass B2 β-lactamases have one tightly coordinated zinc ion Zn2 that is sufficient for maximal enzymatic activity, whereas the binding of a zinc ion Zn1 at another site reduces the enzymatic activity. Compared with B1, the B2 subclass has a Zn1 binding site with one altered residue (Asp116, H118, H196), but retains the same Zn2 binding sequence. Finally, subclass B3 has the same Zn1 binding site as B1 and B2 but differs in the Zn2 coordinating sequence. The B1 and B3 subclasses have a broad substrate spectrum that includes penicillins, cephalosporins and carbapenems, whereas the B2 subclass has a narrow substrate spectrum that includes carbapenems.[211, 212] The classification of subclass B is summarized in Table 3.

Table 3 Classification of B subclasses.*[211]*

Subclass	Substrates	Nr. of Zn^{2+} active site atoms	Zn1 coordinating residues	Zn2 coordinating residues	Representative families
B1a	All β-lactams except monobactams	2	His His His	Asp Cys His	IMP, VIM, SPM, CCrA
B1b	All β-lactams except monobactams	2	His His His	Asp Cys His	NDM
B2	Carbapenems	1	Asp His His	Asp Cys His	CphA, Sfh-1
B3	All β-lactams except monobactams	2	His His His	Asp His His	L1, CAU-1

Although the MBLs exhibit a varying range of amino acid sequences with only 25% identity between some β-lactamases, their overall structures are very similar and contain a characteristic αβ/βα sandwich fold comprising two central β-sheets and five α-helices on the external faces.[212] The enzymes of the B1 subclass contain a flexible L3 loop between β3- and β4-strands that possess hydrophobic side chains. The loop is thought to have an important role in the binding of substrates and inhibitors. It closes over the bound substrate or inhibitor after diffusion into the active site (Figure 53).[213] NDM-1 (New-Delhi MBL) shows lower sequence identity with other MBLs, and the most closely related MBLs are VIM-type (Verona integron-encoded MBL) and IMP-type (imipenemase) enzymes, which show 37% sequence identity with NDM-1.[214] As can be observed in (Figure 53), the overall structure of NDM-1 shares a feature common to other MBLs enzymes, but there are some differences. The L3 loop in NDM-1 was revealed to be more open and hydrophobic than that in IMP-1 and VIM-types. The L3 loop likely plays an important role in the binding of the antibiotics at the active site.[214]

Figure 53 ***Comparison of the structural features of NDM-1 (green) with IMP-1 (cyan), VIM-2 (magenta), and VIM-7 (yellow). (Adapted from Jeon)[212]***

The hydrolyzed carbapenem core of meropenem has extensive non-covalent interactions with the zinc center and the R1 side chain of meropenem when positioned on the active-site cleft. Although the R2 substituent of meropenem does not interact covalently or electrostatically with NDM-1, the latter contains a large active-site cleft which provides accommodation for the bulky R2 group, and therefore assists in the recognition and hydrolysis of meropenem (Figure 54).[215]

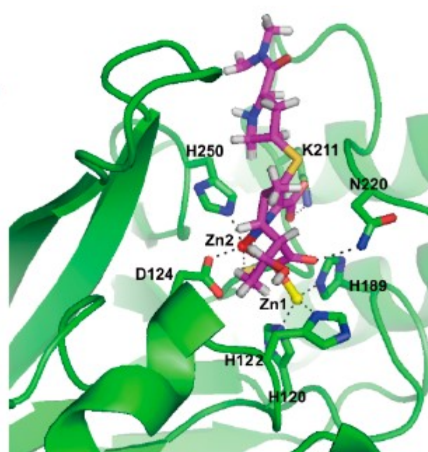

Figure 54 ***Active site of the NDM-1 in complex with meropenem. The residues (H120, H122, D124, H189, K211, N220, and H250) in the active-site cleft are shown as green sticks. Zinc coordination and hydrogen bonding in the active site in NDM-1 are shown as dashed black lines. The hydrolyzed meropenem is shown in magenta. Zn1 and Zn2 are represented as yellow and red spheres, respectively. (Adapted from Jeon)[212]***

Deactivation of β-lactam antibiotics by MBL, like class A β-lactamases, occurs via cleavage of the amide bond of the β-lactam ring.[216-218] The hydrolysis involves three steps: 1) nucleophilic attack on the carbonyl group, 2) lactam C-N bond cleavage, and 3) protonation of the deacylated nitrogen. The process is initiated by the interaction of Zn1 with the β-lactam carbonyl and Zn2 with the carboxylate group on the 5- or 6- membered fused ring. The hydroxide ion is stabilized by both metal centers in a position to attack the C-N carbon. Zn1 acts as a Lewis acid, coordinating to the carbonyl oxygen in the β-lactam ring and lowering electron density at the carbonyl carbon, facilitating nucleophilic substitution. Nucleophilic attack of the hydroxide on the carbonyl carbon leads to formation of a non-covalent tetrahedral

intermediate.[219-221] The C-N bond in the tetrahedral intermediate can be cleaved by coordination and subsequent protonation of nitrogen, likely from a water molecule (Wat) originally bound to Zn2 (Figure 55).[221, 222]

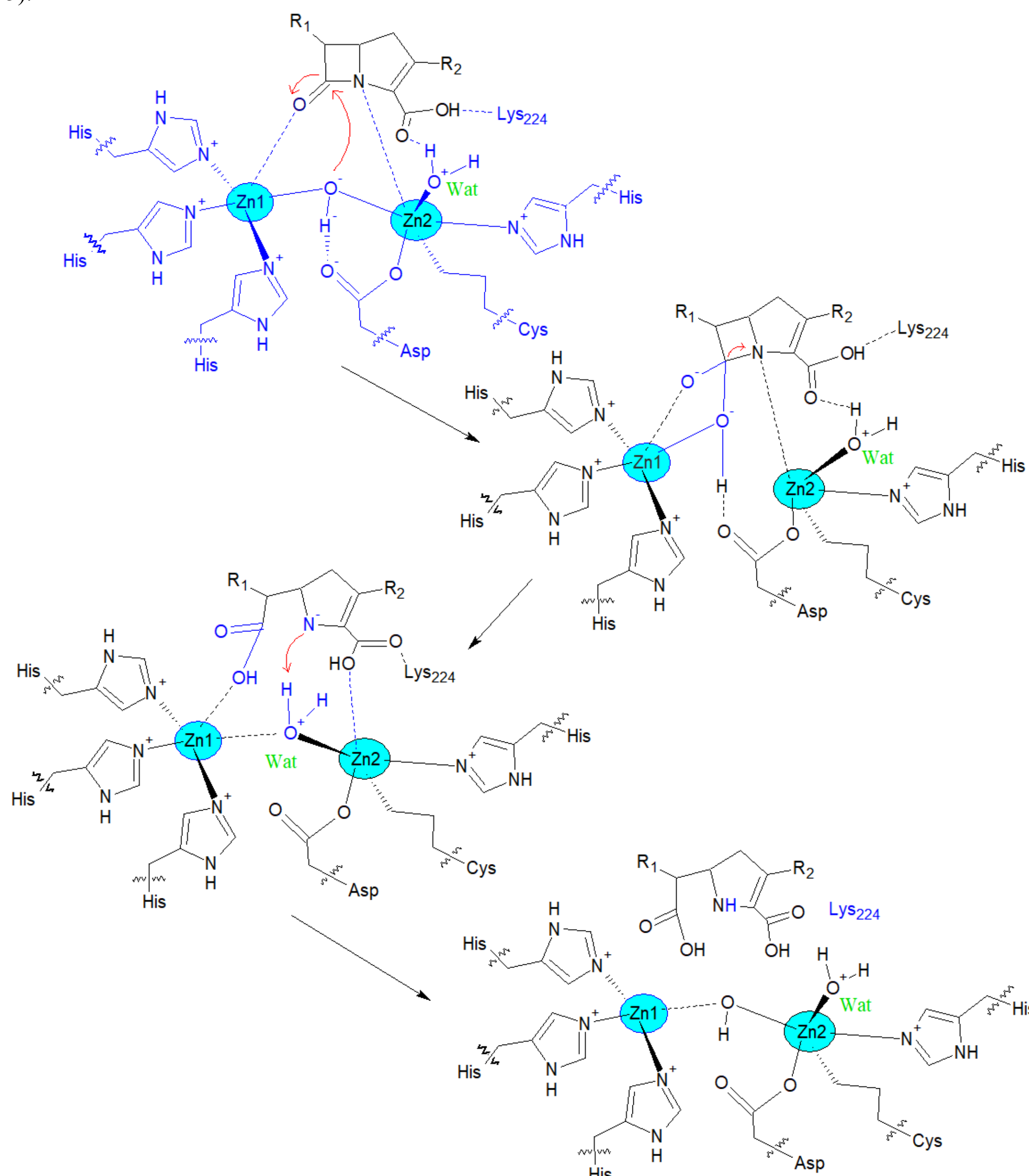

Figure 55 ***Proposed mechanisms for carbapenem hydrolysis by B1 class of MBL.***[219-221]

3.2.5.2.2 Clinically important class B β-lactamases

Metallo-β-lactamases were discovered over forty-five years ago. They were not initially considered a serious clinical problem for antimicrobial therapy because they were only chromosomally encoded in non-pathogenic bacteria.[223] However, the situation changed in 1990, when the IMP- and VIM-type metallo-β-lactamases were isolated in an increasing number of Gram-negative pathogens such as *Enterobacterales*, *P. aeruginosa*, and *A. baumannii.* Moreover, those enzymes were encoded by genes carried on mobile DNA elements. The most common families identified in *Enterobacterales* included the VIM, IMP, and emerging NDM group.[224, 225]

Currently, 48 variants of IMP-type carbapenemases were isolated from clinically important pathogens[226] and reported from more than 30 countries. In 1990, IMP-1 was firstly isolated from

P. aeruginosa in Japan where it is still predominant.[227] The bla_{IMP} was located on a conjugative plasmid of the clinical isolate and afterwards was found on an integron in *Serratia marcescens* and other *Enterobacterales* isolates in Japan.[228, 229] In Europe, the first member of the IMP family was detected a few years later. In an *A. baumannii* isolate a related enzyme, IMP-2, was observed on a class 1 integron.[230] Since then, IMP isolates have been found throughout the world.[231] MBLs are embedded in a variety of genetic elements, most commonly integrons. These integrons are often associated with transposons or plasmids that allow transmission between species.[232] In Europe, there is high incidence of IMP carbapenemase in *P. aeruginosa*, but it is still low in *Enterobacterales*.[233] The high prevalence of IMP-producing *P. aeruginosa* isolates has also been reported by Czech hospitals, specifically.[234]

VIM enzymes also belong to integron-associated MBLs. VIM-1 was first described in Italy in 1997[208] and its allelic variant, VIM-2, was subsequently labeled after first being observed in France in 1996.[235] Both of these MBLs were found in class 1 integrons of *P. aeruginosa* clinical isolates. VIM enzymes are predominant in Europe, especially in Mediterranean countries. VIM-2-like β-lactamases have been mostly associated with *P. aeruginosa,*[236] whereas VIM-1-like and VIM-4-like enzymes have been frequently reported in strains of *Enterobacterales.*[231, 237] Increasing prevalence of VIM-producing *P. aeruginosa* has been reported in the Czech Republic.[234]

The first NDM-1 enzyme was detected in *K. pneumoniae* and *E. coli* from a patient returning to Sweden from India in 2008. Since then NDM-1 has been shown to be present at significant frequency in *Enterobacterales* family in a many countries worldwide.[209] The bla_{NDM-1} gene has been found on several plasmid types and it can be transferred among Gram-negative bacteria by conjugation.[211] The spread of bla_{NDM}-like genes is mostly derived in *Enterobacteriacea,* moreover, the carbapenemases have been reported from *P. aeruginosa* and *Acinetobacter* spp. The main known reservoirs of the carbapenemase is the Indian subcontinent, Middle East and Balkan region.[238] In the Czech Republic, recently an increased occurrence of isolates with different NDM types was reported.[239]

3.2.5.3 Ambler class C β-lactamases

The class C cephalosporinase, AmpC is an important contributor to multiple drug resistance and can be chroosomal or plasmid mediated. It is protective against β-lactams in Gram-negative pathogens such as *Enterobacterales*. Cephalosporinases demonstrate hydrolyzing activity against most penicillins and cephalosporins, including oxyiminocephalosporins, cephamycins, and monobactams. They usually retain susceptibility to carbapenems and in some cases to fourth-generation cephalosporins. However, the production of mutant AmpC enzymes has been described in clinical isolates resistant to fourth-generation cephalosporins, and indirectly a concomitant porin loss in AmpC-producing strains can provide resistance to carbapenems. AmpC β-lactamases are not readily inhibited by β-lactamase inhibitors in comparison with class A β-lactamases.[13]

3.2.5.3.1 Structural Components and Catalytic Mechanism

In comparison to Class A enzymes, Class C β-lactamases have larger active site cavities which may allow them to bind the bulky extended-spectrum cephalosporins (oxyimino β-lactams).[240] Class C β-lactamases have very similar structures with class A β-lactamases. They are also consisted of two main domains where domain 1 has only α-helix and domain 2 comprises an α/β domain.[241] All class C β-lactamases contain 3 active-site motifs, Ser64-X-X-Lys67, Tyr150-X-Asn152, and Lys315-Thr316-Gly317.[242] The active site lies in the center of the enzyme at the left edge of the five-stranded β-sheet with the reactive serine residue.[243] The active site are divided into two subsites- R1 and R2. The R1 subsite refers to the region that accommodates the R1 side chain at C6 (C7) of the β-lactam antibiotics. The R2 subsite interacts with the right part of the β-lactam ring including the R2 side chain at C2 (C3). The R1 subsite is surrounded by the Ω-loop while the R2 subsite is enclosed by the R2-loop containing the α10 and α11 helices (Figure 56).[241, 244]

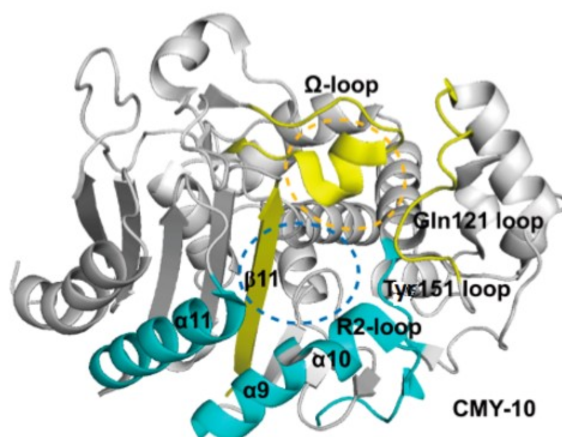

Figure 56 Structure of CMY-10. The R1 subsite is surrounded by the Ω-loop (yellow). The R2 subsie is enclosed by Tyr151 loop, α10 in the R2 loop, and α11 (cyan) (Adapted from Jeon).[212]

The hydrolysis reaction catalyzed by class C β-lactamases consists also of two steps acylation and deacylation, as was observed in class A β-lactamases. During the hydrolyzation, Ser64 residue attacks the carbonyl carbon of the β-lactam ring to form an acyl-enzyme intermediate, which was initiated by the activation of catalytic water by Tyr150.[173, 242] And the second process was a detachment of a hydroxylated substrate from the enzyme, which was associated with proton transfer from the side chain of Lys67 to Ser64. From the viewpoint of substrate deacylation, a key factor of class C β-lactamase is that the deprotonated side chain of Tyr150 is steadily present in the catalytic center of the acyl-enzyme complex and is held by Lys67 and Lys315 cooperatively Figure 57.[245]

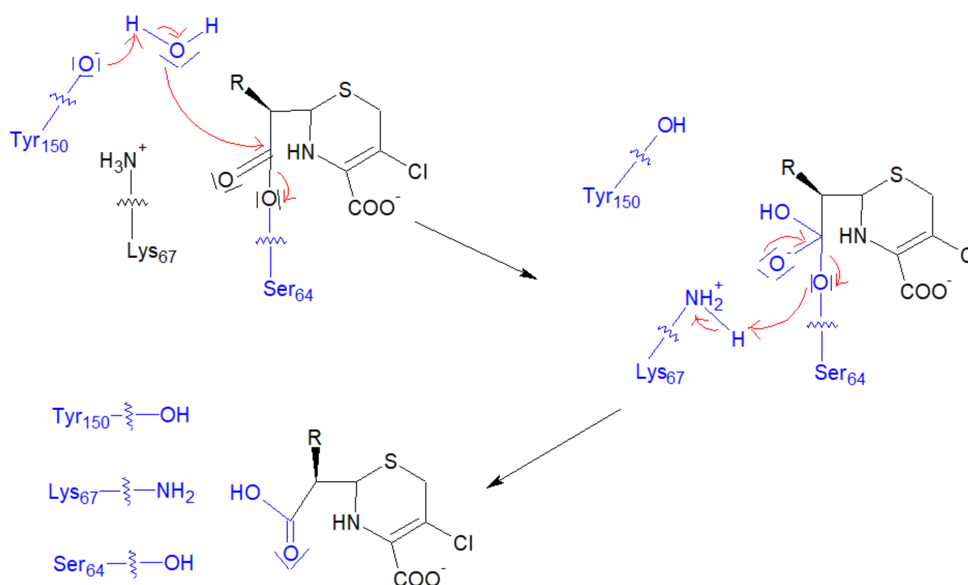

Figure 57 Deacylation mechanism.[245]

3.2.5.3.2 Clinically important class C β-lactamases

AmpC enzymes are chromosomally encoded on particularly important clinical isolates of *Entrerobacteriaceae* family (*Citrobacter freundii, Enterobacter* spp., *Morganella* spp., *Proteus* spp.,

Hafnia alvei, *Yersinia* spp., and *Serratia* spp.) and other Gram-negative organisms (*P. aeruginosa*, *Aeromonas* spp., *A. baumanii).*[246-248]

Plasmid-encoded AmpC genes have been known since 1989. Since then they have been found worldwilde in nosocomial and nonnosocomial isolates. However they are less common than extended-spectrum β-lactamases (ESBLs). In addition, the plasmid-encoded class C β-lactamases have been found in *Klebsiella* spp., *E. coli*, *Salmonella* spp., *Proteus mirabilis, Enterobacter* spp.[212] In comparison with chromosomal enzymes, plasmid-encoded class C enzymes are more problematic because they are transmissible to other bacterial species and are often expressed in large amount.[249] AmpC plasmids can carry additional resistance determinants for non β-lactams such that their acquisition can promote multidrug resistence.[250] AmpC β-lactamases have minor differences in amino acid sequence have given rise to families (CMY, FOX, ACC, LAT, MIR, ACT, MOX, and DHA). These enyzmes are very closely related to chromosomally determined AmpC β-lactamases. For example six current varieties of CMY-1, -8, -9, -10, -11, and -19 are related to chromosomally determined AmpC enzymes in *Aeromonas* spp.[241]

3.2.5.4 Ambler class D β-lactamases

Class D β-lactamases are known as OXAs (oxacillinases) because they are able to hydrolyze the cloxacillin and oxacillin much faster than benzylpenicilin.[184] At present, the OXA enzymes include more than 450 OXA types that are widely disseminated in Gram-negative bacteria. They do not hydrolyze aztreonam or extended-spectrum cephalosporins, they are poorly inhibited by β-lactamase inhibitors such as clavulanic acid,[251] they are classified into narrow- or extended-spectrum enzymes according to their conferred resistance profile to β-lactam antibiotics.[252, 253] Some of the OXA variants possess weak carbapenemase activity. For example OXA-2 β-lactamase is a narrow-spectrum enzyme resistant to penicillins and some early cephalosporins, however it does not hydrolyze carbapenem.[254]

Based on their amino acid sequences the oxacillinases are classified into 12 subgroups: OXA-23, OXA-24/40, OXA-48, OXA-51, OXA-58, OXA-134a, OXA-143, OXA-211, OXA-213, OXA-214, OXA-229, and OXA-235. The first five subgroups have major clinical importance due to their wide occurence in bacterial isolates. The majority of these oxacilinases, except OXA-48, have been identified in various *Acinetobacter* spp.[254]

Before the OXA-23 group was dicovered, the oxacillinases were thought to be a relatively minor group of plasmid-encoded β-lactamases active against penicillins. After the introduction of carbapenem antibiotics and the increase of *Acinetobacter* spp.,mainly *A. baumannii* isolates, an explosion of new OXA enzymes were observed. Over the years, there have been 102 unique OXA sequences identified of which 9 are extended-spectrum β-lactamases and at least 37 are considered to be carbapenemases.[231, 255]

For example OXA-23, OXA-24/40, OXA-48, OXA-58, and OXA-146 are classified as class D carbapenemases[256-261] and OXA-1 from *E. coli,* as well as OXA-10 and OXA-13 from *P. aeruginosa* belong to class D non-carbapenemases.[262-264]

3.2.5.4.1 Structural Components and Catalytic Mechanism

The most efficient enzyme for imipenem hydrolysis belonging to class D carbapenemases is OXA-48. The D class carbapenemases have a similar structure to those of the D class non-carbapenemases. They consist of two main domains, one of them comprised of helices, and the second, a mixed α/β domain including a central six-stranded antiparellel β-sheet. Their active-site motifs contain Ser70-X-X-Lys73, Ser118-X-Val/Ile120, and Lys 216-Thr/Ser217-Gly218.[265] OXA-48 is very similar to non-carbapenemases oxacillinases. They differ in a loop between β5- and β6-strand, which may vary in orientation and length. The length of the β5- β6 loop in OXA-48 is shorter than those in OXA-1, OXA-10, and OXA-13 (Figure 58). The short loop is hypothesized to play a major role in conferring carbapenemase activity.[260]

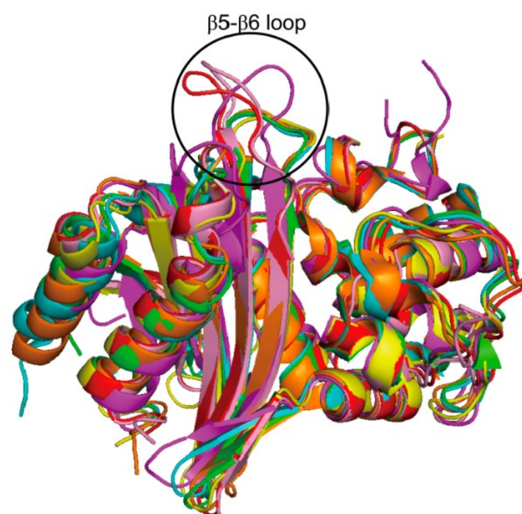

Figure 58 ***Comparison of structural features of class D carbapenemases (OXA-1 magenta, OXA-10 red, and OXA-13 pink, OXA-23 green, OXA-24/40 cyan, OXA-48 yellow, OXA-58 orange. (Adapted from Jeon)***[212]

The hydrolytic reactions catalyzed by class D β-lactamases include acylation and deacylation steps described above for class A β-lactamases (Figure 50 and Figure 51). In brief, the two-step pathway involves the deprotonation of serine by an active site base, activating it for nucleophilic attack on the β-lactam carbonyl. It results in formation of an intermediate where the β-lactam forms an ester linkage with the attacking serine molecule. General base-mediated activation of an active site water molecule then leads to hydrolysis of the ester bond, releasing the product and restoring the serine to its original state.[266] Lohans et. al[267] published an analysis that proposed hydrolysis of the β-lactam ring in ertapenem by OXA-48, producing a β-lactone (Figure 59, bottom right).

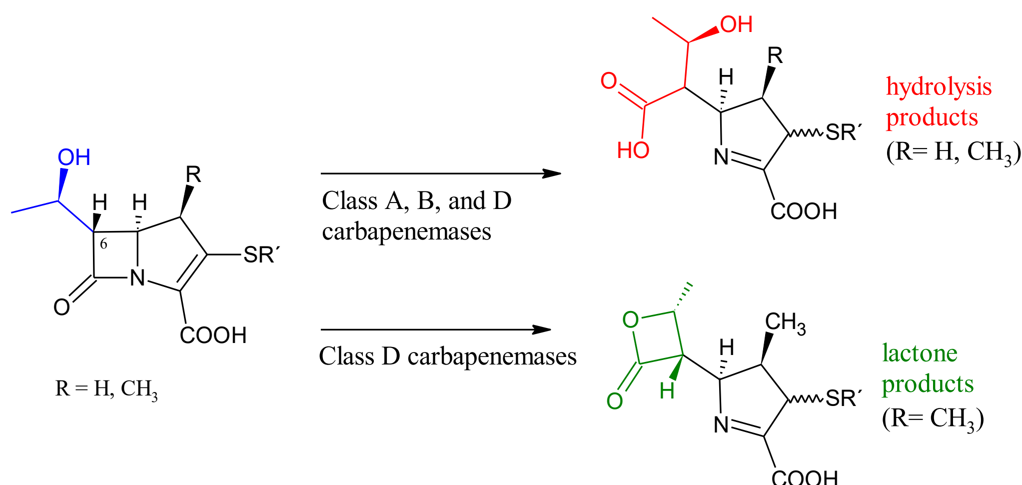

Figure 59 ***Carbapenem-derived products formed by carbapenemases. (Adapted from Lohans).***[267]

β-lactone formation was observed only with meropenem, biapenem, and doripenem. The mechanism proposed by Lohans states that the hydroxyethyl 6R side chain of carbapenem reacts with the carbonyl from the β-lactam ring to form a β-lactone. Imipenem and panipenem did not form the lactone ring, presumably, because the presence of a 1β-methyl substituent is necessary for lactone formation. Lohans also proposed that the methyl substituent destabilizes the conformation of the hydroxyethyl side chain required for hydrolysis of the acyl-enzyme complex. Carbapenems with those groups are more resistant to hydrolysis than carbapenems without the 1β-methyl substituent.[267]

3.2.5.4.2 Clinically important class D β-lactamases

As was mentioned before, the class D carbapenemases have a great clinical importance due to their wide occurrence in bacterial pathogens. The first group of carbapenem resistant OXA type enzymes to be identified in *A. baumannii* was the OXA-23 group. The first isolate was collected in the United Kigndom in 1985,[268] interestingly the same year that imipenem was introduced to the market. Since then, more than 15 variants of the $bla_{\text{OXA-23}}$ gene have been identified.

The second group of OXA-type β-lactamases were OXA-24/40 enzymes, which were reported from isolates recovered as part of an outbreak in Spain in 1997.[269] They were initially found in isolates of *A. baumannii,* though more recent reports have identified $bla_{\text{OXA-24/40}}$ genes on plasmids in other *Acinetobacter* spp. as well as in *K. pneumoniae* and *P. aeruginosa.*[251]

The other group labeled *2d* group by the Bush-Jacoby-Medeiros system, are able to hydrolyze cloxacillin and oxacillin and predominatly occur in *P. aeruginosa*. However, the most common OXA-1 has been found in *E. coli* isolates. Most of the OXA-type enzymes are not able to hydrolyze the extended-spectrum cephalosporins to a significant degree and thus are not regarded as ESBLs. However, OXA-10 weakly hydrolyzes cefotaxime, ceftriaxone, and aztreonam, reducing the susceptibility of most organisms to these antibiotics.[183]

The first OXA-48 was identified in *K. pneumoniae* in Turkey in 2003.[270] Curently, OXA-48 is the most common and widespread in *K. pneumoniae* and other *Enterobacterales* in Turkey, the Middle-East, South Africa and Europe.[271] In the Czech Republic the first isolates with OXA-48 carbapenemase were detected in 2013 from *K. pneumoniae* strains. Interestingly, one of them was aquired from a child without a history of previous hospitalization, moreover, the $bla_{\text{OXA-48}}$ gene was chromosomally encoded. By contrast the second isolate was plasmid-mediated from a patient repatriated from Romania.[272] Isolates producing OXA-48 are among the most difficult carbapenemase producers to identify due to their point mutant analogues with ESBLs. Therefore their true prevalence is difficult to estimate. However, they showed a low level of hydrolytic activity against the carbapenems.

OXA-51 is the largest group among the OXA-type β-lactamases. This group was firstly identified in *A. baumannii* isolate from Argentina in 1996.[273] A large number of variants indicate that they are chromosomally encoded enzymes and have been under selective pressure from antibiotic use.

OXA-58 enzyme was identified in a multidrug resistant *A. baumannii* clinical isolate in France in 2003. OXA-58 has weak activity against penicillin and carbapenems, ceftazidime, cefotaxime, and cefepime.[274] The rest of the OXA subgroups: OXA-134a, OXA-143, OXA-211, OXA-213, OXA-214, OXA-229, and OXA-235 where identified in *Acinetobacter* species.[212]

4. The hypothesis of the dissertation thesis

- The worldwide spread of antimicrobial resistance is one of the greatest health threats of today.
- Understanding the biochemical and genetic basis of resistance is of paramount importance to correctly detect the emergence and spread of resistance.
- Structural studies of β-lactamases and β-lactam antibiotics are important for better understanding of the mechanism of antibiotic resistance.
- Development of rapid and comprehensive diagnostic methods for the detection of infections caused by carbapenem-resistant isolates is crucial for therapy and surveillance.

5. The aims of the dissertation thesis

- Development of a simple and rapid detection of carbapenem-resistant *Enterobacterales* and *Pseudomonas* spp.
- To evaluate a commercial method for detection methicillin-resistant *Staphylococcus aureus* by MALDI-TOF mass spectrometry.
- To perform surveillance of clinical carbapenem-resistant Enterobacterales detected in the Czech Republic.
- To determine the incidence and distribution of carbapenem resistance by molecular-genetic analysis.
- To characterize resistance plasmids and novel genetic elements during an outbreak with carbapenem-producing isolates.

6. Methods

Accurate identification of bacterial isolates is a critical task in clinical microbiology. Most phenotypic methods are labor and material intensive as well as time-consuming. Additionally, phenotypic methods used to identify bacteria require subjective interpretation is of test results. One way to reduce time for microbial identification is with the use of molecular biology techniques. It is important to remember that in order to obtain the most precise identification, classification, and systematics of microorganisms, it is extremely important to choose appropriate techniques, and to have a thorough understanding of the mechanism of their action.

All isolates were rapidly identified by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). It is based on the ionization of the microbial cells with short laser pulses and then acceleration of particles in a vacuum system using an electric field. After ionization, a molecular fingerprint is obtained in the form of a spectral profile, specific for each organism. The spectra are then compared with an existing database, allowing for identification by an automated program.

The sensitivity of the isolates was examined by the broth dilution method and their data were interpreted according to the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[275] Isolates suspected to have carbapenemase activity were tested by a MALDI-TOF MS meropenem hydrolysis assay.[276] Positive isolates were subjected to carbapenemase detection with the use of the double-disk synergy test (DDST) with chelating agent EDTA for detection of MBL producers, phenylboronic acid (PBA) combined with carbapenem for detection of KPC producers, and temocillin disc testing for detection of OXA-48 producers.[277] The positive isolates were subjected to PCR for identification of the genes encoding carbapenemase production. The genetic affinity of the isolates was determined by pulse gel electrophoresis. In addition, multilocus sequence typing (MLST) was performed to determine sequence types of individual isolates.[278-280] The β-lactamase content of certain isolates was determined by isoelectric focusing (IEF). The carbapenemase genes were transferred by conjugation/transformation to a recipient *Escherichia coli* A15 strain and amplification of replicons was performed by PCR for plasmid typing.[281, 282] To define the genetic units of the *bla* genes, the plasmid contents of all carbapenemase-producing strains were analyzed by pulsed-field gel electrophoresis (PFGE) of total DNA digested with S1 nuclease followed by Southern blot hybridization with DIG-labeled probes.[283] Plasmid incompatibility (Inc) groups were determined by PCR-based replicon typing method (PBRT).[284] Whole-genome sequencing with the Illumina MiSeq platform was performed on representative samples of isolated plasmid and chromosomes to verify and complete the obtained data.

All methods used are summarized in the attached table 5.1 by referring to the relevant publications.

Table 5.1 Summary of methods used in publications.

Method	Publication
Species identification by MALDI-TOF MS	1, 2, 3, 4, 5
Broth dilution method	1, 2, 3, 4, 5
Meropenem hydrolysis assay	1, 2, 3, 4
Imipenem hydrolysis assay	1
The double-disc synergy test (DDST)	2, 3
PCR amplification of genes encoding for selected β-lactamases	1, 2, 3, 4
Typing of the isolates by MLST	3, 4
Detection of β-lactamases by IEF	3
Conjugation of plasmid	2, 3, 4
Transformation of plasmid	2, 3, 4
Plasmid DNA extraction	2, 3, 4
Genomic DNA extraction	3
S1 plasmid size profiling by PFGE	2, 3, 4
Southern blot hybridization	2, 3, 4
PCR-based replicon typing (PBRT) method	2, 3, 4
Whole-genome sequencing	2, 3, 4
Sequence analysis by BLAST algorithm	3
Sanger analysis	3
Integron analysis	3

7. List of publications

All publications are focused on the detection of resistant isolates and their further analysis. The first four articles deal with carbapenem-resistant isolates while the last one is concerned with the detection of MRSA. The first four articles have been published in journals with impact factor.

1. **Rotova V**, Papagiannitsis CC, Skalova A, Chudejova K, Hrabak J. Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity. *J Microbiol Methods*. 2017;137:30-33.
2. **Rotova V**, Papagiannitsis CC, Chudejova K, Medvecky M, Skalova A, Adamkova V, Hrabak J. First description of the emergence of Enterobacter asburiae producing IMI-2 carbapenemase in the Czech Republic. *J Glob Antimicrob Resist*. 2017;11:98-99.
3. **Paskova V**, Medvecky M, Skalova A, Chudejova K, Bitar I, Jakubu V, Bergerova T, Zemlickova H, Papagiannitsis CC, Hrabak J. Characterization of NDM-Encoding Plasmids From *Enterobacteriaceae* Recovered From Czech Hospitals. *Front Microbiol*. 2018;9:1549.
4. Papagiannitsis CC, **Paskova V**, Chudejova K, Medvecky M, Bitar I, Jakubu V, Zemlickova H, Jirsa R, Hrabak J. Characterization of pEncl-30969cz, a novel ColE1-like plasmid encoding VIM-1 carbapenemase, from an Enterobacter cloacae sequence type 92 isolate. *Diagn Microbiol Infect Dis*. 2018;91(2):191-193
5. **Paskova V**, Chudejova K, Sramkova A, Kraftova L, Jakubu V, Petinaki EA, Zemlickova H, Neradova K, Papagiannitsis CC, Hrabak J. Insufficient repeatability and reproducibility of MALDI TOF MS based identification of MRSA, *Folia Microbiologica.* 2020, July.

Moreover the High Resolution Carbapenemase (HRC) assay for detection of carbapenemase production by spectrophotometer and MALDI-TOF mass spectrometer was developed. This assay has not been published yet and is the intellectual property of Ing. Jaroslav Hrabák, PhD. and myself Ing. Veronika Pašková, so it is not possible to mention it in more details.

7.1 Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity

Note

Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity

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Publication: Journal of Microbiological Methods, 2017 June, 137, 30-33
http://doi.org/10.1016/j.mimet.2017.04.003

7.1.1 Abstract

A comparison of carbapenem molecules for the detection of carbapenemase-producing bacteria by MALDI-TOF MS showed that imipenem exhibited higher sensitivity (97%) and specificity (100%) scores for *Pseudomonas aeruginosa* than meropenem. However, meropenem was more efficient (98% sensitivity and 100% specificity) against *Enterobacteriaceae*.

Infections caused by carbapenemase-producing *Enterobacteriaceae* and *Pseudomonas spp.* are increasing worldwide, and are associated with high rates of mortality. Therefore, methods like Carba NP test and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) carbapenem hydrolysis assay should be performed for the direct detection of carbapenemase-producing bacteria (Hrabak et al., 2014). Nowadays, several modifications of MALDI-TOF MS carbapenem hydrolysis assay have been described and validated (Hrabak et al., 2011; Burckhardt and Zimmermann, 2011; Kempf et al., 2012). However, most of these modifications have been validated only on *Enterobacteriaceae*, or they exhibit lower efficiency for the detection of carbapenemase-producing *Pseudomonas aeruginosa* mainly due to lower quality of their spectra (Hrabak et al., 2011).

In this study, we aimed to validate the efficiency of imipenem and meropenem, for the MALDI-TOF MS detection of carbapenemase-producing *Enterobacteriaceae* and *P. aeruginosa*.

The methods were tested against a group of 250 *P. aeruginosa* isolates from the collection of the Czech National Reference Laboratory for Antibiotics. The isolates were previously characterized, as described below. For all isolates, susceptibility to carbapenems was determined by using imipenem and meropenem disks and interpreted according to the EUCAST criteria (http://www.eucast.org/). All isolates that were nonsusceptible to at least one carbapenem were screened by PCR for the presence of the clinically important carbapenemase-encoding genes, bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{KPC}, bla_{GES} and $bla_{\mathrm{OXA\text{-}48}}$, as reported previously (Papagiannitsis et al., 2015a). PCR products were sequenced on both strands using an ABI 3500 sequencer (Applied Biosystems, Foster City, CA). All carbapenem non-susceptible isolates that tested negative with molecular assays for the detection of carbapenemase genes were further investigated by a spectrophotometric assay with crude extracts, using imipenem as a substrate, as previously described (Lauretti et al., 1999), in order to exclude the presence of carbapenemase types not included in the molecular

assay. The group included 142 isolates producing IMP- (n=97), VIM- (n=41), and GES-type (n=4) carbapenemases (Table 1). The remaining 108 isolates were non-carbapenemase producers. Furthermore, a group of 124 *Enterobacteriaceae* isolates from collections of the Faculty of Medicine and University Hospital in Plzen (Czech Republic), the National Medicines Institute in Warsaw (Poland) and the Robert Koch Institute in Wernigerode (Germany) was also examined for the imipenem assay. These isolates were tested previously using meropenem assay (Papagiannitsis et al., 2015b).

The MALDI-TOF MS assays were performed essentially as described previously (Knox et al., 2014; Papagiannitsis et al., 2015b). Isolates were inoculated on Mueller-Hinton agar plates (Bio-Rad Laboratories, Prague, Czech Republic) and incubated overnight at 35°C. A bacterial inoculum, equivalent to 3 of the McFarland scale, was prepared in a suspension buffer (20 mM Tris-HCl, 20 mM NaCl, pH 7.0). Then, 1.0 ml of the bacterial inoculum was centrifuged. The pellet was resuspended in 50 µl of a reaction buffer (0.4 mM imipenem in 0.45% NaCl, 0.1 mM $ZnSO_4$ or 0.1 mM meropenem in 20 mM Tris-HCl (pH 7.0), 0.01% sodium dodecyl sulfate, 50 mM NH_4HCO_3). The reaction mixture was incubated at 35°C for 2 h. Then, the reaction mixture was centrifuged, 1 µl of the supernatant was applied on a stainless steel MALDI target plate (MSP 96 target; Bruker Daltonics) and allowed to dry. Each sample was overlaid with 1 µl of matrix solution (3.3 mg/ml of a-cyano-4-hydroxycinnamic acid [HCCA] in 50% ethanol for imipenem assay; or 10 mg/mL of 2,5-dihydroxybenzoic acid [DHB] in 50 % ethanol for meropenem assay). After air drying, spectra were measured within the m/z range 300 to 600 using a microflex LT mass spectrometer with the flexControl 3.4 software (Bruker Daltonics). The analysis of the spectra was performed using the flexAnalysis 3.4 software. For imipenem assay, a negative result was defined as the presence of imipenem (300-m/z peak), whereas a positive result was defined as the complete disappearance of imipenem (Knox et al., 2014). For meropenem assay, an isolate was interpreted as a non-carbapenemase producer if the absence of both decarboxylated products of meropenem (358.5-*m*/*z* and 380.5-*m*/*z* peaks) and the presence of meropenem and/or its sodium salt (384.5-*m*/*z* and 406.5-*m*/*z* peaks) were observed, while an isolate was interpreted as a carbapenemase producer if the presence of at least one of the decarboxylated products of meropenem was detected (Papagiannitsis et al., 2015b).

The results are summarized in Table 1. For *P. aeruginosa* isolates, the imipenem assay gave no false-positive results among non-carbapenemase producers (100% specificity), while it correctly detected 138 (97% sensitivity) carbapenemase-producing isolates. This assay missed one IMP- and three VIM-producing isolates. On the other hand, eight non-carbapenemase-producing isolates were falsely classified as positive by the meropenem assay (93% specificity). This assay correctly detected the production of a carbapenemase in 128 isolates, while it failed to detect five *P. aeruginosa* isolates expressing either IMP (n=3) or VIM (n=2). For nine carbapenemase-producing *P. aeruginosa*, meropenem hydrolysis spectra were of low quality precluding a definitive analysis (90% sensitivity). Imipenem hydrolysis spectra were generally better (Figure 1), allowing an easier interpretation. However, in agreement with the findings of a previous study (Knox et al., 2014), no peaks corresponding to imipenem degradation products were identified.

Table 1. Results of the MALDI-TOF MS meropenem (MER) and imipenem (IMI) hydrolysis assays.[a]

Resistance mechanism	No. of isolates	No. of carbapenemase producers detected using MALDI-TOF MS[b]:		Sensitivity		Specificity	
		MER assay[c]	IMI assay	MER assay[c]	IMI assay	MER assay[c]	IMI assay
Pseudomonas aeruginosa				90%	97%	93%	100%
IMP	97	91	96				
VIM	41	33	38				
GES	4	4	4				
Total carbapenemase producers	**142**	**128**	**138**				

Non-carbapenemase producers	**108**	**8**	**0**				
Enterobacteriaceae				99%	96%	100%	100%
KPC	21	20	21				
VIM	18	18	16				
IMP	1	1	1				
NDM	24	24	24				
OXA-48-like	19	19	18				
Total carbapenemase producers	**83**	**82**	**80**				
Non-carbapenemase producers	**41**	**0**	**0**				

[a] Rows representing the total number of isolates per category are in bold.

[b] Numbers refer to the number of isolates that were interpreted as carbapenemase producers by each method.

[c] Data for the Meropenem assay against *Enterobacteriaceae* isolates are from a previous study (MALDI-TOF BIC assay; Papagiannitsis et al., 2015b).

For *Enterobacteriaceae* isolates, false-positive results were not observed with the imipenem assay (100% specificity) (Table 1). This assay correctly detected eighty carbapenemase-producing *Enterobacteriaceae*, including all KPC-, IMP- and NDM-producing isolates (96% sensitivity). The imipenem assay missed one OXA-48-producing *Klebsiella pneumoniae* and two *Enterobacter cloacae* expressing VIM metallo-β-lactamases.

To our knowledge, this is the first study validating the use of different carbapenem molecules for the MALDI-TOF MS detection of carbapenemase activity. Imipenem assay achieved the best sensitivity (97%) and specificity (100%) scores for *P. aeruginosa*. Whereas, based on the data previously published (Papagiannitsis et al., 2015b), the meropenem assay exhibiting 98% sensitivity and 100% specificity was more efficient against *Enterobacteriaceae*. For meropenem assay, addition of NH_4HCO_3 to the reaction buffer is crucial for the efficient detection of OXA-48-type producers (Papagiannitsis et al., 2015b). Imipenem assay didn't experience problems with the subset of the OXA-48-type producers. However, for this assay, addition of $ZnSO_4$ to the reaction buffer increased its sensitivity for the detection of VIM-producing bacteria (Papagiannitsis unpublished data). This is in accordance with the results of a recent study demonstrating that addition of zinc ions improved detection of metallo-β-lactamase producers by MALDI-TOF MS imipenem hydrolysis assay (Knox and Palombo, 2016). These findings may indicate that distinct strategies must be used for the detection of carbapenemase activity in different pathogen types.

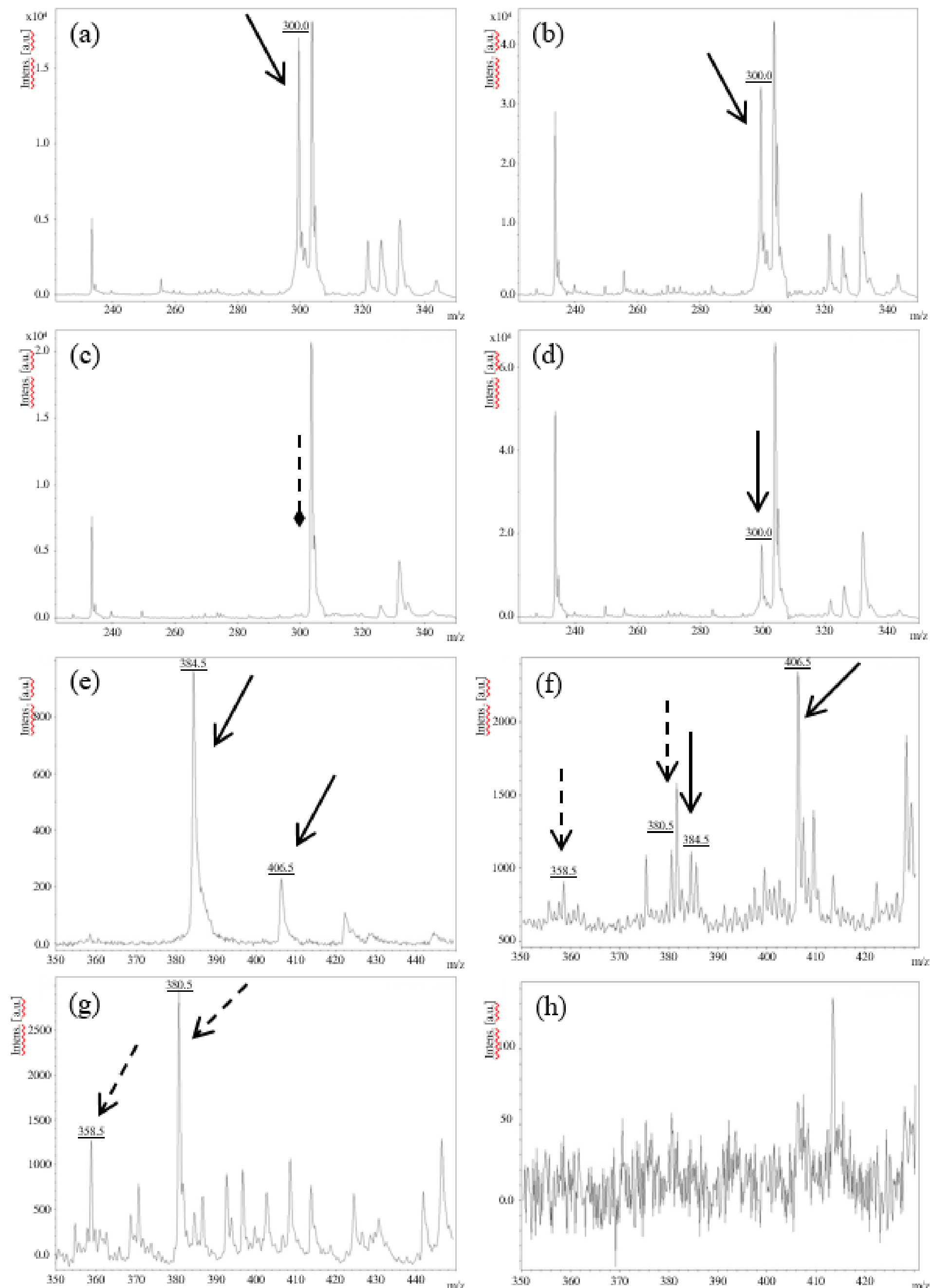

Figure 1. (a to d) Representative mass spectra of MALDI-TOF MS imipenem hydrolysis assay: (a) imipenem, (b) non-carbapenemase-producing *P. aeruginosa*, (c) IMP-producing *P. aeruginosa* interpreted as positive and (d) VIM-producing *P. aeruginosa* interpreted as negative. Peaks corresponding to imipenem are indicated with arrows with solid lines. The absence of imipenem peak is indicated with diamond-shaped arrows with dotted lines for carbapenemase producers. (e to h) Representative mass spectra of MALDI-TOF MS meropenem hydrolysis assay: (e) meropenem, (f) non-carbapenemase-producing *P. aeruginosa* interpreted as positive, (g) VIM-producing *P. aeruginosa* interpreted as positive and (h) IMP-producing *P. aeruginosa* not interpreted due to low quality of spectra. Peaks corresponding to meropenem and its sodium salt are indicated with arrows with solid lines, while peaks corresponding to decarboxylated products of meropenem are shown with arrows with dotted lines.

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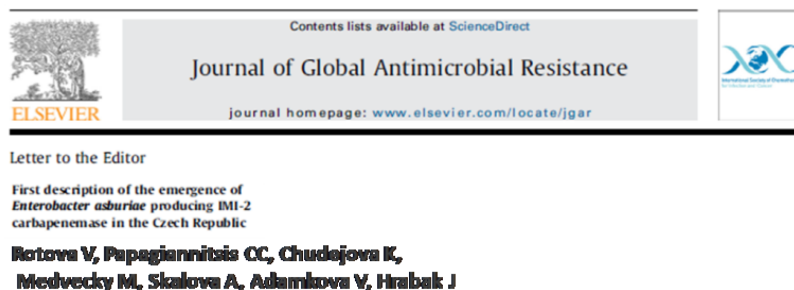
References

1. Burckhardt, I., Zimmermann, S., 2011. Using matrix-assisted laser desorption ionization-time of flight mass spectrometry to detect carbapenem resistance within 1 to 2.5 hours. J. Clin. Microbiol. 49, 3321-3324.
2. Hrabak, J., Walkova, R., Studentova, V., Bergerova, T., 2011. Carbapenemase activity detection by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. 49, 3222-3227.
3. Hrabak, J., Chudackova, E., Papagiannitsis, C.C., 2014. Detection of carbapenemases in *Enterobacteriaceae*: a challenge for diagnostic microbiological laboratories. Clin. Microbiol. Infect. 20, 839-853.
4. Kempf, M., Bakour, S., Flaudrops, C., Berrazeg, M., Brunel, J.M., Drissi, M., Mesli, E., Touati, A., Rolain, J.M., 2012. Rapid detection of carbapenem resistance in *Acinetobacter baumannii* using matrix-assisted laser desorption ionization-time of flight mass spectrometry. PLoS One 7, e31676.
5. Knox, J., Jadhav, S., Sevior, D., Agyekum, A., Whipp, M., Waring L., Iredell, J., Palombo, E., 2014. Phenotypic detection of carbapenemase-producing *Enterobacteriaceae* by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry and the Carba NP test. J. Clin. Microbiol. 52, 4075-4077.
6. Knox, J., Palombo, E., 2017. Performance of a MALDI-TOF MS-based imipenem hydrolysis assay incorporating zinc sulfate. Diagn. Microbiol. Infect. Dis. 87, 258-260.
7. Lauretti, L., Riccio, M.L., Mazzatiol, A., Cornaglia, G., Amicosante, G., Fontana, R., Rossolini, G.M., 1999. Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob. Agents Chemother. 43, 1584-1590.
8. Papagiannitsis, C.C., Izdebski, R., Baraniak, A., Fiett, J., Herda, M., Hrabak, J., Derde, L.P., Bonten, M.J., Carmeli, Y., Goossens, H., Hryniewicz, W., Brun-Buisson, C., Gniadkowski, M.; MOSAR WP2 WP3 and WP5 study groups, 2015. Survey of metallo-β-lactamase-producing *Enterobacteriaceae* colonizing patients in European ICUs and rehabilitation units, 2008-11. J. Antimicrob. Chemother. 70, 1981-1988.
9. Papagiannitsis, C.C., Študentová, V., Izdebski, R., Oikonomou, O., Pfeifer, Y., Petinaki, E., Hrabák, J., 2015. Matrix-assisted laser desorption ionization-time of flight mass spectrometry meropenem hydrolysis assay with NH_4HCO_3, a reliable tool for direct detection of carbapenemase activity. J. Clin. Microbiol. 53, 1731-17

7.2 First description in Czech Republic of emergence of an *Enterobacter asburiæ* producing an IMI-2 carbapenemase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Letter to the Editor

First description of the emergence of *Enterobacter asburiae* producing IMI-2 carbapenemase in the Czech Republic

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7.2.1 IMI-2-producing Enterobacter asburiæ

The acquired class A carbapenemase IMI-1, was originally described in an *Enterobacter cloacæ* isolated in a Californian hospital in 1984.[1] Since their first description, IMI-type carbabenemases have occasionally been detected in *Enterobacteriaceæ* from the USA, Europe, the Far East, and South Africa. Here we report a case of an IMI-2-producing *Enterobacter asburiæ* identified in the Czech Republic.

In 2016, *E. asburiæ* Easb-36567cz was recovered from a patient admitted to a Czech hospital. Easb-36567cz was isolated from a rectal swab during routine screening for carbapenemase-producing *Enterobacteriaceæ* (CPE). Easb-36567cz was resistant to aminopenicillins, aminopenicillin-sulbactam combinations, second-generation cephalosporins, aztreonam, carbapenems and colistin but susceptible to piperacillin-tazobactam, cefotaxime, ceftazidime, and various non-β-lactam antibiotics (Table 1). Carbapenemase production was hypothesized by a positive result in the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) imipenem hydrolysis assay.[2] Easb-36567cz tested negative by the ethylene diamine tetra-acetic acid (EDTA)-meropenem test, whilst the respective boronic acid-meropenem combined-disc test appeared positive indicating production of a class A carbapenemase. PCR and sequencing showed that Easb-36567cz carried $bla_{\text{IMI-2}}$.[3]

Conjugal transfer of $bla_{\text{IMI-2}}$ to rifampin-resistant *Escherichia coli* strain A15 was achieved by mating experiments in mixed-broth cultures, using rifampin (150 mg/L) and ampicillin (50 mg/L) as selective agents. The β-lactam resistance phenotype of Easb-36567cz was transferred (Table 1) at a frequency of ca. 10^{-3} per donor cell. Transconjugants were confirmed to be IMI producers by PCR and MALDI-TOF MS imipenem hydrolysis assay.[2,3] $bla_{\text{IMI-2}}$-positive transconjugant exhibited resistance to aminopenicillins, aminopenicillin-sulbactam combinations, and imipenem, whilst it remained susceptible to the remaining antibiotics tested (Table 1). The plasmid location of the $bla_{\text{IMI-2}}$ gene was demonstrated by S1 nuclease analysis of Easb-36567cz and its transconjugant, followed by hybridization with a digoxigenin-labelled bla_{IMI} probe. Plasmid analysis indicated transfer of a single plasmid (pEasb-36567cz)

of ca. 80 kb that hybridized strongly with the bla_{IMI} probe (data not shown). Plasmid pEasb-36567cz was positive for the FII allele using the replicon typing method.

Plasmid DNA from the IMI-producing transconjugant was extracted using a Qiagen Large-construct kit (Qiagen, Hilden, Germany), and was sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA).Sequencing, assembling of the reads, filling of sequence gaps, and analysis of the plasmid sequence were performed as described previously.[4]

Table 1. Antimicrobial susceptibility of *E. asburiae* and the *E. coli* A15 transconjugant harboring the IMI-2-encoding plasmid pEasb-36567cz.

Isolate	MIC (mg/L) of[a]:																			
	Amp	Ams	Pip	Tzp	Cfz	Cxm	Ctx	Caz	Fep	Atm	Imp	Mem	Etp	Gen	Amk	Tet	Sxt	Cip	Col	Fos
E. asburiæ Eae-36567cz	128	64	16	2	>16	64	0.25	0.5	1	4	>32	>16	>2	1	1	1	0.5	0.12	8	2
E. coli A15 pEasb-36567cz	128	16	8	≤1	>16	4	0.12	0.25	0.25	0.5	>32	1	0.25	0.5	1	1	0.5	≤0.06	0.25	2
E. coli A15 (recipient)	≤1	2	≤1	≤1	2	2	≤0.06	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	0.03	≤0.25	≤0.5	1	≤0.03	≤0.06	0.25	2

[a] Amp, ampicillin; Ams, ampicillin-sulbactam (inhibitor fixed at 4 mg/L); Pip, piperacillin; Tzp, piperacillin-tazobactam (inhibitor fixed at 4 mg/L); Cfz, cefazolin; Cxm, cefuroxime; Ctx, cefotaxime; Caz, ceftazidime; Fep, cefepime; Atm, aztreonam; Imp, imipenem; Mem, meropenem; Etp, ertapenem; Gen, gentamicin; Amk, amikacin; Tet, tetracycline; Sxt, trimethoprim-sulfamethoxazole; Cip, ciprofloxacin; Col, colistin; Fos, fosfomycin.

Sequencing data showed that plasmid pEasb-36567cz is 77,824 bp in size. pEasb-36567cz showed a high degree of similarity to pJF-787 (99% coverage; 99% identity), previously characterized from *Klebsiella variicola* strain H152460787 (GenBank accession no. KX868552). The plasmid backbone was composed of regions responsible for replication (*repA* gene), conjugative transfer (*tra* and *trb* genes), and maintenance (*parAB*and *psiAB* operons, and *klcA* gene) of the plasmid (Figure S1).The LysR-type regulator gene (*imiR-2*) was found upstream ofbla_{IMI-2}. In their genetic environment, IS*Ec36* was detected upstream of*imiR-2*, whilst remnants of mobile elements IS*1*, Tn*3*-like and IS*Eae1* as well as an intact insertion sequence exhibiting 86% identity to IS*Sgsp1* (GenBank accession no. HE578057) were found downstream of bla_{IMI-2} (Figure S1). pEasb-36567cz carried no additional resistance genes.

To our knowledge, this is the first description of an IMI carbapenemase-producing *Enterobacteriaceæ* from the Czech Republic. Of note was that IMI-2-producing *E asburiæ* Easb-36567cz was isolated from a patient with no history of travelling abroad or previous hospitalization. This finding indicates the spread potential of carbapenemase genes via routes that remain largely unknown. The patient was isolated to avoid further spread of CPE and was successfully treated for his main disease.

IMI carbapenemases, together with closely related NMC-A β-lactamase, are mainly found in *Enterobacter* spp. and have remained overall uncommon in the clinical setting, unlike other class A carbapenemase such as the KPC-type enzymes. Similarly to previous reports, the bla_{IMI-2} gene was located in a conjugative plasmid and was linked to mobile elements.[3,5] These features may be involved in the future spread of this emerging resistance mechanism between different members of *Enterobacteriaceæ*. Therefore, accurate detection of IMI-producing *Enterobacteriaceæ*, which exhibit unusual antimicrobial resistance profiles, is of utmost importance since such bacteria can act as hidden sources of clinically important resistance determinants.

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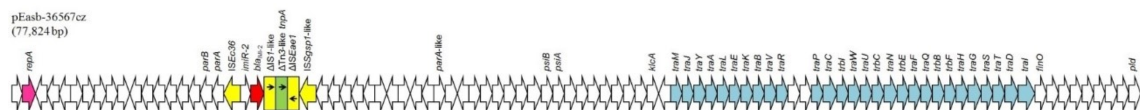

Figure S1. Linear map of the *bla*$_{IMI-2}$-carrying plasmid pEasb-36567cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Resistance genes, IS elements and transposases are shown in red, yellow and green, respectively. The *repA* genes is shaded pink, while *tra* and *trb* genes are shown in light blue. The remaining genes are shown in white.

References

1. Rasmussen BA, Bush K, Keeney D, Yang Y, Hare R, O'Gara C, Medeiros AA. Characterization of IMI-1 beta-lactamase, a class A carbapenem-hydrolyzing enzyme from Enterobacter cloacæ. Antimicrobial agents and chemotherapy. 1996 Sep 1;40(9):2080-6.
2. Rotova V, Papagiannitsis CC, Skalova A, Chudejova K, Hrabak J. Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity. Journal of microbiological methods. 2017 Jun 1;137:30-3.
3. Aubron C, Poirel L, Ash RJ, Nordmann P. Carbapenemase-producing Enterobacteriaceæ, US rivers. Emerging infectious diseases. 2005 Feb;11(2):260.
4. Skalova A, Chudejova K, Rotova V, Medvecky M, Studentova V, Chudackova E, Lavicka P, Bergerova T, Jakubu V, Zemlickova H, Papagiannitsis CC, Hrabak J. Molecular characterization of OXA-48-like-producing Enterobacteriaceæ in the Czech Republic and evidence for horizontal transfer of pOXA-48-like plasmids. Antimicrobial agents and chemotherapy. 2017 Feb 1;61(2):e01889-16.
5. Rojo-Bezares B, Martín C, López M, Torres C, Sáenz Y. First detection of blaIMI-2 gene in a clinical Escherichia coli strain. Antimicrobial agents and chemotherapy. 2012 Feb 1;56(2):1146-7.

7.3 Characterization of NDM-encoding plasmids from *Enterobacteriaceæ* recovered from Czech hospitals

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Characterization of NDM-Encoding Plasmids From *Enterobacteriaceae* Recovered From Czech Hospitals

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7.3.1 Abstract

The aim of the present study was to characterize sporadic cases and an outbreak of NDM-like-producing *Enterobacteriaceæ* recovered from hospital settings, in Czechia. During 2016, 18 *Entrobacteriaceae* isolates including 9 *Enterobacter cloacæ* complex, 4 *Escherichia coli*, 1 *Enterobacter asburiæ*, 1 *Enterobacter intermedius*, 1 *Klebsiella pneumoniæ*, 1 *Klebsiella oxytoca*, and 1 *Raoultella ornithinolytica* that produced NDM-like carbapenemases were isolated from 15 patients. Three of the patients were colonized or infected by two different NDM-like producers. Moreover, an NDM-4-producing *Enterobacter*, isolated in 2012, was studied for comparative purposes. All *Enterobacter* isolates, recovered from the same hospital, were assigned to ST182. Additionally, two *E. coli* belonged to ST167, while the remaining isolates were not clonally related. Thirteen isolates carried $bla_{\text{NDM-4}}$, while six isolates carried $bla_{\text{NDM-1}}$ (n = 3) or $bla_{\text{NDM-5}}$ (n = 3). Almost all isolates carried bla_{NDM}-like-carrying plasmids being positive for the IncX3 allele, except ST58 *E. coli* and ST14 *K. pneumoniæ* isolates producing NDM-1. Analysis of plasmid sequences revealed that all IncX3 bla_{NDM}-like-carrying plasmids exhibited a high similarity to each other and to previously described plasmids, like pNDM-QD28, reported from worldwide. However, NDM-4-encoding plasmids differed from other IncX3 plasmids by the insertion of a Tn*3*-like transposon. On the other hand, the ST58 *E. coli* and ST14 *K. pneumoniæ* isolates carried two novel NDM-1-encoding plasmids, pKpn-35963cz and pEsco-36073cz. Plasmid pKpn-35963cz that was an IncFIB(K) molecule contained an acquired sequence, encoding NDM-1 metallo-β-lactamase (MBL), which exhibited high similarity to the mosaic region of pS-3002cz from an ST11 *K. pneumoniæ* from Czechia. Finally, pEsco-36073cz was a multireplicon A/C_2+R NDM-1-encoding plasmid. Similar to other type 1 A/C_2

plasmids, the bla_{NDM-1} gene was located within the ARI-A resistance island. These findings underlined that IncX3 plasmids have played a major role in the dissemination of bla_{NDM}-like genes in Czech hospitals. In combination with further evolvement of NDM-like-encoding MDR plasmids through reshuffling, NDM-like producers pose an important public threat.

7.3.2 Introduction

Acquired carbapenem-hydrolyzing β-lactamases are resistance determinants of increasing clinical importance in Gram-negative pathogens. Of these, NDM-1 metallo-β-lactamase (MBL) was first described in *Klebsiella pneumoniæ* and *Escherichia coli* isolated in Sweden in 2008 from an Indian patient transferred from a New Delhi hospital.[1] Since then, NDM-1-producing bacteria, including clinical isolates of *Enterobacteriaceæ* and *Acinetobacter baumannii*, have been reported from the Indian subcontinent but also worldwide.[2]

In Czechia, the occurrence of NDM-producing bacteria was rare, with only three sporadic cases being detected during 2011-2013. These cases included an NDM-1-producing *A. baumanni* isolated from a patient repatriated from Egypt, an NDM-4-producing strain of an *Enterobacter* species from a patient previously hospitalized in Sri Lanka and a ST11 *K. pneumoniæ* isolate carrying two NDM-1-encoding plasmids, from Slovakia.[3-5] However, an increase in the isolation frequency of NDM-like-producing *Enterobacteriaceæ* from Czech hospitals was observed, during 2016.

Thus, the aim of the present study was to characterize the NDM-like producers detected in Czech hospitals, during 2016. Also, we describe the complete nucleotide sequences of representative bla_{NDM}-like-carrying plasmids harbored by the studied isolates.

7.3.3 Materials and methods

7.3.3.1 Bacterial isolates and confirmation of carbapenemase production

In 2016, Czech hospitals referred a total of 410 *Enterobacteriaceæ* isolates with a meropenem MIC of >0.125 μg/ml to the National Reference Laboratory for Antibiotics.[6] Species identification was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany). All isolates were tested for carbapenemase production by the MALDI-TOF MS meropenem hydrolysis assay.[7] Isolates that were positive by the MALDI-TOF MS meropenem hydrolysis assay were subjected to metallo-β-lactamase, KPC, and OXA-48 detection using the double-disc synergy test with EDTA, the phenylboronic acid disc test, and the temocillin disc test, respectively.[8-10] Additionally, carbapenemase genes (bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, and bla_{OXA-48}-like) were detected by PCR amplification.[1,11-13] PCR products were sequenced as described below. Isolates positive for bla_{NDM}-like genes were further studied. Moreover, the NDM-4-producing *Enterobacter* isolate, recovered at the University Hospital Pilsen (Pilsen, Czechia) during 2012, was included in this study for comparative purposes.[4]

7.3.3.2 Susceptibility testing

The MICs of piperacillin, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, meropenem, ertapenem, gentamicin, amikacin, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, colistin, and tigecycline were determined by the broth dilution method.[14] Data were interpreted according to the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org).

7.3.3.3 Typing

All isolates were typed by multilocus sequence typing (MLST).[15–18] The databases at:

- https://pubmlst.org/ecloacæ/,

- http://mlst.warwick.ac.uk/mlst/dbs/Ecoli,
- http://bigsdb.web.pasteur.fr/klebsiella and https://pubmlst.org/koxytoca/

were used to assign STs.

7.3.3.4 Detection of β-lactamases

The β-lactamase content of all *bla*$_{NDM}$-like-positive isolates was determined by isoelectric focusing (IEF). Bacterial extracts were obtained by sonication of bacterial cells suspended in 1% glycine buffer and clarified by centrifugation. Sonicated cell extracts were analyzed by IEF in polyacrylamide gels containing ampholytes (pH 3.5 to 9.5; AP Biotech, Piscataway, NJ). The separated β-lactamases were visualized by covering the gel with the chromogenic cephalosporin nitrocefin (0.2 mg/ml; Oxoid Ltd., Basingstoke, United Kingdom).[19]

On the basis of the IEF data, PCR detection of various *bla* genes was performed by the use of primers specific for *bla*$_{TEM-1}$, *bla*$_{OXA-1}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, and *bla*$_{CMY}$, as reported previously.[20–23] Both strands of the PCR products were sequenced using an ABI 377 sequencer (Applied Biosystems, Foster City, CA).

7.3.3.5 Transfer of *bla*$_{NDM}$-like genes

Conjugal transfer of *bla*$_{NDM}$-like genes from the clinical strains was carried out in mixed broth cultures, using the rifampin-resistant *E. coli* A15 laboratory strain as a recipient.[24] Transconjugants were selected on MacConkey agar plates supplemented with rifampin (150 mg/l) and ampicillin (50 mg/l). Plasmid DNA from clinical isolates, which failed to transfer *bla*$_{NDM}$-like by conjugation, was extracted using a Qiagen Maxi kit (Qiagen, Hilden, Germany) and used to transform *E. coli* DH5α cells. The preparation and transformation of competent *E. coli* cells were done using calcium chloride.[25] Transformants were selected on Luria-Bertani agar plates with ampicillin (50 mg/l). Transconjugants or transformants were confirmed to be NDM-like producers by PCR and the MALDI-TOF MS meropenem hydrolysis assay.[1, 7]

7.3.3.6 Plasmid analysis

To define the genetic units of the *bla*$_{NDM}$-like genes, the plasmid contents of all NDM-producing clinical and recombinant strains were analyzed by pulsed-field gel electrophoresis (PFGE) of total DNA digested with S1 nuclease (Promega, Madison, WI, USA).[26] Following PFGE, the DNA was transferred to a BrightStar-Plus positively charged nylon membrane (Applied Biosystems, Foster City, CA) and hybridized with digoxigenin-labeled *bla*$_{NDM}$-like probe.

Plasmid incompatibility (Inc) groups were determined by the PCR-based replicon typing (PBRT) method, using total DNA from transconjugants or transformants.[27, 28] Additionally, the IncR replicon was detected as described previously.[29]

7.3.3.7 Detection of characteristic regions

Based on the results from Illumina sequencing (see below), six PCRs targeting characteristic regions of NDM-4-ncoding IncX3 plasmids and ST182 *Enterobacter* genomes sequenced during this study were designed. The selected regions included: (i) a Tn*3*-like transposon found in NDM-4-encoding IncX3 plasmids, and (ii) four insertions identified in the genome of Encl-922 (see section: Comparative analysis of *Enterobacter* isolates). All NDM-producing clinical or recombinant strains were screened for the presence of the regions described above by the use of specific primers (Table S1).

7.3.3.8 Plasmid and chromosome sequencing

Ten plasmids were selected for complete sequencing. These plasmids were selected as representatives of different origins, plasmid sizes and hospitals. Additionally, *E. cloacæ* isolates Encl-922 and Encl-44578 were also selected for whole genome sequencing. These two isolates were selected as representatives of different isolation periods.

Plasmid DNAs from transconjugants or transformants were extracted using a Qiagen Large-Construct kit (Qiagen, Hilden, Germany). Additionally, the genomic DNAs of *Enterobacter* isolates were extracted using a DNA-Sorb-B kit (Sacace Biotechnologies S.r.l., Como, Italy). Multiplexed DNA libraries were prepared, using the Nextera XT Library Preparation kit, and 300-bp paired-end sequencing was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) using the MiSeq v3 600-cycle Reagent kit. Initial paired-end reads were quality trimmed using the Trimmomatic tool v0.33 with the sliding window size of 4 bp, required average base quality ≥17 and minimum read length of 48 bases Genomic DNA reads of *E. cloacæ* were consequently assembled using the de Bruijn graph-based *de novo* assembler SPAdes v3.9.1, using k-mer sizes 21, 33, 55, 77, 99 and 127.[30,31] For assembly of the plasmids, reads were mapped to the reference *E. coli* K-12 substrain MG 1655 genome (GenBank accession no. U00096) using the BWA-MEM algorithm, in order to filter out the chromosomal DNA.[32] Then, all the unmapped reads were assembled in the same way as described above. The sequence gaps were filled by a PCR-based strategy and Sanger sequencing. For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISfinder database (www-is.biotoul.fr/), and the open reading frame (ORF) finder tool (www.bioinformatics.org/sms/) were utilized. Comparative genome alignments were performed using the Mauve v2.3.1 program.[33]

Antibiotic resistance genes were identified using the ResFinder 2.1 tool (https://cge.cbs.dtu.dk/services/ResFinder/) with an identity threshold of >90%.[34]

7.3.3.9 Comparative analysis of *E. cloacæ* clinical isolates

Comparative genomic analysis of *Enterobacter* clinical strains was based on statistics calculated by QUAST v4.5 and VarScan v2.3.9 tools.[35, 36] All quality trimmed Illumina reads of Encl-922 were mapped to contigs of Encl-44578, employing BWA-MEM algorithm v0.7.12 and SAMtools v1.3, for the format conversions and analysis of the results.[32,37] Then, single nucleotide polymorphisms (SNPs) and indels were detected employing VarScan with parameters set as follows: minimum read depth at a position =6, minimum base quality at a position =20 and minimum variant allele frequency threshold of 0.45. Moreover, SNPs and indels located in a region within 127 bp from any edge of a contig, as well as SNPs and indels harbored by contigs smaller than 2 kb were excluded from further analysis. Remaining SNPs and indels were also manually checked and refined by visualization of mapped data via Tablet v1.14.04.10.[38] Differences in assembly of *E. cloacæ* genomes were inspected using QUAST's Icarus viewer.[39] In order to examine whether SNPs and indels were located in intergenic or coding regions, as well as to find out what are the differences in genetic information between studied isolates, contigs of clinical strains were annotated using Prokka v1.10.[40] Genes harboring SNPs were compared against NCBI's conserved domain database via CD-Search to identify conserved domain hits.[41,42] Finally, sequencing data of clinical strains were examined for the presence of prophage sequences using PHAST web server.[43]

7.3.3.10 Nucleotide sequence accession numbers

The nucleotide sequences of the pEsco-5256cz, pEncl-922cz, pRor-30818cz, pKpn-35963cz, pEsco-36073cz, pEncl-44578cz, pEnas-80654cz, pEnin-51781cz, pEsco-4382cz and pKlox-45574cz plasmids have been deposited in GenBank under accession numbers MG252891, MG252892, MG252893, MG252894, MG252895, MG833402, MG833403, MG833404, MG833405 and MG833406, respectively. Whole genome assemblies of *Enterobacter* isolates were deposited in NCBI under accession number PRJNA432167.

7.3.4 Results

7.3.4.1 Carbapenemase-producing *Enterobacteriaceæ*

A total of 40 *Enterobacteriaceæ* isolates showing carbapenemase activity on MALDI-TOF MS meropenem hydrolysis assay were recovered from Czech hospitals during 2016. PCR screening showed that 18 of the isolates were positive for bla_{NDM}, 14 isolates were positive for $bla_{\text{OXA-48}}$, while the remaining 8 isolates were positive for bla_{KPC}.

7.3.4.2 NDM-like-producing isolates

Altogether, 18 nonrepetitive isolates producing NDM-like carbapenemases were isolated from 15 patients in 2016. Among them, 9 were identified to be *E. cloacæ* complex, 4 were identified to be *E. coli*, while the remaining isolates belonged to unique species (*Enterobacter asburiæ*, *Enterobacter intermedius*, *K. pneumoniæ*, *Klebsiella oxytoca*, and *Raoultella ornithinolytica*). Three of the patients were colonized or infected by two different NDM-like producers (Table 1).

NDM-like producers were collected from five Czech hospitals located in three different Czech cities. In hospital B1, an outbreak that included ten patients diagnosed with NDM-like-producing *Enterobacteriaceæ* lasted the studied period. Additionally, two patients colonized or infected with NDM-like producers were reported in hospital B2. The three remaining cases were identified in three different hospitals. None of the patients, treated in hospital B1, had recently traveled abroad or had been previously hospitalized. The patient treated in hospitals was directly repatriated from a hospital in China, while clinical data weren't available for the remaining patients.

Additionally, the NDM-4-producing *Enterobacter* isolate identified in 2012, was studied.[4]

Table 1. Characteristics of NDM-like-producing *Enterobacteriaceae*

Isolate[a]	Isolation mn/yr (hospital)	Material (infection/ colonization)	ST	β-Lactamase content	Size of NDM-encoding plasmid (kb)[b]	Replicon of NDM-encoding plasmid	Additional resistance markers
E. xiangfangensis							
Encl-922	09/2012 (B1)	Rectal swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55 (53.683)**	IncX3	
Encl-66918	04/2016 (B1)	Rectal swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
Encl-89040	06/2016 (B1)	Bile (infection)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
Encl-44578	07/2016 (B1)	Venous catheter (infection)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55 (53.683)**	IncX3	
Encl-89485○	07/2016 (B1)	Bile (infection)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
Encl-91221	09/2016 (B1)	Throat swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
Encl-93141	10/2016 (B1)	Peritoneal catheter (infection)	ST182	NDM-4, CTX-M-15, OXA-1	**~55**	IncX3	
Encl-98042	11/2016 (B1)	Rectal swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1	**~55**	IncX3	
Encl-98047■	11/2016 (B1)	Rectal swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
Encl-98546	12/2016 (B1)	Rectal swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
E. asburiæ							
Enas-80654○	07/2016 (B1)	Bile (infection)	NA	NDM-4, CTX-M-15	**~55 (53.683)**	IncX3	
E. intermedius							
Enin-51781	10/2016 (B1)	Rectal swab (colonization)	NA	NDM-4, CTX-M-15, OXA-1	**~55 (53.683)**	IncX3	
E. coli							
Esco-14290	06/2016 (B2)	Nasal swab (colonization)	ST167	NDM-5, CTX-M-15, TEM-1	~45	IncX3	
Esco-5256▲	07/2016 (B2)	Bronchoalveolar lavage (infection)	ST167	NDM-5, CTX-M-15, TEM-1	~45 (46.161)	IncX3	
Esco-36073	09/2016 (A1)	Urine (infection)	ST58	NDM-1, CMY-16, OXA-10, CTX-M-15, TEM-1	**~300 (300.958)**	IncR, $IncA/C_2$	*floR*, *tet*(A), *strAB*, *sul2*, *aacA4*, *aphA7*, *dfrA14*, *arr-2*, *cmlA1*, *aadA1*, *aphA6*, *sul1*
Esco-4382■	12/2016 (B1)	Rectal swab (colonization)	ST69	NDM-4, CTX-M-15, TEM-1	**~55 (53.683)**	IncX3	
K. oxytoca							
Klox-45574▲	07/2016 (B2)	Rectal swab (colonization)	ST2	NDM-5	~45 (46.161)	IncX3	
K. pneumoniæ							
Kpn-35963	09/2016 (A2)	Urine catheter (infection)	ST14	NDM-1, SHV-12, CTX-M-15, OXA-1	~150 (161.324)	IncFIB	*aacA4*, *dfrA14*, *mph*(A)

Raoultella ornithinolytica							
Ror-30818	09/2016 (C)	Rectal swab (colonization)	NA	NDM-1, SHV-12, CTX-M-15, OXA-1, TEM-1	~55 (53.051)	IncX3	

[NA] Not applicable.
[a] White circles, black squares, and black triangles each indicate the NDM-like-producing isolates recovered from the same patient.
[b] Data for plasmids found in transconjugants are shown in bold; data for plasmids observed in transformants are underlined.

All 19 NDM-like producers exhibited resistance to piperacillin, piperacillin-tazobactam, cephalosporins and ertapenem (Table S2), while the observed variations in the MICs of aztreonam might reflect the presence of additional resistance mechanisms in some of the isolates. Seventeen of the NDM-like producers also exhibited resistance to ciprofloxacin; 15 were resistant to gentamicin, 13 were resistant to trimethoprim-sulfamethoxazole, 1 was resistant to amikacin and 1 was resistant to colistin, whereas all isolates were susceptible to tigecycline.

The population structure of NDM-like-producing isolates studied by MLST is shown in Table 1. All *Enterobacter* isolates, which were recovered from hospital B1, belonged to ST182. Of note was that the NDM-4-producing *Enterobacter* that was isolated, in 2012, from the patient previously hospitalized in Sri Lanka was also assigned to ST182.[4] ST182 *Enterobacter* isolates were previously identified in Mexico and were associated with the production of NDM-1 enzyme.[44,45] Two of *E. coli*, both of which were from hospital B2, belonged to ST167. *E. coli* ST167 was recently found among NDM-5-producing isolates from different healthcare institutions in China.[46, 47] The two remaining *E. coli* isolates were not clonally related and belonged to different STs (ST58 and ST69). The *K. pneumoniæ* isolate was assigned to the high risk clone ST14, while the *K. oxytoca* isolate was classified into ST2 that belongs to a growing international clonal complex (CC2).[48,49]

Sequencing of the PCR products revealed three bla_{NDM}-type genes encoding the NDM-1, NDM-4 and NDM-5 enzymes (Table 1).[1,50,51] NDM-5 is an NDM-1-related MBL variant that differs from NDM-1 by two amino-acid substitutions, Val88Leu and Met154Leu, the former one being its only change with NDM-4. Thirteen of the isolates, all of which were from hospital B1, were found to produce the NDM-4 MBL (Table 1). The three isolates from hospital B2 produced the NDM-5 enzyme, while the three remaining isolates that were recovered from sporadic cases in three different hospitals expressed NDM-1 carbapenemase. Additionally, most of bla_{NDM}-like-positive isolates were confirmed to coproduce the extended-spectrum β-lactamase CTX-M-15 (n = 18) either alone or along with TEM-1 (n = 13) and/or OXA-1 (n = 13), whereas the *K. pneumoniæ* and *R. ornithinolytica* isolates also expressed the SHV-12 enzyme. The ST58 NDM-1-producing *E. coli* isolate coproduced CMY-16, CTX-M-15, OXA-10 and TEM-1 β-lactamases.

7.3.4.3 bla_{NDM}-like-carrying plasmids

The bla_{NDM}-like genes from all clinical strains were transferred by conjugation (n = 14) or transformation (n = 5) (Table 5). All bla_{NDM}-like-positive recombinants exhibited resistance to piperacillin, piperacillin-tazobactam, cephalosporins and ertapenem, while they remained susceptible to meropenem (Table S2). The three NDM-1-producing recombinants also exhibited resistance to aztreonam. Additionally, most of bla_{NDM}-like-positive recombinants (n = 18) were susceptible to non-β-lactam antibiotics.

Plasmid analysis of NDM-4-producing donor and transconjugant strains revealed the transfer of plasmids, all of which were ~55 kb in size (Table 1). The three NDM-5-producing transformants harbored plasmids of ~45 kb, whereas the three remaining recombinants carried bla_{NDM-1}-positive plasmids of different sizes (~55 kb, ~150 kb and ~300 kb). Replicon typing showed seventeen of the plasmids, including those sizing ~45 kb, and ~55 kb, were positive for the IncX3 allele. The bla_{NDM-1}-positive plasmid of ~300 kb was positive for replicons R and A/C, whereas the one remaining bla_{NDM-1}-carrying plasmid was nontypeable by the PBRT method.[27, 28]

7.3.4.4 Structure of bla_{NDM}-like-carrying plasmids

The complete sequence of bla_{NDM}-like-carrying plasmids representative of different plasmid sizes, replicons, and resistance genes (n = 10) was determined (Table 1). Sequence analysis revealed that all IncX3 bla_{NDM}-like-carrying plasmids exhibited a high similarity to each other and to previously described

NDM-like-encoding plasmids, belonging to IncX3 group, reported from worldwide.[52-54] The *bla*$_{NDM-5}$-positive plasmids, pEsco-5256cz and pKlox-45574cz, were almost identical to NDM-5-encoding plasmid pNDM-QD28 (100% coverage, 99% identity) (GenBank accession no. KU167608) that was characterized from a ST167 *E. coli* in China.[53] Differences among these plasmids consisted in few SNPs (n = 5), almost all located in mobile elements. Similar to pNDM-QD28, no other resistance genes were detected in these plasmids. Compared to other IncX3 NDM-encoding plasmids, all *bla*$_{NDM-4}$-encoding plasmids differed by the insertion of a Tn*3*-like transposon (nt 7108-14624 in pEncl-44578cz) downstream *topB* gene (Figure 1). The Tn*3*-like sequence was composed by the 38-bp inverted repeats (IR) of the transposon, *tnpA*, *tnpR* and two ORFs encoding hypothetical proteins. Target site duplications of 5 bp (GTACC) at the boundaries of the Tn*3*-like element indicated insertion by transposition. Of note was that the sequence of pEncl-922cz, isolated in 2012, was identical to the respective sequences of NDM-4-encoding plasmids recovered in the same hospital, during 2016.[4] PCR screening confirmed the presence of the Tn*3*-like transposon in all NDM-4-encoding IncX3 plasmids, isolated in hospital B1, while Tn*3*-like wasn't detected in the remaining *bla*$_{NDM}$-like-positive plasmids that belonged to IncX3 group. Furthermore, the *bla*$_{NDM-1}$-positive plasmid, pRor-30818cz, harbored an additional 7875-bp sequence (nt 40617-48491 in pRor-30818cz) encoding the extended-spectrum β-lactamase SHV-12 (Figure 1). A similar SHV-12-encoding region was found in the IncX3 *bla*$_{NDM-1}$-positive plasmid pKP04NDM (100% coverage, 99% identity) (GenBank accession no. KU314941) described from a *K. pneumoniæ* isolate in China.

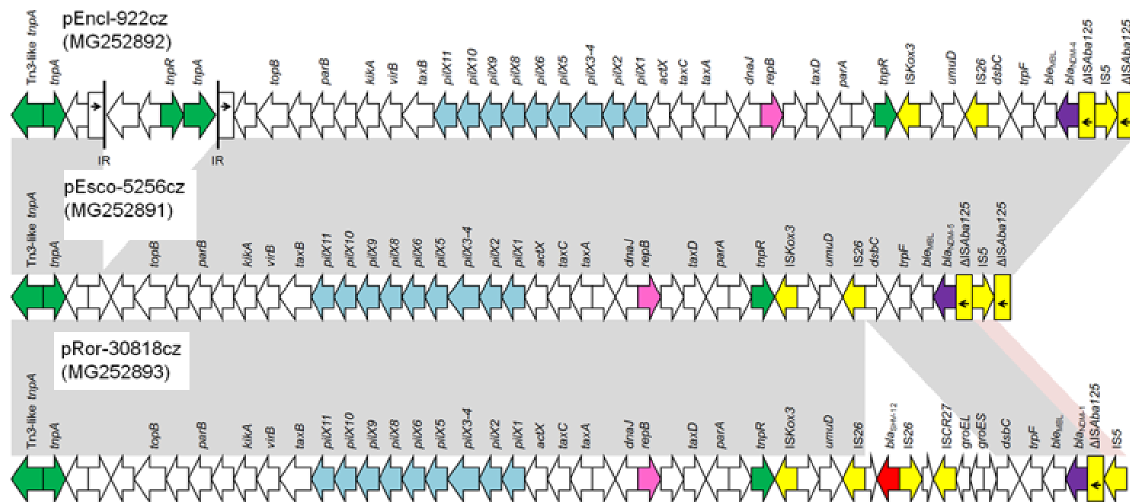


Figure 1. Comparison of linear maps of the NDM-like-encoding IncX3 plasmids pEncl-922cz, pEsco-5256cz, and pRor-30818cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Replicons of the plasmids are shown in pink. *bla*$_{NDM}$-like genes are shaded purple, while other resistance genes are shown in red. IS elements and transposases are shown in yellow and green, respectively. Light blue arrows indicate genes responsible for the conjugative transfer of the plasmids. The remaining genes, including plasmid scaffold regions, are shown in white. Homologous segments (representing ≥99% sequence identity) are indicated by light gray shading, while pink shading shows inverted homologous segments.

The NDM1-encoding plasmid pKpn-35963cz that was nontypeable by the PBRT method was 161324 bp in size.[27] Plasmid pKpn-35963cz was composed of two distinct parts: a contiguous plasmid backbone of 115998 bp (nt 1-58655 and 103982-161324) and an acquired sequence of 45326 bp (nt 58656-103981). The plasmid backbone, which shared similarities with the respective regions of plasmid p1605752FIB (GenBank accession no. CP022125) recovered from a pan-resistant isolate of *K. pneumoniæ* from the United States, harbored regions responsible for replication [*repB* gene; IncFIB(K) replicon], conjugative transfer (*tra* and *trb* genes) and plasmid maintenance (*vagCD*, *psiAB*, *umuCD* and *parAB* operons, and *ssb* gene) (Figure 2). The acquired sequence of pKpn-35963cz contained a 17836-bp segment (nt 77360-95195) encoding NDM-1, which was similar to the mosaic region of pS-3002cz (99% identity). pS-3002cs was characterized from an ST11 *K. pneumoniæ* isolate identified in Czechia.[5] The acquired

sequence of pKpn-35963cz contained two additional segments that have also been described in pS-3002cz. The first segment (nt 65518-72935) included genes encoding an EcoRII methylase and EcoRII endonuclease, and the class 1 integron In191 carrying the *dfrA14* resistance gene. The second segment (nt 101342-103981) contained fragments of transposons Tn*1000* (ΔTn*1000*) and Tn*1331* (ΔTn*1331*). ΔTn*1331* comprised *tnpR* and *aacA4* resistance gene. Furthermore the acquired sequence of pKpn-35963cz carried a macrolide resistance operon [*mph*(A)], and regions encoding OXA-1 and CTX-M-15 β-lactamases (Figure 2). In the acquired sequence of pKpn-35963cz, intact and truncated copies of several mobile elements that may have been implicated in the formation of this region were found.

The plasmid pEsco-36073cz, which encoded the NDM-1 carbapenemase, is 300,958 bp in size. The plasmid showed a complex structure, being composed of sequences of diverse origin (Figure 3). A 170314-bp sequence (nt 232204-300958 and 1-101559) resembled the type 1 A/C_2 plasmid pRH-1238 (94% coverage, 99% identity) (Figure 3), characterized from a *Salmonella enterica* serovar Corvallis strain isolated from a migratory wild bird in Germany.[55] Analysis of A/C_2-associated sequence by the core gene PMLST (cgPMLST) scheme indicated that it belonged to cgST3.4.[56] The A/C_2 backbone was composed of regions responsible for replication (*repA* gene), conjugative transfer (Tra1 and Tra2 regions), and plasmid maintenance (*higBA* and *parAB* operons and *xerD*- and *kfrA*-like genes). Apart from the backbone, pEsco-36073cz carried the bla_{CMY-2}-like-containing region, and the ARI-B and ARI-A resistance islands, as previously described in other type 1 A/C_2 MDR plasmids.[57,58] The bla_{NDM-1} gene was located within ARI-A, in a genetic environment similar to those previously identified in pRH-1238.[55] However, unlike in pRH-1238, the ARI-A of pEsco-36073cz lacked the macrolide resistance determinant *mphA-mel-repAciN*. Furthermore, a class 1 integron with *aacA4* and *aphA1* gene cassettes was located between *resI* and *resII* sites of the Tn*1696* module. The ARI-A of pEsco-36073cz also carried a new integron, In1459, whose variable region comprised the *dfrA14*, *arr-2*, *cmlA1*, bla_{OXA-10}, *aadA1* cassettes. Additionally, pEsco-36073cz included fragments resembling the backbone of the recently described IncR plasmid pKP1780, and sequences previously found in the plasmid pPSP-a3e and in the chromosomes of several Gram-negative rods.[59, 60] Genes encoding for resistance to arsenate, cooper and mercury were identified in the three remaining acquired regions of pEsco-36073cz.

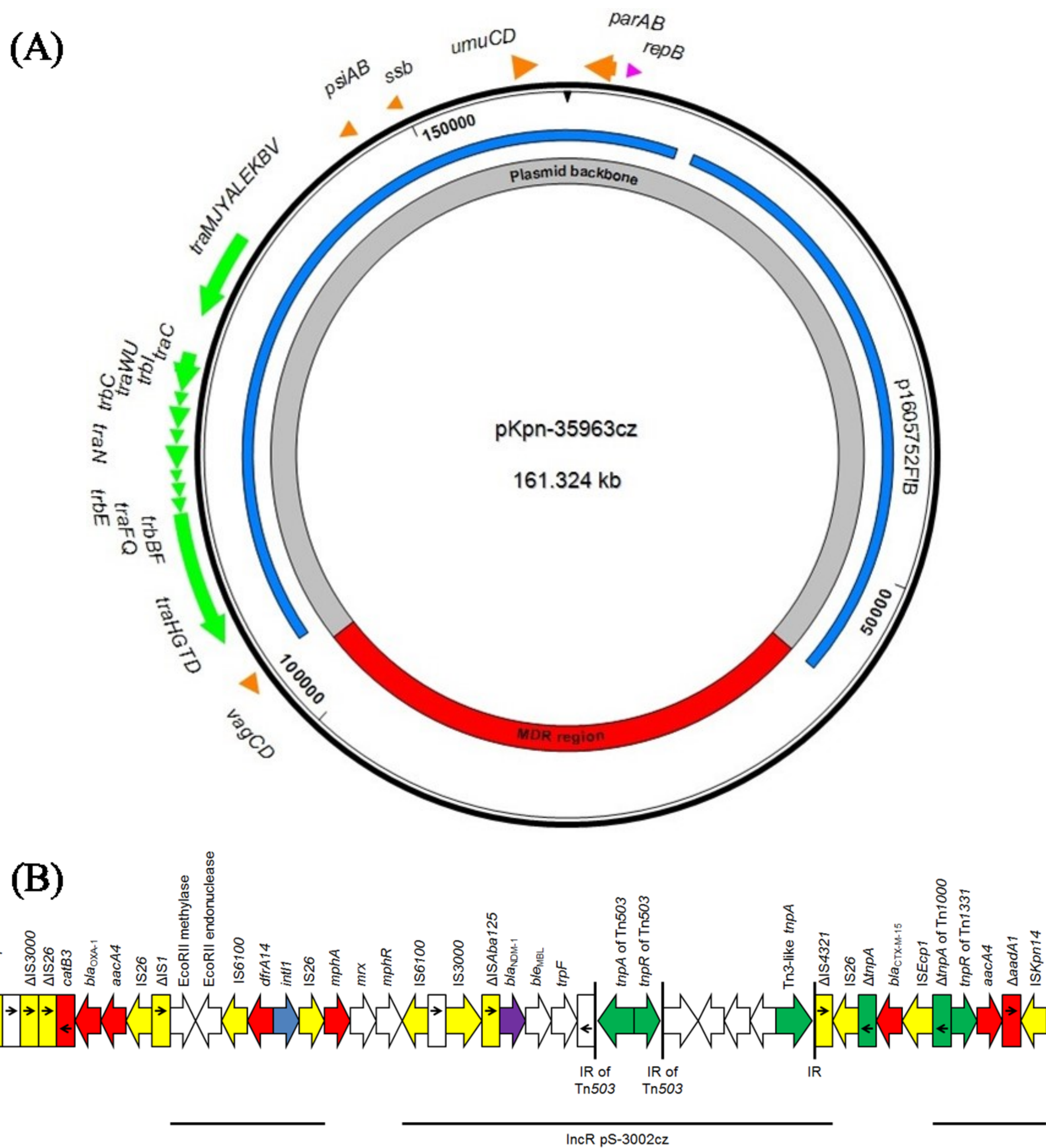


Figure 2. (A) Overview of the plasmid pKpn-35963cz. The innermost circles show the main regions of the plasmids. Similarities with other plasmids are shown in the next circle; each color represents a unique plasmid. In the outer circle, indicative genes and the direction of transcription are shown by arrows. Replicons of the plasmid are indicated as pink arrows. Genes responsible for plasmid transfer and maintenance are shown in green and orange, respectively. (B) Linear map of the multidrug resistance region (MDR) of the plasmid pKpn-35963cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). *bla*$_{NDM}$-like genes are shaded purple, while other resistance genes are shown in red. IS elements and transposases are shown in yellow and green, respectively. *intI1* genes are shaded blue. The remaining genes are shown in white. Thin lines below the map correspond to highly similar sequences from other plasmids.

7.3.4.5 Comparative analysis of *Enterobacter* isolates

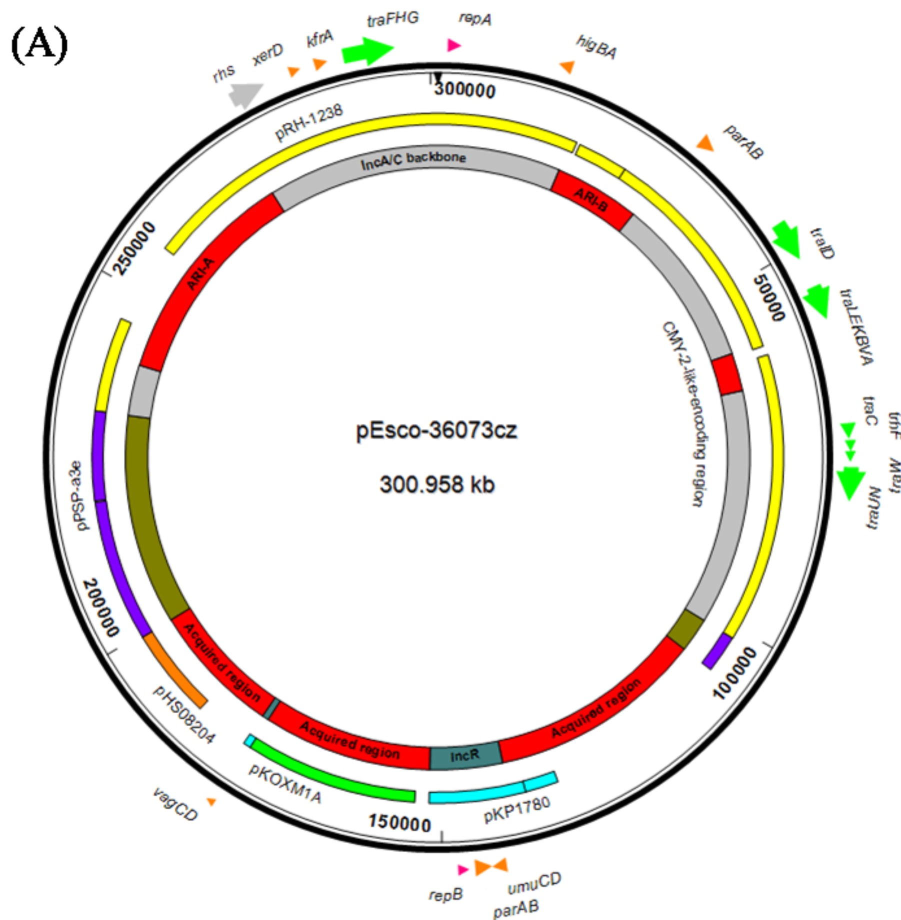
'*In silico*' *hsp60* typing of the genome sequences showed that both isolates belonged to the recently recognized *E. xiangfangensis* species.[61, 62]

Since all *Enterobacter* isolates belonged to the same ST and carried the same IncX3 *bla*$_{NDM-4}$-carrying plasmid, the WGS data of clinical strains Encl-922 and Encl-44578 were compared, using

QUAST and VarScan tools, in order to examine the phylogenetic relationship of the isolates recovered in 2012 and 2016.

Comparative analysis of *Enterobacter* clinical isolates revealed that the genome of Encl-922 exhibited extensive similarity (99.87% identity) to the genome of Encl-44578. Sixteen SNPs were identified in the genome of Encl-922, compared to that of Encl-44578, five of which were located within prophage regions (Table 2). Interestingly, Encl-922 harbored three large insertions of 8,933 bp (nt 439392-448324 in node 2), of 17,903 bp (nt 17786-35688 in node 32) and of 13,165 bp (nt 1-13165 in node 27; prophage sequence PHAGE_Salmon_SPN3UB_NC_019545). Additionally, Encl-922 harbored an insertion of 33-bp sequence (AACCCTCTCCCCAAAGGGGAGAGGGGACGATTA) located in an intergenic region. Moreover, Encl-922 showed a single nucleotide (G) deletion leading to CDS annotation change of general stress protein 39 to putative oxidoreductase YghA. Analysis of whole genome sequencing (WGS) data by PHAST web server found five intact prophage sequences (PHAGE_Haemop_HP2_NC_003315, PHAGE_Salmon_SPN3UB_NC_019545, PHAGE_Entero_mEp390_NC_019721, PHAGE_Pseudo_PPpW_3_NC_023006, and PHAGE_Salmon_SP_004_NC_021774) and one questionable prophage region (PHAGE_Entero_SfI_NC_027339), in both Enterobacter isolates. However, Encl-922 included one additional incomplete prophage region (PHAGE_Salmon_SPN3UB_NC_019545), which was absent from the Encl-44578 genome.

Screening by PCR and sequencing identified that all Enterobacter isolates, recovered during 2016, didn't harbor any of the four mentioned insertions. Thus, this finding indicated that Enterobacter isolates from 2016 differed from Encl-922.



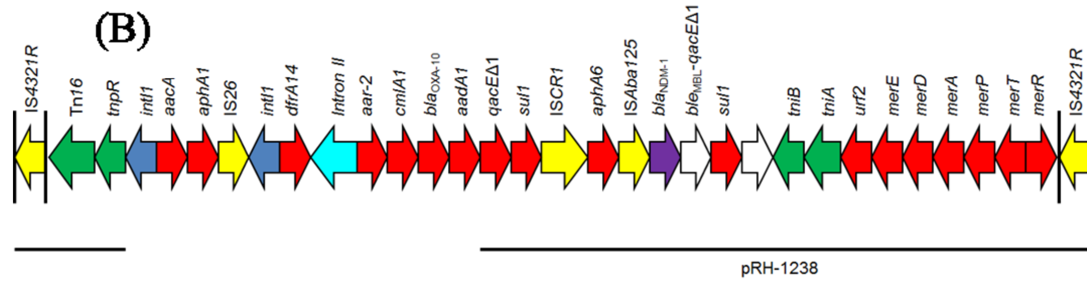


Figure 3. (A) Overview of the plasmid pEsco-36073cz. The innermost circles show the main regions of the plasmids. Similarities with other plasmids are shown in the next circle; each color represents a unique plasmid. In the outer circle, indicative genes and the direction of transcription are shown by arrows. Replicons of the plasmid are indicated as pink arrows. Genes responsible for plasmid transfer and maintenance are shown in green and orange, respectively. (B) Linear map of the ARI-A resistance island of the plasmid pEsco-36073cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). bla_{NDM}-like genes are shaded purple, while other resistance genes are shown in red. IS elements and transposases are shown in yellow and green, respectively. intI1 genes are shaded blue; teal blue arrow indicates the group II intron. The remaining genes are shown in white. Thin lines below the map correspond to highly similar sequences from other plasmids.

Table 2. Summary table of sixteen SNPs found between the genomes of *Enterobacter* isolates Encl-44578 (reference) and Encl-922 (query).

PROKKA name	Conserved domain classification	Enzyme Commision number	Contig	SNP	Gene length (aa)	aa substitution
–[a]	–	–	2	T64623G	-	-
–[a]	–	–	7	88097G	-	-
Methyl viologen resistance protein SmvA	MFS transporter	–	8	T51220C	496	M293T
D-amino acid dehydrogenase small subunit	D-amino acid dehydrogenase	1.4.99.1	23	A46893G	432	S395S
NADP-dependent malic enzyme	NADP-dependent malic enzyme	1.1.1.40	2	A296564G	759	N584N
Glyoxylate/hydroxypyruvate reductase A	Glyoxylate/hydroxypyruvate reductase A	1.1.1.79	4	G113111A	312	R267H
Ribonuclease E	Ribonuclease E	3.1.26.12	4	T156395C	1035	H685R
Hypothetical protein	–	–	38	C784A	369	T239N
Hypothetical protein	Similar to protein YjaG	–	39	A24170G	196	I61V
Low-affinity gluconate transporter	Low-affinity gluconate transporter	–	6	T100479C	421	S277P
Arabinose operon regulatory protein	DNA-binding transcriptional regulator	–	12	A66284G	281	N193S
Anaerobic dimethyl sulfoxide reductase chain B	DMSO_dmsB family protein	–	35	T1976G	205	K120Q
Tail length tape measure protein	COG5281 and Phage_HK97_TLTM domain-containing protein	–	5	G24809A	1154	L824L
Tail length tape measure protein	COG5281 and Phage_HK97_TLTM domain-containing protein	–	5	T24845C	1154	A836A
Tail length tape measure protein	COG5281 and Phage_HK97_TLTM domain-containing protein	–	5	C24893A	1154	G852G
Terminase-like family protein	P family protein	–	26	G7615T	589	R485L

[a] The first two SNPs are located in intergenic regions.

7.3.5 Discussion

The present study investigated sporadic cases and an outbreak of NDM-like-producing *Enterobacteriacœ* recovered from Czech hospitals, during 2016. Specifically, 12 NDM-4-producing isolates, which belonged to *E. xiangfangensis* (n = 9), *E. asburiæ* (n = 1), *E. intermedius* (n = 1) and *E. coli* species, 3 NDM-5 producers of *E. coli* (n = 2) and *K. oxytoca* (n = 1) species, and one *E. coli*, one *K. pneumoniæ* and one *R. ornithinolytica* producing NDM-1 MBL were characterized.

The setting that was most affected was hospital B1, in which an outbreak of NDM-4-producing ST182 *E. xiangfangensis* isolates took place. Of note was that the *Enterobacter*, isolated in 2012 from a

patient who had been previously hospitalized in Sri Lanka, also belonged to ST182 and harbored an IncX3 *bla*$_{NDM-4}$-positive plasmid being identical to respective plasmids characterized from *Enterobacter* isolates recovered from patients treated in hospital B1 (Table 1), during 2016.[4] However, comparative genome analysis revealed the presence of four insertions in the genome of *Enterobacter* Encl-922 isolate. These insertions were not found in the genomic DNA of *Enterobacter* isolates from 2016, suggesting a second insertion event of NDM-4-producing *Enterobacter* isolates in Czech hospitals.

In three of the patients, two different NDM-like producers were identified during their hospitalization, supposing the *in vivo* horizontal transfer of *bla*$_{NDM}$-like-carrying plasmids. Sequencing and PCR screening data revealed the presence of the same *bla*$_{NDM-4}$- or *bla*$_{NDM-5}$-carrying plasmid in these isolates (Table 1). These results confirmed the hypothesis of the *in vivo* horizontal transfer of *bla*$_{NDM}$-like-carrying plasmids.

Results from Illumina sequencing showed that IncX3 plasmids have played a major role in the dissemination of *bla*$_{NDM}$-like genes in Czech hospitals, which is in agreement with the findings from previous studies from worldwide.[52-54] In the current study, three *bla*$_{NDM}$-type genes, encoding the NDM-1, NDM-4, and NDM-5 enzymes, were associated with IncX3 plasmids exhibiting high similarity to each other. Considering also the fact that NDM-1, NDM-4 and NDM-5 differ by one or two amino-acid substitutions may indicate the possibility that *bla*$_{NDM}$-like genes encoding NDM-1-related variants have evolved in the same plasmid type. Additionally, Illumina data showed the presence of a unique sequence, a Tn*3*-like transposon, in sequenced *bla*$_{NDM-4}$-carrying plasmids. PCR confirmed the presence of the Tn*3*-like sequence in all transconjugants, carrying *bla*$_{NDM-4}$-positive plasmids. Thus, the PCR targeting the Tn*3*-like sequence was able to distinguish *bla*$_{NDM-4}$-positive plasmids from other IncX3 plasmids carrying *bla*$_{NDM-1}$ or *bla*$_{NDM-5}$. On the other hand, two of the sporadic isolates carried novel NDM-1-encoding plasmids. Plasmid pKpn-35963cz that was an IncFIB(K) molecule contained an acquired sequence, encoding NDM-1 MBL, which exhibited high similarity to the mosaic region of pS-3002cz from an ST11 *K. pneumoniæ* from Czechia.[5] Whereas plasmid pEsco-36073cz was a multireplicon A/C_2+R NDM-1-encoding plasmid, being a fusion derivative of sequences of diverse origin. Similar to other type 1 A/C_2 plasmids (Villa et al., 2015; Harmer and Hall, 2015), the *bla*$_{NDM-1}$ gene was located within the ARI-A resistance island.[55, 57]

In conclusion, the data presented here contribute to the current knowledge of NDM-like-producing *Enterobacteriaceæ*. In agreement with previous studies, our findings punctuate that NDM-like producers constitute an important public threat, mainly due to the rapid horizontal transfer of IncX3 *bla*$_{NDM}$-carrying plasmids but, also, due to further evolvement of NDM-like-encoding MDR plasmids via reshuffling.

Table S1. Oligonucleotide primers.

Name	Sequence (5'–3')	Usage	Reference
pNDM-F	CGTGGCTCTTGTCATGCTGA	Tn*3*-like segment mapping with pNDM4-R	This study
pNDM4-R	TAACGACAAAGATCAGGAGCA	Tn*3*-like segment mapping with pNDM-F	This study
pNDM4-F	TGCAGGTTCGCTGAAGCTG	Tn3-like segment mapping with pNDM-R	This study
pNDM-R	AGGGAAGTAGTCTCTGATATCT	Tn3-like segment mapping with pNDM4-F	This study
922.9k-F	GATCGGCAGTAGAGGTGGA	9-kb segment mapping with 922.9k-R	This study
922.9k-R	TACCAAAGCAACAGCTGACG	9-kb segment mapping with 922.9k-F	This study
922.13k-F	GCTCTACAGCAGCGTTCCAG	13-kb segment mapping with 922.13k-R	This study
922.13k-R	AGTGGGAAAGCGTTGCAGATC	13-kb segment mapping with 922.13k-F	This study
922.18k-F	TGATACATGAACAAGGCAGATG	18-kb segment mapping with 922.18k-R	This study
922.18k-R	GTGCGATCAATGGTTAACTCA	18-kb segment mapping with 922.18k-F	This study
922.33b-F	ATGGCGAAACTGCCCTCGA	33-bb insertion mapping with 922.33b-R	This study
922.33b-R	TCTGTCTGAACGTGCTGGCT	33-bb insertion mapping with 922.33b-R	This study

Table S2. Antimicrobial susceptibility of NDM-like-producing clinical and recombinant strains.

Isolate	MIC (mg/l) of:[a]															
	Pip	Tzp	Ctx	Caz	Fep	Atm	Mem	Etp	Gen	Amk	Cmp	Tet	Sxt	Cip	Col	Tgc
Encl-922	>128	>128	>8	>16	>16	>16	4	>2	16	2	16	>32	>4	>8	0.25	1
Trc Encl-922	128	128	>8	>16	16	≤0.12	1	2	≤0.5	≤0.25	4	0.5	≤0.03	≤0.06	0.25	0.25
Encl-66918	>128	>128	>8	>16	>16	>16	4	>2	16	2	16	>32	>4	>8	0.25	1
Trc Encl-66918	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-89040	>128	>128	>8	>16	>16	>16	2	>2	16	4	16	>32	>4	>8	0.25	1
Trc Encl-89040	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-44578	>128	>128	>8	>16	>16	>16	2	>2	16	1	16	>32	>4	>8	0.25	1
Trc Encl-44578	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-89485	>128	>128	>8	>16	>16	>16	4	>2	16	1	16	>32	>4	>8	0.25	1
Trc Encl-89485	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-91221	>128	>128	>8	>16	>16	>16	4	>2	>32	1	16	>32	>4	>8	0.25	1
Trc Encl-91221	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-93141	>128	>128	>8	>16	>16	>16	4	>2	16	1	16	>32	0.5	>8	≤0.12	1
Trc Encl-93141	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-98042	>128	>128	>8	>16	>16	>16	2	>2	32	1	16	>32	1	>8	0.25	1
Trc Encl-98042	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-98047	>128	>128	>8	>16	>16	>16	2	>2	16	1	16	>32	>4	>8	0.25	1
Trc Encl-98047	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-98546	>128	>128	>8	>16	>16	>16	16	>2	16	1	16	>32	0.25	0.12	0.25	1
Trc Encl-98546	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Enas-80654	>128	>128	>8	>16	>16	0.5	16	>2	≤0.25	≤0.5	16	8	0.12	8	>16	1
Trc Enas-80654	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Enin-51781	>128	128	>8	>16	>16	>16	2	>2	16	1	16	>32	1	1	0.25	1
Trc Enin-51781	128	128	>8	>16	16	≤0.12	1	2	≤0.5	≤0.25	4	0.5	≤0.03	≤0.06	0.25	0.25
Esco-36073	>128	>128	>8	>16	>16	16	2	>2	8	4	>32	>32	>4	≤0.06	0.25	0.25
Trc Esco-36073	>128	>128	>8	>16	>16	16	2	>2	2	2	>32	32	>4	≤0.06	0.25	0.25
Esco-4382	>128	>128	>8	>16	>16	≤0.12	1	>2	32	1	4	>32	>4	>8	0.25	0.25
Trc Esco-4382	128	128	>8	>16	16	≤0.12	1	2	≤0.5	≤0.25	4	0.5	≤0.03	≤0.06	0.25	0.25
Esco-5256	>128	>128	>8	>16	>16	1	8	>2	≤0.25	≤0.5	4	>32	>4	>8	≤0.12	0.12
Trf Esco-5256	128	128	>8	>16	>16	≤0.12	2	>2	≤0.5	≤0.25	2	0.5	≤0.03	≤0.06	0.25	0.25
Esco-14290	>128	>128	>8	>16	>16	>16	2	2	16	1	16	>32	>4	>8	0.25	0.12
Trf Esco-14290	128	128	>8	>16	>16	≤0.12	2	>2	≤0.5	≤0.25	2	0.5	≤0.03	≤0.06	0.25	0.25
Klox-45574	>128	>128	>8	>16	>16	>16	4	>2	≤0.25	≤0.5	>32	>32	>4	2	≤0.12	0.5
Trf Klox-45574	128	128	>8	>16	>16	≤0.12	2	>2	≤0.5	≤0.25	2	0.5	≤0.03	≤0.06	0.25	0.25
Kpn-35963	>128	>128	>8	>16	>16	>16	2	>2	0.5	4	16	>32	1	>8	0.25	0.5
Trf Kpn-35963	>128	64	>8	>16	8	>16	0.5	2	0.5	4	1	16	0.25	≤0.06	0.25	0.25
Ror-30818	>128	>128	>8	>16	>16	>16	8	>2	>32	>64	>32	4	>4	>8	0.25	1
Trf Ror-30818	>128	>128	>8	>16	16	>16	1	>2	0.5	4	1	16	0.25	≤0.06	0.25	0.25

[a] Pip, piperacillin; Tzp, piperacillin-tazobactam (inhibitor fixed at 4 mg/l); Ctx, cefotaxime; Caz, ceftazidime; Fep, cefepime; Atm, aztreonam; Mem, meropenem; Etp, ertapenem; Gen, gentamicin; Amk, amikacin; Cmp, chloramphenicol; Tet, tetracycline; Sxt, trimethoprim-sulfamethoxazole; Cip, ciprofloxacin; Col, colistin; Tgc, tigecycline.

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References

1. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR. Characterization of a new metallo-β-lactamase gene, blaNDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniæ sequence type 14 from India. Antimicrobial agents and chemotherapy. 2009 Dec 1;53(12):5046-54.
2. Nordmann P, Poirel L, Walsh TR, Livermore DM. The emerging NDM carbapenemases. Trends in microbiology. 2011 Dec 1;19(12):588-95.
3. Hrabák J, Štolbová M, Študentová V, Fridrichová M, Chudáčková E, Zemlickova H. NDM-1 producing Acinetobacter baumannii isolated from a patient repatriated to the Czech Republic from Egypt, July 2011. Eurosurveillance. 2012 Feb 16;17(7):20085.
4. Papagiannitsis CC, Studentova V, Chudackova E, Bergerova T, Hrabak J, Radej J, Novak I. Identification of a New Delhi metallo-β-lactamase-4 (NDM-4)-producing Enterobacter cloacæ from a Czech patient previously hospitalized in Sri Lanka. Folia microbiologica. 2013 Nov 1;58(6):547-9.
5. Studentova V, Dobiasova H, Hedlova D, Dolejska M, Papagiannitsis CC, Hrabak J. Complete nucleotide sequences of two NDM-1-encoding plasmids from the same sequence type 11 Klebsiella pneumoniæ strain. Antimicrobial agents and chemotherapy. 2015 Feb 1;59(2):1325-8.
6. European Committee on Antimicrobial Susceptibility Testing. EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. EUCAST, Basel, Switzerland: http://www. eucast. org/clinical_breakpoints. 2013 Dec.
7. Rotova V, Papagiannitsis CC, Skalova A, Chudejova K, Hrabak J. Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity. Journal of microbiological methods. 2017 Jun 1;137:30-3.
8. Glupczynski Y, Huang TD, Bouchahrouf W, de Castro RR, Bauraing C, Gérard M, Verbruggen AM, Deplano A, Denis O, Bogaerts P. Rapid emergence and spread of OXA-48-producing carbapenem-resistant Enterobacteriaceæ isolates in Belgian hospitals. International journal of antimicrobial agents. 2012 Feb 1;39(2):168-72.
9. Lee K, Lim YS, Yong D, Yum JH, Chong Y. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-β-lactamase-producing isolates of Pseudomonas spp. and Acinetobacter spp. Journal of clinical microbiology. 2003 Oct 1;41(10):4623-9.
10. Doi Y, Potoski BA, Adams-Haduch JM, Sidjabat HE, Pasculle AW, Paterson DL. Simple disk-based method for detection of Klebsiella pneumoniæ carbapenemase-type β-lactamase by use of a boronic acid compound. Journal of clinical microbiology. 2008 Dec 1;46(12):4083-6.
11. Poirel L, Héritier C, Tolün V, Nordmann P. Emergence of oxacillinase-mediated resistance to imipenem in Klebsiella pneumoniæ. Antimicrobial agents and chemotherapy. 2004 Jan 1;48(1):15-22.
12. Ellington MJ, Kistler J, Livermore DM, Woodford N. Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases. Journal of Antimicrobial Chemotherapy. 2006 Dec 21;59(2):321-2.
13. Naas T, Cuzon G, Villegas MV, Lartigue MF, Quinn JP, Nordmann P. Genetic structures at the origin of acquisition of the β-lactamase blaKPC gene. Antimicrobial agents and chemotherapy. 2008 Apr 1;52(4):1257-63.
14. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clinical Microbiology and Infection. 2003 Aug;9(8):ix-xv.
15. Miyoshi-Akiyama T, Hayakawa K, Ohmagari N, Shimojima M, Kirikae T. Multilocus sequence typing (MLST) for characterization of Enterobacter cloacæ. PloS one. 2013 Jun 11;8(6):e66358.
16. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden MC, Ochman H, Achtman M. Sex and virulence in Escherichia coli: an evolutionary perspective. Molecular microbiology. 2006 Jun 1;60(5):1136-51.
17. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. Multilocus sequence typing of Klebsiella pneumoniæ nosocomial isolates. Journal of clinical microbiology. 2005 Aug 1;43(8):4178-82.
18. Herzog, K.A., Schneditz, G., Leitner, E., Feierl, G., Hoffmann, K.M., Zollner-Schwetz, I., Krause, R., Gorkiewicz, G., Zechner, E.L. and Högenauer, C., 2014. Genotypes of Klebsiella oxytoca isolates from patients with nosocomial pneumonia are distinct from those of isolates from patients with antibiotic-associated hemorrhagic colitis. Journal of clinical microbiology, 52(5), pp.1607-1616.
19. Papagiannitsis CC, Študentová V, Jakubů V, Španělová P, Urbášková P, Žemličková H, Hrabák J, Czech Participants of European Antimicrobial Resistance Surveillance Network. High prevalence of ST131 among

CTX-M-producing Escherichia coli from community-acquired infections, in the Czech Republic. Microbial Drug Resistance. 2015 Feb 1;21(1):74-84.

20. Palucha A, Mikiewicz B, Hryniewicz W, Gniadkowski M. Concurrent outbreaks of extended-spectrum β-lactamase-producing organisms of the family Enterobacteriaceæ in a Warsaw hospital. Journal of Antimicrobial Chemotherapy. 1999 Oct 1;44(4):489-99.

21. Pérez-Pérez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. Journal of clinical microbiology. 2002 Jun 1;40(6):2153-62.

22. Woodford N, Fagan EJ, Ellington MJ. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. Journal of antimicrobial chemotherapy. 2005 Nov 10;57(1):154-5

23. Coque TM, Novais Â, Carattoli A, Poirel L, Pitout J, Peixe L, Baquero F, Cantón R, Nordmann P. Dissemination of clonally related Escherichia coli strains expressing extended-spectrum β-lactamase CTX-M-15. Emerging infectious diseases. 2008 Feb;14(2):195.

24. Vatopoulos AC, Philippon A, Tzouvelekis LS, Komninou Z, Legakis NJ. Prevalence of a transferable SHV-5 type β-lactamase in clinical isolates of Klebsiella pneumoniæ and Escherichia coli in Greece. Journal of antimicrobial chemotherapy. 1990 Nov 1;26(5):635-48.

25. Cohen SN, Chang AC, Hsu L. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. Proceedings of the National Academy of Sciences. 1972 Aug 1;69(8):2110-4.

26. Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. Analytical biochemistry. 1995 Apr 1;226(2):235-40.

27. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of plasmids by PCR-based replicon typing. Journal of microbiological methods. 2005 Dec 1;63(3):219-28.

28. Johnson TJ, Bielak EM, Fortini D, Hansen LH, Hasman H, Debroy C, Nolan LK, Carattoli A. Expansion of the IncX plasmid family for improved identification and typing of novel plasmids in drug-resistant Enterobacteriaceæ. Plasmid. 2012 Jul 31;68(1):43-50.

29. García-Fernández A, Fortini D, Veldman K, Mevius D, Carattoli A. Characterization of plasmids harbouring qnrS1, qnrB2 and qnrB19 genes in Salmonella. Journal of Antimicrobial Chemotherapy. 2008 Nov 11;63(2):274-81.

30. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014 Apr 1;30(15):2114-20.

31. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. Journal of computational biology. 2012 May 1;19(5):455-77.

32. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv preprint arXiv:1303.3997. 2013 Mar 16.

33. Darling AE, Mau B, Perna NT. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PloS one. 2010 Jun 25;5(6):e11147.

34. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. Identification of acquired antimicrobial resistance genes. Journal of antimicrobial chemotherapy. 2012 Jul 10;67(11):2640-4.

35. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for genome assemblies. Bioinformatics. 2013 Feb 19;29(8):1072-5.

36. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, Miller CA, Mardis ER, Ding L, Wilson RK. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome research. 2012 Mar 1;22(3):568-76.

37. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. The sequence alignment/map format and SAMtools. Bioinformatics. 2009 Aug 15;25(16):2078-9.

38. Milne I, Stephen G, Bayer M, Cock PJ, Pritchard L, Cardle L, Shaw PD, Marshall D. Using Tablet for visual exploration of second-generation sequencing data. Briefings in bioinformatics. 2012 Mar 24;14(2):193-202.

39. Mikheenko A, Valin G, Prjibelski A, Saveliev V, Gurevich A. Icarus: visualizer for de novo assembly evaluation. Bioinformatics. 2016 Jul 4;32(21):3321-3.

40. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014 Mar 18;30(14):2068-9.

41. Marchler-Bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, Gwadz M. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic acids research. 2016 Nov 28;45(D1):D200-3.

42. Marchler-Bauer A, Bryant SH. CD-Search: protein domain annotations on the fly. Nucleic acids research. 2004 Jul 1;32(suppl_2):W327-31.

43. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. PHAST: a fast phage search tool. Nucleic acids research. 2011 Jun 14;39(suppl_2):W347-52.

44. Torres-González P, Bobadilla-del Valle M, Tovar-Calderón E, Leal-Vega F, Hernández-Cruz A, Martínez-Gamboa A, Niembro-Ortega MD, Sifuentes-Osornio J, Ponce-de-León A. Outbreak caused by Enterobacteriaceæ harboring NDM-1 metallo-β-lactamase carried in an IncFII plasmid in a tertiary care hospital in Mexico City. Antimicrobial agents and chemotherapy. 2015 Nov 1;59(11):7080-3.

45. Bocanegra-Ibarias P, Garza-González E, Morfín-Otero R, Barrios H, Villarreal-Treviño L, Rodríguez-Noriega E, Garza-Ramos U, Petersen-Morfin S, Silva-Sanchez J. Molecular and microbiological report of a hospital outbreak of NDM-1-carrying Enterobacteriaceæ in Mexico. PloS one. 2017 Jun 21;12(6):e0179651.

46. Yang P, Xie Y, Feng P, Zong Z. blaNDM-5 carried by an IncX3 plasmid in Escherichia coli sequence type 167. Antimicrobial agents and chemotherapy. 2014 Dec 1;58(12):7548-52.

47. Zhang LP, Xue WC, Meng DY. First report of New Delhi metallo-β-lactamase 5 (NDM-5)-producing Escherichia coli from blood cultures of three leukemia patients. International Journal of Infectious Diseases. 2016 Jan 1;42:45-6.

48. Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS microbiology reviews. 2011 Sep 1;35(5):736-55.

49. Izdebski R, Fiett J, Urbanowicz P, Baraniak A, Derde LP, Bonten MJ, Carmeli Y, Goossens H, Hryniewicz W, Brun-Buisson C, Brisse S. Phylogenetic lineages, clones and β-lactamases in an international collection of Klebsiella oxytoca isolates non-susceptible to expanded-spectrum cephalosporins. Journal of Antimicrobial Chemotherapy. 2015 Aug 27;70(12):3230-7.

50. Hornsey M, Phee L, Wareham DW. A novel variant, NDM-5, of the New Delhi metallo-β-lactamase in a multidrug-resistant Escherichia coli ST648 isolate recovered from a patient in the United Kingdom. Antimicrobial agents and chemotherapy. 2011 Dec 1;55(12):5952-4.

51. Nordmann P, Boulanger AE, Poirel L. NDM-4 metallo-β-lactamase with increased carbapenemase activity from Escherichia coli. Antimicrobial agents and chemotherapy. 2012 Apr 1;56(4):2184-6.

52. Krishnaraju M, Kamatchi C, Jha AK, Devasena N, Vennila R, Sumathi G, Vaidyanathan R. Complete sequencing of an IncX3 plasmid carrying blaNDM-5 allele reveals an early stage in the dissemination of the blaNDM gene. Indian journal of medical microbiology. 2015 Jan 1;33(1):30.

53. Zhu YQ, Zhao JY, Xu C, Zhao H, Jia N, Li YN. Identification of an NDM-5-producing Escherichia coli sequence type 167 in a neonatal patient in China. Scientific Reports. 2016 Jul 13;6:29934.

54. Pál T, Ghazawi A, Darwish D, Villa L, Carattoli A, Hashmey R, Aldeesi Z, Jamal W, Rotimi V, Al-Jardani A, Al-Abri SS. Characterization of NDM-7 carbapenemase-producing Escherichia coli isolates in the Arabian Peninsula. Microbial Drug Resistance. 2017 Oct 1;23(7):871-8.

55. Villa L, Guerra B, Schmoger S, Fischer J, Helmuth R, Zong Z, Garcia-Fernandez A, Carattoli A. IncA/C plasmid carrying blaNDM-1, blaCMY-16, and fosA3 in a Salmonella enterica serovar Corvallis strain isolated from a migratory wild bird in Germany. Antimicrobial agents and chemotherapy. 2015 Oct 1;59(10):6597-600.

56. Hancock SJ, Phan MD, Peters KM, Forde BM, Chong TM, Yin WF, Chan KG, Paterson DL, Walsh TR, Beatson SA, Schembri MA. Identification of IncA/C plasmid replication and maintenance genes and development of a plasmid multilocus sequence typing scheme. Antimicrobial agents and chemotherapy. 2017 Feb 1;61(2):e01740-16.

57. Harmer CJ, Hall RM. The A to Z of A/C plasmids. Plasmid. 2015 Jul 31;80:63-82.

58. Papagiannitsis CC, Dolejska M, Izdebski R, Giakkoupi P, Skálová A, Chudějová K, Dobiasova H, Vatopoulos AC, Derde LP, Bonten MJ, Gniadkowski M. Characterisation of IncA/C2 plasmids carrying an In416-like integron with the blaVIM-19 gene from Klebsiella pneumoniæ ST383 of Greek origin. International journal of antimicrobial agents. 2016 Feb 1;47(2):158-62.

59. Papagiannitsis CC, Miriagou V, Giakkoupi P, Tzouvelekis LS, Vatopoulos AC. Characterization of pKP1780, a novel IncR plasmid from the emerging Klebsiella pneumoniæ ST147, encoding the VIM-1 metallo-β-lactamase. Journal of Antimicrobial Chemotherapy. 2013 May 27;68(10):2259-62.

60. Conlan S, Thomas PJ, Deming C, Park M, Lau AF, Dekker JP, Snitkin ES, Clark TA, Luong K, Song Y, Tsai YC. Single-molecule sequencing to track plasmid diversity of hospital-associated carbapenemase-producing Enterobacteriaceæ. Science translational medicine. 2014 Sep 17;6(254):254ra126.

61. Hoffmann H, Roggenkamp A. Population genetics of the nomenspecies Enterobacter cloacæ. Applied and environmental microbiology. 2003 Sep 1;69(9):5306-18.

62. Gu CT, Li CY, Yang LJ, Huo GC. Enterobacter xiangfangensis sp. nov., isolated from Chinese traditional sourdough, and reclassification of Enterobacter sacchari Zhu et al. 2013 as Kosakonia sacchari comb. nov. International

journal of systematic and evolutionary microbiology. 2014 Aug 1;64(8):2650-6.

7.4 Characterization of pEncl-30969cz, a novel ColE1-like plasmid encoding VIM-1 carbapenemase, from an *Enterobacter cloacae* sequence type 92 isolate

Note

Characterization of pEncl-30969cz, a novel ColE1-like plasmid encoding VIM-1 carbapenemase, from an *Enterobacter cloacae* sequence type 92 isolate

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7.4.1 Abstract

A VIM-1-producing ST92 *Enterobacter cloacae* was isolated in a Czech hospital. $\textit{bla}_{\text{VIM-1}}$ was part of the class 1 integron In110 carried by a Tn*1721*-like transposon. Tn*1721*-like was located on a ColE1-like plasmid, pEncl-30969cz (33,003 bp). Target site duplications at the boundaries of Tn*1721*-like suggested its transposition into the pEncl-30969cz backbone.

The emergence and spread of carbapenemase-producing *Enterobacteriaceae* (CPE) have caused a public health crisis of global dimensions (Nordmann et al., 2011). In Europe, during the period 2001-2008, VIM producers prevailed among CPE (Tzouvelekis et al., 2012). However, in the Czech Republic, the isolation frequency of *Enterobacteriaceae* producing VIM-type metallo-β-lactamases (MβLs) has been low, with only sporadic cases being detected (Hrabak et al., 2013; Papousek et al., 2017). The aim of the present study was to report a case of a VIM-1-producing *Enterobacter cloacae* isolate (Encl-30969cz) of Czech origin. We also describe the sequence of pEncl-30969cz, a novel VIM-1-encoding plasmid, harbored by Encl-30969cz.

Encl-30969cz was recovered from the sputum of a patient, with a chronic pulmonary disease, who was treated in a Czech hospital, in August of 2015. Encl-30969cz was resistant to piperacillin, piperacillin-tazobactam combination, cephalosporins and ertapenem but susceptible to aztreonam, meropenem, tigecycline and colistin (Table 1), as determined by broth dilution method (EUCAST 2003) and interpreted according to EUCAST criteria (http://www.eucast.org/). Carbapenemase production was hypothesized due to a positive result in the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) meropenem hydrolysis assay (Rotova et al., 2017). Ecl-35771cz was MβL positive with the EDTA-meropenem test (Lee et al., 2003). PCR screening for various MβL-encoding genes (Ellington et al., 2007; Yong et al., 2009) followed by sequencing revealed the presence of the $bla_{\text{VIM-1}}$ gene in Encl-30969cz.

The multilocus sequence typing (MLST) analysis (Miyoshi-Akiyama et al., 2013) classified the isolate into ST92. ST92 was originally identified among CTX-M-15-producing *E. cloacae* isolates from a rehabilitation unit located in Israel (Izdebski et al., 2015).

Attempts to transfer β-lactam resistance from Encl-30969cz to rifampin-resistant *Escherichia coli* strain A15 by conjugation were unsuccessful. Plasmid DNA from Encl-30969cz was extracted using a Qiagen Maxikit (Qiagen, Hilden, Germany) and used to transform *E. coli* DH5α cells. Transformants were selected on Luria-Bertani agar plates with ampicillin (50 μg/ml), and confirmed to carry bla_{VIM} by PCR (Ellington et al., 2007). $bla_{\text{VIM-1}}$-positive transformant exhibited resistance to penicillin, penicillin-sulbactam combination, cephalosporins and carbapenems (Table 1). The plasmid location of $bla_{\text{VIM-1}}$ gene was demonstrated by the S1 nuclease analysis (Barton et al., 1995) of Encl-30969cz and its transformant, followed by hybridization with a digoxigenin labeled bla_{VIM} probe. Plasmid analysis indicated transfer of a single plasmid (pEncl-30969cz) of approximately 30 kb that hybridized strongly with the bla_{VIM} probe. Plasmid pEncl-30969cz was nontypeable by PCR-based replicon typing (PBRT) (Carattoli et al., 2005).

Plasmid DNA from the VIM-producing *E. coli* transformant was extracted using a Qiagen Large-Construct Kit (Qiagen, Hilden, Germany), and was sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Sequencing, assembling of the reads, filling of sequence gaps, and analysis of the plasmid sequence were performed as described previously (Skalova et al., 2017).

Plasmid pEncl-30969cz is 33,003 bp in size (Figure 1). The plasmid included a contiguous sequence of 2,499 bp (nt 1 to 2499) sharing extensive similarity with the backbone of the ColE1-like plasmid pPIGDM1 (Mikiewicz et al., 1997). A consensus sequence for the ColE1 replication origin (*oriV*) (Tomizawa et al., 1997) was located at positions 1146 to 1148. Additionally, the pEncl-30969cz replication region included putative regions containing the RNA transcripts RNAII (nt 1145 to 1663) and RNAI (nt 1559 to 1663) that control the initiation of DNA replication and the plasmid copy number (Polisky, 1988). Downstream of *oriV*, an open reading frame (ORF) encoding Rom protein was present (nt 40 to 225). The Rom protein enhances the interaction of the RNA I inhibitor with its target, thus resulting in a reduction of the replication initiation frequency (Cesareni et al., 1991).

In the remaining part of pEncl-30969cz (nt 2500 to 33003), a Tn*1721*-like transposon structure was identified (Figure 1). The Tn*1721*-like sequence of pEncl-30969cz contained the VIM-1-encoding integron In110 (Lombardi et al., 2002), whose variable region comprised the $bla_{\text{VIM-1}}$, *aacA4* and *aadA1* cassettes. The IRi of In110 was located within the *tnpM* gene of the transposon, while the 3´CS was intact. Downstream (108 bp) of *orf5*, a Tn*21* fragment consisting of IS*1326*, *tniB*Δ1, *tniA* and part of the *mer* operon (Δ*merE*) was found. A Tn*3*-like transposon consisting of IRs of the transposon, *tnpA*, six ORFs, and *tnpR* was identified adjacent to Δ*merE*. Two copies of an IS*5075* element, which was shown previously to target the IRs of Tn*21*-like transposons (Partridge & Hall, 2003), disrupted the IRs of the Tn*3*-like. It is likely that insertion of the Tn*3*-like transposon deleted the remaining part of the Tn*21 mer* module. Next to the Tn*3*-like, pEncl-30969cz included a 4173-bp segment (nt 4097 to 8269) comprising of two ORFs, a *qnrB*-like gene conferring resistance to quinolones (Halova et al., 2014), and a truncated ORF. A second 777-bp fragment of Tn*1721* (Allmeier et al., 1992), comprising the IR of the transposon and a truncated *orf1* that lacked 889 bp of its 5´end due to insertion of an IS*26*, was found at the boundary of the plasmid backbone. Target site duplications of 5 bp (TTTTC) were found at the boundaries of Tn*1721*-like transposon, suggesting its transposition into the pEncl-30969cz backbone.

Patient's clinical data indicated that VIM-1 producer could be described as hospital-acquired, since the isolate was recovered on month after patient's hospitalization for the treatment of his chronic disease. The patient was isolated, in order to avoid further spread of CPE. In addition, surveillance cultures from patients treated in the same department, from August 2015 till December 2015, were negative for the presence of VIM-producing *Enterobacteriaceae* indicating the success of isolations precautions.

In conclusion, this study presents the complete nucleotide sequence of a novel ColE1-like plasmid pEncl-30969cz, encoding VIM-1 carbapenemase. Sequencing data suggested that pEncl-30969cz might have evolved by acquisition of a Tn*1721*-like transposon carrying the VIM-1-encoding integron In110 by a ColE1-type plasmid. This finding underscores the important role of mobile genetic elements in the dissemination of resistance determinants, such as *bla*$_{\text{VIM-1}}$.

Nucleotide sequence accession numbers.

The nucleotide sequence of the plasmid pEncl-30969cz has been assigned GenBank accession number MG049738.

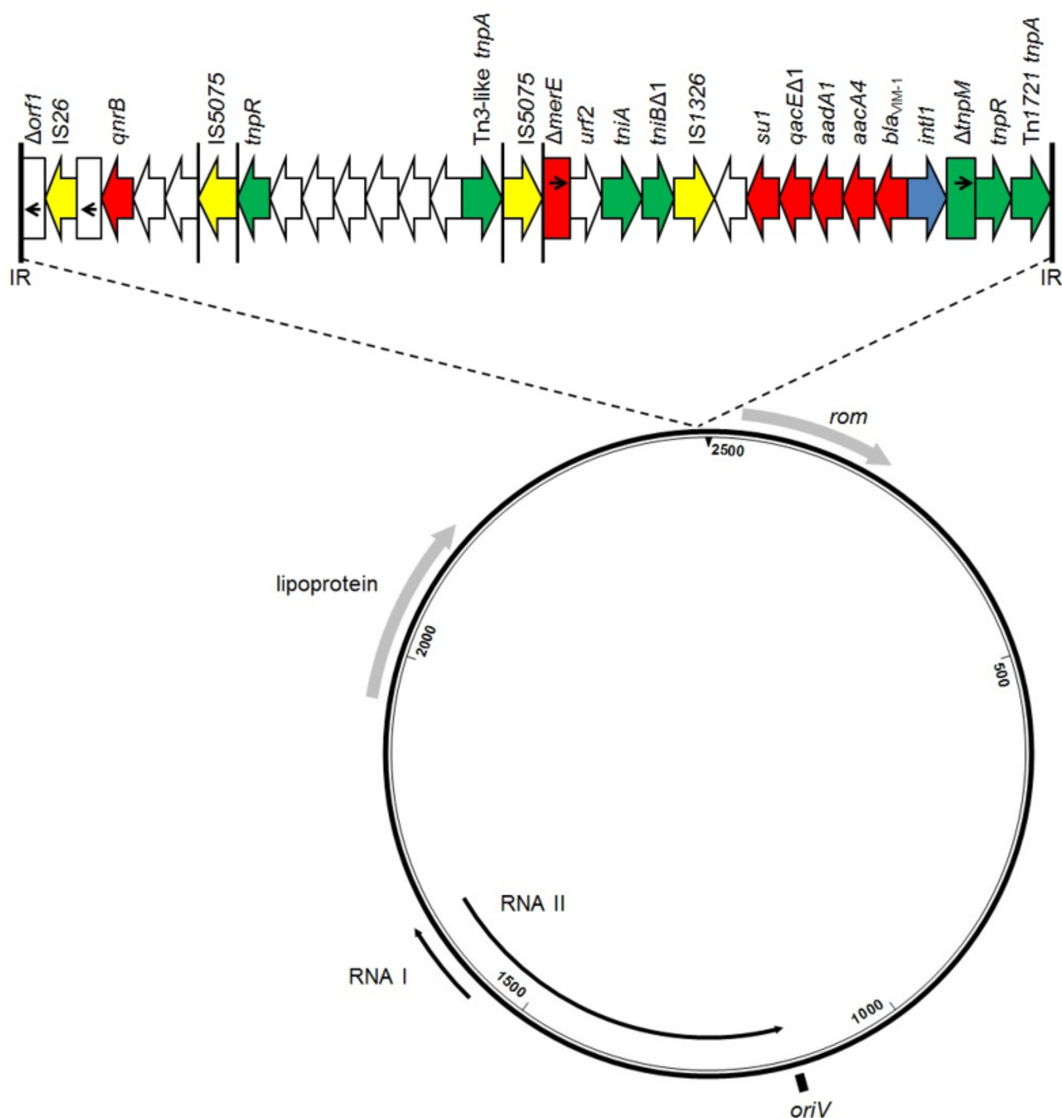

Figure 1. Map of the blaVIM-1-carrying plasmid pEncl-30969cz. Arrows show the direction of transcription of open reading frames (ORFs) and regulatory elements, while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Resistance genes, IS elements and transposases are shown in red, yellow and green, respectively. The intI1 gene is shaded blue, while the remaining genes are shown in white.

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References

1. Allmeier H, Cresnar B, Greck M, Schmitt R. Complete nucleotide sequence of Tn*1721*: gene organization and a novel gene product with features of a chemotaxis protein. Gene 1992; 111:11-20.
2. Barton BM, Harding GP, Zuccarelli AJ. A general method for the detecting and sizing large plasmids. Anal Biochem 1995; 226:235-40.
3. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 2005; 63:219-28.
4. Cesareni G, Helmer-Citterich M, Castagnoli L. Control of ColE1 plasmid replication by antisense RNA. Trends Genet 1991; 7:230-5.
5. Ellington MJ, Kistler J, Livermore DM, Woodford N. Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases. J Antimicrob Chemother 2007; 59:321-2.
6. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Clin Microbiol Infect 2003; 9:1-7.
7. Halova D, Papousek I, Jamborova I, Masarikova M, Cizek A, Janecko N, Oravcova V, Zurek L, Clark AB, Townsend A, Ellis JC, Literak I. Plasmid-mediated quinolone resistance genes in *Enterobacteriaceae* from American crows: high prevalence of bacteria with variable *qnrB* genes. Antimicrob Agents Chemother 2014; 58:1257-8.
8. Hrabak J, Papagiannitsis CC, Studentova V, Jakubu V, Fridrichová M, Zemlickova H, Czech Participants of European Antimicrobial Resistance Surveillance Network. Carbapenemase-producing *Klebsiella pneumoniae* in the Czech Republic in 2011. Euro Surveill 2013; 18:pii=20626.
9. Izdebski R, Baraniak A, Herda M, Fiett J, Bonten MJ, Carmeli Y, Goossens H, Hryniewicz W, Brun-Buisson C, Gniadkowski M; MOSAR WP2, WP3 and WP5 Study Groups. MLST reveals potentially high-risk international clones of *Enterobacter cloacae*. J Antimicrob Chemother 2015; 70:48-56.
10. Lee K, Lim YS, Yong D, Yum JH, Chong Y. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-β-lactamase-producing isolates of *Pseudomonas spp.* and *Acinetobacter spp*. J Clin Microbiol 2003;41:4623-9.
11. Lombardi G, Luzzaro F, Docquier JD, Riccio ML, Perilli M, Coli A, Amicosante G, Rossolini GM, Toniolo A. Nosocomial infections caused by multidrug-resistant isolates of *Pseudomonas putida* producing VIM-1 metallo-beta-lactamase. J Clin Microbiol 2002; 40:4051-5.
12. Mikiewicz D, Wrobel B, Wegrzyn G, Plucienniczak A. Isolation and characterization of a ColE1-like plasmid from *Enterobacter agglomerans* with a novel variant of *rom* gene. Plasmid 1997; 38:210-9.
13. Miyoshi-Akiyama T, Hayakawa K, Ohmagari N, Shimojima M, Kirikae T. Multilocus sequence typing (MLST) for characterization of Enterobacter cloacae. PLoS One 2013; 8:e66358.
14. Nordmann P, Poirel L, Walsh TR, Livermore DM. The emerging NDM carbapenemases. Trends Microbiol 2011; 19:588-95.
15. Papousek I, Papagiannitsis CC, Medvecky M, Hrabak J, Dolejska M. Complete nucleotide sequences of two VIM-1-encoding plasmids from *Klebsiella pneumoniae* and *Leclercia adecarboxylata* isolates of Czech origin. Antimicrob Agents Chemother 2017; 61:pii=e02648-16.
16. Partridge SR, Hall RM. The IS*1111* family members IS*4321* and IS*5075* have subterminal inverted repeats of Tn*21* family transposons. J Bacteriol 2003; 185:6371-84.
17. Polisky B. ColE1 replication control circuitry: sense from antisense. Cell 1988; 55:929-32.
18. Rotova V, Papagiannitsis CC, Skalova A, Chudejova K, Hrabak J. Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity. J Microbiol Methods 2017; 137:30-3.
19. Skalova A, Chudejova K, Rotova V, Medvecky M, Studentova V, Chudackova E, Lavicka P, Bergerova T, Jakubu V, Zemlickova H, Papagiannitsis CC, Hrabak J. Molecular characterization of OXA-48-like-producing *Enterobacteriaceae* in the Czech Republic: evidence for horizontal transfer of pOXA-48-like plasmids. Antimicrob Agents Chemother 2017; 61:e01889-16.

20. Tomizawa JI, Ohmori H, Bird RE. Origin of replication of colicin E1 plasmid DNA. Proc Natl Acad Sci USA 1997; 74:1865-9.
21. Tzouvelekis LS, Markogiannakis A, Psichogiou M, Tassios PT, Daikos GL. Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: an evolving crisis of global dimensions. Clin Microbiol Rev 2012; 25:682-707.
22. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR. Characterization of a new metallo-β-lactamase gene, $bla_{\mathrm{NDM-1}}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob Agents Chemother 2009; 53:5046-54.

7.5 Insufficient repeatability and reproducibility of MALDI-TOF MS-based identification of MRSA

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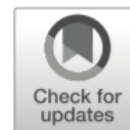
ORIGINAL ARTICLE



Insufficient repeatability and reproducibility of MALDI-TOF MS-based identification of MRSA

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Keywords: PSM, MRSA, MALDI-TOF MS

Running title: MRSA detection using MALDI-TOF MS

7.5.1 Abstract

Rapid identification of methicillin-resistant *Staphylococcus aureus* (MRSA) is essential for proper initial antibiotic therapy and timely set up of hygienic measures. Recently, detection of MRSA using MALDI-TOF mass spectrometer mediated by the peptide - phenol soluble modulin (PSM-mec), linked to the class A *mec* gene complex present in SCC*mec* cassettes types II, III, and VIII of MRSA strains, has been commercially available. We present here a multicentre study on MALDI-TOF MS detection of MRSA evincing a poor repeatability and reproducibility of the assay. The sensitivity of the assay varies between 50 and 90% in strains carrying psm_{MEC} and psm_{δ} genes encoding for PSM-mec and δ-toxin (a member of the PSM peptide family), respectively. No false positive results were found. The very major error calculation was 30% and the major error achieved 0%. Interlaboratory repeatability varies between 0 and 100%. No significant difference was observed with the use of different cultivation media. Our data showed a poor sensitivity of the method excluding it from the use in routine laboratory testing.

7.5.2 Introduction

Rapid and accurate test distinguishing methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) strains is essential for the appropriate treatment of infections caused by this pathogen, especially in life-threatening diseases, such as pneumonia or sepsis. Moreover, infections caused by MRSA significantly increase mortality and extra economic costs required for the therapy (deKraker et al. 2011; Marshall et al. 2004). Thus, a timely intervention in infection control is necessary (EUCAST 2017).

Resistance to methicillin/oxacillin in *Staphylococcus aureus* is due to an additional production of altered penicillin-binding proteins PBP2a or PBP2c with a low affinity for β-lactam antibiotics (Hanssen et al. 2006; Liu et al. 2016). Genes (*mecA*, *mecC*) encoding for those enzymes are carried on a genomic island Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (Ballhausen et al. 2014). The MRSA strains are resistant to all anti-staphylococcal β-lactams except novel cephalosporins such as ceftaroline, and ceftobiprole (Purello et al. 2016). The virulent potential of *S. aureus* differs significantly among various strains due to the structure of their genomes (e.g., mobile genetic elements – phages, plasmids, transposons). As well as SCC*mec* elements may contain other genes encoding for virulence factors (Novick et al. 2001; Rozgonyi et al. 2007). So far, only several different types and subtypes of variants of the SCC*mec* have been detected. The presence of the SCC elements is not specific only for MRSA clones but is also found in other strains carrying various genes, e.g., responsible for fusidic acid, capsule gene cluster, and a mercury resistance operon (Lakhundi and Zhang 2018). However, significant diversity of that genomic island has been observed between healthcare-associated (HA-MRSA) and community-associated (CA-MRSA) MRSA. HA-MRSA isolates predominantly carry types I, II and III SCC*mec*, and usually possess resistance to many classes of non–β-lactam antimicrobials. Whereas CA-MRSA isolates carry SCC*mec* type IV or type V that do not show any multidrug-resistant phenotype (Sunagar et al. 2016; Xie et al. 2016). Recently, a novel SSC*mec* XI element containing *mecC* gene, a homolog of *mecA* gene, was described (García-Álvarez et al. 2011; Shore et al. 2011). The *mecC* exhibits 70% identity to *mecA* gene on the nucleotide level and their PBP2c resp. PBP2a shares 63% identity at the amino acid level. Both proteins are responsible for the same level of resistance to methicillin/oxacillin (Ballhausen et al. 2014).

In the recent years, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) revolutionized the procedure of bacteria and yeasts identification in clinical microbiology (Fedorko et al. 2012; Lagacé-Wiens et al. 2012; Stevenson et al. 2010). This technology can also be used for other applications in microbiological laboratories, especially for detection of antibiotic resistance (Hrabak et al. 2013). Antibiotic resistance mechanisms can be detected by functional assays measuring enzymatic activity that leads to antibiotic resistance (i.e., modification of antibiotic molecule, alteration of target site), or by the detection of resistance-specific peaks from standard spectra used for taxonomic identification. The latter approach is the most promising as no further processing is required.

Several studies demonstrated the ability of MALDI-TOF MS to identify MRSA based on divergent spectral profiles (Du et al. 2002; Edwards-Jones et al. 2000; Josten et al. 2014). The first studies, published by Edwards-Jones et al. (2000), Walker et al. (2002) and Du et al. (2002) employed the protein/peptide fingerprinting to distinguish between MSSA and MRSA using different types of matrix and specimen preparation. However, any of the above-mentioned methods, do not detect any MRSA-specific proteins, such as PBP2a or its fragment, but are more or less clone-specific (Hrabák et al. 2013). Thus, they cannot be applied in routine microbiological laboratories.

Study conducted by Josten et al. in 2014 showed the first successful detection of some MRSA-specific proteins. The assay applied in Josten's study was based on the detection of a small peptide – phenol-soluble modulin (PSM-mec) that is encoded on SCC*mec* cassette II, III, and VIII, corresponding to the peak of 2415 *m/z* (Josten et al. 2014). MRSA usually produces another member of PSM family, the δ-toxin, a haemolysin disturbing the host cellular integrity, that is observed as the peak with *m/z* of 3007 in the MS spectra. The production of δ-toxin is regulated by accessory gene regulator *agr*. Production of δ-toxin, however, is not MRSA-specific, therefore it cannot be used solely for MRSA identification (Gagnaire et al. 2012). PSMs belong to the small-peptide-toxins, which have been considered as a crucial virulence determinant that promotes pro-inflammatory processes and lysis of human cells (Wang et al. 2007; Kretschmer 2010). Furthermore, some PSMs facilitate biofilm formation. The PSM-mec gene can also be found in methicillin-resistant *S. epidermidis* (MRSE) (Chatterjee et al. 2011).

Based on our laboratory experience, routine use of MALDI-TOF MS based analysis of the same MRSA strains obtained from the same or different cultures spotted multiple times on Maldi-target commonly yields various results, hence the MALDI-TOF MS based detection of MRSA expressed low reproducibility. Thus, we decided to perform a multicentre study using previously well-characterized isolates to determine intra- and interlaboratory reproducibility and repeatability of that assay.

7.5.3 Materials and Methods

Thirty-five previously characterized MRSA isolates were obtained from National Reference Laboratory (NRL) for Antibiotics, National Institute of Public Health, Prague, Czech Republic and University Hospital of Larissa, Larissa, Greece. All strains carrying SCC*mec* type I (n=7), type II (n=10), type III (n=10), type IV (n=6) and type V (n=2) were tested with MALDI-TOF MS using MBT Subtyping Software Module (Bruker Daltonics, Germany). All isolates were blindly processed in three laboratories (University Hospital in Pilsen, NRL for Antibiotics in Prague and University Hospital in Hradec Kralove) following a protocol recommended for routine identification of bacteria (Bruker Daltonics, Germany). Bacteria were cultivated on blood agar (OXOID, Czech Republic) and on MH agar with 4 % of NaCl at 37 °C overnight. The cell material was directly deposited on a target plate with a toothpick creating a confluent layer of bacteria by a technician experienced with MALDI-TOF MS-based identification. Each sample was overlaid with 1 µL of matrix consisting of α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile with 2.5 % trifluoroacetic acid (Bruker Daltonics, Germany; Merck, Czech Republic). The spectra were acquired using microFlex™ mass spectrometer (Bruker Daltonics, Germany) in the positive mode. Calibration was performed using Bacterial Test Standard (Bruker Daltonics, Germany) according to the manufacturer recommendation. Spectra were automatically interpreted by Compass software (4.1) with a licensed MBT Subtyping Module. The software allows automatic identification of respective peaks (PSM-mec and δ-toxin) based on pre-processing of the spectra and determination of significant peaks. The spectra were also manually analysed by two independent researchers using flexAnalysis software 3.4 (Bruker Daltonics, Germany) detecting the peaks of PSM-mec (2413 ± 3.00 *m/z*) and δ–toxin (3006 ± 3.00 *m/z*).

Each isolate from the same culture was processed in triplets. Spots with low identification scores were re-evaluated. For the determination of the method repeatability, six isolates of SCC*mec* I type (n=1), SCC*mec* II type (n=2) and SCC*mec* III type (n=3) were selected and subsequently tested in all three laboratory centres. Moreover, all spectra were analysed automatically as well as manually.

Sensitivity, specificity, reproducibility and repeatability of the method was determined according to Watson and Petrie (Watson et al. 2010). Very major error was calculated as the percentage of measurements giving negative results in MRSA strains to a total number of MRSA strains carrying SCC*mec* II and III.

7.5.4 Results and Discussion

In the first part of this study performed at the University Hospital in Pilsen, 35 isolates of *S. aureus* methicillin resistant (MRSA) were examined in triplicates to determine sensitivity and specificity of the assay. The results were confirmed as positive if at least one out of three spots was indicated as putative MRSA based on the presence of PSM-mec and δ-toxin. Compass software automatic analysis did not detect any false-positive results in any of the tested SCCmec groups. In the group possessing SCC*mec* type II, nine out of ten isolates were correctly identified as MRSA (90 % sensitivity). On the other hand, only 50% of strains harbouring SCC*mec* III cassette were correctly identified as MRSA.

The very major error was calculated to 30 %. Identical results were obtained by manual analysis by a researcher who was not acquainted with the results from the automatic measurement. The sensitivity and specificity results are summarized in Table 1.

Additionally, to evaluate the repeatability of the assay across the centres, six selected strains were analysed in 12 replicates. The repeatability of the method for PSMs varies between 0 % and 100 % (see Table 2). Better results were achieved with δ-toxin in all of the centres (especially in SCC-mec II and III) (see Table 2). These results were expected, as δ-toxin provides strong signal in MALDI-TOF MS spectra, however, this is not MRSA specific. δ-toxin has been described as epidemiological marker by many authors, e.g. Gagnaire et al. (2012) and Otto (2014) and its production has been linked to prediction of virulence potential of the *Staphylococcus aureus* strains and the function of the accessory gene regulator A [249] that is defective in glycopeptide intermediate

sensitive strains (GISA, hGISA). However, function of this specific toxin as a marker determining the virulence should be further examined (Otto 2014).

All false negative samples automatically evaluated with MBT Subtyping Software Module did not contain PSM-mec specific peak that would be detectable even with manual analysis (Figure 1).

The expression of *psm* can be regulated in various ways to produce very small amount of the protein (Otto 2014). Thus, different types of cultivation media were tested, particularly MH Agar with 4 % NaCl. Addition of NaCl is commonly used in selective media to increase sensitivity for MRSA identification. However, there was no difference in subsequent identification of MRSA strains grown on various cultivation media (data not published). This finding suggests that the poor reproducibility is unlikely due to different expression of psm_{mec}. After manual interpretation of the spectra, we found that the peak of PSM-mec may be suppressed by other peaks with high intensity in the rich area (*m/z* between 3 000 and 10 000) as indicated by higher sensitivity for detection of δ-toxin. In this case, the desired information could not be extracted from the raw spectra used for taxonomic identification in routine laboratories. Hence, to increase the sensitivity of the method, the measurement parameters require optimisation for lower molecular mass.

Although the MALDI-TOF MS-based method for MRSA detection seemed promising (Josten et al. 2014; Jang et al. 2017; Rhoads et al. 2016; Schuster et al. 2018) as no specific sample preparation is required, and the peaks can be identified in the spectra during routine bacterial identification there are some drawbacks excluding this method from the use in routine laboratory. The main disadvantage is represented by the disability to detect MRSA strains possessing other SCC*mec* types. In the areas with occurrence of strains carrying this specific SCC*mec* type, the assay could help with rapid detection of MRSA. Recently, more studies have been published focusing on the development of assay for differentiation and typing of HA-MRSA from CA-MRSA based on the identification of molecular determinants (Jakckson et al. 2005; Majcherczyk 2006; Shah et al. 2011; Wolters et al. 2011).

7.5.5 Conclusions

Our results showed poor robustness of the method. Based on the data, even though no false-positive results were detected, the method is not suitable for the use in routine laboratory as IVD. Reliable detection of MRSA using MALDI-TOF MS still remains a challenge for clinical microbiology.

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References

Ballhausen B, Kriegeskorte A, Schleimer N, Peters G, Becker K (2014) The mecA homolog mecC confers resistance against β-lactams in Staphylococcus aureus irrespective of the genetic strain background. Antimicrob Agents Chemother 58: 3791-3798.

Chatterjee SS, Chen L, Joo HS, Cheung GYC, Kreiswirth BN, Otto M (2011) Distribution and regulation of the mobile genetic element-encoded phenol-soluble modulin PSM-mec in methicillin-resistant *Staphylococcus aureus*. PLoS ONE 6: e28781.

Du Z, Yang R, Guo Z, Song Y, Wang J (2002) Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Chem 74: 5487-5491.

Edwards-Jones V, Claydon MA, Evason DJ, Walker J, Fox AJ, Gordon DB (2000) Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. J Med Microbiol 49: 295-300.

European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2017) EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance, Version 2.0. Available at http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_170711.pdf

Fedorko DP, Stock F, Murray PR, Drake SK (2012) Identification of clinical isolates of anaerobic bacteria using matrix-assisted laser desorption ionization-time of flight mass spectrometry. Eur. J. Clin Microbiol Infect Dis 31: 2257-2262.

García-Álvarez L, Holden MTG, Lindsay H, Webb CR, Brown DFJ, Curran MD, Walpole E, Brooks K, Pickard DJ, Teale C, Parkhill J, Bentley SD, Edwards GF, Girvan EK, Kearns AM, Pichon B, Hill RLR, Larsen AR, Skov RL, Peacock SJ, Maskell DJ, Holmes MA (2011) Meticillin-resistant *Staphylococcus aureus* with a novel mecA homologue in human and bovine populations in the UK and Denmark: A descriptive study. Lancet Infect Dis 11: 595-603.

Gagnaire J, Dauwalder O, Boisset S, Khau D, Freydière AM, Ader F, Bes M, Lina G, Tristan A, Reverdy ME, Marchand A, Geissmann T, Benito Y, Durand G, Charrier JP, Etienne J, Welker M, Van Belkum A, Vandenesch F (2012) Detection of Staphylococcus aureus delta-toxin production by whole-cell MALDI-TOF mass spectrometry. PLoS One 7: e40660.

Hanssen AM, Ericson Sollid JU (2006) SCCmec in staphylococci: Genes on the move. FEMS Immunol Med Microbiol 46: 8-20.

Hrabák J, Chudacková E, Walková R (2013) Matrix-assisted laser desorption ionization-time of flight (MALDITOF) mass spectrometry for detection of antibiotic resistance mechanisms: From research to routine diagnosis. Clin Microbiol Rev 26: 103-114.

Jackson KA, Edwards-Jones V, Sutton CW, Fox AJ (2005) Optimisation of intact cell MALDI method for fingerprinting of methicillin-resistant *Staphylococcus aureus*. J Microbiol Methods 62: 273-284.

Jang KS, Park M, Lee JY, Kim JS (2017) Mass spectrometric identification of phenol-soluble modulins in the ATCC® 43300 standard strain of methicillin-resistant *Staphylococcus aureus* harboring two distinct phenotypes. Eur J Clin Microbiol Infect Dis 36: 1151-1157.

Josten M, Dischinger J, Szekat C, Reif M, Al-Sabti N, Sahl HG, Parcina M, Bekeredjian-Ding I, Bierbaum G (2014) Identification of agr-positive methicillin-resistant *Staphylococcus aureus* harbouring the class A mec complex by MALDI-TOF mass spectrometry. Int J Med Microbiol 304: 1018-1023.

de Kraker MEA, Davey PG, Grundmann H (2011) Mortality and hospital stay associated with resistant *Staphylococcus aureus* and Escherichia coli bacteremia: Estimating the burden of antibiotic resistance in Europe. PLoS Med 8: e1001104 .

Kretschmer D, Gleske AK, Rautenberg M, Wang R, Köberle M, Bohn E, Schöneberg T, Rabiet MJ, Boulay F, Klebanoff SJ, Van Kessel KA, Van Strijp JA, Otto M, Peschel A (2010) Human formyl peptide receptor 2 senses highly pathogenic *Staphylococcus aureus*. Cell Host Microb 7: 463-473.

Lagacé-Wiens PRS, Adam HJ, Karlowsky JA, Nichol KA, Pang PF, Guenther J, Webb AA, Miller C, Alfa MJ (2012) Identification of blood culture isolates directly from positive blood cultures by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry and a commercial extraction system: Analysis of performance, cost, and turnaround time. J Clin Microbiol 50: 3324-3328.

Lakhundi S, Zhang K (2018) Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology. Clin Microbiol Rev 12: pii=e00020-18

Liu J, Chen D, Peters BM, Li L, Li B, Xu Z, Shirliff ME (2016) Staphylococcal chromosomal cassettes mec (SCCmec): A mobile genetic element in methicillin-resistant *Staphylococcus aureus*. Microb Pathog 101: 56-67.

Majcherczyk PA, McKenna T, Moreillon P, Vaudaux P (2006) The discriminatory power of MALDI-TOF mass spectrometry to differentiate between isogenic teicoplanin-susceptible and

teicoplanin-resistant strains of methicillin-resistant *Staphylococcus aureus*. FEMS Microbiol Lett 255: 233-239.

Marshall C, Kossmann T, Wesselingh S, Spelman D (2004) Methicillin-resistant Staphylococcus aureus and beyond: What's new in the world of the 'golden staph'? ANZ J Surg 74: 465-469.

Novick RP, Schlievert P, Ruzin A (2001) Pathogenicity and resistance islands of staphylococci. Microbes Infect 3: 585-594.

Otto M (2014) Phenol-soluble modulins. Int J Med Microbiol 304: 164-169.

Purrello SM, Garau J, Giamarellos E, Mazzei T, Pea F, Soriano A, Stefani S (2016) Methicillin-resistant *Staphylococcus aureus* infections: A review of the currently available treatment options. J Glob Antimicrob Resist 7: 178-186.

Rhoads DD, Wang H, Karichu J, Richter SS (2016) The presence of a single MALDI-TOF mass spectral peak predicts methicillin resistance in staphylococci. Diag Microbiol Infect Dis 86: 257-261.

Rozgonyi F, Kocsis E, Kristóf K, Nagy K (2007) Is MRSA more virulent than MSSA? Clin Microbiol Infect 13: 843-845.

Schuster D, Josten M, Janssen K, Bodenstein I, Albert C, Schallenberg A, Gajdiss M, Sib E, Szekat C, Kehl K, Parčina M, Hischebeth GTR, Bierbaum G (2018) Detection of methicillin-resistant coagulase-negative staphylococci harboring the class A mec complex by MALDI-TOF mass spectrometry. Int J Med Microbiol 308: 522-526.

Shah HN, Rajakaruna L, Ball G, Misra R, Al-Shahib A, Fang M, Gharbia SE (2011) Tracing the transition of methicillin resistance in sub-populations of *Staphylococcus aureus*, using SELDI-TOF Mass Spectrometry and Artificial Neural Network Analysis. Syst Appl Microbiol 34: 81-86.

Shore AC, Deasy EC, Slickers P, Brennan G, O'Connell B, Monecke S, Ehricht R, Coleman DC (2011) Detection of staphylococcal cassette chromosome mec type XI carrying highly divergent mecA, mecI, mecR1, blaZ, and ccr genes in human clinical isolates of clonal complex 130 methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 55: 3765-3773.

Stevenson LG, Drake SK, Shea YR, Zelazny AM, Murray PR (2010) Evaluation of matrix-assisted laser desorption ionization - Time of flight mass spectrometry for identification of clinically important yeast species. J Clin Microbiol 48: 3482-3486.

Sunagar R, Hegde NR, Archana GJ, Sinha AY, Nagamani K, Isloor S (2016) Prevalence and genotype distribution of methicillin-resistant Staphylococcus aureus (MRSA) in India. J Glob Antimicrob Resist 7: 46-52.

Walker J, Fox AJ, Edwards-Jones V, Gordon DB (2002) Intact cell mass spectrometry (ICMS) used to type methicillin-resistant *Staphylococcus aureus*: Media effects and inter-laboratory reproducibility. J Microbiol Methods 48: 117-126.

Wang R, Braughton KR, Kretschmer D, Bach THL, Queck SY, Li M, Kennedy AD, Dorward DW, Klebanoff SJ, Peschel A, DeLeo FR, Otto M (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med 13: 1510-1514.

Watson PF, Petrie A (2010) Method agreement analysis: A review of correct methodology. Theriogenology 73: 1167-1179.

Wolters M, Rohde H, Maier T, Belmar-Campos C, Franke G, Scherpe S, Aepfelbacher M, Christner M (2011) MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. Int J Med Microbiol 301: 64-68.

Xie X, Bao Y, Ouyang N, Dai X, Pan K, Chen B, Deng Y, Wu X, Xu F, Li H, Huang S (2016) Molecular epidemiology and characteristic of virulence gene of community-acquired and hospital-acquired methicillin-resistant *Staphylococcus aureus* isolates in Sun Yat-sen Memorial hospital, Guangzhou, Southern China. BMC Infect Dis 16: 339.

Table 4. Detection of MRSA by MALDI-TOF mass spectrometry in strains possessing different SCCmec cassettes. SCCmec I, IV, V – do not produce PSM-mec peptide, thus they cannot be identified as MRSA using mentioned method. SCCmec II and III produce PSM-mec and δ-toxin, therefore they should be indicated as putative MRSA. All strains were processed in triplicates. The peaks were automatically identified by Compass software (4.1) with a licensed MBT Subtyping Module and in case of positivity determined as "putative MRSA".

SCCmec	No. of isolates	Identified as MRSA	Identified as MSSA	Sensitivity (%)	Specificity (%)
I	7	0	7	100	100
II	10	9	1	90	100
III	10	5	5	50	100
IV	6	0	6	100	100
V	2	0	2	100	100

Table 5. Interlaboratory reproducibility of MALDI-TOF MS-based MRSA identification using detection of PSM-mec (2413 ± 3.00 m/z) and δ-toxin (3006 ± 3.00 m/z) measured in three centers. The peaks were manually identified in mass spectra.

Isolate	SCC*mec*	PSM-mec			δ-toxin		
		Pilsen (%)	Prague (%)	Hradec Kralove (%)	Pilsen (%)	Prague (%)	Hradec Kralove (%)
2388	I	0	0	0	83	100	8
1764	II	0	92	100	100	100	100
1840	II	0	0	33	92	16	33
1087	III	92	100	75	100	100	42
1287	III	33	0	8	58	100	41
2783	III	50	0	0	58	100	41

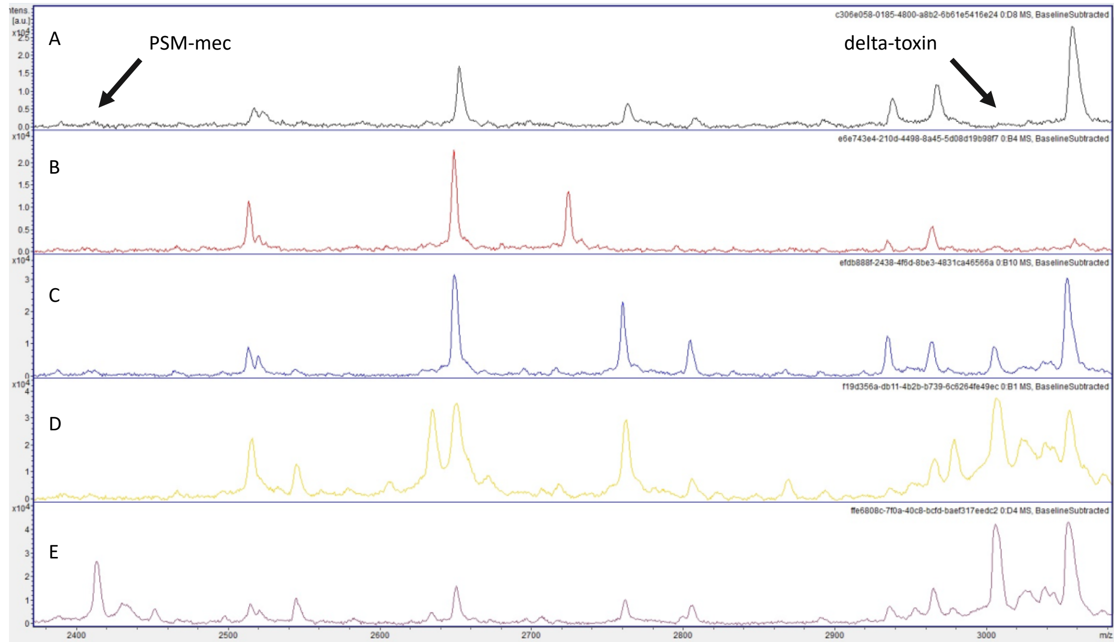

Figure 1. Mass spectra acquired by automatic measurement. A, B – strains expressing no PSM-mec neither δ-toxin; C, D – strains expressing δ-toxin only; E – strain identified as MRSA showing both proteins (PSM-mec, δ-toxin).

8. Discussion

The publications are focused on the early detection of infectious disease agents and their antibiotic resistance. Each of the publications is concentrated on different problematic areas, from the development and validation of a method for the detection of carbapenemase activity, molecular-epidemiological characterization of carbapenemase positive isolates detected in the Czech Republic, to the validation of commercial automatic program on MALDI-TOF MS-based identification of MRSA.

8.1 Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity

The aim of the recent study was a comparison of the efficiency of imipenem and meropenem hydrolysis assay for the detection of carbapenemase-producing *Pseudomonas aeruginosa* and *Enterobacterales* by MALDI-TOF mass spectrometer.

In the study, 250 *P. aeruginosa* isolates with carbapenemase activity were tested including 97 IMP, 41 VIM, and 4 GES. The remaining 108 isolates were non-carbapenemase producers. Additionally, 83 isolates with carbapenemase activity from *Enterobacteriacea* family were tested. The group included 21 KPC, 18 VIM, 1 IMP, 24 NDM, and 19 OXA-48 carbapenemases and 41 non-carbapenemase producers.

The preparation of all isolates was identical for both species of bacteria. The composition of reaction buffer of carbapenems differed and thus different matrices had to be used for detection on MALDI-TOF MS.

The analysis spectra were measured within the m/z range 300 to 600. For the imipenem hydrolysis assay, a negative result was defined as the presence of the imipenem peak at 300 m/z, indicating that bacteria with no carbapenemase activity was measured. A positive result was defined as the complete disappearance of the imipenem peak indicating the presence of carbapenemase.[285] For the meropenem hydrolysis assay, non-carbapenemase producers were detected with the presence of meropenem and its sodium salt (384.5 m/z and 406.5 m/z peaks), while in cases of carbapenemase producers at least one of the decarboxylated products of meropenem was observed (358.5 m/z and 380.5 m/z peaks).[286]

The validation supported the high efficiency of both assays. For *Enterobacterales* isolates, both assays showed high sensitivity (99 % with the meropenem assay and 96 % with the imipenem assay) and 100 % specificity. For *P. aeruginosa* isolates, the sensitivity of the meropenem assay was lower than the imipenem assay with 90% vs. 97%, respectively. The specificity was also lower with the meropenem assay. Therefore, the meropenem assay is more suitable for analysis of *Enterobacterales* family, while the imipenem is more effective with *P. aeruginosa*. It has been shown that the addition of NH_4HCO_3 to the reaction buffer is essential for the detection of OXA-48 carbapenemases in the meropenem assay, while for the imipenem assay, the addition of bicarbonate was not required, though the addition of $ZnSO_4$ was necessary for higher sensitivity of the detection of VIM carbapenemases. These findings suggest that different strategies must be used to detect cabapenemase activity in different types of pathogens.

8.2 The first detection of IMI-2-producing *Enterobacter asburiæ* in the Czech Republic

IMI carbapenemases, named after their ability to hydrolyze imipenem, are Ambler class A enzymes. IMI was first identified on an *Enterobacter cloacae* isolate from the USA in 1984, and subsequently, small numbers of IMI-positive isolates in some states of Asia, Europe, and the USA have been identified.

In 2016, the first case of IMI-2-producing *Enterobacter asburiae*, sample Easb-36567cz, was identified in Czechia. Plasmid analysis has estimated the IncFII plasmid as approximately 80 kb in size. The plasmid showed a high degree of similarity to the pJF-787 plasmid, previously found in a *Klebsiella variicola* strain from the United Kingdom. The plasmid was mainly composed of regions responsible for replication, conjugative transfer and maintenance genes for the plasmid. The LysR-type transcriptional

regulator, IS*Ec36* element and other mobile elements IS*1,* Tn-3-like transposase, and IS*Eae1* were also found.

Easb-36567cz was isolated from a patient with no previous hospitalization and without travel history. This suggests the spread of carbapenemase genes by pathways that remain largely unknown. Moreover, the bla_{IMI-2} gene was placed in the conjugative plasmid linked to mobile genetic elements. This type of resistence mechanism has the potential to spread between strains of *Enterobacteriacea* family bacteria. The proliferation of these resistence genes is crucial to monitor because the bacteria can act as hidden sources of clinically important resistance determinants.

8.3 The dissemination of NDM-like producing *Enterobacteriaceæ* in the Czech Republic

Acquired NDM metallo-β-lactamases are emerging resistance determinants in clinically relevant Gram-negative species. The first case of NDM-1 has been described from *Klebsiella pneumoniae* and *Escherichia coli* isolated in Sweden in 2008 from patient transferred from a New-Delhi hospital. Since then, the bla_{NDM-1} gene has been widely identified in *Enterobacterales* and *Acinetobacter baumannii* from India to various countries worldwide.

The aim of the present study was to characterize NDM carbapenemases isolated from *Enterobacterales* during an outbreak in 2016. Until then, the occurrence of NDM carbapenemases was rare, with only three sporadic cases recorded from 2011 to 2013. During 2016, an increased number of NDM-producing *Enterobacterales* were observed in several Czech hospitals.

In the same year, 18 NDM-producing *Enterobacterales* were isolated from 15 patients hospitalized in 5 different Czech hospitals (B1, B2, A1, A2, and C) in three cities. 9 isolates of *Enterobacter cloacae* complex, four of *Escherichia coli*, and one of each species of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter asburiae*, *Enterobacter intermedius*, and *Raoultella ornithinolytica* were identified. In addition, three of the patients were infected by two different pathogens. For comparison, one NDM-4 producing *Enterobacter* isolate (Encl-922) was identified in 2012 from a patient repatriated from Sri Lanka. In 2016 an outbreak was reported in one hospital (B1) and four sporadic cases were discovered in four different hospitals. None of the patients treated in hospital B1 had been previously hospitalized or traveled abroad. Only one patient was repatriated from a hospital in China, while for the remaining patients the clinical data were not available.

All twelve isolates (nine of *E. cloacae*, one *E. asburiae,* one *E. intermedia*, and one *E. coli*) obtained from hospital B1 during the outbreak were identified as NDM-4 carbapenemase producers. It is noteworthy, that the study by MLST has shown that all *E. cloacae* isolates belong to ST182 as well as the *Enterobacter* isolate from 2012. *Enterobacter* ST182 isolates associated with the production of NDM-1 carbapenemase were recently found in Mexico.[287] Plasmid analysis of all NDM-4 producing transconjugants carried the IncX3 plasmid, roughly 55kb in size. Comparative analysis of genomes of *Enterobacter* isolates - the isolate from 2012 (Encl-922) and the isolate from the 2016 outbreak (Encl-44578) was performed. The analysis exhibited high similarity of their genomes. However, isolate Encl-922 had an additional four insertion sequences in contrast with the genome of the Encl-44578 isolate. This finding indicates that *Enterobacter* isolates from 2016 differed from Encl-922.

From hospital B2 three isolates (two *E. coli*, and one *K. oxytoca*) were obtained with expressed NDM-5 carbapenemase from two patients. Both *E. coli* isolates belong to ST167 and have been repeatedly reported in China.[288, 289] The last isolate, *K. oxytoca* was assigned to a growing international clonal complex ST2.[290] All three NDM-producing transformants harbored plasmids of 45 kb size and were positive for the IncX3 allele.

The last remaining isolates (*E. coli*, *K. pneumoniæ*, and *R. ornithinolytica*) that were recovered from sporadic cases in three different hospitals expressed NDM-1 carbapenemase. The *E. coli* isolate included a 300kb bla_{NDM-1}-positive plasmid, a fusion derivative of IncA/C and IncR replicons. The *K. pneumoniae* isolate carried a 161 kb bla_{NDM-1}-positive plasmid exhibiting high similarity to the IncFIB plasmid and the mosaic region of pS-3002cz from an ST11 *K. pneumoniae* from Czechia.[291] And finally, *R. ornithinolytica* isolate was positive for the IncX3 plasmid, 55 kb in size.

Two patients from hospitals B1 and B2 have been shown to have two distinct NDM-producing strains, suggesting a horizontal gene transfer mechanism. These findings indicate that NDM like producers pose an important public threat, mainly due to the rapid horizontal transfer of IncX3 bla_{NDM}-carrying plasmids and due to the further evolution of NDM like encoding MDR plasmids by reshuffling.

8.4 Characterization of pEncl-30969cz, a novel ColE1-like plasmid encoding VIM-1 carbapenemase, from an *Enterobacter cloacae* sequence type 92 isolate

VIM-1carbapenemase has been frequently detected among *Enterobacteriacea*, in Europe. However, the isolation frequency of *Enterobacteriacea* producing VIM-carbapenemases has been rare with just a few sporadic cases detected in the Czech Republic.[292, 293] The aim of the study was to report a case of a VIM-1-producing *Enterobacter cloacae* isolate (Encl-30969cz) of Czech origin with a description of the sequences of pEncl-30969cz novel VIM-1-encoding plasmid.

The isolate was collected from a patient with chronic pulmonary disease, who was treated in a Czech hospital in 2015. The MLST analysis classified the isolate into ST92, which was originally identified in *E. coli* producing CTX-M-15 β-lactamase in Israel in 2015.[294] Plasmid analysis of VIM-producing transformant pEncl-30969 demonstrated a size of 33 kb. The plasmid contains a sequence of approximately 2,5 bp, which shares extensive similarity with the backbone of the ColE1-like plasmid pPIGM1.[295] The plasmid replication region included regions with RNA transcripts for control of the initiation of DNA replication and the copy number of the plasmid.[296] In addition, an open reading frame (ORF) encoding a Rom protein was present, which enhances the interaction of the RNA I inhibitor with its target, resulting in a reduction in the frequency of replication initiation.[297] In the remaining part of the plasmid a Tn*1721*-like transposon was identified, containing integron In*110*,[298] whose variable region comprised the bla_{VIM-1}, *aacA4*, and *aadA1* cassettes. Downstream of *orf5*, a Tn*21* fragment was found consisting of IS*1326*, *tniB*Δ1, *tniA* and part of the *mer* operon (Δ*merE*). In addition, Tn*3*-like transposon consisting of IRs of the transposon was found. Two copies of an IS*5075* element, which was shown previously to target the IRs of Tn*21*-like transposons[299] disrupted the IRs of the Tn*3*-like transposon. Next, a segment comprising two ORFs, a *qnrB*-like gene with resistance to quinolones,[300] and a truncated ORF was identified. The second part of Tn*1721*-like transposon[301] comprising the IR of the transposon and a truncated *orf1* that missed 889 bp of its 5´end due to insertion of an IS*26*, was found at the boundary of the plasmid backbone.

Plasmid analysis suggests that pEncl-30969cz could be developed by obtaining a Tn*1721*-like transposon carrying the integron In*110* encoding VIM-1 with a ColE1-type plasmid. This finding highlights the important role of mobile genetic elements in the spread of resistance determinants such as bla_{VIM-1}.

8.5 Insufficient repeatability and reproducibility of MALDI-TOF MS-based identification of MRSA

The spread of methicillin-resistant *Staphylococcus aureus* (MRSA) strains in hospitals and other health care systems remains an important issue worldwide. Therefore, rapid identification of MRSA is essential for proper antibiotic treatment and timely adjustment of hygienic measures. At present, many methods for MRSA identification are available. In our study, we target a rapid detection method of MRSA based on the specific phenol soluble modulin-mec peak (PSM-mec) detection by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The PSM-mec peptide is encoded by staphylococcal cassette chromosome mec (SCCmec) types II, III, and VIII.[302] These types of genomic islands have been observed mainly in healthcare-associated MRSA (HA-MRSA) in contrast with community-associated MRSA (CA-MRSA). CA-MRSA isolates carry SCC*mec* type IV or V, that do not show any multi-drug resistant phenotype.[303, 304] In addition, some types of MRSA produce another member of the PMS family, the δ-toxin, a hemolysin that disturbs the integrity of the host cell. Therefore, δ-toxin can not be used solely for MRSA identification.[305] MALDI-TOF MS should be able to distinguish between MSSA and MRSA isolates based on divergent peptide/protein fingerprinting.

In the current study, we intended to evaluate the sensitivity, specificity, reproducibility and repeatability of the MALDI-TOF MS method thirty-five previously characterized MRSA isolates. All strains carrying SCC*mec* type I (n=7), type II (n=10), type (n=10), type V (n=2) were tested with MALDI-TOF MS using MBT Subtyping Software Module (Bruker Daltonics, Germany). All isolates were blindly processed in three laboratories (University Hospital in Pilsen, NRL for antibiotics in Prague and University Hospital in Hradec Kralove). Spectra were automatically interpreted by Compass software (4.1) with a licensed MBT Subtyping Module. The software allows automatic identification of respective peaks (PSM-mec and δ-toxin). The spectra were also manually analyzed for detection of the peaks of PSM-mec (2413 ± 3) and δ-toxin (3006 ± 3) by two independent researchers.

First, MRSA were examined in triplicates to determine the sensitivity and specificity of the assay. The automatic analysis did not detect any false-positive results in any of the tested SCC*mec* types. SCC*mec* type II allowed 90% sensitivity, while SCCmec III was limited to a lower sensitivity (50 %). All SCC*mec* types showed 100% specificity. The repeatability of the method varies between 0% and 100%. For the reproducibility test, different types of cultivation media were used due to the observation of expression of PSM. The test does not prove a difference in subsequent identification of MRSA isolates. This finding suggests that poor reproducibility is unlikely due to differential psm_{mec} expression. Based on the data, the method is not suitable for routine use in the laboratories.

9. Conclusion

This thesis briefly summarizes our understanding of the history not only of the development of new β-lactam antibiotics but also of a complex family of enzymes, evaluates the mechanisms by which these hydrolases inactivate β-lactam antibiotics, and highlights current challenges to β-lactam efficacy, notably, carbapenemases. The most effective carbapenemases, in terms of carbapenem hydrolysis and geographical spread are KPC, VIM, IMP, NDM and OXA-48 types. Carbapenem resistance in Gram-negative pathogens has dramatically limited treatment options while Gram-positive bacteria have still reliable alternatives to carbapenem (e.g. glycopeptides, daptomycin). Carbapenemase-producing Gram-negatives, in particular, are resistant to all or almost all β-lactams, while commonly carrying at the same time genes encoding for other resistance mechanisms. Therefore, older agents, such as polymyxins and fosfomycin, which were rarely implemented in the past because of efficacy and/ or toxicity concerns, together with the newer tigecycline, have become last-resort choices.

Currently, we are witnessing the constant development of substrate specificity corresponding to each newly introduced β-lactam antibiotics. The long history and extensive literature of β-lactamase research might suggest a mature field lacking substance for discovery science. We would instead argue that technological advances, enzymes recently identified through the explosion in microbial sequencing information, and the response of the bacterial population to the selection pressure imposed by traditional and novel β-lactam based therapies, together create rich opportunities both for fundamental biochemistry research and innovative drug discovery that are driven by the pressing need to overcome antimicrobial resistance. The future of research in this problematic area needs to be open to new approaches and especially to new technologies.

10. Attachements

10.1 Curriculum vitæ

Name Maiden name	Veronika Pašková, Ing (M.Sc.) Rotová
Date & Place of Birth	17th June, 1990, Pilsen, Czech Republic
Address	Únětice 50, 336 01 Blovice
Sex	Woman
Position	2016-2019 Ph.D. student – Department of Clinical Microbiology, Biomedical Center, Faculty of Medicine in Pilsen, Charles University
Work Address and Contacts	Alej Svobody 80, 323 00, Pilsen, Czech Republic
Education	2009–2015 Faculty of Chemical Technology,University of chemistry and technology Prague; specialization - Drugs manufacturing 2000–2008 grammar school, Gymnázium Blovice
Language Skills	Czech – mother tongue English – advanced German – basic Spanish – basic
Technical Skills	Microbiology: Experienced in Microbiology techniques. Isolation, identification and characterization of bacteria (mainly *Enterobacteriaceæ*, *Pseudomonas* spp.); experienced in Clinical microbiology, especially in bacteriology and molecular-genetic diagnostics, molecular epidemiology. Molecular Biology: Experienced in molecular biology techniques, e.g., real time quantitative PCR, RT-PCR and related molecular biology works based on DNA amplification, DNA sequencing, southern blotting, hybridization, transformation, conjugation, PFGE, MLST. Protein Analysis: Isoelectric focusing, MALDI-TOF mass spectrometry techniques.
H-Index	4+1 (23.6.2020, Scopus)
Sum of Times Cited without Self-Citations	61 (23.6.2020, Scopus)
Number of Publications in Journals with IF	9
Publications	See list of publications

Professional experiences	Posters (Abstracts in Proceedings): European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2018 ➢ Poster – Characterization of NDM-like-producing *Enterobacteriaceæ* isolated in Czech hospitals (Paskova V., Bitar I., Medvecky M., Skalova A., Chudejova K., Jakubu V., Bergerova T., Zemlickova H., Papagiannitsis CC, Hrabak J.) European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2017 ➢ ePoster – Evaluation and validation of HRC assay for detection and confirmation of carbapenemases in *Enterobacteriaceæ* (Rotova V., Skalova A., Chudejova K., Papagiannitsis C. C., Hrabak J.) ➢ Poster – Molecular characterization of MBL-producing *Pseudomonas æruginosa* isolates in Czech hospitals (Papagiannitsis C. C., Chudejova K., Medvecky M., Skalova A., Rotova V., Jakubu V., Zemlickova H., Hrabak J.) ➢ Poster – Automatic deposition of bacteria and yeast on MALDI target using MALDI Colonyst robot (Hrabak J., Chudejova K., Rotova V., Papagiannitsis C. C., Bohac M., Skalova A., Bergerova T.) European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2016 ➢ ePoster – Molecular epidemiological analysis of OXA-48 producing *Enterobacteriaceæ* in the Czech Republic with an evidence of horizontal gene transfer (Skalova A., Chudejova K., Rotova V., Bergerova T., Jakubu V., Zemlickova H., Papagiannitsis C. C., Hrabak J.)

10.2 List of publications

Publications	Impact factor (Web of science)	Citations (Web of Science, 23.6.2020)	Citations (Scopus, 23.6.2020)
Skalova A, Chudejova K, Rotova V, Medvecky M, Studentova V, Chudackova E, Lavicka P, Bergerova T, Jakubu V, Zemlickova H, Papagiannitsis CC, Hrabak J. Molecular characterization of OXA-48-like-producing Enterobacteriaceæ in the Czech Republic and evidence for horizontal transfer of pOXA-48-like plasmids. Antimicrobial Agents and Chemotherapy. 2017 Feb 1;61(2):e01889-16.	4.302	27	31
Rotova V, Papagiannitsis CC, Skalova A, Chudejova K, Hrabak J. Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity. Journal of Microbiological Methods. 2017 Jun 1;137:30-3.	1.79	18	18
Chudejova K, Bohac M, Skalova A, Rotova V, Papagiannitsis CC, Hanzlickova J, Bergerova T, Hrabak J. Validation of a novel automatic deposition of bacteria and yeasts on MALDI target for MALDI-TOF MS-based identification using MALDI Colonyst robot. PloS one. 2017 Dec 29;12(12):e0190038.	2.806	3	4
Papagiannitsis CC, Medvecky M, Chudejova K, Skalova A, Rotova V, Spanelova P, Jakubu V, Zemlickova H, Hrabak J. Molecular Characterization of Carbapenemase-Producing Pseudomonas æruginosa of Czech Origin and Evidence for Clonal Spread of Extensively Resistant Sequence Type 357 Expressing IMP-7 Metallo-β-Lactamase. Antimicrobial Agents and Chemotherapy. 2017 Dec 1;61(12):e01811-17.	4.302	7	8
Rotova V, Papagiannitsis CC, Chudejova K, Medvecky M, Skalova A, Adamkova V, Hrabak J. First description of the emergence of Enterobacter asburiæ producing IMI-2 carbapenemase in the Czech Republic. Journal of Global Antimicrobial Resistance. 2017 Oct 10;11:98.	1.276	4	4
Chudejova K, Rotova V, Skalova A, Medvecky M, Adamkova V, Papagiannitsis CC, Hrabak J. Emergence of sequence type 252 Enterobacter cloacæ producing GES-5 carbapenemase in a Czech hospital. Diagnostic Microbiology and Infectious Disease. 2018 Feb 1;90(2):148-50.	2.401	0	1
Paskova V, Medvecky M, Skalova A, Chudejova K, Bitar I, Jakubu V, Bergerova T, Zemlickova H, Papagiannitsis CC, Hrabak J. Characterization of NDM-encoding plasmids from Enterobacteriaceæ recovered from Czech hospitals. Frontiers in Microbiology.2018; 9, JUL, 1549.	4.076	18	19
Papagiannitsis CC, Paskova V, Chudejova K, Medvecky M, Bitar I, Jakubu V, Zemlickova H, Jirsa R, Hrabak J. Characterization of pEncl-30969cz, a novel ColE1-like plasmid encoding VIM-1 carbapenemase, from an Enterobacter cloacae sequence type 92 isolate. Diagnostic Microbiology and Infectious Disease. 2018; 91(2), pp. 191-193	2.401	0	0
Paskova V, Chudejova K, Sramkova A, Kraftova L, Jakubu V, Petinaki EA, Zemlickova H, Neradova K, Papagiannitsis CC, Hrabak J. Insufficient repeatability and reproducibility of maldi tof ms based identification of MRSA. Folia Microbiologica. 2020, 65, Jul	1,730	0	0

11. References

1. Giguère, S.; Katzung, B. G., *Basic and clinical pharmacology* 11 edition ed.; McGraw-Hill Medical: New York, 2011.
2. Shaw, D. H.; Ihle, S. L., β-Lactam Antimicrobials. In *Small animal Critical Care Medicine*, Wiley-Blackwell: 2014.
3. Dalhoff, A.; Thomson, C. J., The art of fusion: From penams and cephems to penems. *Chemotherapy* **2003,** *49* (3), 105-120.
4. Sauvage, E.; Kerff, F.; Terrak, M.; Ayala, J. A.; Charlier, P., The penicillin-binding proteins: Structure and role in peptidoglycan biosynthesis. *FEMS Microbiology Reviews* **2008,** *32* (2), 234-258.
5. Drawz, S. M.; Bonomo, R. A., Three Decades of beta-Lactamase Inhibitors. *Clinical Microbiology Reviews* **2010,** *23* (1), 160-+.
6. Sauvage, E.; Terrak, M., Glycosyltransferases and transpeptidases/penicillin-binding proteins: Valuable targets for new antibacterials. *Antibiotics* **2016,** *5* (1).
7. Edoo, Z.; Arthur, M.; Hugonnet, J. E., Reversible inactivation of a peptidoglycan transpeptidase by a β-lactam antibiotic mediated by β-lactam-ring recyclization in the enzyme active site. *Scientific Reports* **2017,** *7* (1).
8. Lincová. D; Farghali. H, *Základní a aplikovaná farmakologie*. Galén: 2002; Vol. 2.
9. Patrick, G. L., *An Introduction to Medicinal Chemistry*. 5 ed.; Oxford University Press: 2013.
10. Bush, K.; Bradford, P. A., beta-Lactams and beta-Lactamase Inhibitors: An Overview. *Cold Spring Harbor Perspectives in Medicine* **2016,** *6* (8), 22.
11. Lagacé-Wiens, P.; Rubinstein, E., Adverse reactions to β-lactam antimicrobials. *Expert Opinion on Drug Safety* **2012,** *11* (3), 381-399.
12. Wilke, M. S.; Lovering, A. L.; Strynadka, N. C. J., beta-lactam antibiotic resistance: a current structural perspective. *Current Opinion in Microbiology* **2005,** *8* (5), 525-533.
13. Poole, K., Resistance to beta-lactam antibiotics. *Cell. Mol. Life Sci.* **2004,** *61* (17), 2200-2223.
14. Fleming, A., On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. 1929. *Bulletin of the World Health Organization* **2001,** *79* (8), 780-790.
15. Chain, E.; Florey, H. W.; Adelaide, M. B.; Gardner, A. D.; Oxfd, D. M.; Heatley, N. G.; Jennings, M. A.; Orr-Ewing, J.; Sanders, A. G., PENICILLIN AS A CHEMOTHERAPEUTIC AGENT. *The Lancet* **1940,** *236* (6104), 226-228.
16. Mandell, Antimicrobial agents: Penicillin, Cephalosporins, and other beta-lactam antibiotics. In *The pharmacological basis of therapeutics*, McGraw-Hill Education: 1996.
17. Crowfoot, D.; Bunn, C. W.; Rogers-Low, B. W.; Turner, A. J., The X-ray crystallographic investigation of the structure of penicillin. In *The Chemistry of Penicillin*, 2015; pp 310-366.
18. Rolinson, G. N., Forty years of β-lactam research. *Journal of Antimicrobial Chemotherapy* **1998,** *41* (6), 589-603.
19. Sheehan, J. C., THE CHEMISTRY OF SYNTHETIC AND SEMISYNTHETIC PENICILLINS. In *Annals of the New York Academy of Sciences*, 1967; Vol. 145, pp 216-223.
20. Jevons, M. P., “Celbenin” -resistant Staphylococci. *British Medical Journal* **1961,** *1* (5219), 124-125.
21. Hawkey, P. M., Molecular epidemiology of clinically significant antibiotic resistance genes. *British Journal of Pharmacology* **2008,** *153* (SUPPL. 1), S406-S413.
22. Giguère, S.; Prescott, J. F.; Dowling, M. D., *Antimicrobial Therapy in Veterinary Medicine*. 5 ed.; Wiley-Blackwell: 2013.
23. Boothe, D. M., *Small Animal Clinical Pharmacology and Therapeutics*. 2 ed.; Saunders: 2011.
24. Llarrull, L. I.; Testero, S. A.; Fisher, J. F.; Mobashery, S., The future of the β-lactams. *Current Opinion in Microbiology* **2010,** *13* (5), 551-557.
25. Papp-Wallace, K. M.; Endimiani, A.; Taracila, M. A.; Bonomo, R. A., Carbapenems: Past, present, and future. *Antimicrobial Agents and Chemotherapy* **2011,** *55* (11), 4943-4960.
26. Wade, K. C.; Benjamin, D. K., Clinical Pharmacology of Anti-Infective Drugs. In *Infectious Diseases of the Fetus and Newborn Infant*, 2011; pp 1160-1211.
27. Daehne, W.; Frederiksen, E.; Gundersen, E.; Lund, F.; Morch, P.; Petersen, H. J.; Roholt, K.; Tybring, L.; Godtfredsen, W. O., Acyloxymethyl esters of ampicillin. *J. Med. Chem.* **1970,** *13* (4), 607-612.
28. Maddison, J. E.; Waston, D. J.; Elliott, J., Antibacterial drugs. In *Small Animal Clinical Pharmacology*, 2 ed.; Elsevier Health Sciences: 2008.
29. Wright, A. J., The penicillins. *Mayo Clinic Proceedings* **1999,** *74* (3), 290-307.
30. Nathwani, D.; Wood, M. J., Penicillins: A Current Review of their Clinical Pharmacology and Therapeutic Use. *Drugs* **1993,** *45* (6), 866-894.
31. Bodey, G. P.; Le Blanc, B., Piperacillin: in vitro evaluation. *Antimicrobial Agents and Chemotherapy* **1978,** *14* (1), 78-87.
32. Fu, K. P.; Neu, H. C., Azlocillin and mezlocillin: new ureido penicillins. *Antimicrobial Agents and Chemotherapy* **1978,** *13* (6), 930-938.
33. White, G. W.; Malow, J. B.; Zimelis, V. M.; Pahlavanzadeh, H.; Panwalker, A. P.; Jackson, G. G., COMPARATIVE INVITRO ACTIVITY OF AZLOCILLIN, AMPICILLIN, MEZLOCILLIN, PIPERACILLIN, AND TICARCILLIN, ALONE AND IN COMBINATION WITH AN AMINOGLYCOSIDE. *Antimicrobial Agents and Chemotherapy* **1979,** *15* (4), 540-543.
34. Newton, G. G. F.; Abraham, E. P., Cephalosporin C, a new antibiotic containing sulphur and D-α-aminoadipic acid [1]. *Nature* **1955,** *175* (4456), 548.
35. Hodgkin, D. C.; Maslen, E. N., The x-ray analysis of the structure of cephalosporin C. *The Biochemical journal* **1961,** *79*, 393-402.

36. Velasco, J.; Adrio, J. L.; Moreno, M. A.; Diez, B.; Soler, G.; Barredo, J. L., Environmentally safe production of 7-aminodeacetoxycephalosporanic acid (7-ADCA) using recombinant strains of Acremonium chrysogenum. *Nature Biotechnology* **2000,** *18* (8), 857-861.
37. Shahid, M.; Sobia, F.; Singh, A.; Malik, A.; Khan, H. M.; Jonas, D.; Hawkey, P. M., Beta-lactams and beta-lactamase-inhibitors in current-or potential-clinical practice: A comprehensive update. *Critical Reviews in Microbiology* **2009,** *35* (2), 81-108.
38. Saez-Llorens, X.; McCracken G.H, Jr., Antimicrobial and antiinflammatory treatment of Bacterial meningitis. *Infectious Disease Clinics of North America* **1999,** *13* (3), 619-636.
39. Murray, P.; Rosenthal, K.; Pfaller, M., *Medical Microbiology*. 8 ed.; Elsevier: 2015.
40. Bilman, B. F.; Turhanoğlu, M., Antimicrobial activity of Ceftaroline and Ceftobiprole tested against MRSA isolates from Turkey, in 2016. *IOSR Journal of Pharmacy and Biological Sciences* **2016,** *11*, 52-56.
41. Zhanel, G. G.; Chung, P.; Adam, H.; Zelenitsky, S.; Denisuik, A.; Schweizer, F.; Lagacé-Wiens, P. R. S.; Rubinstein, E.; Gin, A. S.; Walkty, A.; Hoban, D. J.; Lynch, J. P.; Karlowsky, J. A., Ceftolozane/tazobactam: A novel cephalosporin/β-lactamase inhibitor combination with activity against multidrug-resistant gram-negative bacilli. *Drugs* **2014,** *74* (1), 31-51.
42. Wise, R., The pharmacokinetics of the oral cephalosporins—a review. *Journal of Antimicrobial Chemotherapy* **1990,** *26*, 13-20.
43. Norrby, S. R., Side Effects of Cephalosporins. *Drugs* **1987,** *34* (2), 105-120.
44. Madaan, A.; Li, J. T. C., Cephalosporin allergy. *Immunology and Allergy Clinics of North America* **2004,** *24* (3), 463-476.
45. Fernandes, R.; Amador, P.; Prudêncio, C., β-Lactams: Chemical structure, mode of action and mechanisms of resistance. *Reviews in Medical Microbiology* **2013,** *24* (1), 7-17.
46. Neu, H. C., β-lactam antibiotics: Structural relationships affecting in vitro activity and pharmacologic properties. *Reviews of Infectious Diseases* **1986,** *8*, 237-259.
47. Medeiros, A. A., Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. *Clinical Infectious Diseases* **1997,** *24* (1 SUPPL.), S19-S45.
48. Giordano, P. A.; Elston, D.; Akinlade, B. K.; Weber, K.; Notario, G. F.; Busman, T. A.; Cifaldi, M.; Nilius, A. M., Cefdinir vs. cephalexin for mild to moderate uncomplicated skin and skin structure infections in adolescents and adults. *Current Medical Research and Opinion* **2006,** *22* (12), 2419-2428.
49. Fung-Tomc, J. C.; Huczko, E.; Stickle, T.; Minassian, B.; Kolek, B.; Denbleyker, K.; Bonner, D.; Kessler, R., Antibacterial activities of cefprozil compared with those of 13 oral cephems and 3 macrolides. *Antimicrobial Agents and Chemotherapy* **1995,** *39* (2), 533-538.
50. Sader, H. S.; Jones, R. N., Historical overview of the cephalosporin spectrum: Four generations of structural evolution. *Antimicrobic Newsletter* **1992,** *8* (12), 75-82.
51. Spencer, J. L.; Siu, F. Y.; Flynn, E. H.; Jackson, B. G.; Sigal, M. V.; Higgins, H. M.; Chauvette, R. R.; Andrews, S. L.; Bloch, D. E., Chemistry of cephalosporin antibiotics. 8. Synthesis and structure-activity relationships of cephaloridine analogues. *Antimicrobial Agents and Chemotherapy* **1966,** *6*, 573-580.
52. Alexander, D. C.; Jensen, S. E., Investigation of the Streptomyces clavuligerus cephamycin C gene cluster and its regulation by the CcaR protein. *Journal of Bacteriology* **1998,** *180* (16), 4068-4079.
53. Laurent, G.; Durant, F.; Frere, J. M.; Klein, D.; Ghuysen, J. M., Des-, syn- and anti-oxyimino-delta 3-cephalosporins. Intrinsic reactivity and reaction with RTEM-2 serine beta-lactamase and D-alanyl-D-alanine-cleaving serine and Zn2+-containing peptidases. *The Biochemical journal* **1984,** *218* (3), 933-937.
54. Brown, M. J.; Sharma, P.; Mir, F. A.; Bennett, P. N., *Clinical Pharmacology*. 12 ed.; Elsevier: 2018.
55. Curtis, N. A. C.; Orr, D.; Ross, G. W.; Boulton, M. G., Affinities of penicillins and cephalosporins for the penicillin-binding proteins of Escherichia coli K-12 and their antibacterial activity. *Antimicrobial Agents and Chemotherapy* **1979,** *16* (5), 533-539.
56. Nomura, H.; Minami, I.; Hitaka, T.; Fugono, T., Semisynthetic β-lactam antibiotics. 8 structure-activity relationships of α-sulfocephalosporins. *The Journal of Antibiotics* **1976,** *29* (9), 928-936.
57. Fisher, J. F.; Meroueh, S. O.; Mobashery, S., Bacterial resistance to β-lactam antibiotics: Compelling opportunism, compelling opportunity. *Chemical Reviews* **2005,** *105* (2), 395-424.
58. Lovering, A. L.; Gretes, M. C.; Safadi, S. S.; Danel, F.; De Castro, L.; Page, M. G. P.; Strynadka, N. C. J., Structural insights into the anti-methicillin-resistant Staphylococcus aureus (MRSA) activity of ceftobiprole. *J. Biol. Chem.* **2012,** *287* (38), 32096-32102.
59. Kurazono, M.; Ida, T.; Yamada, K.; Hirai, Y.; Maruyama, T.; Shitara, E.; Yonezawa, M., In vitro activities of ME1036 (CP5609), a novel parenteral carbapenem, against methicillin-resistant Staphylococci. *Antimicrobial Agents and Chemotherapy* **2004,** *48* (8), 2831-2837.
60. Laudano, J. B., Ceftaroline fosamil: A new broad-spectrum cephalosporin. *Journal of Antimicrobial Chemotherapy* **2011,** *66* (SUPPL.3), iii11-iii18.
61. Hughes, D. L., Patent Review of Manufacturing Routes to Fifth-Generation Cephalosporin Drugs. Part 2, Ceftaroline Fosamil and Ceftobiprole Medocaril. *Organic Process Research and Development* **2017,** *21* (6), 800-815.
62. Yao, H.; Liu, J.; Xu, S.; Zhu, Z.; Xu, J., The structural modification of natural products for novel drug discovery. *Expert Opinion on Drug Discovery* **2017,** *12* (2), 121-140.
63. Zhanel, G. G.; Sniezek, G.; Schweizer, F.; Zelenitsky, S.; Lagacé-Wiens, P. R. S.; Rubinstein, E.; Gin, A. S.; Hoban, D. J.; Karlowsky, J. A.,

Ceftaroline: A novel broad-spectrum cephalosporin with activity against meticillin-resistant staphylococcus aureus. *Drugs* **2009,** *69* (7), 809-831.
64. Lemaire, S.; Glupczynski, Y.; Duval, V.; Joris, B.; Tulkens, P. M.; Van Bambeke, F., Activities of ceftobiprole and other cephalosporins against extracellular and intracellular (THP-1 macrophages and keratinocytes) forms of methicillin-susceptible and methicillin-resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **2009,** *53* (6), 2289-2297.
65. O'Connor, K. M.; Paauw, D. S., *Pharmacologic Therapy*. Elsevier: 2016.
66. Ceftolozane sulfate with tazobactam sodium. *Aust. Prescr.* **2016,** *39* (3), 106-107.
67. Giacobbe, D. R.; Bassetti, M.; De Rosa, F. G.; Del Bono, V.; Grossi, P. A.; Menichetti, F.; Pea, F.; Rossolini, G. M.; Tumbarello, M.; Viale, P.; Viscoli, C.; on behalf of, I.-S., Ceftolozane/tazobactam: place in therapy. *Expert Rev. Anti-Infect. Ther.* **2018,** *16* (4), 307-320.
68. Cefiderocol (Fetroja) - A New IV Cephalosporin for Complicated UTI. *Med. Lett. Drugs Ther.* **2020,** *62* (1597), 65-68.
69. Mollmann, U.; Heinisch, L.; Bauernfeind, A.; Kohler, T.; Ankel-Fuchs, D., Siderophores as drug delivery agents: application of the "Trojan Horse" strategy. *Biometals* **2009,** *22* (4), 615-624.
70. Sykes, R. B.; Bonner, D. P., Aztreonam: The first monobactam. *The American Journal of Medicine* **1985,** *78* (2 SUPPL. 1), 2-10.
71. Sykes, R. B.; Bonner, D. P., Discovery and development of the monobactams. *Reviews of Infectious Diseases* **1985,** *7 Suppl 4*, S579-593.
72. Bonner, D. P.; Sykes, R. B., Structure activity relationships among the monobactams. *Journal of Antimicrobial Chemotherapy* **1984,** *14* (4), 313-327.
73. Nordmann, P.; Poirel, L., Emerging carbapenemases in Gram-negative aerobes. *Clinical Microbiology and Infection* **2002,** *8* (6), 321-331.
74. Wang, X.; Zhang, F.; Zhao, C.; Wang, Z.; Nichols, W. W.; Testa, R.; Li, H.; Chen, H.; He, W.; Wang, Q.; Wang, H., In vitro activities of ceftazidime-avibactam and aztreonam-avibactam against 372 gram-negative bacilli collected in 2011 and 2012 from 11 teaching hospitals in China. *Antimicrobial Agents and Chemotherapy* **2014,** *58* (3), 1774-1778.
75. Bräse, S., *Privileged Scaffolds in Medicinal Chemistry: Design, Synthesis, Evaluation*. 1 ed.; Royal Society of Chemistry: 2015.
76. Page, M. G. P.; Dantier, C.; Desarbre, E., In vitro properties of BAL30072, a novel siderophore sulfactam with activity against multiresistant gram-negative bacilli. *Antimicrobial Agents and Chemotherapy* **2010,** *54* (6), 2291-2302.
77. Brown, A. G., Clavulanic acid, a novel β-lactamase inhibitor - A case study in drug discovery and development. *Drug Design and Delivery* **1986,** *1* (1), 1-21.
78. Payne, D.J., Cramp, R., Winstanley, D.J., Knowles, D.J., Comparative activities of clavulanic acid, sulbactam, and tazobactam against clinically important beta-lactamases, *Antimicrobial Agents and Chemotherapy **1994,** 38 (4), 767-772.*
79. Steward, C. D.; Rasheed, J. K.; Hubert, S. K.; Biddle, J. W.; Raney, P. M.; Anderson, G. J.; Williams, P. P.; Brittain, K. L.; Oliver, A.; McGowan J.E, Jr.; Tenover, F. C., Characterization of clinical isolates of Klebsiella pneumoniae from 19 laboratories using the National Committee for Clinical Laboratory Standards extended-spectrum β-lactamase detection methods. *Journal of Clinical Microbiology* **2001,** *39* (8), 2864-2872.
80. Neu, H. C., β-Lactamases, β-lactamase inhibitors, and skin and skin-structure infections. *Journal of the American Academy of Dermatology* **1990,** *22* (5), 896-904.
81. Klein, J. O., Amoxicillin/clavulanate for infections in infants and children: Past, present and future. *Pediatric Infectious Disease Journal* **2003,** *22* (8 SUPPL.), S139-S148.
82. Soto, E.; Shoji, S.; Muto, C.; Tomono, Y.; Marshall, S., Population pharmacokinetics of ampicillin and sulbactam in patients with community-acquired pneumonia: Evaluation of the impact of renal impairment. *British Journal of Clinical Pharmacology* **2014,** *77* (3), 509-521.
83. Lister, P. D., β-Lactamase inhibitor combinations with extended-spectrum penicillins: Factors influencing antibacterial activity against enterobacteriaceae and Pseudomonas aeruginosa. *Pharmacotherapy* **2000,** *20* (9 II), 213S-218S.
84. Coleman, K., Diazabicyclooctanes (DBOs): A potent new class of non-β-lactam β-lactamase inhibitors. *Current Opinion in Microbiology* **2011,** *14* (5), 550-555.
85. Ehmann, D. E.; Jahić, H.; Ross, P. L.; Gu, R. F.; Hu, J.; Durand-Réville, T. F.; Lahiri, S.; Thresher, J.; Livchak, S.; Gao, N.; Palmer, T.; Walkup, G. K.; Fisher, S. L., Kinetics of avibactam inhibition against class A, C, and D β-lactamases. *J. Biol. Chem.* **2013,** *288* (39), 27960-27971.
86. Ehmann, D. E.; Jahić, H.; Ross, P. L.; Gu, R. F.; Hu, J.; Kern, G.; Walkup, G. K.; Fisher, S. L., Avibactam is a covalent, reversible, non-β-lactam β-lactamase inhibitor. *Proc. Natl. Acad. Sci. U. S. A.* **2012,** *109* (29), 11663-11668.
87. King, A. M.; King, D. T.; French, S.; Brouillette, E.; Asli, A.; Alexander, J. A. N.; Vuckovic, M.; Maiti, S. N.; Parr, T. R.; Brown, E. D.; Malouin, F.; Strynadka, N. C. J.; Wright, G. D., Structural and Kinetic Characterization of Diazabicyclooctanes as Dual Inhibitors of Both Serine-β-Lactamases and Penicillin-Binding Proteins. *ACS Chemical Biology* **2016,** *11* (4), 864-868.
88. Biedenbach, D. J.; Kazmierczak, K.; Bouchillon, S. K.; Sahm, D. F.; Bradford, P. A., In vitro activity of aztreonam-avibactam against a global collection of Gram-negative pathogens from 2012 and 2013. *Antimicrobial Agents and Chemotherapy* **2015,** *59* (7), 4239-4248.
89. Flamm, R. K.; Farrell, D. J.; Sader, H. S.; Jones, R. N., Antimicrobial activity of ceftaroline combined with avibactam tested against bacterial organisms isolated from acute bacterial skin and skin structure infections in United States medical centers (2010-2012). *Diagnostic Microbiology and Infectious Disease* **2014,** *78* (4), 449-456.
90. Li, H.; Estabrook, M.; Jacoby, G. A.; Nichols, W. W.; Testa, R. T.; Bush, K., In vitro susceptibility of characterized β-lactamase-producing strains tested with avibactam combinations.

Antimicrobial Agents and Chemotherapy **2015,** *59* (3), 1789-1793.
91. Livermore, D. M., Current epidemiology and growing resistance of Gram-negative pathogens. *Korean Journal of Internal Medicine* **2012,** *27* (2), 128-142.
92. Livermore, D. M.; Mushtaq, S.; Warner, M.; Woodford, N., Activity of OP0595/β-lactam combinations against Gram-negative bacteria with extended-spectrum, AmpC and carbapenem-hydrolysing β-lactamases. *Journal of Antimicrobial Chemotherapy* **2015,** *70* (11), 3032-3041.
93. Mushtaq, S.; Vickers, A.; Woodford, N.; Haldimann, A.; Livermore, D. M., Activity of nacubactam (RG6080/OP0595) combinations against MBL-producing Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy* **2019,** *74* (4), 953-960.
94. Kiener, P. A.; Waley, S. G., Reversible inhibitors of penicillinases. *Biochem. J.* **1978,** *169* (1), 197-204.
95. Lapuebla, A.; Abdallah, M.; Olafisoye, O.; Cortes, C.; Urban, C.; Quale, J.; Landman, D., Activity of meropenem combined with RPX7009, a novel β-lactamase inhibitor, against gram-negative clinical isolates in New York City. *Antimicrobial Agents and Chemotherapy* **2015,** *59* (8), 4856-4860.
96. Queenan, A. M.; Bush, K., Carbapenemases: The versatile β-lactamases. *Clinical Microbiology Reviews* **2007,** *20* (3), 440-458.
97. Rolinson, G. N., Naturally-occurring β-lactamase inhibitors with antibacterial activity. *Journal of Antibiotics* **1976,** *29* (6), 668-669.
98. Kahan, J. S.; Kahan, F. M.; Goegelman, R.; Currie, S. A.; Jackson, M.; Stapley, E. O.; Miller, T. W.; Miller, A. K.; Hendlin, D.; Woodruff, H. B.; Birnbaum, J.; Mochales, S.; Hernandez, S., Thienamycin, a new β-lactam antibiotic i. discovery, taxonomy, isolation and physical properties. *The Journal of Antibiotics* **1979,** *32* (1), 1-12.
99. Bonfiglio, G.; Russo, G.; Nicoletti, G., Recent developments in carbapenems. *Expert Opinion on Investigational Drugs* **2002,** *11* (4), 529-544.
100. Graham, D. W.; Ashton, W. T.; Barash, L.; Brown, J. E.; Brown, R. D.; Canning, L. F.; Chen, A.; Springer, J. P.; Rogers, E. F., Inhibition of The Mammalian β-Lactamase Renal Dipeptidase (dehydropeptidase-I) By (Z)-2-(Acylamino)-3-substituted-propenoic Acids. *J. Med. Chem.* **1987,** *30* (6), 1074-1090.
101. Kahan, F. M.; Kropp, H.; Sundelof, J. G.; Birnbaum, J., Thienamycin: Development of imipenem-cilastatin. *Journal of Antimicrobial Chemotherapy* **1983,** *12* (SUPPL. D), 1-35.
102. Fukasawa, M.; Sumita, Y.; Harabe, E. T.; Tanio, T.; Nouda, H.; Kohzuki, T.; Okuda, T.; Matsumura, H.; Sunagawa, M., Stability of meropenem and effect of 1β-methyl substitution on its stability in the presence of renal dehydropeptidase I. *Antimicrobial Agents and Chemotherapy* **1992,** *36* (7), 1577-1579.
103. Sunagawa, M.; Matsumura, H.; Inoue, T.; Fukasawa, M.; Kato, M., A novel carbapenem antibiotic, SM-7338 structure-activity relationships. *The Journal of Antibiotics* **1990,** *43* (5), 519-532.
104. Bush, K., Proliferation and significance of clinically relevant β-lactamases. In *Annals of the New York Academy of Sciences*, 2013; Vol. 1277, pp 84-90.
105. Woodward, R. B., Penems and related substances. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **1980,** *289* (1036), 239-250.
106. Bassetti, M.; Nicolini, L.; Esposito, S.; Righi, E.; Viscoli, C., Current status of newer carbapenems. *Current Medicinal Chemistry* **2009,** *16* (5), 564-575.
107. Goa, K. L.; Noble, S., Panipenem/betamipron. *Drugs* **2003,** *63* (9), 913-925.
108. Queenan, A. M.; Shang, W.; Flamm, R.; Bush, K., Hydrolysis and inhibition profiles of β-lactamases from molecular classes A to D with doripenem, imipenem, and meropenem. *Antimicrobial Agents and Chemotherapy* **2010,** *54* (1), 565-569.
109. Rodloff, A. C.; Goldstein, E. J. C.; Torres, A., Two decades of imipenem therapy. *Journal of Antimicrobial Chemotherapy* **2006,** *58* (5), 916-929.
110. Nix, D. E.; Majumdar, A. K.; DiNubile, M. J., Pharmacokinetics and pharmacodynamics of ertapenem: An overview for clinicians. *Journal of Antimicrobial Chemotherapy* **2004,** *53* (SUPPL. 2), ii23-ii28.
111. Riera, E.; Cabot, G.; Mulet, X.; García-Castillo, M.; del Campo, R.; Juan, C.; Cantón, R.; Oliver, A., Pseudomonas aeruginosa carbapenem resistance mechanisms in Spain: Impact on the activity of imipenem, meropenem and doripenem. *Journal of Antimicrobial Chemotherapy* **2011,** *66* (9), 2022-2027.
112. Collins, V. L.; Marchaim, D.; Pogue, J. M.; Moshos, J.; Bheemreddy, S.; Sunkara, B.; Shallal, A.; Chugh, N.; Eiseler, S.; Bhargava, P.; Blunden, C.; Lephart, P. R.; Memon, B. I.; Hayakawa, K.; Abreu-Lanfranco, O.; Chopra, T.; Munoz-Price, L. S.; Carmeli, Y.; Kaye, K. S., Efficacy of ertapenem for treatment of bloodstream infections caused by extended-spectrum-β-lactamase-producing Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy* **2012,** *56* (4), 2173-2177.
113. Dagan, R.; Velghe, L.; Rodda, J. L.; Klugman, K. P., Penetration of meropenem into the cerebrospinal fluid of patients with inflamed meninges. *Journal of Antimicrobial Chemotherapy* **1994,** *34* (1), 175-179.
114. Hugonnet, J. E.; Tremblay, L. W.; Boshoff, H. I.; Barry Iii, C. E.; Blanchard, J. S., Meropenem-clavulanate is effective against extensively drug-resistant Mycobacterium tuberculosis. *Science* **2009,** *323* (5918), 1215-1218.
115. Villegas-Estrada, A.; Lee, M.; Hesek, D.; Vakulenko, S. B.; Mobashery, S., Co-opting the cell wall in fighting methicillin-resistant Staphylococcus aureus: Potent inhibition of PBP 2a by two anti-MRSA β-Lactam Antibiotics. *J. Am. Chem. Soc.* **2008,** *130* (29), 9212-9213.
116. Lin, J.; Nishino, K.; Roberts, M. C.; Tolmasky, M.; Aminov, R. I.; Zhang, L. X., Mechanisms of antibiotic resistance. *Front. Microbiol.* **2015,** *6*, 3.
117. Ahmed, A.; Azim, A.; Gurjar, M.; Baronia, A. K., Current concepts in combination antibiotic therapy for critically ill patients. *Indian J Crit Care Med* **2014,** *18* (5), 310-4.
118. Abraham, E. P.; Chain, E., An enzyme from bacteria able to destroy penicillin [1]. *Nature* **1940,** *146* (3713), 837.

119. Tang, S. S.; Apisarnthanarak, A.; Hsu, L. Y., Mechanisms of β-lactam antimicrobial resistance and epidemiology of major community- and healthcare-associated multidrug-resistant bacteria. *Advanced Drug Delivery Reviews* **2014,** *78*, 3-13.
120. Adler, M. Mechanisms and Dynamics of Carbapenem Resistance in Escherichia coli. Uppsala University, Uppsala, 2014.
121. Grundmann, H.; Livermore, D. M.; Giske, C. G.; Canton, R.; Rossolini, G. M.; Campos, J.; Vatopoulos, A.; Gniadkowski, M.; Toth, A.; Pfeifer, Y.; Jarlier, V.; Carmeli, Y.; Mittermayer, H.; Goossens, M.; Tihic, N.; Proevska, Y.; Tambic, A.; Pieridou-Bagatzouni, D.; Zemlickova, H.; Hrabak, J.; Hammerum, A. M.; Ivanova, M.; Jalavi, J.; Pfeifer, Y.; Vatopoulos, A.; Toth, A.; Hardarson, H.; Wee Boo, T.; Colodner, R.; Carmeli, Y.; Monaco, M.; Garcia-Fernandez, A.; Balode, A.; Miciuleviciene, J.; Caruana, P.; Skov, G.; Samuelsen, O.; Hryniewicz, W.; Gniadkowski, M.; Caniça, M.; Manageiro, V.; Codita, I.; Mueller-Premru, M.; Kolman, J.; Campos, J.; Oteo Iglesias, J.; Cantón, R.; Edquist, P. J.; Giske, C.; Droz, S.; Livermore, D.; Leverstein - van Hall, M. A.; Huijsdens, X.; Grundmann, H., Carbapenem-non-susceptible Enterobacteriaceae in Europe: Conclusions from a meeting of national experts. *Eurosurveillance* **2010,** *15* (46).
122. Chambers, H. F.; DeLeo, F. R., Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nature Reviews Microbiology* **2009,** *7* (9), 629-641.
123. D'Costa, V. M.; McGrann, K. M.; Hughes, D. W.; Wright, G. D., Sampling the antibiotic resistome. *Science* **2006,** *311* (5759), 374-377.
124. Fisher, J. F.; Mobashery, S., β-Lactam resistance mechanisms: Gram-positive bacteria and mycobacterium tuberculosis. *Cold Spring Harbor Perspectives in Medicine* **2016,** *6* (5).
125. Pitout, J. D. D.; Sanders, C. C.; Sanders Jr, W. E., Antimicrobial resistance with focus on β-lactam resistance in gram- negative bacilli. *American Journal of Medicine* **1997,** *103* (1), 51-59.
126. Davies, J.; Davies, D., Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews* **2010,** *74* (3), 417-433.
127. Hawkey, P. M., Antibiotic resistance. In *Brenner's Online Encyclopedia of Genetics*, 1 ed.; Academic Press: 2001.
128. Nordmann, P.; Dortet, L.; Poirel, L., Carbapenem resistance in Enterobacteriaceae: here is the storm! *Trends Mol Med* **2012,** *18* (5), 263-72.
129. Navarro, F., Acquisition and horizontal diffusion of β-lactam resistance among clinically relevant microorganisms. *International Microbiology* **2006,** *9* (2), 79-81.
130. Bello-López, J. M.; Cabrero-Martínez, O. A.; Ibáñez-Cervantes, G.; Hernández-Cortez, C.; Pelcastre-Rodríguez, L. I.; Gonzalez-Avila, L. U.; Castro-Escarpulli, G., Horizontal Gene Transfer and Its Association with Antibiotic Resistance in the Genus Aeromonas spp. *Microorganisms* **2019,** *7* (9), 363.
131. Weldhagen, G. F., Integrons and β-lactamases - A novel perspective on resistance. *Int. J. Antimicrob. Agents* **2004,** *23* (6), 556-562.
132. Franco, M. R. G.; Caiaffa-Filho, H. H.; Burattini, M. N.; Rossi, F., Metallo-beta-lactamases among imipenem-resistant Pseudomonas aeruginosa in a Brazilian university hospital. *Clinics* **2010,** *65* (9), 825-829.
133. Patel, G.; Bonomo, R. A., "Stormy waters ahead": Global emergence of carbapenemases. *Front. Microbiol.* **2013,** *4* (MAR).
134. Walsh, F., The multiple roles of antibiotics and antibiotic resistance in nature. *Front. Microbiol.* **2013,** *4* (AUG).
135. Silhavy, T. J.; Kahne, D.; Walker, S., The bacterial cell envelope. *Cold Spring Harbor perspectives in biology* **2010,** *2* (5).
136. Masi, M.; Réfregiers, M.; Pos, K. M.; Pagès, J. M., Mechanisms of envelope permeability and antibiotic influx and efflux in Gram-negative bacteria. *Nature Microbiology* **2017,** *2* (3).
137. Džidić, S.; Šušković, J.; Kos, B., Antibiotic resistance mechanisms in bacteria: Biochemical and genetic aspects. *Food Technology and Biotechnology* **2008,** *46* (1), 11-21.
138. Kojima, S.; Nikaido, H., Permeation rates of penicillins indicate that Escherichia coli porins function principally as nonspecific channels. *Proc. Natl. Acad. Sci. U. S. A.* **2013,** *110* (28), E2629-E2634.
139. Nikaido, H.; Zgurskaya, H. I., Antibiotic efflux mechanisms. *Current Opinion in Infectious Diseases* **1999,** *12* (6), 529-536.
140. Webber, M. A.; Piddock, L. J. V., The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy* **2003,** *51* (1), 9-11.
141. Van Veen, H. W.; Konings, W. N., Drug efflux proteins in multidrug resistant bacteria. *Biological Chemistry* **1997,** *378* (8), 769-777.
142. Langton, K. P.; Henderson, P. J. F.; Herbert, R. B., Antibiotic resistance: Multidrug efflux proteins, a common transport mechanism? *Natural Product Reports* **2005,** *22* (4), 439-451.
143. Trias, J.; Nikaido, H., Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy* **1990,** *34* (1), 52-57.
144. Terzi, H. A.; Kulah, C.; Atasoy, A. R.; Ciftci, I. H., Investigation of oprd porin protein levels in carbapenem-resistant Pseudomonas aeruginosa isolates. *Jundishapur Journal of Microbiology* **2015,** *8* (12).
145. Van Bambeke, F.; Balzi, E.; Tulkens, P. M., Antibiotic efflux pumps. *Biochem. Pharmacol.* **2000,** *60* (4), 457-470.
146. Lomovskaya, O.; Warren, M. S.; Lee, A.; Galazzo, J.; Fronko, R.; Lee, M.; Blais, J.; Cho, D.; Chamberland, S.; Renau, T.; Leger, R.; Hecker, S.; Watkins, W.; Hoshino, K.; Ishida, H.; Lee, V. J., Identification and characterization of inhibitors of multidrug resistance efflux pumps in Pseudomonas aeruginosa: Novel agents for combination therapy. *Antimicrobial Agents and Chemotherapy* **2001,** *45* (1), 105-116.
147. Poole, K., Multidrug efflux pumps and antimicrobial resistance in Pseudomonas aeruginosa and related organisms. *Journal of Molecular Microbiology and Biotechnology* **2001,** *3* (2), 255-263.
148. Gotoh, N.; Tsujimoto, H.; Poole, K.; Yamagishi, J. I.; Nishino, T., The outer membrane protein OprM of Pseudomonas aeruginosa is encoded by oprK of the mexA-mexB-oprK multidrug

resistance operon. *Antimicrobial Agents and Chemotherapy* **1995,** *39* (11), 2567-2569.
149. Köhler, T.; Epp, S. F.; Curty, L. K.; Pechère, J. C., Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of Pseudomonas aeruginosa. *Journal of Bacteriology* **1999,** *181* (20), 6300-6305.
150. Vargiu, A. V.; Nikaido, H., Multidrug binding properties of the AcrB efflux pump characterized by molecular dynamics simulations. *Proc. Natl. Acad. Sci. U. S. A.* **2012,** *109* (50), 20637-20642.
151. Eicher, T.; Cha, H. J.; Seeger, M. A.; Brandstätter, L.; El-Delik, J.; Bohnert, J. A.; Kern, W. V.; Verrey, F.; Grütter, M. G.; Diederichs, K.; Pos, K. M., Transport of drugs by the multidrug transporter AcrB involves an access and a deep binding pocket that are separated by a switch-loop. *Proc. Natl. Acad. Sci. U. S. A.* **2012,** *109* (15), 5687-5692.
152. Hung, L. W.; Kim, H. B.; Murakami, S.; Gupta, G.; Kim, C. Y.; Terwilliger, T. C., Crystal structure of AcrB complexed with linezolid at 3.5 Å resolution. *Journal of Structural and Functional Genomics* **2013,** *14* (2), 71-75.
153. Spratt, B. G., Resistance to antibiotics mediated by target alterations. *Science* **1994,** *264* (5157), 388-393.
154. Ballhausen, B.; Kriegeskorte, A.; Schleimer, N.; Peters, G.; Becker, K., The mecA homolog mecC confers resistance against β-lactams in Staphylococcus aureus irrespective of the genetic strain background. *Antimicrobial Agents and Chemotherapy* **2014,** *58* (7), 3791-3798.
155. Lambert, P. A., Bacterial resistance to antibiotics: Modified target sites. *Advanced Drug Delivery Reviews* **2005,** *57* (10), 1471-1485.
156. Blair, J. M. A.; Webber, M. A.; Baylay, A. J.; Ogbolu, D. O.; Piddock, L. J. V., Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology* **2015,** *13* (1), 42-51.
157. Bonomo, R. A., β-Lactamases: A focus on current challenges. *Cold Spring Harbor Perspectives in Medicine* **2017,** *7* (1).
158. Ambler, R. P., The structure of beta-lactamases. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **1980,** *289* (1036), 321-331.
159. Bush, K., Recent developments in β-lactamase research and their implications for the future. *Clinical Infectious Diseases* **1988,** *10* (4), 681-690.
160. Hall, B. G.; Barlow, M., Revised Ambler classification of β-lactamases [1]. *Journal of Antimicrobial Chemotherapy* **2005,** *55* (6), 1050-1051.
161. Tooke, C. L.; Hinchliffe, P.; Bragginton, E. C.; Colenso, C. K.; Hirvonen, V. H. A.; Takebayashi, Y.; Spencer, J., β-Lactamases and β-Lactamase Inhibitors in the 21st Century. *J. Mol. Biol.* **2019,** *431* (18), 3472-3500.
162. Bush, K., The ABCD's of beta-lactamase nomenclature. *J. Infect. Chemother.* **2013,** *19* (4), 549-559.
163. Bush, K.; Jacoby, G. A., Updated Functional Classification of beta-Lactamases. *Antimicrobial Agents and Chemotherapy* **2010,** *54* (3), 969-976.
164. Perez, F.; Endimiani, A.; Hujer, K. M.; Bonomo, R. A., The continuing challenge of ESBLs. *Current Opinion in Pharmacology* **2007,** *7* (5), 459-469.
165. Bush, K.; Fisher, J. F., Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from gram-negative bacteria. In *Annual Review of Microbiology*, 2011; Vol. 65, pp 455-478.
166. Voulgari, E.; Poulou, A.; Koumaki, V.; Tsakris, A., Carbapenemase-producing Enterobacteriaceae: Now that the storm is finally here, how will timely detection help us fight back? *Future Microbiology* **2013,** *8* (1), 27-39.
167. Johnson, A. P.; Woodford, N., Global spread of antibiotic resistance: The example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. *Journal of Medical Microbiology* **2013,** *62* (PART4), 499-513.
168. Lynch, J. P.; Clark, N. M.; Zhanel, G. G., Evolution of antimicrobial resistance among Enterobacteriaceae (focus on extended spectrum β-lactamases and carbapenemases). *Expert Opinion on Pharmacotherapy* **2013,** *14* (2), 199-210.
169. Drawz, S. M.; Bonomo, R. A., Three decades of β-lactamase inhibitors. *Clinical Microbiology Reviews* **2010,** *23* (1), 160-201.
170. Matagne, A.; Lamotte-Brasseur, J.; Frere, J. M., Catalytic properties of class A beta-lactamases: efficiency and diversity. *Biochem. J.* **1998,** *330*, 581-598.
171. Wang, F.; Cassidy, C.; Sacchettini, J. C., Crystal structure and activity studies of the Mycobacterium tuberculosis beta-lactamase reveal its critical role in resistance to beta-lactam antibiotics. *Antimicrobial Agents and Chemotherapy* **2006,** *50* (8), 2762-2771.
172. Philippon, A.; Slama, P.; Deny, P.; Labia, R., A Structure-Based Classification of Class A beta-Lactamases, a Broadly Diverse Family of Enzymes. *Clinical Microbiology Reviews* **2016,** *29* (1), 29-57.
173. Chen, Y.; Minasov, G.; Roth, T. A.; Prati, F.; Shoichet, B. K., The deacylation mechanism of AmpC beta-lactamase at ultrahigh resolution. *J. Am. Chem. Soc.* **2006,** *128* (9), 2970-2976.
174. Damblon, C.; Raquet, X.; Lian, L. Y.; LamotteBrasseur, J.; Fonze, E.; Charlier, P.; Roberts, G. C. K.; Frere, J. M., The catalytic mechanism of beta-lactamases: NMR titration of an active-site lysine residue of the TEM-1 enzyme. *Proc. Natl. Acad. Sci. U. S. A.* **1996,** *93* (5), 1747-1752.
175. Lietz, E. J.; Truher, H.; Kahn, D.; Hokenson, M. J.; Fink, A. L., Lysine-73 is involved in the acylation and deacylation of beta-lactamase. *Biochemistry* **2000,** *39* (17), 4971-4981.
176. Meroueh, S. O.; Fisher, J. F.; Schlegel, H. B.; Mobashery, S., Ab initio QM/MM study of class A beta-lactamase acylation: Dual participation of Glu166 and Lys73 in a concerted base promotion of Ser70. *J. Am. Chem. Soc.* **2005,** *127* (44), 15397-15407.
177. Vandavasi, V. G.; Weiss, K. L.; Cooper, J. B.; Erskine, P. T.; Tomanicek, S. J.; Ostermann, A.; Schrader, T. E.; Ginell, S. L.; Coates, L., Exploring the Mechanism of beta-Lactam Ring Protonation in the Class A beta-lactamase Acylation Mechanism Using

Neutron and X-ray Crystallography. *J. Med. Chem.* **2016,** *59* (1), 474-479.
178. King, A. M. Discovery and characterization of novel beta-lactamase inhibitors. McMaster University 2016.
179. Datta, N.; Kontomichalou, P., PENICILLINASE SYNTHESIS CONTROLLED BY INFECTIOUS R FACTORS IN ENTEROBACTERIACEAE. *Nature* **1965,** *208* (5007), 239-+.
180. Rice, L. B.; Bonomo, R. A., beta-lactamases: which ones are clinically important? *Drug Resist. Update* **2000,** *3* (3), 178-189.
181. Tzouvelekis, L. S.; Bonomo, R. A., SHV-type beta-lactamases. *Curr. Pharm. Design* **1999,** *5* (11), 847-864.
182. Neu, H. C., THE NEW BETA-LACTAMASE-STABLE CEPHALOSPORINS. *Ann. Intern. Med.* **1982,** *97* (3), 408-419.
183. Paterson, D. L.; Bonomo, R. A., Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews* **2005,** *18* (4), 657-+.
184. Bush, K.; Jacoby, G. A.; Medeiros, A. A., A FUNCTIONAL CLASSIFICATION SCHEME FOR BETA-LACTAMASES AND ITS CORRELATION WITH MOLECULAR-STRUCTURE. *Antimicrobial Agents and Chemotherapy* **1995,** *39* (6), 1211-1233.
185. Baraniak, A.; Fiett, J.; Hryniewicz, W.; Nordmann, P.; Gniadkowski, M., Ceftazidime-hydrolysing CTX-M-15 extended-spectrum beta-lactamase (ESBL) in Poland. *Journal of Antimicrobial Chemotherapy* **2002,** *50* (3), 393-396.
186. Tzouvelekis, L. S.; Tzelepi, E.; Tassios, P. T.; Legakis, N. J., CTX-M-type beta-lactamases: an emerging group of extended-spectrum enzymes. *Int. J. Antimicrob. Agents* **2000,** *14* (2), 137-142.
187. Yu, W. L.; Pfaller, M. A.; Winokur, P. L.; Jones, R. N., Cefepime MIC as a predictor of the extended-spectrum beta-lactamase type in Klebsiella pneumoniae, Taiwan. *Emerg. Infect. Dis* **2002,** *8* (5), 522-524.
188. Bush, K.; Macalintal, C.; Rasmussen, B. A.; Lee, V. J.; Yang, Y. J., KINETIC INTERACTIONS OF TAZOBACTAM WITH BETA-LACTAMASES FROM ALL MAJOR STRUCTURAL CLASSES. *Antimicrobial Agents and Chemotherapy* **1993,** *37* (4), 851-858.
189. Hawkey, P. M.; Jones, A. M., The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy* **2009,** *64* (4), 3-10.
190. Yigit, H.; Queenan, A. M.; Anderson, G. J.; Domenech-Sanchez, A.; Biddle, J. W.; Steward, C. D.; Alberti, S.; Bush, K.; Tenover, F. C., Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of Klebsiella pneumoniae. *Antimicrobial Agents and Chemotherapy* **2001,** *45* (4), 1151-1161.
191. Naas, T.; Nordmann, P.; Vedel, G.; Poyart, C., Plasmid-mediated carbapenem-hydrolyzing beta-lactamase KPC in a Klebsiella pneumoniae isolate from France. *Antimicrobial Agents and Chemotherapy* **2005,** *49* (10), 4423-4424.
192. Chen, Y.; McReynolds, A.; Shoichet, B. K., Re-examining the role of Lys67 in class C beta-lactamase catalysis. *Protein Sci.* **2009,** *18* (3), 662-669.
193. Munoz-Price, L. S.; Poirel, L.; Bonomo, R. A.; Schwaber, M. J.; Daikos, G. L.; Cormican, M.; Cornaglia, G.; Garau, J.; Gniadkowski, M.; Hayden, M. K.; Kumarasamy, K.; Livermore, D. M.; Maya, J. J.; Nordmann, P.; Patel, J. B.; Paterson, D. L.; Pitout, J.; Villegas, M. V.; Wang, H.; Woodford, N.; Quinn, J. P., Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases. *Lancet Infect Dis* **2013,** *13* (9), 785-796.
194. Bratu, S.; Landman, D.; Haag, R.; Recco, R.; Eramo, A.; Alam, M.; Quale, J., Rapid spread of carbapenem-resistant Klebsiella pneumoniae in New York City - A new threat to our antibiotic armamentarium. *Arch. Intern. Med.* **2005,** *165* (12), 1430-1435.
195. Poirel, L.; Weldhagen, G. F.; Naas, T.; De Champs, C.; Dove, M. G.; Nordmann, P., GES-2, a class A beta-lactamase from Pseudomonas aeruginosa with increased hydrolysis of imipenem. *Antimicrobial Agents and Chemotherapy* **2001,** *45* (9), 2598-2603.
196. Naas, T.; Vandel, L.; Sougakoff, W.; Livermore, D. M.; Nordmann, P., CLONING AND SEQUENCE-ANALYSIS OF THE GENE FOR A CARBAPENEM-HYDROLYZING CLASS-A BETA-LACTAMASE, SME-1, FROM SERRATIA-MARCESCENS S6. *Antimicrobial Agents and Chemotherapy* **1994,** *38* (6), 1262-1270.
197. Rasmussen, B. A.; Bush, K.; Keeney, D.; Yang, Y. J.; Hare, R.; Ogara, C.; Medeiros, A. A., Characterization of IMI-1 beta-lactamase, a class A carbapenem-hydrolyzing enzyme from Enterobacter cloacae. *Antimicrobial Agents and Chemotherapy* **1996,** *40* (9), 2080-2086.
198. Aubron, C.; Poirel, L.; Ash, R. J.; Nordmann, P., Carbapenemase-producing Enterobacteriaceae, US rivers. *Emerg. Infect. Dis* **2005,** *11* (2), 260-264.
199. Yu, Y. S.; Du, X. X.; Zhou, Z. H.; Chen, Y. G.; Li, L. J., First isolation of bla(IMI-2) in an Enterobacter cloacae clinical isolate from China. *Antimicrobial Agents and Chemotherapy* **2006,** *50* (4), 1610-1611.
200. Rotova, V.; Papagiannitsis, C. C.; Chudejova, K.; Medvecky, M.; Skalova, A.; Adamkova, V.; Hrabak, J., First description of the emergence of Enterobacter asburiae producing IMI-2 carbapenemase in the Czech Republic. *Journal of Global Antimicrobial Resistance* **2017,** *11*, 98-99.
201. Yang, Y. J.; Wu, P. J.; Livermore, D. M., BIOCHEMICAL-CHARACTERIZATION OF A BETA-LACTAMASE THAT HYDROLYZES PENEMS AND CARBAPENEMS FROM 2 SERRATIA-MARCESCENS ISOLATES. *Antimicrobial Agents and Chemotherapy* **1990,** *34* (5), 755-758.
202. Naas, T.; Dortet, L.; Iorga, B. I., Structural and Functional Aspects of Class A Carbapenemases. *Curr. Drug Targets* **2016,** *17* (9), 1006-1028.
203. Bush, K., The ABCD's of β-lactamase nomenclature. *J. Infect. Chemother.* **2013,** *19* (4), 549-559.
204. Rasmussen, B. A.; Gluzman, Y.; Tally, F. P., CLONING AND SEQUENCING OF THE CLASS-B BETA-LACTAMASE GENE (CCRA) FROM BACTEROIDES-FRAGILIS TAL3636. *Antimicrobial Agents and Chemotherapy* **1990,** *34* (8), 1590-1592.

205. Walsh, T. R.; Hall, L.; Assinder, S. J.; Nichols, W. W.; Cartwright, S. J.; Macgowan, A. P.; Bennett, P. M., SEQUENCE-ANALYSIS OF THE L1 METALLO-BETA-LACTAMASE FROM XANTHOMONAS-MALTOPHILIA. *Biochim. Biophys. Acta-Gene Struct. Expression* **1994,** *1218* (2), 199-201.
206. Docquier, J. D.; Pantanella, F.; Giuliani, F.; Thaller, M. C.; Amicosante, G.; Galleni, M.; Frere, J. M.; Bush, K.; Rossolini, G. M., CAU-1, a subclass B3 metallo-beta-lactamase of low substrate affinity encoded by an ortholog present in the Caulobacter crescentus chromosome. *Antimicrobial Agents and Chemotherapy* **2002,** *46* (6), 1823-1830.
207. Osano, E.; Arakawa, Y.; Wacharotayankun, R.; Ohta, M.; Horii, T.; Ito, H.; Yoshimura, F.; Kato, N., MOLECULAR CHARACTERIZATION OF AN ENTEROBACTERIAL METALLO BETA-LACTAMASE FOUND IN A CLINICAL ISOLATE OF SERRATIA-MARCESCENS THAT SHOWS IMIPENEM RESISTANCE. *Antimicrobial Agents and Chemotherapy* **1994,** *38* (1), 71-78.
208. Lauretti, L.; Riccio, M. L.; Mazzariol, A.; Cornaglia, G.; Amicosante, G.; Fontana, R.; Rossolini, G. M., Cloning and characterization of bla(VIM), a new integron-borne metallo-beta-lactamase gene from a Pseudomonas aeruginosa clinical isolate. *Antimicrobial Agents and Chemotherapy* **1999,** *43* (7), 1584-1590.
209. Yong, D.; Toleman, M. A.; Giske, C. G.; Cho, H. S.; Sundman, K.; Lee, K.; Walsh, T. R., Characterization of a New Metallo-beta-Lactamase Gene, bla(NDM-1), and a Novel Erythromycin Esterase Gene Carried on a Unique Genetic Structure in Klebsiella pneumoniae Sequence Type 14 from India. *Antimicrobial Agents and Chemotherapy* **2009,** *53* (12), 5046-5054.
210. Toleman, M. A.; Simm, A. M.; Murphy, T. A.; Gales, A. C.; Biedenbach, D. J.; Jones, R. N.; Walsh, T. R., Molecular characterization of SPM-1, a novel metallo-beta-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. *Journal of Antimicrobial Chemotherapy* **2002,** *50* (5), 673-679.
211. Palzkill, T., Metallo-beta-lactamase structure and function. In *Antimicrobial Therapeutics Reviews: The Bacterial Cell Wall as an Antimicrobial Target*, Bush, K., Ed. Blackwell Science Publ: Oxford, 2013; Vol. 1277, pp 91-104.
212. Jeon, J. H.; Lee, J. H.; Lee, J. J.; Park, K. S.; Karim, A. M.; Lee, C. R.; Jeong, B. C.; Lee, S. H., Structural Basis for Carbapenem-Hydrolyzing Mechanisms of Carbapenemases Conferring Antibiotic Resistance. *Int. J. Mol. Sci.* **2015,** *16* (5), 9654-9692.
213. Malabanan, M. M.; Amyes, T. L.; Richard, J. P., A role for flexible loops in enzyme catalysis. *Curr. Opin. Struct. Biol.* **2010,** *20* (6), 702-710.
214. Zhang, H. M.; Hao, Q., Crystal structure of NDM-1 reveals a common beta-lactam hydrolysis mechanism. *Faseb J.* **2011,** *25* (8), 2574-2582.
215. King, D. T.; Worrall, L. J.; Gruninger, R.; Strynadka, N. C. J., New Delhi Metallo-beta-Lactamase: Structural Insights into beta-Lactam Recognition and Inhibition. *J. Am. Chem. Soc.* **2012,** *134* (28), 11362-11365.
216. Wang, Z. G.; Fast, W.; Valentine, A. M.; Benkovic, S. J., Metallo-beta-lactamase: structure and mechanism. *Curr. Opin. Chem. Biol.* **1999,** *3* (5), 614-622.
217. Wang, Z. G.; Fast, W.; Benkovic, S. J., On the mechanism of the metallo-beta-lactamase from Bacteroides fragilis. *Biochemistry* **1999,** *38* (31), 10013-10023.
218. Park, H.; Brothers, E. N.; Merz, K. M., Hybrid QM/MM and DIFT investigations of the catalytic mechanism and inhibition of the dinuclear zinc metallo-beta-lactamase CcrA from Bacteroides fragilis. *J. Am. Chem. Soc.* **2005,** *127* (12), 4232-4241.
219. Crowder, M. W.; Spencer, J.; Vila, A. J., Metallo-beta-lactamases: Novel weaponry for antibiotic resistance in bacteria. *Accounts Chem. Res.* **2006,** *39* (10), 721-728.
220. Llarrull, L. I.; Fabiane, S. M.; Kowalski, J. M.; Bennett, B.; Sutton, B. J.; Vila, A. J., Asp-120 locates Zn2 for optimal metallo-ss-lactamase activity. *J. Biol. Chem.* **2007,** *282* (25), 18276-18285.
221. Simona, F.; Magistrato, A.; Dal Peraro, M.; Cavalli, A.; Vila, A. J.; Carloni, P., Common Mechanistic Features among Metallo-beta-lactamases A COMPUTATIONAL STUDY OF AEROMONAS HYDROPHILA CphA ENZYME. *J. Biol. Chem.* **2009,** *284* (41), 28164-28171.
222. Meini, M. R.; Llarrull, L. I.; Vila, A. J., Overcoming differences: The catalytic mechanism of metallo-beta-lactamases. *FEBS Lett.* **2015,** *589* (22), 3419-3432.
223. Lim, H. M.; Pene, J. J.; Shaw, R. W., CLONING, NUCLEOTIDE-SEQUENCE, AND EXPRESSION OF THE BACILLUS-CEREUS 5/B/6 BETA-LACTAMASE-II STRUCTURAL GENE. *Journal of Bacteriology* **1988,** *170* (6), 2873-2878.
224. Walsh, T. R.; Toleman, M. A.; Poirel, L.; Nordmann, P., Metallo-beta-lactamases: the quiet before the storm? *Clinical Microbiology Reviews* **2005,** *18* (2), 306-+.
225. Nordmann, P.; Poirel, L.; Walsh, T. R.; Livermore, D. M., The emerging NDM carbapenemases. *Trends Microbiol.* **2011,** *19* (12), 588-595.
226. Nordmann, P.; Poirel, L., The difficult-to-control spread of carbapenemase producers among Enterobacteriaceae worldwide. *Clinical Microbiology and Infection* **2014,** *20* (9), 821-830.
227. Watanabe, M.; Iyobe, S.; Inoue, M.; Mitsuhashi, S., TRANSFERABLE IMIPENEM RESISTANCE IN PSEUDOMONAS-AERUGINOSA. *Antimicrobial Agents and Chemotherapy* **1991,** *35* (1), 147-151.
228. Arakawa, Y.; Murakami, M.; Suzuki, K.; Ito, H.; Wacharotayankun, R.; Ohsuka, S.; Kato, N.; Ohta, M., A NOVEL INTEGRON-LIKE ELEMENT CARRYING THE METALLO-BETA-LACTAMASE GENE BLA(IMP). *Antimicrobial Agents and Chemotherapy* **1995,** *39* (7), 1612-1615.
229. Ito, H.; Arakawa, Y.; Ohsuka, S.; Wacharotayankun, R.; Kato, N.; Ohta, M., Plasmid-mediated dissemination of the metallo-β-lactamase gene bla(IMP) among clinically isolated strains of Serratia marcescens. *Antimicrobial Agents and Chemotherapy* **1995,** *39* (4), 824-829.
230. Riccio, M. L.; Franceschini, N.; Boschi, L.; Caravelli, B.; Cornaglia, G.; Fontana, R.;

Amicosante, G.; Rossolini, G. M., Characterization of the metallo-beta-lactamase determinant of Acinetobacter baumannii AC-54/97 reveals the existence of bla(IMP) allelic variants carried by gene cassettes of different phylogeny. *Antimicrobial Agents and Chemotherapy* **2000,** *44* (5), 1229-1235.
231. Queenan, A. M.; Bush, K., Carbapenemases: the versatile beta-lactamases. *Clinical Microbiology Reviews* **2007,** *20* (3), 440-458.
232. Mojica, M. F.; Bonomo, R. A.; Fast, W., B1-Metallo-beta-Lactamases: Where Do We Stand? *Curr. Drug Targets* **2016,** *17* (9), 1029-1050.
233. Albiger, B.; Glasner, C.; Struelens, M. J.; Grundmann, H.; Monnet, D. L.; European Survey, C., Carbapenemase-producing Enterobacteriaceae in Europe: assessment by national experts from 38 countries, May 2015. *Eurosurveillance* **2015,** *20* (45), 17-34.
234. Papagiannitsis, C. C.; Medvecky, M.; Chudejova, K.; Skalova, A.; Rotova, V.; Spanelova, P.; Jakubu, V.; Zemlickova, H.; Hrabak, J.; Czech Participants European, A., Molecular Characterization of Carbapenemase-Producing Pseudomonas aeruginosa of Czech Origin and Evidence for Clonal Spread of Extensively Resistant Sequence Type 357 Expressing IMP-7 Metallo-beta-Lactamase. *Antimicrobial Agents and Chemotherapy* **2017,** *61* (12), 15.
235. Poirel, L.; Naas, T.; Nicolas, D.; Collet, L.; Bellais, S.; Cavallo, J. D.; Nordmann, P., Characterization of VIM-2, a carbapenem-hydrolyzing metallo-beta-lactamase and its plasmid- and integron-borne gene from a Pseudomonas aeruginosa clinical isolate in France. *Antimicrobial agents and chemotherapy* **2000,** *44* (4), 891-897.
236. Patel, G.; Bonomo, R. A., Status report on carbapenemases: challenges and prospects. *Expert Rev. Anti-Infect. Ther.* **2011,** *9* (5), 555-570.
237. Ktari, S.; Arlet, G.; Mnif, B.; Gautier, V.; Mahjoubi, F.; Ben Jmeaa, M.; Bouaziz, M.; Hammami, A., Emergence of multidrug-resistant Klebsiella pneumoniae isolates producing VIM-4 metallo-beta-lactamase, CTX-M-15 extended-spectrum beta-lactamase, and CMY-4 AmpC beta-lactamase in a Tunisian university hospital. *Antimicrobial Agents and Chemotherapy* **2006,** *50* (12), 4198-4201.
238. Dortet, L.; Poirel, L.; Nordmann, P., Worldwide Dissemination of the NDM-Type Carbapenemases in Gram-Negative Bacteria. *Biomed Res. Int.* **2014**, 12.
239. Paskova, V.; Medvecky, M.; Skalova, A.; Chudejova, K.; Bitar, I.; Jakubu, V.; Bergerova, T.; Zemlickova, H.; Papagiannitsis, C. C.; Hrabak, J., Characterization of NDM-Encoding Plasmids From Enterobacteriaceae Recovered From Czech Hospitals. *Front. Microbiol.* **2018,** *9*, 12.
240. Lobkovsky, E.; Moews, P. C.; Liu, H.; Zhao, H.; Frere, J. M.; Knox, J. R., Evolution of an enzyme activity: crystallographic structure at 2-A resolution of cephalosporinase from the ampC gene of Enterobacter cloacae P99 and comparison with a class A penicillinase. *Proc. Natl. Acad. Sci. U. S. A.* **1993,** *90* (23), 11257-11261.
241. Jacoby, G. A., AmpC beta-Lactamases. *Clinical Microbiology Reviews* **2009,** *22* (1), 161-+.
242. Galleni, M.; Lamotte-Brasseur, J.; Raquet, X.; Dubus, A.; Monnaie, D.; Knox, J. R.; Frère, J. M., The enigmatic catalytic mechanism of active-site serine β-lactamases. *Biochem. Pharmacol.* **1995,** *49* (9), 1171-1178.
243. Knox, J. R.; Moews, P. C.; Frere, J. M., Molecular evolution of bacterial β-lactam resistance. *Chemistry and Biology* **1996,** *3* (11), 937-947.
244. Kim, J. Y.; Jung, H. I.; An, Y. J.; Hun, J. H.; Suh, P. G.; Lee, H. S.; Lee, S. H.; Cha, S. S., Structural basis for the extended substrate spectrum of CMY-10, a plasmid-encoded class C beta-lactamase. *Mol. Microbiol.* **2006,** *60* (4), 907-916.
245. Hata, M.; Tanaka, Y.; Fujii, Y.; Neya, S.; Hoshino, T., A theoretical study on the substrate deacylation mechanism of class C beta-lactamase. *J. Phys. Chem. B* **2005,** *109* (33), 16153-16160.
246. Philippon, A.; Arlet, G.; Jacoby, G. A., Plasmid-determined AmpC-type beta-lactamases. *Antimicrobial Agents and Chemotherapy* **2002,** *46* (1), 1-11.
247. Lee, S. H.; Kim, J. Y.; Lee, G. S.; Cheon, S. H.; An, Y. J.; Jeong, S. H.; Lee, K. J., Characterization of bla(CMY-11), an AmpC-type plasmid-mediated beta-lactamase gene in a Korean clinical isolate of Escherichia coli. *Journal of Antimicrobial Chemotherapy* **2002,** *49* (2), 269-273.
248. Walther-Rasmussen, J.; Hoiby, N., Plasmid-borne AmpC beta-lactamases. *Can. J. Microbiol.* **2002,** *48* (6), 479-493.
249. Marchese, A.; Arlet, G.; Schito, G. C.; Lagrange, P. H.; Philippon, A., Characterization of FOX-3, an AmpC-type plasmid-mediated beta-lactamase from an Italian isolate of Klebsiella oxytoca. *Antimicrobial Agents and Chemotherapy* **1998,** *42* (2), 464-467.
250. Rodriguez-Martinez, J. M.; Pascual, A.; Garcia, I.; Martinez-Martinez, L., Detection of the plasmid-mediated quinolone resistance determinant qnr among clinical isolates of Klebsiella pneumoniae producing AmpC-type beta-lactamase. *Journal of Antimicrobial Chemotherapy* **2003,** *52* (4), 703-706.
251. Evans, B. A.; Amyes, S. G. B., OXA beta-Lactamases. *Clinical Microbiology Reviews* **2014,** *27* (2), 241-263.
252. Nordmann, P.; Guibert, M., Extended-spectrum beta-lactamases in Pseudomonas aeruginosa. *Journal of Antimicrobial Chemotherapy* **1998,** *42* (2), 128-131.
253. Walther-Rasmussen, J.; Hoiby, N., OXA-type carbapenemases. *Journal of Antimicrobial Chemotherapy* **2006,** *57* (3), 373-383.
254. Poirel, L.; Naas, T.; Nordmann, P., Diversity, Epidemiology, and Genetics of Class D beta-Lactamases. *Antimicrobial Agents and Chemotherapy* **2010,** *54* (1), 24-38.
255. Nordmann, P.; Naas, T.; Poirel, L., Global Spread of Carbapenemase-producing Enterobacteriaceae. *Emerg. Infect. Dis* **2011,** *17* (10), 1791-1798.
256. Smith, C. A.; Antunes, N. T.; Toth, M.; Vakulenko, S. B., Crystal Structure of Carbapenemase OXA-58 from Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy* **2014,** *58* (4), 2135-2143.
257. Kaitany, K. C. J.; Klinger, N. V.; June, C. M.; Ramey, M. E.; Bonomo, R. A.; Powers, R. A.;

Leonard, D. A., Structures of the Class D Carbapenemases OXA-23 and OXA-146: Mechanistic Basis of Activity against Carbapenems, Extended-Spectrum Cephalosporins, and Aztreonam. *Antimicrobial Agents and Chemotherapy* **2013,** *57* (10), 4848-4855.
258. Smith, C. A.; Antunes, N. T.; Stewart, N. K.; Toth, M.; Kumarasiri, M.; Chang, M.; Mobashery, S.; Vakulenko, S. B., Structural basis for carbapenemase activity of the OXA-23 β-lactamase from Acinetobacter baumannii. *Chem. Biol.* **2013,** *20* (9), 1107-1115.
259. Schneider, K. D.; Ortega, C. J.; Renck, N. A.; Bonomo, R. A.; Powers, R. A.; Leonard, D. A., Structures of the Class D Carbapenemase OXA-24 from Acinetobacter baumannii in Complex with Doripenem. *J. Mol. Biol.* **2011,** *406* (4), 583-594.
260. Docquier, J. D.; Calderone, V.; De Luca, F.; Benvenuti, M.; Giuliani, F.; Bellucci, L.; Tafi, A.; Nordmann, P.; Botta, M.; Rossolini, G. M.; Mangani, S., Crystal Structure of the OXA-48 beta-Lactamase Reveals Mechanistic Diversity among Class D Carbapenemases. *Chem. Biol.* **2009,** *16* (5), 540-547.
261. Santillana, E.; Beceiro, A.; Bou, G.; Romero, A., Crystal structure of the carbapenemase OXA-24 reveals insights into the mechanism of carbapenem hydrolysis. *Proc. Natl. Acad. Sci. U. S. A.* **2007,** *104* (13), 5354-5359.
262. Sun, T.; Nukaga, M.; Mayama, K.; Braswell, E. H.; Knox, J. R., Comparison of beta-lactamases of classes A and D: 1.5-angstrom crystallographic structure of the class D OXA-1 oxacillinase. *Protein Sci.* **2003,** *12* (1), 82-91.
263. Pernot, L.; Frenois, F.; Rybkine, T.; L'Hermite, G.; Petrella, S.; Delettre, J.; Jarlier, V.; Collatz, E.; Sougakoff, W., Crystal structures of the class D beta-lactamase OXA-13 in the native form and in complex with meropenem. *J. Mol. Biol.* **2001,** *310* (4), 859-874.
264. Paetzel, M.; Danel, F.; De Castro, L.; Mosimann, S. C.; Page, M. G. P.; Strynadka, N. C. J., Crystal structure of the class D β-lactamase OXA-10. *Nature Structural Biology* **2000,** *7* (10), 918-925.
265. Couture, F.; Lachapelle, J.; Levesque, R. C., PHYLOGENY OF LCR-1 AND OXA-5 WITH CLASS-A AND CLASS-D BETA-LACTAMASES. *Mol. Microbiol.* **1992,** *6* (12), 1693-1705.
266. Schneider, K. D.; Bethel, C. R.; Distler, A. M.; Hujer, A. M.; Bonomo, R. A.; Leonard, D. A., Mutation of the Active Site Carboxy-Lysine (K70) of OXA-1 beta-Lactamase Results in a Deacylation-Deficient Enzyme. *Biochemistry* **2009,** *48* (26), 6136-6145.
267. Lohans, C. T.; van Groesen, E.; Kumar, K.; Tooke, C. L.; Spencer, J.; Paton, R. S.; Brem, J.; Schofield, C. J., A New Mechanism for beta-Lactamases: Class D Enzymes Degrade 1 beta-Methyl Carbapenems through Lactone Formation. *Angew. Chem.-Int. Edit.* **2018,** *57* (5), 1282-1285.
268. Lyon, J. A., Imipenem/cilastatin: The first carbapenem antibiotic. *Drug Intelligence and Clinical Pharmacy* **1985,** *19* (12), 894-899.
269. Bou, G.; Oliver, A.; Martinez-Beltran, J., OXA-24, a novel class D beta-lactamase with carbapenemase activity in an Acinetobacter baumannii clinical strain. *Antimicrobial Agents and Chemotherapy* **2000,** *44* (6), 1556-1561.
270. Poirel, L.; Heritier, C.; Tolun, V.; Nordmann, P., Emergence of oxacillinase-mediated resistance to imipenem in Klebsiella pneumoniae. *Antimicrobial Agents and Chemotherapy* **2004,** *48* (1), 15-22.
271. Ma, L.; Wang, J. T.; Wu, T. L.; Siu, L. K.; Chuang, Y. C.; Lin, J. C.; Lu, M. C.; Lu, P. L., Emergence of OXA-48-Producing Klebsiella pneumoniae in Taiwan. *Plos One* **2015,** *10* (9), 9.
272. Skalova, A.; Chudejova, K.; Rotova, V.; Medvecky, M.; Studentova, V.; Chudackova, E.; Lavicka, P.; Bergerova, T.; Jakubu, V.; Zemlickova, H.; Papagiannitsis, C. C.; Hrabak, J., Molecular Characterization of OXA-48-Like- Producing Enterobacteriaceae in the Czech Republic and Evidence for Horizontal Transfer of pOXA-48-Like Plasmids. *Antimicrobial Agents and Chemotherapy* **2017,** *61* (2), 10.
273. Brown, S.; Young, H. K.; Amyes, S. G. B., Characterisation of OXA-51, a novel class D carbapenemase found in genetically unrelated clinical strains of Acinetobacter baumannii from Argentina. *Clinical Microbiology and Infection* **2005,** *11* (1), 15-23.
274. Poirel, L.; Marque, S.; Heritier, C.; Segonds, C.; Chabanon, G.; Nordmann, P., OXA-58, a novel class D beta-lactamase involved in resistance to carbapenems in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy* **2005,** *49* (1), 202-208.
275. Testing, E. C. o. A. S., EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. July 2017.
276. Hrabak, J.; Chudackova, E.; Walkova, R., Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry for Detection of Antibiotic Resistance Mechanisms: from Research to Routine Diagnosis. *Clinical Microbiology Reviews* **2013,** *26* (1), 103-114.
277. Hrabak, J.; Chudackova, E.; Papagiannitsis, C. C., Detection of carbapenemases in Enterobacteriaceae: a challenge for diagnostic microbiological laboratories. *Clinical Microbiology and Infection* **2014,** *20* (9), 839-853.
278. Miyoshi-Akiyama, T.; Hayakawa, K.; Ohmagari, N.; Shimojima, M.; Kirikae, T., Multilocus Sequence Typing (MLST) for Characterization of Enterobacter cloacae. *Plos One* **2013,** *8* (6), 10.
279. Wirth, T.; Falush, D.; Lan, R. T.; Colles, F.; Mensa, P.; Wieler, L. H.; Karch, H.; Reeves, P. R.; Maiden, M. C. J.; Ochman, H.; Achtman, M., Sex and virulence in Escherichia coli: an evolutionary perspective. *Mol. Microbiol.* **2006,** *60* (5), 1136-1151.
280. Diancourt, L.; Passet, V.; Verhoef, J.; Grimont, P. A. D.; Brisse, S., Multilocus sequence typing of Klebsiella pneumoniae nosocomial isolates. *Journal of Clinical Microbiology* **2005,** *43* (8), 4178-4182.
281. Vatopoulos, A. C.; Philippon, A.; Tzouvelekis, L. S.; Komninou, Z.; Legakis, N. J., PREVALENCE OF A TRANSFERABLE SHV-5 TYPE BETA-LACTAMASE IN CLINICAL ISOLATES OF KLEBSIELLA-PNEUMONIAE

AND ESCHERICHIA-COLI IN GREECE. *Journal of Antimicrobial Chemotherapy* **1990,** *26* (5), 635-648.
282. Cohen, S. N.; Chang, A. C. Y.; Hsu, L., NONCHROMOSOMAL ANTIBIOTIC RESISTANCE IN BACTERIA - GENETIC TRANSFORMATION OF ESCHERICHIA-COLI BY R-FACTOR DNA. *Proc. Natl. Acad. Sci. U. S. A.* **1972,** *69* (8), 2110-&.
283. Barton, B. M.; Harding, G. P.; Zuccarelli, A. J., A GENERAL-METHOD FOR DETECTING AND SIZING LARGE PLASMIDS. *Anal. Biochem.* **1995,** *226* (2), 235-240.
284. Carattoli, A.; Bertini, A.; Villa, L.; Falbo, V.; Hopkins, K. L.; Threlfall, E. J., Identification of plasmids by PCR-based replicon typing. *Journal of Microbiological Methods* **2005,** *63* (3), 219-228.
285. Knox, J.; Jadhav, S.; Sevior, D.; Agyekum, A.; Whipp, M.; Waring, L.; Iredell, J.; Palombo, E., Phenotypic detection of carbapenemase-producing Enterobacteriaceae by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry and the Carba NP test. *Journal of clinical microbiology* **2014,** *52* (11), 4075-4077.
286. Papagiannitsis, C. C.; Študentová, V.; Izdebski, R.; Oikonomou, O.; Pfeifer, Y.; Petinaki, E.; Hrabák, J., Matrix-assisted laser desorption ionization-time of flight mass spectrometry meropenem hydrolysis assay with NH4HCO3, a reliable tool for direct detection of carbapenemase activity. *Journal of clinical microbiology* **2015,** *53* (5), 1731-1735.
287. Bocanegra-Ibarias, P.; Garza-González, E.; Morfín-Otero, R.; Barrios, H.; Villarreal-Treviño, L.; Rodríguez-Noriega, E.; Garza-Ramos, U.; Petersen-Morfin, S.; Silva-Sanchez, J., Molecular and microbiological report of a hospital outbreak of NDM-1-carrying Enterobacteriaceae in Mexico. *PloS one* **2017,** *12* (6), e0179651-e0179651.
288. Yang, P.; Xie, Y.; Feng, P.; Zong, Z., blaNDM-5 carried by an IncX3 plasmid in Escherichia coli sequence type 167. *Antimicrobial agents and chemotherapy* **2014,** *58* (12), 7548-7552.
289. Zhu, Y.-Q.; Zhao, J.-Y.; Xu, C.; Zhao, H.; Jia, N.; Li, Y.-N., Identification of an NDM-5-producing Escherichia coli Sequence Type 167 in a Neonatal Patient in China. *Scientific reports* **2016,** *6*, 29934-29934.
290. Izdebski, R.; Fiett, J.; Urbanowicz, P.; Baraniak, A.; Derde, L. P. G.; Bonten, M. J. M.; Carmeli, Y.; Goossens, H.; Hryniewicz, W.; Brun-Buisson, C.; Brisse, S.; Gniadkowski, M.; on behalf of the MOSAR WP2, W.; Groups, W. S.; Herda, M.; Dautzenberg, M. J.; Adler, A.; Kazma, M.; Navon-Venezia, S.; Malhotra-Kumar, S.; Lammens, C.; Legrand, P.; Chalfine, A.; Giamarellou, H.; Petrikkos, G. L.; Balode, A.; Dumpis, U.; Stammet, P.; Aragão, I.; Esteves, F.; Martí, A. T.; Lawrence, C.; Salomon, J.; Paul, M.; Lerman, Y.; Rossini, A.; Salvia, A.; Samso, J. V.; Fierro, J.; on behalf of the MOSAR WP2, W.; Groups, W. S., Phylogenetic lineages, clones and β-lactamases in an international collection of Klebsiella oxytoca isolates non-susceptible to expanded-spectrum cephalosporins. *Journal of Antimicrobial Chemotherapy* **2015,** *70* (12), 3230-3237.
291. Studentova, V.; Dobiasova, H.; Hedlova, D.; Dolejska, M.; Papagiannitsis, C. C.; Hrabak, J., Complete nucleotide sequences of two NDM-1-encoding plasmids from the same sequence type 11 Klebsiella pneumoniae strain. *Antimicrobial agents and chemotherapy* **2015,** *59* (2), 1325-1328.
292. Hrabak, J.; Papagiannitsis, C. C.; Studentova, V.; Jakubu, V.; Fridrichova, M.; Zemlickova, H.; Czech Participants European, A., Carbapenemase-producing Klebsiella pneumoniae in the Czech Republic in 2011. *Eurosurveillance* **2013,** *18* (45), 6-12.
293. Papousek, I.; Papagiannitsis, C. C.; Medvecky, M.; Hrabak, J.; Dolejska, M., Complete Nucleotide Sequences of Two VIM-1-Encoding Plasmids from Klebsiella pneumoniae and Leclercia adecarboxylata Isolates of Czech Origin. *Antimicrobial agents and chemotherapy* **2017,** *61* (5), e02648-16.
294. Izdebski, R.; Baraniak, A.; Herda, M.; Fiett, J.; Bonten, M. J. M.; Carmeli, Y.; Goossens, H.; Hryniewicz, W.; Brun-Buisson, C.; Gniadkowski, M.; on behalf of the MOSAR WP2, W.; groups, W. s., MLST reveals potentially high-risk international clones of Enterobacter cloacae*. *Journal of Antimicrobial Chemotherapy* **2014,** *70* (1), 48-56.
295. Mikiewicz, D.; Wrobel, B.; Wegrzyn, G.; Plucienniczak, A., Isolation and characterization of a ColE1-like plasmid from Enterobacter agglomerans with a novel variant of rom gene. *Plasmid* **1997,** *38* (3), 210-219.
296. Polisky, B., ColE1 replication control circuitry: Sense from antisense. *Cell* **1988,** *55* (6), 929-932.
297. Cesareni, G.; Helmer-Citterich, M.; Castagnoli, L., Control of ColE1 plasmid replication by antisense RNA. *Trends in Genetics* **1991,** *7* (7), 230-235.
298. Lombardi, G.; Luzzaro, F.; Docquier, J.-D.; Riccio, M. L.; Perilli, M.; Colì, A.; Amicosante, G.; Rossolini, G. M.; Toniolo, A., Nosocomial infections caused by multidrug-resistant isolates of pseudomonas putida producing VIM-1 metallo-beta-lactamase. *Journal of clinical microbiology* **2002,** *40* (11), 4051-4055.
299. Partridge, S. R.; Hall, R. M., The IS1111 family members IS4321 and IS5075 have subterminal inverted repeats and target the terminal inverted repeats of Tn21 family transposons. *Journal of bacteriology* **2003,** *185* (21), 6371-6384.
300. Halová, D.; Papousek, I.; Jamborova, I.; Masarikova, M.; Cizek, A.; Janecko, N.; Oravcova, V.; Zurek, L.; Clark, A. B.; Townsend, A.; Ellis, J. C.; Literak, I., Plasmid-mediated quinolone resistance genes in Enterobacteriaceae from American crows: high prevalence of bacteria with variable qnrB genes. *Antimicrobial agents and chemotherapy* **2014,** *58* (2), 1257-1258.
301. Allmeier, H.; Cresnar, B.; Greck, M.; Schmitt, R., Complete nucleotide sequence of Tn1721: gene organization and a novel gene product with features of a chemotaxis protein. *Gene* **1992,** *111* (1), 11-20.
302. Chatterjee, S. S.; Chen, L.; Joo, H.-S.; Cheung, G. Y. C.; Kreiswirth, B. N.; Otto, M., Distribution and regulation of the mobile genetic element-encoded phenol-soluble modulin PSM-mec in methicillin-resistant Staphylococcus aureus. *PloS one* **2011,** *6* (12), e28781-e28781.

303. Sunagar, R.; Hegde, N. R.; Archana, G. J.; Sinha, A. Y.; Nagamani, K.; Isloor, S., Prevalence and genotype distribution of methicillin-resistant Staphylococcus aureus (MRSA) in India. *Journal of Global Antimicrobial Resistance* **2016,** *7*, 46-52.
304. Xie, X.; Bao, Y.; Ouyang, N.; Dai, X.; Pan, K.; Chen, B.; Deng, Y.; Wu, X.; Xu, F.; Li, H.; Huang, S., Molecular epidemiology and characteristic of virulence gene of community-acquired and hospital-acquired methicillin-resistant Staphylococcus aureus isolates in Sun Yat-sen Memorial hospital, Guangzhou, Southern China. *BMC infectious diseases* **2016,** *16*, 339-339.
305. Gagnaire, J.; Dauwalder, O.; Boisset, S.; Khau, D.; Freydière, A.-M.; Ader, F.; Bes, M.; Lina, G.; Tristan, A.; Reverdy, M.-E.; Marchand, A.; Geissmann, T.; Benito, Y.; Durand, G.; Charrier, J.-P.; Etienne, J.; Welker, M.; Van Belkum, A.; Vandenesch, F., Detection of Staphylococcus aureus delta-toxin production by whole-cell MALDI-TOF mass spectrometry. *PloS one* **2012,** *7* (7), e40660-e40660.
306. Karaiskos I, Lagou S, Pontikis K, Rapti V, Poulakou G. The "Old" and the "New" Antibiotics for MDR Gram-Negative Pathogens: For Whom, When, and How. Front Public Health. 2019;7:151. Published 2019 Jun 11. doi:10.3389/fpubh.2019.00151